TESIS DOCTORAL

Programa de Doctorado en Química Fina

Nuevos disolventes supramoleculares funcionales para procesos de extracción analíticos

New functional supramolecular solvents for analytical extraction processes

Directoras:

Ana María Ballesteros Gómez

Noelia Caballero Casero

Autora:

Soledad González Rubio

Córdoba, abril 2023

TITULO: *Nuevos disolventes supramoleculares funcionales para procesos de extracción analíticos*

AUTOR: *Soledad González Rubio*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

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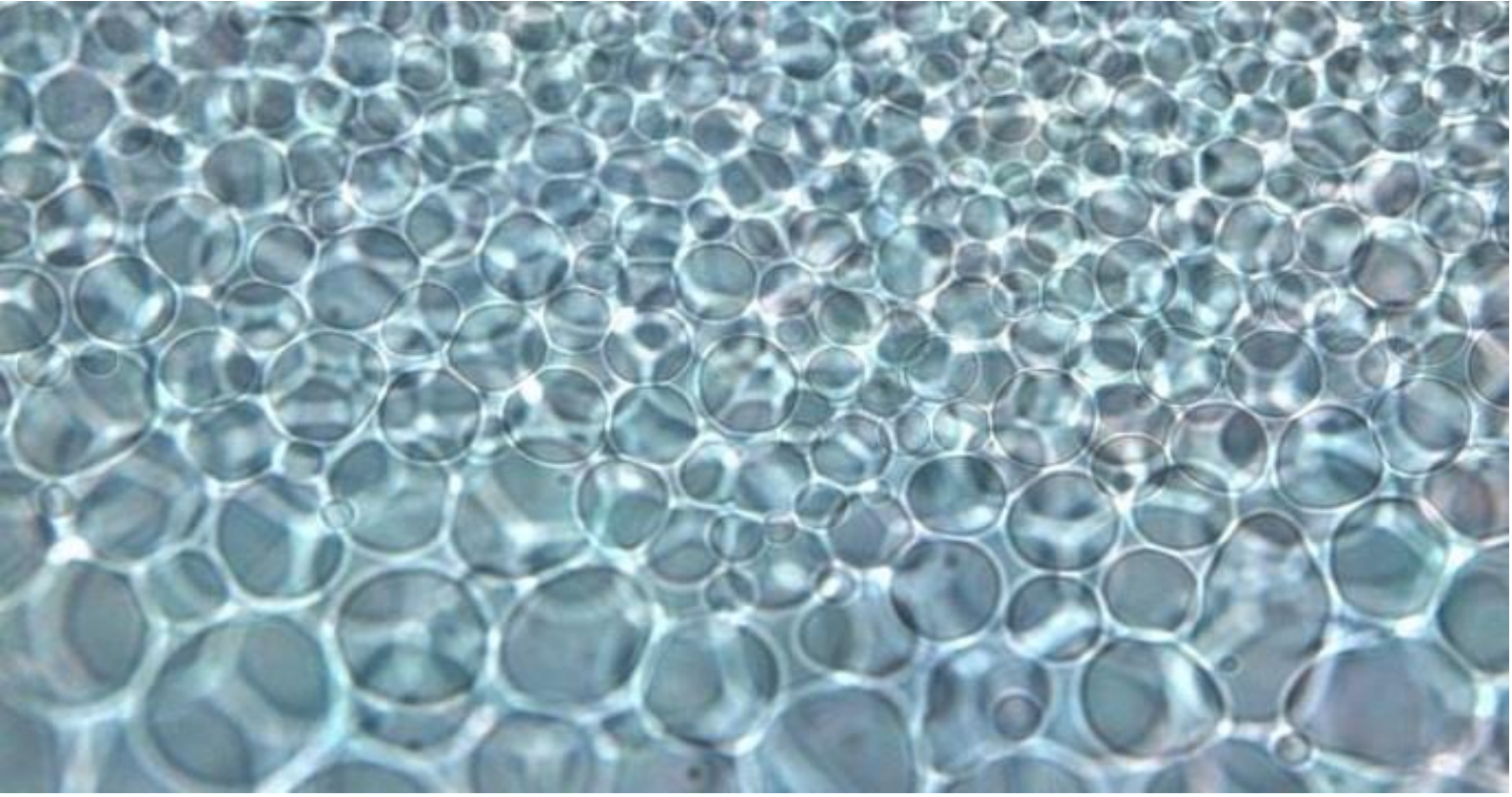
ucopress@uco.es



Tesis Doctoral

NUEVOS DISOLVENTES SUPRAMOLECULARES FUNCIONALES PARA PROCESOS DE EXTRACCIÓN ANALÍTICOS

Soledad González Rubio

UNIVERSIDAD D CÓRDOBA

2023

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Trabajo presentado, para optar al grado de Doctora, por

Soledad González Rubio

que lo firma en Córdoba, marzo de 2023

Firmado:

GONZALEZ RUBIO SOLEDAD - 31008591Z
Digitally signed by GONZALEZ RUBIO SOLEDAD - 31008591Z
Date: 2023.03.22 17:21:19 +01'00'

Soledad González Rubio

Graduada en Química

con el VºBº de las directoras,

Ana Mª Ballesteros Gómez y Noelia Caballero Casero

Firmado:

BALLESTEROS GOMEZ ANA MARIA - 30968640Z
Digitally signed by BALLESTEROS GOMEZ ANA MARIA - 30968640Z
Date: 2023.03.23 08:24:32 +01'00'

Ana Mª Ballesteros Gómez

Profesora Titular del Departamento de Química Analítica de la Universidad de Córdoba

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Fecha: 2023.03.22 17:14:13 +01'00'

Noelia Caballero Casero

Contratada Juan de la Cierva del Departamento de Química Analítica de la Universidad de Córdoba

Ana Mª Ballesteros Gómez, profesora Titular del Departamento de Química Analítica de la Universidad de Córdoba, y Noelia Caballero Casero, contratada Juan de la Cierva del Departamento de Química Analítica de la Universidad de Córdoba, en calidad de directoras de la Tesis Doctoral presentada por Soledad González Rubio, graduada en Química,

CERTIFICAN: Que la citada Tesis Doctoral, titulada Nuevos disolventes supramoleculares funcionales para procesos de extracción analíticos, se ha realizado en los laboratorios del Departamento de Química Analítica de la Universidad deCórdoba y que, a su juicio, reúne todos los requisitos exigidos a este tipo de trabajos.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, marzo de 2023.

BALLESTEROS GOMEZ ANA MARIA - 30968640Z
Digitally signed by BALLESTEROS GOMEZ ANA MARIA - 30968640Z
Date: 2023.03.23 08:27:37 +01'00'

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Fecha: 2023.03.22 17:14:38 +01'00'

Firmado:

Ana Mª Ballesteros Gómez

Profesora Titular del Departamento de Química Analítica de la Universidad de Córdoba

Firmado:

Noelia Caballero Casero

Contratada Juan de la Cierva del Departamento de Química Analítica de la Universidad de Córdoba

Mediante la presentación y defensa de esta Memoria de Tesis se opta a la obtención de la mención de Doctorado Internacional habida cuenta de que se reúnen los requisitos exigidos:

1. La doctoranda ha realizado una estancia de tres meses en la Norwegian University of Science and Technology (Noruega) bajo la supervisión de los doctores, Veerle Jaspers y Alexandros G. Asimakopoulos, donde han desarrollado dos de las investigaciones que constituyen esta Tesis.

2. Parte de esta Memoria, en concreto los objetivos, el contenido y las conclusiones, se ha redactado y será presentada en inglés.

3. La Tesis cuenta con informes favorables de dos expertos doctores pertenecientes a instituciones de educación superior no españolas.

4. Uno de los miembros del tribunal evaluador de la Tesis es un experto doctor perteneciente a una institución de educación superior no española.

TÍTULO DE LA TESIS: Nuevos disolventes supramoleculares funcionales para procesos de extracción analíticos. DOCTORANDA: Soledad González Rubio. INFORME RAZONADO DE LAS DIRECTORAS

DE LA TESIS: Las investigaciones desarrolladas en esta Tesis Doctoral han tenido como objetivos generales la síntesis y caracterización de disolventes supramoleculares (SUPRASs) y el desarrollo de estrategias innovadoras para la determinación de compuestos en un amplio intervalo de polaridad en diferentes tipos de muestras. Los SUPRASs fueron producidos a partir de disoluciones acuosas de anfifilos de simple o doble cabeza polar mediante coacervación inducida por sales o por disolventes orgánicos, y mostraron un elevado potencial para ser usados en procesos de separación de compuestos de diversa naturaleza. Estas investigaciones han supuesto un avance importante en el diseño de nuevos disolventes. Los métodos propuestos para el análisis de contaminantes y drogas en agua, alimentos y orina, previa a su determinación por cromatografía de líquidos y espectrometría de masas de baja o alta resolución, ofrecen procedimientos de tratamiento de muestra simples, rápidos y con bajo consumo de reactivos. Estas metodologías constituyen herramientas muy útiles para ser aplicadas en estudios a gran escala, que permitan poner en marcha procedimientos de prevención o remediación cuando sea necesario. Los resultados de las investigaciones realizadas se han materializado en 7 artículos científicos publicados en revistas indexadas, 1 de ellas situadas en el primer decil dentro de su categoría y las otras 6 en el primer cuartil (JCR). La

doctoranda también es coautora de otros dos artículos científicos. Además, ha realizado 12 contribuciones a congresos científicos (5 nacionales y 7 internacionales). En concreto, ha presentado 3 comunicaciones orales y 9 pósteres, uno de los cuales obtuvo el premio a la mejor comunicación oral del congreso. En base a la originalidad de las investigaciones desarrolladas y expuestas en esta Memoria, así como la formación científica adquirida por Dª. Soledad González Rubio, se autoriza la presentación de esta Tesis Doctoral. Córdoba, marzo de 2023.

Firma de las directoras

BALLESTEROS GOMEZ ANA MARIA - 30968640Z
Digitally signed by BALLESTEROS GOMEZ ANA MARIA - 30968640Z
Date: 2023.03.23 08:29:49 +01'00'

CABALLERO CASERO NOELIA - 44353553P
Firmado digitalmente por CABALLERO CASERO NOELIA - 44353553P
Fecha: 2023.03.22 17:15:06 +01'00'

Fdo.: Ana Mª Ballesteros Gómez

Fdo.: Noelia Caballero Casero

INFORME SOBRE LOS INDICIOS DE CALIDAD DE LA TESIS

A continuación, se detallan las publicaciones que se aportan como indicios de calidad de esta Tesis Doctoral.

1. A new sample treatment strategy based on simultaneous supramolecular solvent and dispersive solid-phase extraction for the determination of ionophore coccidiostats in all legislated foodstuffs
- Autores: Soledad González-Rubio, Diego García-Gómez, Ana María Ballesteros-Gómez, Soledad Rubio.
- Revista: Food Chemistry 326 (2020) 126987.
- Índice de impacto de la revista: 7.27 (JCR 2020).
- Área temática: Food Science & Technology.
- Posición revista/nº total de revistas: primer cuartil (Q1) 7/143.

2. Bioaccumulation potential of bisphenols and benzophenone UV filters: A multiresidue approach in raptor tissues
- Autores: Soledad González-Rubio, Kristine Vike-Jonas, Susana V. González, Ana Ballesteros-Gómez, Christian Sonne, Rune Dietz, David Boertmann, Lars Maltha Rasmussen,
Veerle L.B. Jaspers, Alexandros G. Asimakopoulos.
- Revista: Science of the Total Environment 741 (2020) 140330.
- Índice de impacto de la revista: 9.701 (JCR 2020).
- Área temática: Environmental Sciences.
- Posición revista/nº total de revistas: Q1 25/274

3. A review on contaminants of emerging concern in European raptors (2002–2020)
- Autores: Soledad González-Rubio, A, Ana Ballesteros-Gómez, Alexandros G. Asimakopoulos, Veerle L.B. Jaspers.
- Revista: Science of the Total Environment 760 (2021) 143337.
- Índice de impacto de la revista: 10.754 (JCR 2021).
- Área temática: Environmental Sciences.
- Posición revista/nº total de revistas: Q1 26/279

4. Double-headed amphiphile-based sponge droplets: synthesis, characterization
and potential for the extraction of compounds over a wide polarity range
- Autores: Soledad González-Rubio, Ana Ballesteros-Gómez, Diego García-Gómez, Soledad Rubio.
- Revista: Talanta 239 (2022) 123108.

- Índice de impacto de la revista: 6.556 (JCR, 2021).
- Área temática: Chemistry, Analytical.
- Posición revista/nº total de revistas: Q1 11/87.

5. A comprehensive study on the performance of different retention mechanisms in sport drug testing by liquid chromatography tandem mass spectrometry
- Autores: Soledad González-Rubio, Ana Ballesteros-Gómez, Soledad Rubio.
- Revista: Journal of Chromatography B 1178 (2021) 122821.
- Índice de impacto de la revista: 1.911 (JCR 2021).
- Área temática: Analytical, Chemistry.
- Posición revista/nº total de revistas: Segundo cuartil (Q2) 37/79.

6. Cubosomic Supramolecular Solvents: Synthesis, Characterization, and Potential for High-Throughput Multiclass Testing of Banned Substances in Urine
- Autores: Soledad González-Rubio, Ana Ballesteros-Gómez, Gloria Muñoz,Soledad Rubio.
- Revista: Analytical Chemistry, 94 (9) (2022) 4103-4111.
- Índice de impacto de la revista: 8.008 (JCR 2021).
- Área temática: Analytical, Chemistry.
- Posición revista/nº total de revistas: Q1 7/87

7. Supramolecular solvents for making comprensive liquid-liquid microextraction in multiclass screening methods based on liquid chromatography-high resolution mass spectrometry.
-Autores: Soledad González-Rubio, Noelia Caballero-Casero, Ana Ballesteros-Gómez, Darío Cuervo, Gloria Muñoz, Soledad Rubio.
-Enviado a publicación a Journal of Chromatography A.

Agradecimientos

Son muchas las personas que han pasado por distintas etapas de mi tesis. Personas que han hecho de ésta una experiencia inolvidable la cual siempre voy a recordar. A ellas les quiero agradecer que hayan formado parte de esta aventura. También siento que debo hacer una pequeña reflexión sobre la importancia de la ciencia. Sobre cómo ésta avanza y cambia la vida de las personas. Lección que me tocó aprender en el año 2020 cuando, después de una serie de pruebas, me diagnosticaron esclerosis múltiple. La esclerosis múltiple es una enfermedad crónica, neurodegenerativa y de origen autoinmune. Actualmente, no se conocen su causa ni cura. Sin embargo, es la enfermedad neurológica que más avances ha visto en los últimos 30 años y cada vez se consigue, con más éxito, frenar su progresión y cronificarla. Desde fármacos inyectables que se comercializaron por primera vez a mediados de los 90 hasta terapias con células madre que se están estudiando en la actualidad. Hoy día se dispone de hasta 15 fármacos de distinta eficacia que cambian el curso natural de la enfermedad. También han sido claves las investigaciones llevadas a cabo sobre el efecto beneficioso que supone una alimentación saludable y una práctica deportiva regular. Esto ha favorecido la mejora del pronóstico de los pacientes, que hace no mucho era poco alentadora. Dicen que las salas de espera de neurología han pasado de estar llenas de gente en silla de ruedas a gente con prisa por ir a trabajar. Que las mujeres afectadas planifiquen su embarazo, proyectos de trabajo… en definitiva, proyectos de vida. Sigamos invirtiendo en ciencia para cambiar vidas, no participar de ella con fines lucrativos y seguir construyendo juntos un futuro mejor. Que, como científicos, nunca olvidemos que ésta sea nuestra motivación real. Mi primer agradecimiento quería dedicarlo, pues, a las personas que con su trabajo, esfuerzo y formación producen avances en la ciencia. Gracias, porque a ellas les debo un futuro más esperanzador.

A mis compañeros de laboratorio, a los que estuvieron en el grupo (Francesca, Encarni) y a los que están (mis pupus María Jesús y Lourdes, Nouman, Luis, Celia, Cristina, Carmen). Gracias por tantas risas, buenos momentos y conversaciones.

A las personas que conocí en Noruega, que me hicieron sentir como en casa. Especialmente, gracias Veerle y mi Patri.

Gracias a todos los que no formáis parte del grupo, pero sí lo sois de forma especial (Juan, Laura, Francis, Guille).
Gracias Diego, por ser la primera persona que me introdujo en el laboratorio. Por los momentos y risas que compartimos al lado del cromatógrafo y las videollamadas en la distancia. Una parte de mí te echa mucho de menos.
Gracias Ana y Noelia, por vuestra ayuda, cariño y disponibilidad desinteresadas. Por salvarme de alguna que otra. Ya sabéis que sois más que directoras de tesis para mí.
A Isabel del SCAI. Gracias por tu ayuda, consejos y empatía. Eres una gran persona y una excelente profesional.
Gracias Lola y Loreto, por vuestro cariño.
Gracias Loles, por las conversaciones y apoyo incondicional. Te quiero.
A mis amigos, por quedaros en momentos de tempestad. Os quiero.
A mis hermanas Raquel y Bea, a las que adoro. Por haber sido luz en la oscuridad. Os quiero.
A mi padre, por su generosidad, cariño y besos. Te quiero.
A mi madre, por creer y apostar por mí. Por tu positividad, buen humor y amor incondicional. Por los paseos durante las sesiones de entrenamiento. Me conformo con ser una cuarta parte de lo buena persona y profesional que eres. Te quiero.
Y por supuesto, difícil de entender, pero gracias a la esclerosis múltiple por ser la mayor profesora de vida. Por enseñarme a darle importancia a las cosas que realmente la tienen, por plantearme mi rumbo profesional, por saber escuchar a mi cuerpo y por ser la primera prioridad. Por cuidarme y ejercitarme. Soy fiel creyente de que las cosas pasan por algo, de ver el vaso medio lleno y de que de todas las situaciones difíciles podemos sacar nuestra mejor versión.

Gracias a todos por haber sido parte del camino.

A las 3 millones de personas afectadas por EM en el mundo, porque sueñen con la cura en un futuro no muy lejano.

A Acodem, por recibirme con los brazos abiertos.

A mi familia argentina, por sentiros tan cerca a pesar de la distancia.

A Lolo, por devolverme la ilusión con sus charlas sobre la neuroplasticidad, desmarcarse del resto, creer en mí y ser el punto de inflexión en mi recuperación.

Índice

OBJETIVOS

Actualmente, los laboratorios de control de sustancias reguladas, como los laboratorios de análisis de contaminantes en alimentos y medioambiente o sustancias dopantes, se enfrentan diariamente a la determinación simultánea de una amplia variedad de compuestos químicos con estructuras, grupos funcionales y propiedades fisicoquímicas muy diferentes. La determinación de multicomponentes es también esencial en otras áreas de investigación, tales como monitorización ambiental, metabolómica, exposómica, etc., en las que es deseable diferentes tipos de matrices en áreas como el control de contaminantes en distintos tipos de alimentos incluidos en la legislación, o la evaluación del riesgo de exposición humana a sustancias químicas a partir de varias fuentes. Es por ello que el desarrollo e implementación de estrategias que permitan analizar de forma rápida y simple una amplia variedad de compuestos en varios tipos de matrices se ha convertido en un objetivo prioritario. El uso de métodos de tratamiento de muestra que permitan la extracción eficiente de compuestos en un amplio intervalo de polaridad y la eliminación eficaz de interferencias son aspectos relevantes a considerar en estas estrategias.

El objetivo general de las investigaciones que se presentan en esta memoria ha sido el desarrollo de metodologías innovadoras para el cribado y/o cuantificación de sustancias reguladas de interés para laboratorios de control agroalimentario y dopaje, así como de contaminantes emergentes en matrices ambientales. Para su desarrollo se planteó que estas metodologías deberían cumplir al menos las siguientes características: (i) capacidad para el análisis de multicomponentes en un amplio intervalo de polaridad; (ii) capacidad para el análisis de diferentes tipos de matrices de naturaleza líquida o sólida cuando las regulaciones vigentes así lo requirieran; (iii) prestaciones analíticas y operacionales mejoradas con respecto a las metodologías vigentes; (iv) resolución de algunos de los retos actuales a los que se enfrentan los laboratorios de control de sustancias reguladas, o avances en el

conocimiento sobre la distribución de contaminantes en matrices ambientales; (v) incremento de la sostenibilidad de los procesos analíticos.

Con estas características en mente, las investigaciones programadas se centraron en tres áreas: (a) biomonitorización animal y monitorización medioambiental de contaminantes emergentes; (b) control de sustancias reguladas en laboratorios agroalimentarios; (c) cribado y confirmación de sustancias prohibidas en laboratorios acreditados de control de dopaje.

Para abordar los retos presentes en los problemas seleccionados, se establecieron las estrategias que se comentan a continuación:

(1) Desarrollo y caracterización de nuevos disolventes supramoleculares (SUPRASs) que permitieran ampliar el intervalo de polaridad de los compuestos extraíbles mediante extracción líquido-líquido. Para este fin, se programó el desarrollo de SUPRAS a partir de compuestos anfifílicos con doble grupo polar. La hipótesis de trabajo fue que estos anfifilos deberían formar disolventes supramoleculares conteniendo una región hidrófila de gran extensión debido a la mayor superficie ocupada por los grupos polares y al mayor contenido de agua que previsiblemente se incorporaría. La hipótesis estaba basada en el hecho de que estos anfifilos forman agregados coloidales que contienen un número pequeño de monómeros y que debido al impedimento estérico entre sus grupos polares y a que estos agregados son más desordenados, ya que las cadenas hidrocarbonadas están plegadas, se esperaba una mayor incorporación de agua en su estructura.

(2) Uso de la combinación de SUPRASs con propiedades de acceso restringido (SUPRAS-RAM) para la exclusión de macromoléculas con otras estrategias de eliminación de interferencias, preferentemente extracción en fase sólida dispersiva. El fin de estas estrategias fue el desarrollo de metodologías aplicables a una amplia variedad de matrices de naturaleza sólida y líquida.

(3) Establecimiento de las colaboraciones necesarias en aquellos campos en los que el grupo de investigación no tenía suficiente conocimiento sobre el trabajo práctico

desarrollado por los laboratorios de control de sustancias reguladas, o en aquellas aplicaciones que requerían un enfoque multidisciplinar. En este sentido, se establecieron dos colaboraciones de gran interés para el desarrollo de esta Tesis. La primera es la colaboración con el laboratorio de control del dopaje de Madrid, que se ha articulado a través de un proyecto financiado por el organismo internacional Partnership for Clean Competition, y en el que la doctoranda ha sido la responsable de la ejecución de las actividades investigadoras. La segunda colaboración se ha realizado en el marco de la acción COST CA16224 (European Raptor Biomonitoring Facility), en el que la doctoranda obtuvo una beca para la realización de una estancia en la Norwegian University of Science and Technology (NTNU) en Trondheim, Noruega, en el año 2019 bajo la supervisión de Veerle L.B. Jaspers y Alexandros G. Asimakopoulos de los departamentos de Biología y Química Analítica, respectivamente, y que tuvo como objetivo una revisión crítica y un desarrollo metodológico sobre la biomonitorización de contaminantes emergentes en aves rapaces.

Paralelamente, un objetivo fundamental en el desarrollo de esta Tesis ha sido la formación de la doctoranda a través de actividades complementarias a la labor investigadora, tales como la redacción de artículos científicos, la asistencia y presentación de comunicaciones en congresos nacionales e internacionales, divulgación científica, asistencia a seminarios organizados periódicamente por el grupo de investigación, etc.

OBJECTIVES

The simultaneous determination of chemical compounds with different structures, functional groups and physicochemical properties is a main goal for testing laboratories, especially for those that carry out analyses of contaminants in foods, in the environment and in antidoping samples. Multicomponent determination is important in research areas, such as environmental monitoring, metabolomics, exposomics, etc., in which the determination of a great number of compounds in a single analysis is essential. Furthermore, there is a great interest in the development of methods that can be applicable to very different matrices. In this sense, some interesting fields are the control of contaminants in foods or the human risk assessment to chemicals from different sources. For this reason, the development and implementation of strategies that allow the fast and simple analysis of a high number of compounds in different matrices has become a main aim. An important aspect to consider is the use of treatment methods that allows the efficient extraction of compounds in a wide polarity range, as well as the simultaneous removal of interferences.

The main objective of the research carried out in this thesis was the development of innovative methodologies for the screening and/or quantification of regulated substances of interest in food, in the environment and in antidoping control laboratories. Emerging contaminants from environmental samples have also been taken into account.

The features that these methodologies should comply are the following: (i) the ability of multicomponent analysis of compounds in a wide polarity range; (ii) the ability of the analysis of different liquid or solid matrices with regulated contaminants (iii) enhanced analytical and operational features with regard to current methodologies (iv) potential to solve some of the challenges that control laboratories face, or the advances in the knowledge on the contaminant distribution in environmental matrices (v) the improvement of the sustainability of the analytical procedures.

The developed research was focused on the following areas: (a) biomonitoring and environmental monitoring of emerging contaminants (b) control of regulated substances in food laboratories (c) screening and confirmation of prohibited substances in antidoping control laboratories.

These issues were addresed taking into account the following strategies:

(1) The development and characterization of new supramolecular solvents (SUPRASs) that allowed to increase the polarity range of the compounds of interest in liquid-liquid extraction. For this purpose, the development of SUPRASs based on double-headed amphiphiles was carried out. The hypothesis of this study was that these amphiphiles produce supramolecular solvents with a higher hydrophilicity region. This is due to a greater surface occupied by the polar head groups and a higher water content incorporated in their structure. This hypothesis was supported by the fact that these amphiphiles form colloidal aggregates that contains a low number of monomers and that because of the steric hindrance between their polar head groups and of the messier structures that these aggregates offer, since the hydrocarbon chains remain folded, a higher water content is expected in these SUPRAS.

(2) The use of SUPRAS combined with restricted access material properties (SUPRAS-RAM) for the exclusion of macromolecules with other interference removal strategies, such as the dispersive solid phase extraction, was employed for the development of applicable methodologies to a wide variety of solid and liquid matrices.

(3) The establishment of the required collaboration in those fields in which the research group didn't have enough knowledge, such as with control laboratories for the determination of regulated substances, or in those areas in which a multidisciplinary focus was needed. Accordingly, two studies of this Thesis were carried out. The first one was performed with the Antidoping Control Laboratory from Madrid, that was funded by the Partnership for Clean Competition. In this study the PhD student was responsible of the corresponding research activities.

The second collaboration was carried out with a COST Action (CA16224, European Raptor Biomonitoring Facility). For this purpose, the PhD student obtained a grant for a stay at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway, in 2019, The study was supervised by the professors Veerle L.B. Jaspers and Alexandros G. Asimakopoulos from the Biology and Analytical Chemistry Departments, respectively. The main objectives of this stay were to perform of a critical review and to develop a method related to the biomonitoring of emerging contaminants in raptors.

Additionally, a main aim on the development of this Doctoral Thesis was the formation of the PhD student through complementary activities, such as the writing of research articles, the attendance and presentation of contributions in national and international conferences, the scientific dissemination, the attendance to seminars periodically organized by the research group, etc.

CONTENIDO

La memoria de esta Tesis Doctoral se ha estructurado en las secciones que se comentan a continuación. En la primera sección (Introducción) se discute la necesidad de desarrollar estrategias de tratamiento de muestra que permitan el análisis de multicomponentes en los laboratorios de control de sustancias reguladas y en diferentes áreas de investigación, y se analizan las prestaciones ofrecidas por los tratamientos de muestras basadas en el uso de disolventes orgánicos convencionales. A continuación, se discute la necesidad de desarrollar nuevos disolventes, más sostenibles y con prestaciones más innovadoras en relación al análisis de multicomponentes y de poner a punto métodos independientes de la matriz de la muestra. En este contexto se analizan las prestaciones ofrecidas por los líquidos iónicos y disolventes eutécticos. Finalmente, y dado que los disolventes supramoleculares constituyen la base de las innovaciones propuestas en esta Memoria, se describen en la Introducción diferentes aspectos teóricos de los mismos y se presentan sus principales aportaciones en el análisis de multicomponentes. A continuación, distribuidos en cuatro bloques (Bloques A-D),se presentan los artículos científicos derivados de las investigaciones realizadas (Capítulos I-VII). Seguidamente, se han recapitulado las Conclusiones más relevantes de los diferentes estudios realizados. Por último, en los Anexos, se recogen otras publicaciones científicas en las cuales la doctoranda es coautora y que están relacionadas con la temática de la Tesis, así como las comunicaciones presentadas en congresos científicos y las actividades de divulgación realizadas. Acontinuación, se resume el contenido de cada uno de los cuatro bloques.

Bloque A. Biomonitorización de contaminantes emergentes en aves rapaces europeas

Este bloque incluye dos capítulos (I-II) y son el resultado de la estancia de la doctoranda en los Departamentos de Biología y Química Analítica de la Norwegian University of Science and Technology (NTNU), en Trondheim, Noruega, en el marco de la acción COST CA16224. El capítulo I es una revisión crítica de los estudios de biomonitorización de contaminantes emergentes realizados en aves

rapaces europeas en el periodo 2002-2020. La revisión se centra en las especies de aves rapaces investigadas, los tipos y concentraciones de contaminantes emergentes hallados, las matrices analizadas y las metodologías empleadas. Asimismo, se destacan los retos prioritarios que deberían abordarse, tanto desde un punto de vista de avance en el conocimiento sobre la distribución de contaminantes emergentes en aves rapaces, que son consideradas como centinelas de la contaminación ambiental por su posición en la cadena trófica, como desde el punto de vista de la necesidad de estrategias más eficientes y sostenibles para la determinación de contaminantes emergentes en este tipo de matrices. En el Capítulo II se muestran y discuten los resultados obtenidos en el estudio de la biomonitorización de bisfenoles y benzofenonas en diferentes tejidos de varias aves rapaces encontradas muertas en Francia y Groenlandia. Los tejidos analizados fueron músculo, riñón, hígado, cerebro, glándula sebácea y tejido adiposo. Se investigó la presencia y distribución en los diferentes tejidos de 8 bisfenoles y 5 benzofenonas. Este estudio se considera pionero en la detección de ambos tipos de contaminantes en aves rapaces.

Bloque B. Desarrollo de plataformas genéricas de tratamiento de muestra para el control de contaminantes en alimentos

Este bloque incluye el Capítulo III que recoge las investigaciones realizadas con el objetivo de desarrollar plataformas genéricas de tratamiento de muestra aplicables a la determinación de contaminantes en una amplia variedad de matrices. Para ello, las metodologías aplicadas deben ser independientes del tipo de matriz analizado. Con este fin, se desarrolla un método analítico basado en el uso combinado de (i) un disolvente supramolecular con propiedades de material de acceso restringido (SUPRAS-RAM), formado a partir de mezclas de agua, THF y hexanol y (ii) extracción en fase sólida dispersiva (dSPE) utilizando una amina primaria-secundaria (PSA) y sulfato de magnesio. El método se aplicó en la determinación de coccidiostatos ionóforos en todas las matrices de origen animal legisladas (riñón,

hígado, músculo, grasa, leche y huevo). El método se validó de acuerdo a lasdirectrices de la Comisión Europea (2002/657/EC).

Bloque C. Desarrollo de plataformas de tratamiento de muestra para la monitorización ambiental de contaminantes en un amplio intervalo de polaridad El siguiente bloque incluye el Capítulo IV que recoge las investigaciones dirigidas a ampliar el intervalo de polaridad de los contaminantes que pueden extraerse utilizando disolventes supramoleculares. Para ello se sintetizan SUPRASs a partirde disoluciones coloidales de anfifilos de doble cabeza polar (concretamente el 1,2-decanediol, en mezclas de THF y agua). Se establecen las condiciones experimentales para la formación del SUPRAS, se estudia el volumen disolvente que se genera en función de las condiciones de síntesis, y se determina la composición química y las propiedades fisicoquímicas de los coacervados obtenidos. Asimismo, se caracterizan las nanoestructuras que conforman el SUPRAS utilizando microscopía óptica y electrónica. Por último, se han evaluado sus propiedades extractivas, mediante la determinación de compuesto perfluorados de distinta polaridad (log P en el intervalo 0,4-11,6) en muestras de agua ambiental.

Bloque D. Desarrollo de plataformas de tratamiento de muestra para el análisis demulticomponentes en control antidopaje

Este bloque contiene tres capítulos (Capítulos V-VII) dirigidos al desarrollo de plataformas de tratamiento de muestra para el análisis de multicomponentes en control antidopaje. Las sustancias prohibidas por la Agencia Mundial Antidopaje (World Antidoping Agency, WADA) incluyen 10 grupos de compuestos y sus metabolitos que en total suman más de 400 sustancias. Además de estos compuestos, están prohibidas todas las sustancias con estructura o efecto semejantes a las que están incluidas en la lista. Todas las investigaciones recogidasen este bloque se han desarrollado en colaboración con el Laboratorio de Control del Dopaje de Madrid. En el Capítulo V, se muestran los resultados obtenidos en

un amplio estudio dirigido a determinar el efecto de seis fases estacionarias y cuatro tipos de fases móviles en la separación de 93 sustancias prohibidas (log P en el intervalo -2,4 a 9,2) pertenecientes a los 10 grupos de sustancias prohibidas por WADA. Los parámetros investigados incluyeron factores de retención, factores de asimetría, tiempo de desarrollo cromatográfico y efectos matriz. A partir de los resultados obtenidos, se discuten y proponen las condiciones cromatográficas óptimas para la detección y cuantificación de las sustancias prohibidas por WADA. En el Capítulo VI se describe el desarrollo de SUPRASs a partir de 1,2-hexanediol en disoluciones acuosas salinas (1 M, Na_2SO_4). Se determinan los diagramas de fases para delimitar las condiciones experimentales para la formación del SUPRAS, se estudia el volumen disolvente que se genera en función de las condiciones de síntesis, y se determina la composición química y las propiedades fisicoquímicas de los coacervados obtenidos. Asimismo, se caracterizan las nanoestructuras que conforman el SUPRAS utilizando microscopía óptica y electrónica. Se demuestra que el SUPRAS, integrado por estructuras cubosómicas que tienen un elevado contenido de agua, extrae eficientemente 93 sustancias prohibidas (log P en el intervalo -2,4 a 9,2) pertenecientes a los 10 grupos de sustancias prohibidas por WADA, a la vez que reduce eficazmente los efectos matriz en cromatografía de líquidos acoplada a espectrometría de masas con fuente de ionización electrospray (LC-ESI-MS/MS.)En el último capítulo (Capítulo VII) la estrategia se amplía al cribado de sustancias prohibidas mediante cromatografía de gases acoplada a tiempo de vuelo con fuente de ionización electrospray (LC-ESI-TOF). Todos los métodos se han validado de acuerdo a la normativa establecida por WADA.

SUMMARY

This Doctoral Thesis is structured in various sections. In the Introduction, the need of developing sample treatment methodologies that allows the analysis of regulated multicomponents in control laboratories and in other research areas is discussed. The analysis of the benefits offered using sample treatments that are based on the use of conventional organic solvents are also commented. Next, the need of developing new sustainable solvents with innovative features is discussed. These features are related to the analysis of multicomponents and to methods that are independent to the sample matrix. The advantages provided by ionic liquids and eutectic solvents are also discussed. Finally, since supramolecular solvents are the basis of this Thesis, their theoretical aspects and their contribution in the multicomponent analysis field are shown in the Introduction as well. Next, the content of the Thesis is distributed along four different blocks (Blocks A-D) in which the different scientific articles derived from this research are included (Chapters I-VII). Subsequently, the most relevant conclusions of the different studies are recapitulated. Finally, in the Annexes, other scientific publications in which the PhD student is co-author and which are related to the subject of this Thesis, as well as communications presented at scientific conferences, are included. The content of each of the blocks is summarized below.

Block A. Biomonitoring of emerging contaminants in European raptors.

This block includes two chapters (I-II) and are the result of the stay of the PhD student at the Biology and Analytical Chemistry Departments of the Norwegian University of Science and Technology (NTNU), in Trondheim (Norway), within the framework of the COST Action CA16224. The Chapter I is a critical review on the biomonitoring of emerging contaminants in European raptors, in the period 2002-2020. The review discusses the species of raptors that have been investigated, the type and the concentration levels of contaminants found, the analyzed

matrices and the methodologies that were used. Likewise, some priority challenges that should be addressed are highlighted. On one hand the knowledge advance in the distribution of the emerging contaminants in raptors, which are consider sentinels of the environment pollution due to their position in the food chain, is described. On the other hand, the need of establishing more efficient and sustainable methodologies for the determination of emerging contaminants in these types of matrices is also described. Chapter II covers the results of the study on the biomonitoring of bisphenols and benzophenones in tissues of raptors found dead in France and Greenland. The analyzed tissues were muscle, kidney, liver, brain, preen land and fat and the presence and distribution of 8 bisphenols and 5 UV-filter benzophenones were studied. This is the first study that analyze both types of contaminants in raptors.

Block B. Development of generic sample treatment platforms for the control of contaminants in foods.

This block includes Chapter III and it shows the research activities carried out with the aim of developing generic sample treatment platforms that are applicableto the determination of contaminants in a wide variety of matrices. For this purpose, the applied methodologies should be independent of the analyzed matrices. The developed analytical method is based on the combined use of (i) a supramolecular solvent with restricted access material properties (SUPRAS- RAM), formed from mixtures of water, THF and hexanol and (ii) dispersive solid phase extraction (dSPE) using a primary-secondary amine (PSA) and magnesium sulfate. The method was applied to the determination of ionophore coccidiostats in all the legislated matrices (kidney, liver, muscle, fat, milk and egg). The method was also validated in accordance with the European Commission (2002/657/EC).

Block C. Development of sample treatment platforms for the environmental monitoring of contaminants in a wide polarity range.

The following block includes Chapter IV, which shows the research activities that were carried out to increase the polarity range of the contaminants that can be extracted with supramolecular solvents (SUPRASs). For this purpose, the SUPRASs were formed from colloidal solutions of double-headed amphiphiles (1,2-decanediol in THF and water mixtures). The experimental conditions for the SUPRAS formation were established. The generated solvent volume according to the established synthetic conditions, the chemical composition and the physicochemical properties of these coacervates were also studied. Likewise, the nanostructures of the SUPRAS were characterized with optic and electronic microscopy. Finally, their extractive properties were studied by the determination of perfluorinated compounds with different polarities (log P in the range 0.4-11.6) in environmental water samples.

Block D. Development of sample treatment platforms for the analysis of multicomponents in antidoping control.

This block includes three chapters (Chapters V-VII) focused on the development

of sample treatment platforms for the analysis of multicomponents in antidoping control. The substances that are included in the Prohibited List of the World Antidoping Agency (WADA), contain 10 compound groups and their metabolites that involve a total of 400 compounds. In addition to these compounds, all the susbstances that have a similar structure or effect are also included in this List. All the investigations collected in this block were developed in cooperation with the Antidoping Control Laboratory of Madrid (Spain). Chapter V shows the results obtained in a wide study on the effect of six stationary and four mobile phases for the separation of 93 banned substances (log P in the range -2.4 to 9.2) corresponding to the 10 WADA groups of prohibited substances. The investigated parameters include retention factors, assymetry factors, total chromatographic run time and matrix effects. From these results, the optimal chromatographic conditions for the detection and quantification of the substances prohibited by WADA are discussed.

In Chapter VI, the development of SUPRASs formed from 1,2-hexanediol molecules contained in salty water solutions (1 M, Na_2SO_4) is described. The phase diagrams are determined to define the experimental conditions for SUPRAS formation. The solvent volume that is produced on the basis of the different synthetic conditions established is also studied. The chemical composition and the physicochemical properties of the coacervates obtained shown too. Likewise, the nanostructures are characterized by using optic and electronic microscopy. Thus, the formation of a SUPRAS made of cubosomic structures was demonstrated. These structures have a high water content and are able to efficiently extract 93 prohibited substances (log P in the range -2.4 to 9.2) belonging to the 10 groups of substances banned by WADA. At the same time, these SUPRASs efficiently remove matrix effects in LC-ESI-MS/MS. In the last chapter (Chapter VII), this strategy is extended to the screening of prohibited substances with LC-ESI-TOF. All the methods were validated in accordance to theregulations established by WADA.

INTRODUCCIÓN

1. Estrategias de preparación de muestra para la determinación de multicomponentes

La determinación simultánea de una amplia variedad de compuestos químicos con estructuras, grupos funcionales y propiedades fisicoquímicas muy diferentes es en la actualidad una actividad relevante en los laboratorios de control de sustancias reguladas, como los laboratorios de análisis de contaminantes en alimentos [1] y medioambiente [2] o sustancias dopantes [3]. La determinación de multicomponentes es también esencial en áreas de investigación tales como epidemiología, metabolómica, exposómica, etc., en las que es deseable la determinación de tantos tóxicos como sea posible en un único análisis [4,5].

En la actualidad, las técnicas más utilizadas en los laboratorios de análisis de control de sustancias reguladas y laboratorios de investigación son la cromatografía de líquidos (LC) y de gases (GC) acoplada a espectrometría de masas (MS). Las técnicas cromatográficas han evolucionado de forma drástica en las últimas décadas en relación a su eficacia, resolución y reducción del tiempo de análisis [6]. La cromatografía de líquidos es más versátil que la cromatografía de gases para el análisis de multicomponentes, dada la amplia variedad de fases estacionarias disponibles y el mayor intervalo de polaridad de los compuestos a los que puede aplicarse. Por otro lado, el acoplamiento de LC con MS es en la actualidad robusto y se han desarrollado múltiples analizadores de masas cuya sensibilidad se ha incrementado de forma notable en los últimos años. Entre los analizadores de masas de baja resolución destacan el cuadrupolar (Q), la trampa iónica (IT), el triple cuadrupolo (QQQ) y el cuadrupolo-trampa iónica lineal (QTRAP). Los analizadores de alta resolución más utilizados en los laboratorios

analíticos son el de tiempo de vuelo (TOF), el cuadrupolar-tiempo de vuelo (QTOF) y el orbitrap [7].

En LC-MS, la etapa de ionización de los analitos es fundamental. La forma en la que éstos se ionizan depende de las características físico-química de los mismos, fundamentalmente de su polaridad y peso molecular. En la Figura 1, se muestra de forma comparativa la aplicabilidad de las tres fuentes de ionización más importantes en LC-MS: ionización por electrospray a presión atmosférica (API-ES o ESI), ionización química a presión atmosférica (APCI) y fotoionización a presión atmosférica (APPI).

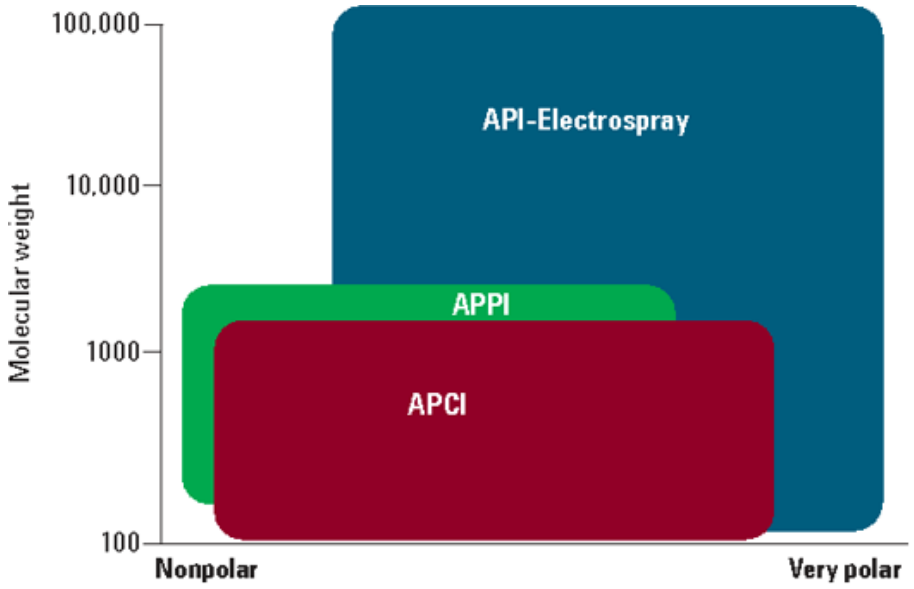


Figura 1. Aplicabilidad de diferentes fuentes de ionización en LC-MS en función de su polaridad y peso molecular [8]

Para análisis de multicomponentes, la fuente de ionización más versátil es ESI dada su elevada sensibilidad y el amplio intervalo de polaridad y peso molecular en el que es aplicable. Sólo en el caso de análisis de compuestos poco polares, no ionizables por ESI, se recomienda el uso de APCI. Sin embargo, uno de los problemas más relevantes de ESI es la supresión de la ionización del analito por

coelución del mismo con otros componentes de la matriz, los cuales originan cambios en la tensión superficial de la gota de aerosol, modificaciones del pH, formación de pares iónicos, etc. Una buena separación cromatográfica eliminaría el efecto de los componentes que coeluyen, pero en el caso de análisis de multicomponentes las coeluciones serán difíciles de evitar. Es por ello que la mayoría de los procedimientos de preparación de muestra actuales se desarrollan con el objetivo de proporcionar extractos compatibles con LC-ESI-MS [9].

Los métodos para la determinación de multicomponentes, además de proporcionar resultados fiables para que cualquier decisión basada en ellos pueda ser tomada con confianza, deberían permitir el análisis rápido de un elevado número de muestras y ser económica y ambientalmente sostenibles. En este sentido, uno de los mayores retos de los métodos disponibles para la determinación de multicomponentes es cómo reducir las interferencias provocadas por la matriz de la muestra a la vez que se evita la pérdida de compuestos que tienen muy diferentes propiedades físicoquímicas. Así, idealmente, los procesos implicados en el tratamiento de la muestra deberían reducirse al mínimo para evitar la pérdida de analitos. Sin embargo, esto se traducirá en la mayoría de los casos en importantes efectos matriz con lo que se comprometerá la sensibilidad de los métodos analíticos resultantes [10]. Actualmente, las tres principales estrategias que se utilizan en la preparación de muestra para la determinación de multicomponentes son la extracción en fase sólida (SPE), la extracción con disolvente (que puede ser líquido-líquido, LLE o sólido-líquido, SLE) y la dilución de la matriz (dilute-and-shoot, D&S) [11].

Los métodos D&S presentan una serie de ventajas para el análisis de multicomponentes. Así, el procedimiento para la preparación de la muestra es simple, rápido y económico. Sin embargo, debido a que es necesario diluir la muestra hasta que los efectos de la matriz son aceptables, la sensibilidad obtenida mediante esta estrategia es limitada y por tanto sólo es aplicable a muestras en las que los compuestos de interés están a concentraciones elevadas. Otro

inconveniente que presenta el método D&S es que los componentes de la matriz para un mismo tipo de muestra (ej. orina, suero o agua superficial, etc.) presentan una elevada variabilidad y por tanto los efectos matriz no son constantes y difíciles de predecir [12]. Dichas interferencias, además de supresión de la ionización, provocan desplazamientos en los tiempos de retención y modificaciones en los picos cromatográficos [13].

La extracción en fase sólida (SPE) es actualmente la técnica preferida para el tratamiento de muestras en análisis de multicomponentes debido a la amplia variedad de adsorbentes disponibles, lo que permite establecer distintos mecanismos de interacción con compuestos con muy diferentes características físico-químicas. Los grupos de compuestos que pueden analizarse simultáneamente dependen del tipo de adsorbente seleccionado [14]. Así, si se requiere la extracción simultánea de compuestos de baja y alta polaridad, se suele usar un cartucho multicapa que combina varios tipos de fases adsorbentes o bien, se analizan diferentes alícuotas de la muestra en paralelo con diferentes fases adsorbentes [15]. La principal desventaja de la SPE es que solo es aplicable a la extracción de muestras líquidas, o a la purificación de muestras sólidas que previamente hayan sido extraídas con un disolvente. Por otro lado, los tratamientos de muestra basados en SPE son complejos, lentos, caros y poco sostenibles. Así, todas las etapas requeridas (acondicionamiento, limpieza, elución), requieren el uso disolventes orgánicos (ej. un volumen total de 20-30 ml por muestra) y, por lo general, los extractos tienen que ser concentrados mediante evaporación [16]. Habitualmente, los efectos originados por los componentes de la matriz no se eliminan en su totalidad y producen supresión de la ionización, particularmente importante cuando se usa LC-ESI-MS. Esto hace que la mayoría de las veces sea necesario el uso de la calibración con ajuste matricial o técnicas adicionales de limpieza de muestra para la eliminación de interferencias [17].

El uso de disolventes orgánicos para el tratamiento de muestras líquidas (LLE) y sólidas (SLE) tiene como ventaja su simplicidad. Sin embargo, la SLE basada en el uso de disolventes orgánicos convencionales no es adecuada para la extracción de analitos con un amplio intervalo de polaridad, dado que el mecanismo de solubilización dependerá de la polaridad del disolvente utilizado. Usando mezclas de disolventes puede ampliarse el intervalo de polaridad, pero la composición de las mezclas estará determinada por la miscibilidad entre los diferentes disolventes. Además, los volúmenes de disolvente orgánico que se requieren para alcanzar rendimientos de extracción adecuados son elevados y normalmente son necesarias extracciones repetitivas [18]. Desafortunadamente, la técnica de LLE utilizando disolventes orgánicos presenta importantes limitaciones para la extracción de multicomponentes con muy diferentes propiedades físico-químicas. Ello se debe a que la mayoría de las muestras de interés contienen un elevado porcentaje de agua y por tanto sólo podrán utilizarse disolventes apolares, lo que restringe la aplicación a compuestos no polares y medianamente polares.

Se están dedicando muchos esfuerzos en los últimos años a la búsqueda de métodos de preparación de muestra para el análisis de multicomponentes que reduzcan o eliminen algunas de las limitaciones de los métodos actuales y cuyo uso sea compatible con el análisis cromatográfico de muestras líquidas y sólidas [19]. Desde la introducción del concepto de la Química Verde en 1999 por Anastas [20], en los últimos años, se ha hecho un gran esfuerzo en incorporar sus principios en el proceso analítico, dando lugar a la creación de la Química Analítica Verde [21]. Las estrategias aplicadas, basadas en estos principios, son la reducción (o incluso la eliminación) de los disolventes orgánicos y otros reactivos tóxicos en el proceso analítico, la miniaturización y automatización del método analítico, la disminución en el consumo de energía, la reducción de desechos y la reutilización de disolventes y materiales [22]. Por ello, en los últimos años, se están centrando los esfuerzos en desarrollar metodologías de tratamientos de muestra analíticas más sostenibles, debido a que las metodologías de tratamiento clásicas requieren el uso de grandes

volúmenes de disolventes orgánicos volátiles y tóxicos. Por tanto, la sustitución de disolventes orgánicos convencionales por alternativas menos tóxicas es de gran importancia cuando se diseñan métodos analíticos sostenibles [23]. Los avances más importantes en este contexto han sido la miniaturización en SPE y LLE y el uso de energías auxiliares en SLE.

La miniaturización de SPE (μSPE) ha permitido la reducción o eliminación del disolvente orgánico y la reducción del adsorbente al orden de miligramos. Las técnicas μSPE se clasifican en técnicas confinadas y no confinadas. En las técnicas confinadas, el adsorbente está retenido en cartuchos especiales o puntas de micropipetas [24]. En las técnicas no confinadas el adsorbente no está retenido en ningún soporte y los analitos interaccionan con la fase extractante mediante procesos de dispersión [25]. Entre los formatos de μSPE, la microextracción en fase sólida (SPME) es la más popular. Fue la primera técnica que propuso una reducción efectiva del adsorbente sólido [26]. Consiste en una fibra de sílice fundida que está recubierta de una fase extractante (polimérica principalmente) seguida de una desorción térmica o elución con disolvente de los analitos. El fácil manejo de la SPME y su disponibilidad comercial ha permitido su implementación a nivel mundial. Además, en los últimos años se han desarrollado diferentes diseños y configuraciones mejorados de la SPME. Entre esas mejoras, la microextracción en capa fina supone una buena alternativa ya que el área superficial de los adsorbentes se ha incrementado [27]. Además, en los últimos años se han desarrollado fases extractantes preparadas con nanomateriales o materiales de alta eficacia que han permitido el incremento de la cantidad de analito adsorbido [28].

Otra técnica relacionada con la SPME es la extracción por adsorción en barras agitadoras (SBSE) que se basa en el uso de un imán situado en el interior de una barra de vidrio que a su vez está recubierta de una fibra de polidimetilsiloxano. Las extracciones se llevan a cabo por inmersión de la barra en una muestra líquida o situando la misma en el espacio cabeza por encima de la muestra líquida o sólida,

cuando los analitos son volátiles o semivolátiles. Esta técnica presenta menores límites de detección que la SPME, debido a que tiene mayor cantidad de fase extractante. Por otro lado, la agitación de la muestra favorece la transferencia de masa durante la extracción, lo que hace que el equilibrio se alcance más rápidamente [29]. Desde que se aprobó el uso de la SBSE, se han desarrollado diferentes configuraciones en las que se han propuesto como fases extractantes membranas [30], sorbentes monolíticos [31] y discos [32].

La microextracción en fase líquida (MFL) abarca una gran variedad de técnicas LLE miniaturizadas en las que el volumen de fase extractante se reduce a pocos mililitros (normalmente ≤100 μL). Su uso supone además ventajas adicionales, como una mayor sensibilidad debido al incremento de los factores de concentración [33]. En función de si la fase extractante está en contacto directo con la muestra líquida o no, las versiones miniaturizadas de la MFL se pueden clasificar en confinadas y no confinadas. En las confinadas la fase extractante y la muestra están separadas por una membrana, un polímero o una combinación de polímero y disolvente; y en las no confinadas hay un contacto directo entre ambas fases. Las técnicas confinadas más utilizadas en la actualidad son la MFL de fibra hueca (MFL-FH) [34] y la MFL de electromembrana (MFL-EM) [35].

En la figura 2 se muestra el fundamento de la MFL-FH. Esta técnica se puede llevar a cabo de dos formas: en dos y tres fases. En la configuración de dos fases se inmoviliza un disolvente inmiscible para el disolvente que contiene la muestra (las fases aceptora y donadora, respectivamente) en una fibra hueca porosa. En la configuración de tres fases, lo que se inmoviliza en los poros de la fibra es la fase donadora mientras que la fase aceptora se introduce en el interior de la fibra hueca [36]. Este último sistema, por lo tanto, se localiza en un recipiente que contiene la muestra. La transferencia de los compuestos de interés a la fase aceptora se incrementa con el uso de un agitador magnético [37].

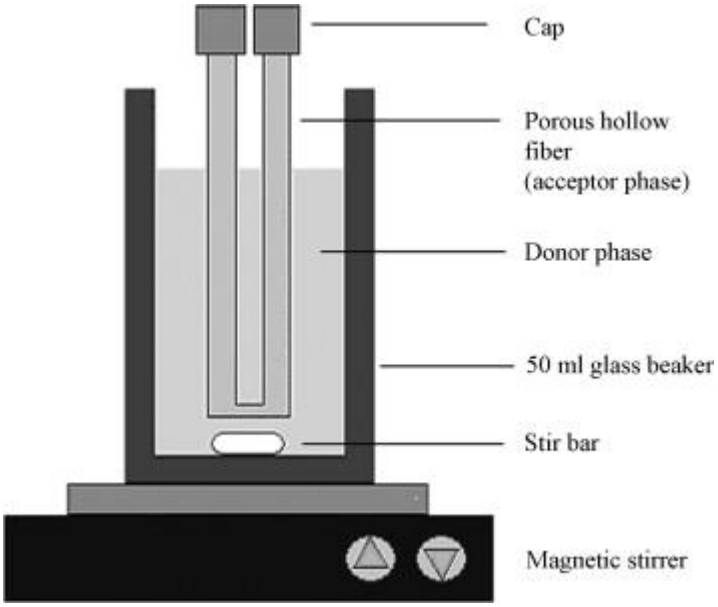


Figura 2. Esquema del fundamento de la MFL-FH [38].

Por el contrario, la MFL-EM es una técnica de microextracción cuyo objetivo es la extracción de cationes y aniones aplicando un campo eléctrico alrededor de una membrana líquida soportada [39]. En la Figura 3 se muestra una ilustración de esta técnica. Tanto la MFL-FH como la MFL-EM proporcionan elevada selectividad así como el enriquecimiento de los analitos. Sin embargo, su uso para el análisis de multicomponentes tiene los mismos problemas que los especificados para la LLE convencional.

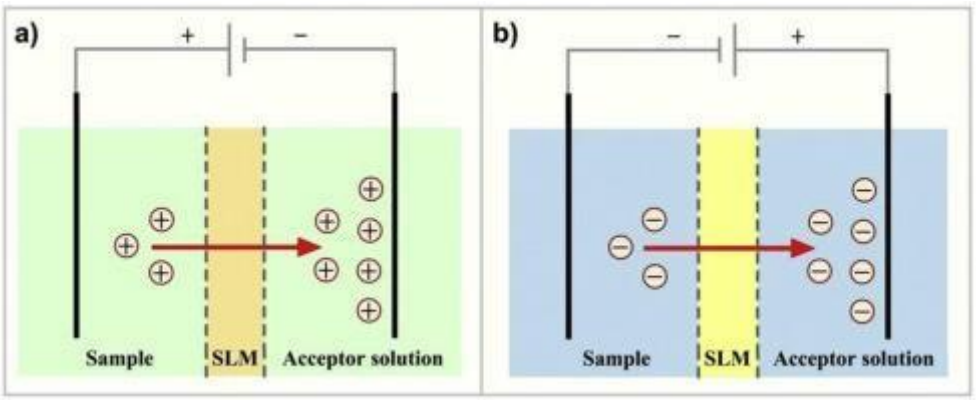


Figura 3. Esquema del fundamento de la MFL-EM [39].

La técnica no confinada más conocida es la microextracción con una gota (SDME). Jeannot y Cantwell [40], y He y Lee [41] propusieron por primera vez el uso de esta técnica con una microjeringa como dispositivo. En la SDME, una gota de disolvente (entre 1 y 20 μL) se coloca en la punta de una jeringa y se sumerge directamente en la muestra acuosa. A continuación, la gota se recoge con la jeringa y se analiza mediante cromatografía de líquidos o de gases. Las técnicas basadas en SDME han evolucionado y actualmente se dispone de diferentes formatos. La jeringa se puede sumergir en un flujo continuo de muestra, puede extraer compuestos volátiles con espacio de cabeza, etc. La figura 4 muestra las distintas configuraciones disponibles. El inconveniente de la SDME es que la superficie-volumen de una sola gota no favorece una extracción rápida. Por ello, la tendencia actual es dispersar el disolvente en la matriz de la muestra para maximizar el contacto entre el analito y la fase extractante [42].

La disminución del consumo de disolventes orgánicos en la extracción sólido-líquido (SLE) se ha abordado mediante el aumento de la eficiencia de extracción con el uso de energías auxiliares que producen el incremento de la presión y/o temperatura (ej. extracción asistida por microondas, extracción acelerada por ultrasonidos y extracción con líquidos presurizados) [43-45].

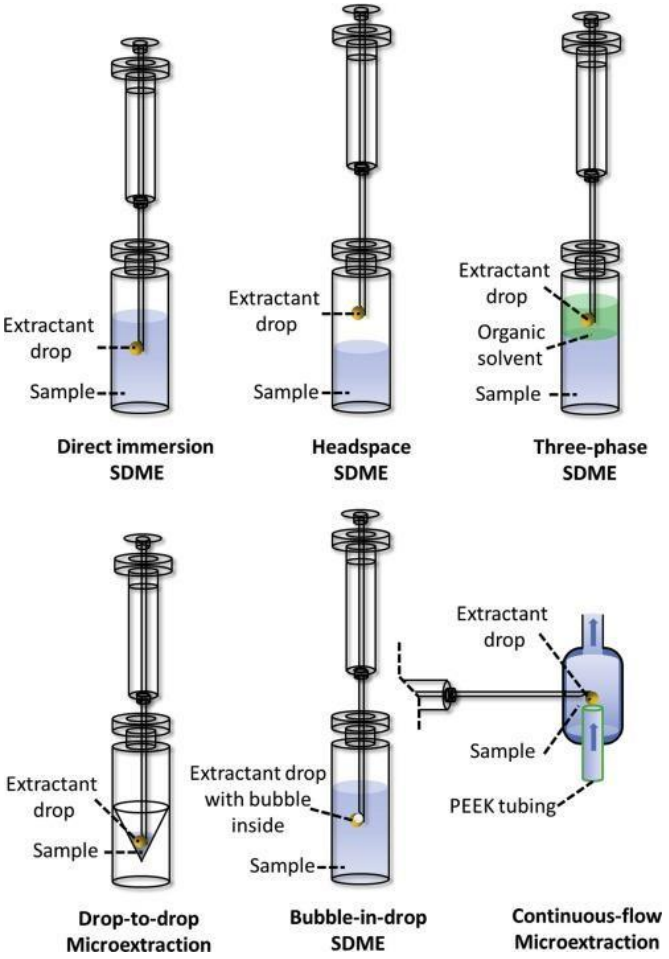


Figura 4. Formatos de SDME disponibles en la actualidad [46]

2. Disolventes verdes en técnicas de microextracción analíticas

La investigación sobre disolventes verdes alternativos a los disolventes orgánicos generalmente usados en las técnicas de microextracción es una tendencia actual, no sólo para hacer estas técnicas más ecológicas, sino también para implementar procesos más eficientes e innovadores. Para que un disolvente sea considerado verde, debe cumplir los siguientes criterios [47]:

- Tener disponibilidad a nivel mundial y capacidad de producción que no muestre grandes fluctuaciones interanuales.
- Tener un coste competitivo y relativamente constante.
- Ser reciclable en todos los procesos químicos usando procesos eco-eficientes.
- Presentar pureza disponible en varios grados.
- Sintetizarse mediante procesos sostenibles.
- Tener baja o nula toxicidad.
- Ser biodegradable sin producción de metabolitos tóxicos.
- Presentar características fisicoquímicas (viscosidad, polaridad, densidad, etc.) similares o superiores a los disolventes convencionales.
- Ser estable térmica y (electro) químicamente.
- No ser inflamable.
- Poder transportarse y almacenarse de forma segura.
- Ser renovable en relación con las fuentes utilizadas para su producción.

Los siguientes apartados pretenden ofrecer una breve descripción de los tipos de disolventes verdes más investigados para su uso en técnicas de microextracción analíticas: los líquidos iónicos [48] y los disolventes eutécticos [49]. Dado que en esta tesis doctoral se ha investigado el desarrollo y aplicación de disolventes supramoleculares, éstos se expondrán de forma más detallada en el apartado 3.

2.1 Líquidos iónicos

Los líquidos iónicos (ILs) son sales cuyos puntos de fusión están por debajo de los 100ºC. Los ILs están formados por iones asimétricos y voluminosos, por lo que presenta fuerzas atractivas más débiles que las sales iónicas convencionales. En los ILs tienen gran importancia los enlaces de hidrógeno y las fuerzas de van der Waals. La mayoría de los ILs tiene una estructura compuesta por un catión orgánico y un anión inorgánico poliatómico. La mayor parte de los cationes tienen naturaleza aromática y son iones de amonio (ej. cationes de tipo alquilimidazolio, alquilamonio, N-alquilpiridinio o N,N-dialquil pirrolidinio), siendo los cationes de tipo imidazolio los más estudiados [48]. En la Figura 5 se muestran los principales cationes y aniones usados para la síntesis de ILs.

Los ILs presentan diferentes propiedades que son relevantes para su uso en técnicas de microextracción. Así, presentan una presión de vapor a temperatura ambiente muy baja y, por lo tanto, no son volátiles. Por otro lado, tiene una elevada estabilidad térmica y una viscosidad y solubilidad en agua variables y modulables, dependiendo de la combinación catión/anión de su estructura [50]. Estas propiedades físicoquímicas hace que los ILs sean disolventes apropiados en una gran variedad de aplicaciones (bio)analíticas [51]. Además, la incorporación de grupos funcionales de diferente polaridad en la estructura del IL, promueve diferentes interacciones con los solutos, permitiendo elevada capacidad de solvatación para compuestos polares y no polares. A pesar de todas estas características, su clasificación como disolventes verdes sigue siendo controvertida [52]. Se ha demostrado que algunos líquidos iónicos son tóxicos y no biodegradables. Además, su síntesis es, por lo general, compleja y costosa. A pesar de los esfuerzos realizados para la producción de ILs degradables y menos tóxicos, el coste de producción sigue siendo elevado y, por tanto, su aplicación a gran escala sigue siendo limitada.

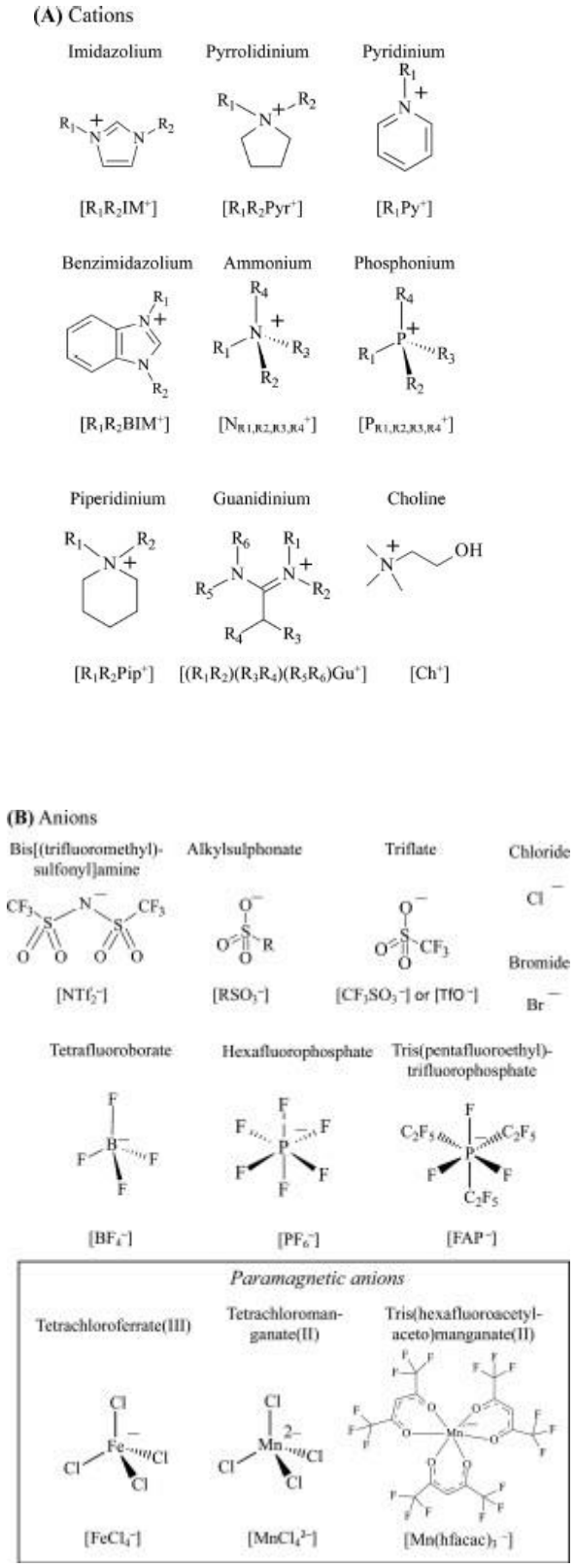


Figura 5. Principales cationes y aniones usados para la síntesis de ILs aplicados en técnicas de microextracción [53]

Las propiedades modulables de los ILs hacen que estos disolventes sean especialmente atractivos en la microextracción líquido-líquido dispersiva (DLLME). La combinación de la DLLME con ILs (IL-DLLME) y sus respectivas modalidades se basan en la adición de un líquido iónico que es miscible o no con la muestra acuosa y de un agente dispersante (ej. un disolvente, temperatura, microondas, ultrasonidos, etc.). En la Figura 6 se muestra el número de publicaciones relacionadas con el uso de la IL-DLLME y sus distintas modalidades durante el período 2008-2017. Como se puede observar, la mayoría de las publicaciones se centran en la IL-DLLME clásica, en la que el agente dispersante es un disolvente orgánico. Sin embargo, existe también un número considerable de publicaciones relacionadas con el uso de diferentes estrategias para dispersar el líquido iónico en la muestra.

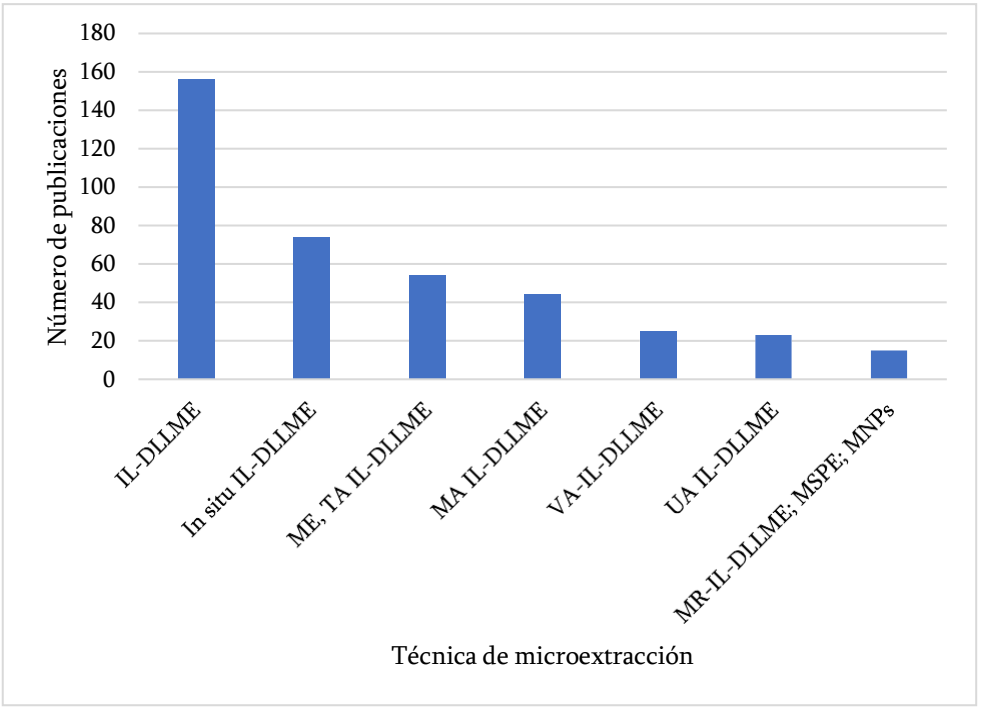


Figura 6. Número de trabajos publicados entre 2008 y 2017 relacionados conel uso de ILs en DLLME y sus variantes [54]

Hasta la fecha, la mayoría de las técnicas IL-DLLME se han aplicado a la extracción y preconcentración de compuestos orgánicos no polares y medianamente polares a partir de muestras acuosas ambientales. Para analitos polares como las aminas biogénicas, es necesario un paso previo de derivatización de las mismas [55]. IL-DLLME también se ha aplicado al tratamiento de muestras biológicas líquidas como orina [56], y a alimentos sólidos como plátanos [57], uvas de mesa y ciruelas [58] u hojas de plantas [59], previa disolución de la muestra mediante digestión. Las principales ventajas del uso de ILs en DLLME son los elevados rendimientos de extracción y factores de enriquecimiento obtenidos, así como la sencillez de operación. Sin embargo, su principal inconveniente es la elevada viscosidad de los ILs, lo que requiere la dilución del extracto, previo a la medida, con un disolvente con el que sea miscible, disminuyendo de esta forma la sensibilidad del método.

En los últimos años, se ha propuesto el uso de ILs magnéticos (MILs) en técnicas de microextracción. Estos disolventes se forman incorporando un componente paramagnético en el catión o en el anión que forman el IL (ver como ejemplo la estructura de algunos aniones paramagnéticos en la figura 5 B) [60]. Los MILs responden a la presencia de un campo magnético externo y han encontrado diversas aplicaciones en DLLME, donde se recuperan tras la extracción con un imán. Inicialmente, los componentes paramagnéticos de los MILs eran hidrófilos y, por tanto, su uso se limitó a la extracción de compuestos procedentes de muestras hidrófobas (ej. aceites). Posteriormente, se ha extendido su aplicación a muestras acuosas, previa modificación de la estructura del ion paramagnético con grupos hidrófobos [60]. Un problema adicional de los MILs es que se requiere la disolución de los analitos antes de su inyección en el equipo. Las aplicaciones más interesantes de los MILs-DLLME son la determinación de biomoléculas en muestras biológicas (ej. aislamiento de ADN [61], extracción de células de E. coli en disoluciones acuosas [62], o de estriol y estrona en muestras de orina [63]).

2.2 Disolventes eutécticos

Los disolventes eutécticos (DES) están formados por un aceptor (HBA) y un donador (HBD) de enlaces de hidrógeno a diferentes ratios [64]. La mezcla resultante tiene un punto de fusión más bajo que la de los componentes iniciales. La disminución en el punto de fusión está relacionada con la magnitud de las interacciones específicas que ocurren entre HBA y HBD; cuanto mayor es la energía de interacción, menor es el punto de fusión del DES. Las sales de amonio o de fósforo cuaternarias suelen usarse como HBA, mientras que los alcoholes, ácidos carboxílicos o aminas se usan como HBD. En la Figura 7 se muestran los principales tipos de HBA y HBD usados para la síntesis de DES aplicados en técnicas de microextracción.

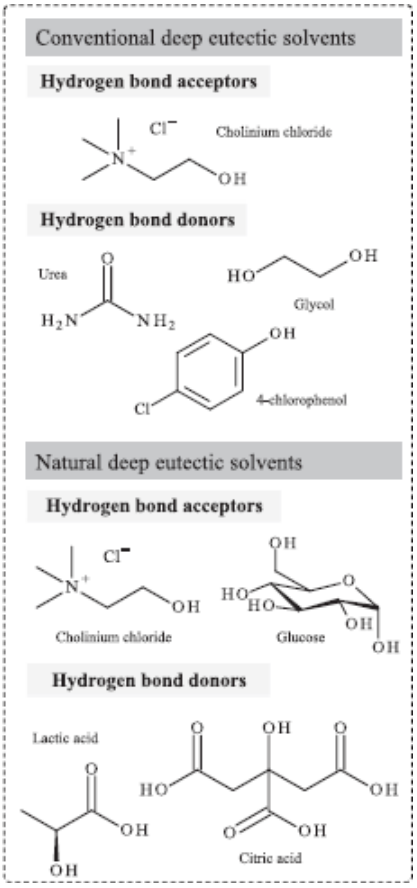


Figura 7. Principales HBA y HBD usados para la síntesis de DES aplicados en técnicas de microextracción [53].

De acuerdo a su solubilidad en agua, los DES se clasifican en hidrófilos e hidrófobos. El primer DES hidrofóbico se sintetizó en 2015 [65] y por lo tanto la aplicaciones de DES en técnicas de microextracción en fase líquida, aunque se han incrementado en los últimos años, son aún reducidas [66]. Las características de los DES son muy semejantes a las de los ILs en términos de viscosidad, polaridad y densidad, las cuales pueden modularse. Sin embargo, respecto a los ILs convencionales, los DES se obtienen a partir de procesos sintéticos más económicos y presentan menor toxicidad. Recientemente, se ha usado una gran variedad de productos naturales, como azúcares o ácidos orgánicos, para la preparación de los DES (NADESs) (ver figura 7) [67]. Los NADESs presentan perfiles toxicológicos más seguros y mayor biodegradabilidad que los ILs.

El uso de los DES en técnicas de microextracción analítica ha crecido de forma exponencial en los últimos años. En la Figura 8 se muestra el número de artículos publicados en el período 2015-2020. Al igual que ocurre con los ILs, los DES se han utilizado preferentemente en DLLME, usando diferentes estrategias para su dispersión en la muestras. Entre estas estrategias destacan, la extracción asistida por vortex [68], o el uso de ultrasonidos [69] o una corriente de aire [70]. En 2019, Altunay et al. [71] usaron por primera vez un NADES para su aplicación en DLLME. Aunque los DES ofrecen una serie de ventajas para su aplicación en DLLME, están lejos de considerase disolventes ideales. Su principal limitación es la elevada viscosidad que presentan y, como ocurre con los ILs, es difícil el análisis directo de los extractos sin previa dilución de los mismos. Esto disminuye, por lo tanto, la sensibilidad de los métodos.

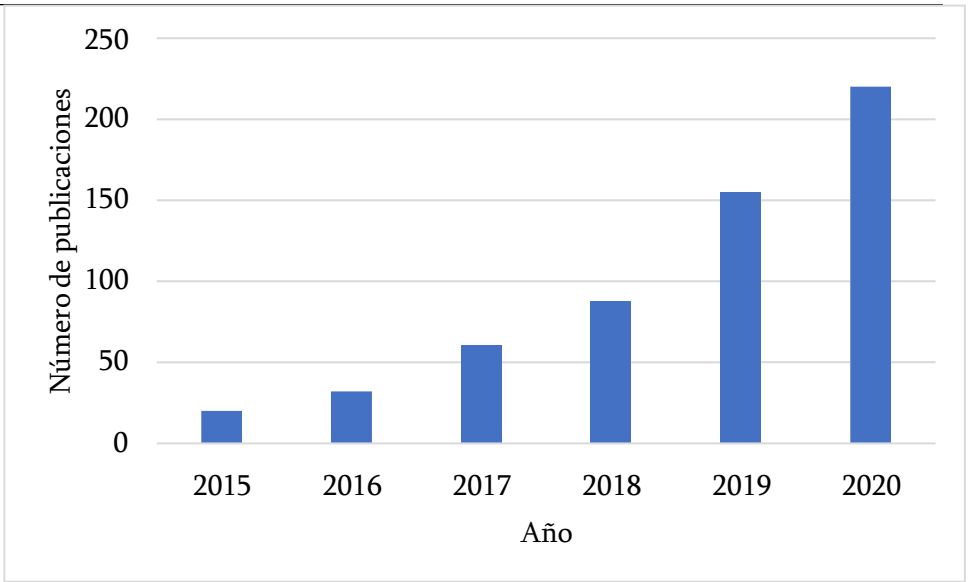


Figura 8. Número de trabajos publicados entre 2015 y 2020 relacionados conel uso de DESs en técnicas de microextracción analítica [72].

3. Disolventes supramoleculares

Los disolventes supramoleculares (SUPRAS) constituyen una alternativa interesante a los disolventes orgánicos en procesos de extracción analítica dado que sus características permiten el desarrollo de tratamiento de muestras innovadores no abordables por los disolventes convencionales. Dado que los SUPRAS constituyen el eje principal de las investigaciones desarrolladas en esta tesis doctoral, en este apartado se realizará una descripción detallada de los mismos, centrándonos en aquellas características que son de interés para su aplicación en técnicas de microextracción analíticas.

3.1 Síntesis de los SUPRAS

Los disolventes supramoleculares (SUPRAS) son líquidos nanoestructurados que se producen a partir de moléculas anfifílicas mediante procesos espontáneos deautoensamblaje y coacervación [73].

El procedimiento general para la síntesis de los SUPRAS consta de dos etapas que se muestran en la Figura 9. Inicialmente, se prepara un sistema coloidal acuoso u orgánico disolviendo una molécula anfifílica a una concentración superior a su concentración de agregación crítica (cac). Las moléculas anfifílicas se asocian de forma espontánea produciendo agregados supramoleculares que normalmente consisten en micelas acuosas, micelas inversas o vesículas.

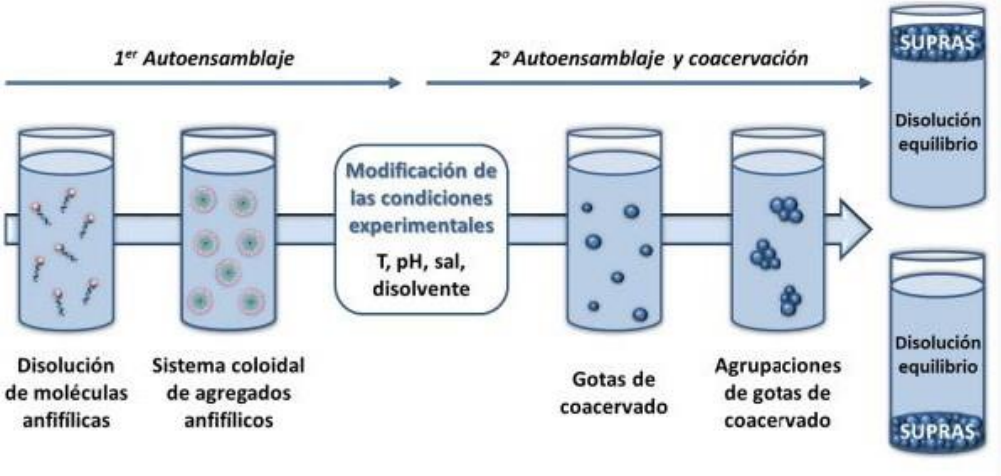


Figura 9. Esquema del procedimiento general de síntesis de los disolventes supramoleculares.

A continuación, se modifican las condiciones experimentales en el sistema coloidal con la finalidad de incrementar el tamaño de los agregados supramoleculares, lo que es esencial para que se produzca la coacervación. Existen varios mecanismos para producir la coacervación, incluyendo cambios en el pH o la temperatura del sistema coloidal, o la adición de diferentes agentes coacervantes (sales orgánicas oinorgánicas, hidrótopos, un disolvente en el que el anfifilo es poco soluble, etc.). Elcrecimiento de los agregados supramoleculares en el sistema coloidal induce la formación de gotitas de coacervado que se asocian para formar conglomerados cuyadensidad es diferente a la del sistema coloidal. De esta forma, los conglomerados sedimentan o floculan como una nueva fase líquida (SUPRAS) donde las gotitas de coacervado permanecen como entidades individuales. El SUPRAS está en equilibrio con la disolución de la que se separó y contiene el anfifilo a la cac.

Una vez definido el agente de coacervación, las condiciones experimentales requeridas para la formación de SUPRAS se pueden delimitar fácilmente estudiando el diagrama de fases del anfifilo respecto al agente que induce la coacervación [74]. Estos diagramas de fases constan de dos o más regiones cuya extensión depende de la estructura del compuesto anfifílico, el valor del agente coacervante y los componentes de la matriz de la muestra [74]. La Figura 10a muestra como ejemplo el diagrama de fases obtenido para el sistema hexanol-THF que coacerva en la presencia de agua. El SUPRAS también puede formarse en la presencia de orina (Fig. 10b), dado que el contenido en agua de esta matriz biológica es de aproximadamente el 95%. Este comportamiento permite que el SUPRAS pueda sintetizarse directamente en la orina para determinar componentes de la misma. En este caso, los componentes de la matriz de la orina amplían la región de coacervación del hexanol.

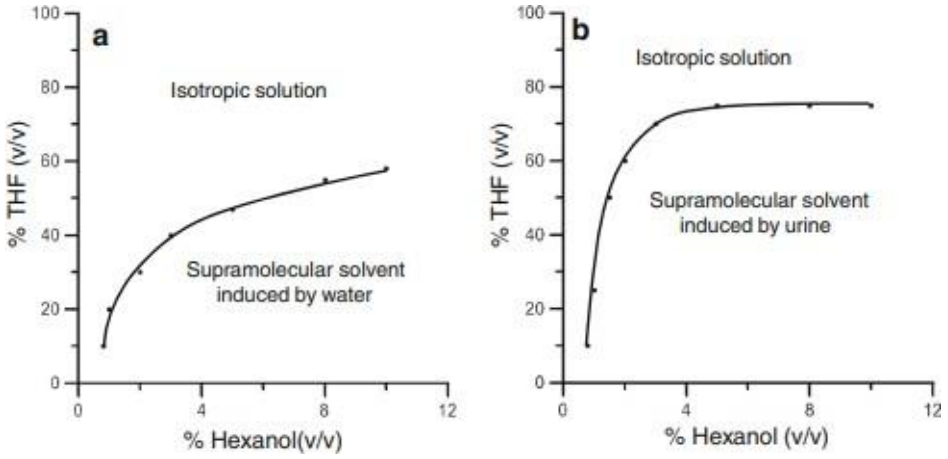


Figura 10. Diagramas de fases de SUPRAS formados en mezclas ternarias de (a) hexanol-THF-agua y (b) hexanol-THF-orina [74]

3.2 Mecanismos de formación de los SUPRAS

Como se ha comentado previamente, los SUPRAS están constituidos por agregados supramoleculares formados mediante fenómenos de autoensamblaje y coacervación. La formación de los agregados en ambas etapas (Figura 9) ocurre a través del balance de las interacciones de atracción y repulsión entre las moléculas de anfifilo-disolvente y anfifilo-anfifilo. El SUPRAS se formará siempre que se favorezca las interacciones anfifilo-anfifilo (Figura 11). Por lo tanto, la solvofobicidad (no afinidad por el disolvente) de una parte de la estructura del anfifilo es la responsable de la agregación. En disoluciones acuosas, el efecto hidrófobo de las cadenas hidrocarbonadas del anfifilo conduce a la agregación mientras la repulsión entre los grupos cabeza es la responsable de que la agregación se pare cuando el agregado alcanza un tamaño determinado. Así, para incrementar el tamaño de los agregados, requerido para la obtención de coacervados, es esencial reducir la repulsión de las cabeza polares de los anfifilos [75]. El proceso de agregación es reversible ya que todas las interacciones producidas son no covalentes (Figura 11); fundamentalmente interacciones iónicas, de dispersión y puentes de hidrógeno, pero también otras menos exploradas como puentes de halógeno [76] e hidrofobicidad polar [77].

El mecanismo de formación de los SUPRAS depende fundamentalmente del carácter del grupo polar; neutro o iónico [73]. En la Tabla 1 se muestra la estructura de las principales moléculas anfifílicas usadas para la formación de los SUPRAS aplicados en microextracciones analíticas. En el caso de anfifilos no iónicos, el principal mecanismo responsable de la coacervación es la desolvatación de los grupos polares, lo que causa la reducción del área que ocupa la cabeza polar permitiendo mayor empaquetamiento (Figura 12). La desolvatación puede ocurrir mediante el incremento de la temperatura. Esta estrategia fue la primera que se utilizó en extracciones analíticas por Watanabe y colaboradores [78] y al fenómeno se le denominó cloud point o punto de nube.

La desolvatación también puede ocurrir mediante la adición de sales, hidrótopos, o un disolvente en el que el anfifilo es poco soluble [79].

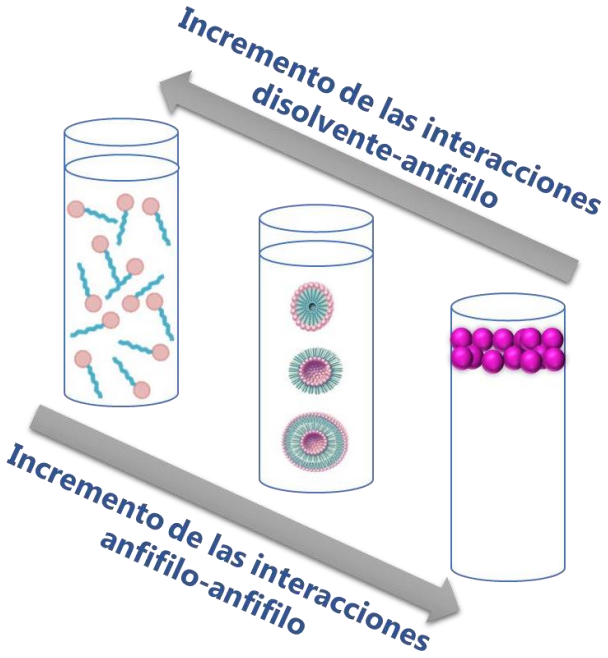
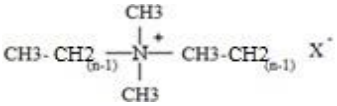
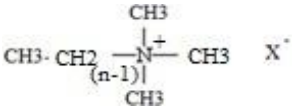
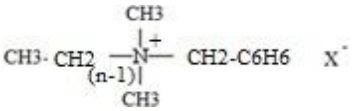
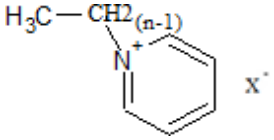
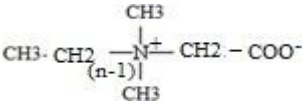
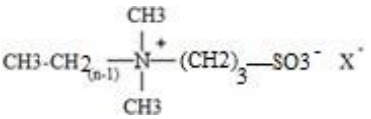


Figura 11. Representación esquemática de la reversibilidad de los agregados en función del tipo de interacción predominante.

Figura 12. Principal mecanismo de coacervación de anfifilos no iónicos.

Tabla 1. Principales anfifilos utilizados en microextracciones analíticas

Grupo polar	Grupo estructural	Fórmula molecular	n	x
No iónicos	Alquil etoxilatos	CH_3-$(CH_2)_{n-1}$-$O(CH_2CH_2O)_xH$	12-16	1-23
	Alquilfenol etoxilados	CH_3-$(CH_2)_{n-1}$-C_6H_6-$O(CH_2CH_2O)_xH$	8,9	1-23
	Alcanoles	CH_3-$(CH_2)_n$-OH	6-14	
Aniónicos	Alquilbenceno sulfonatos	CH3-(CH2)n-m-3-CH-(CH2)m-CH3	10-14	
	Alquil sulfonatos	CH3-(CH2)n-1-SO3- Na+	10-18	
	Alquiletoxi sulfatos	CH3-(CH2)n-1(OCH2CH2)x-OSO3- Na+	12-18	
	Alquil sulfatos	CH3-(CH2)n-1-OSO3- Na+	10-18	
Catiónicos	Sales de dialquil dimetilamonio		12-18	
	Sales de alquil trimetilamonio		12-16	
	Sales de alquildimetil bencilamonio		12-16	
	Sales de alquilpiridinio		12-16	
Anfóteros	Alquilbetaínas		10-16	
	Alquil sulfobetaínas		10-16	

El principal mecanismo para la coacervación de anfifilos iónicos es la neutralización del grupo polar mediante la adición de sales orgánicas o inorgánicas e hidrótopos [80]. En el caso de anfifilos ionizables, la neutralización de la carga puede llevarse a cabo mediante ajuste del pH del sistema coloidal [81]. En todos los casos, la neutralización reduce la repulsión efectiva entre las cabezas polares y permite mayor empaquetamiento de anfifilos en el agregado (Fig. 13).

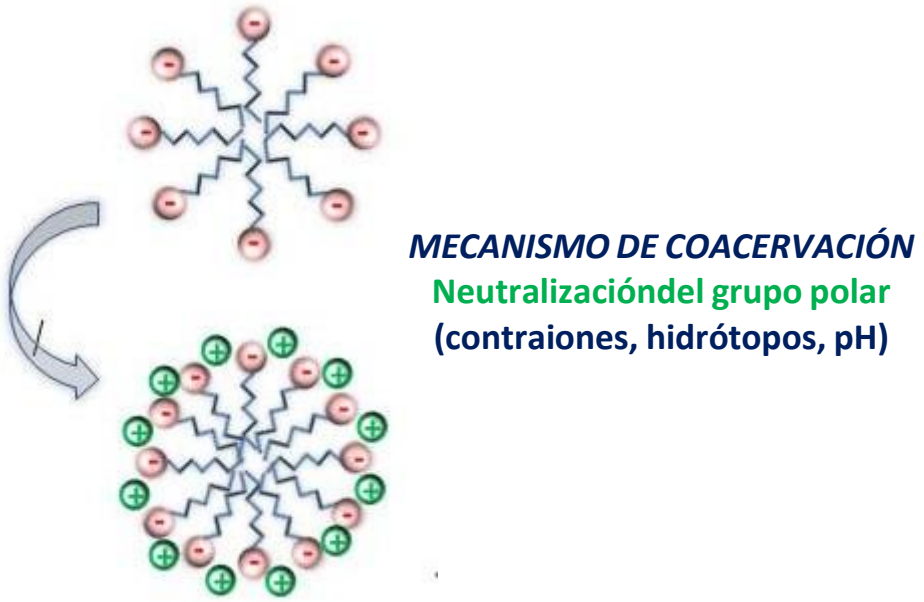


Figura 13. Principal mecanismo de coacervación de anfifilos iónicos

El mecanismo de coacervación de anfifilos zwitteriónicos, cuya estructura tiene carga positiva y negativa y por lo tanto es globalmente neutra, no se ha elucidado. Los SUPRAS basados en anfifilos zwitteriónicos descritos hasta la fecha se forman disminuyendo la temperatura del sistema coloidal por debajo de un valor crítico [82]. Este comportamiento es inesperado si se considera la elevada polaridad de los grupos zwitteriónicos, y se ha explicado en base a que, aunque las interacciones electrostáticas deben están implicadas en las interacciones intra- e inter-agregados, éstas son de corto rango y sus efectos son cualitativamente diferentes a las de los anfifilos iónicos.

3.3 Composición química y estructura de los SUPRAS

Los SUPRAS están generalmente formados por el anfifilo y agua, y pueden incluir otros ingredientes utilizados como agentes coacervantes (sales inorgánicas y orgánicas, disolventes orgánicos (si el anfifilo es insoluble en agua), etc [83]. La composición química de los SUPRAS generalmente se mantiene constante si no se modifican las condiciones experimentales en las que se lleva a cabo la coacervación. Ya que el volumen de SUPRAS muestra en la mayoría de los casos una dependencia lineal con la cantidad de anfifilo usada en la síntesis, cuanto menor sea la cantidad del mismo, mayor será el factor de concentración teórico alcanzado cuando el SUPRAS se aplica a procesos de extracción [79].

Por otro lado, la dependencia de la composición química del SUPRAS con la concentración/magnitud del agente coacervante es muy variable y raramente lineal. Así, para SUPRAS inducidos por la temperatura, éstos contienen menor cantidad de agua y por tanto la concentración de anfifilo aumenta a medida que se incrementa el valor de la temperatura por encima del punto de nube (cloud point) [83]. Para anfifilos ionizables (e.g. alquil sulfatos y sulfonatos), cuya coacervación se lleva a cabo mediante protonación del grupo polar, la concentración de anfifilo en el SUPRAS se incrementa a medida que aumenta la acidez del medio. La concentración de anfifilos iónicos en el SUPRAS también se incrementa al aumentar la cantidad de sal utilizada como agente coacervante. Como ejemplo, en la Tabla 2 se muestra la composición química de SUPRAS formados a partir de sistemas coloidales acuosos de ramnolípidos utilizando NaCl como agente coacervante [84]. Se observa que al aumentar la concentración de NaCl utilizada en la síntesis, aumenta la concentración de ramnolípido y sal en el SUPRAS y disminuye el contenido de agua.

Por otro lado, la composición química del SUPRAS se mantiene constante variando la concentración de anfifilo utilizada para la síntesis (Tabla 2). Por lo tanto, la composición química del SUPRAS, y como resultado, el volumen generado del mismo, puede modelarse, lo que afectará a los factores de preconcentración que pueden alcanzarse. Estos factores pueden predecirse a través de ecuaciones derivadas experimentalmente, que relacionan el volumen de SUPRAS producido en función de las condiciones en las que se lleva a cabo la coacervación.

Tabla 2. Composición química de SUPRAS de ramnolípidos sintetizadoscon diferentes concentraciones de anfifilo y agente coacervante

Condiciones síntesis	Composición del SUPRAS		
[NaCl] (M)	H_2O±SD (%,w/w)[a]	Ramnolípido±SD (%, w/w)	Salt±SD (%,w/w)
1.00	77±2	19±3	4.5±0.3
1.25	73±5	24±5	5.3±0.2
1.50	68±4	31±4	6.0±0.1
1.75	64±5	33±4	6.5±0.1
2.00	60±3	37±3	7.0±0.4
2.25	55±3	40±2	7.2±0.6
Ramnolípido±SD (%, w/v) (NaCl = 2M)	H_2O±SD (%,w/w)	Ramnolípido±SD (%,w/w)	Salt±SD (%,w/w)
2.7	58±4	38±1	6.8±0.4
4.5	59.9±0.8	37±3	7.0±0.2
5.4	60±4	38±2	7.0±0.3
6.3	60.7±0.4	38±4	7.1±0.1
9.0	60.9±0.9	37±1	7.1±0.7

a porcentaje en peso

En relación a la estructura de los SUPRAS, la elucidación de cómo se organizan los anfifilos en los mismos es de gran importancia para entender los mecanismos de coacervación, que es la base para poder modelar las propiedades y funcionalidades de los SUPRAS.

La caracterización estructural de los SUPRAS implica en primer lugar verificar si la fase líquida obtenida es realmente un coacervado. Para ello, se comprueba mediante microscopía óptica si está formada por gotitas de coacervado, cuyo tamaño generalmente se encuentra en el intervalo comprendido entre 1 y 100 μm. En la figura 14 se muestra un ejemplo de microfotografía óptica obtenida para un SUPRAS formado a partir de ramnolípidos [84].

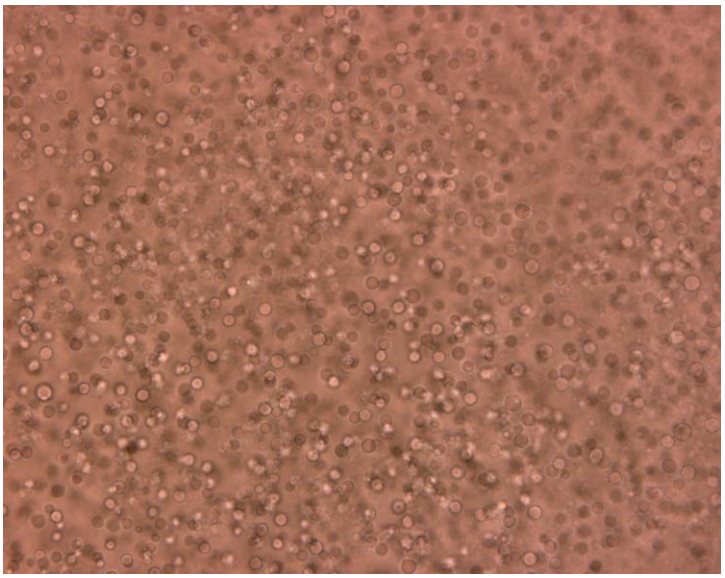


Figura 14. Microfotografía óptica obtenida para un SUPRAS producido a partir deuna mezcla de ramnolípido (4.5% w/v) 0.85 M y acetato amónico [84]

La elucidación de la nanoestructura del SUPRAS dentro de las gotas de coacervado es muy compleja y las técnicas más utilizadas hasta la fecha han sido diferentes modalidades de microscopía electrónica de barrido (SEM) y de transmisión (TEM). Sin embargo, éstas requieren de condiciones de operación (fijado, alto vacío, etc.) en las que es difícil mantener la integridad de los agregados, los cuales están unidos mediante enlaces no covalentes.

En este sentido, la técnica con mayor potencial es freeze-fracture-SEM. Esta técnica es esencial para la observación de líquidos ya que elimina la necesidad de métodos de preparación convencionales, como la fijación química y el secado en puntos críticos, y permite la observación de muestras en su estado hidratado natural. Para ello, la muestra se congela rápidamente, se fractura y se recubre generalmente de platino para aumentar el contraste y la conductividad. En la figura 15 se muestran de forma esquemática las estructuras más frecuentemente encontradas en SUPRAS, y en la Figura 16 se puede ver una microfotografía obtenida mediante FF-SEM para un SUPRAS con estructura hexagonal [85]

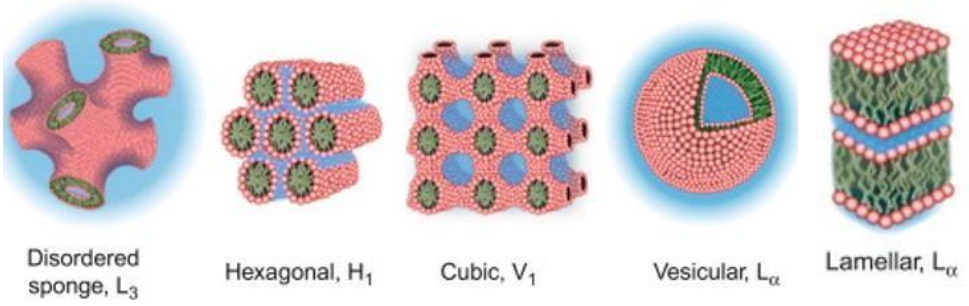


Figura 15. Nanoestructuras en las que frecuentemente los anfifilos se asocian enlos SUPRAS.

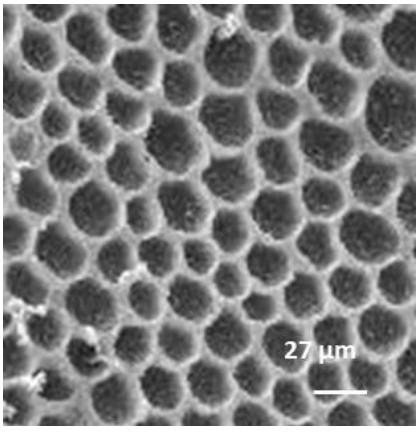


Figura 16. Microfotografías obtenidas mediante FF-SEM para SUPRAS constituidos por alcanoles, tetrahidrofurano y agua [86].

3.4 Propiedades de los SUPRAS relevantes en procesos de microextracción

Los SUPRAS tienen características intrínsecas que les diferencian de los disolventes moleculares y que fundamentalmente derivan del carácter anfifílico de las moléculas que los constituyen, las nanoestructuras que éstas forman y el carácter reversible de las mismas. Estas características son de gran interés para su aplicación en procesos de microextracción. Así, los SUPRAS ofrecen [73]:

-Microambientes de diferente polaridad, en los que los solutos que exhiben un intervalo de polaridad amplio pueden solubilizarse simultáneamente. Por lo tanto, en comparación con los disolventes orgánicos convencionales, los SUPRAS tienen la capacidad de extraer sustancias a través de mecanismos mixtos (por ejemplo, mediante enlaces de hidrógeno e interacciones dipolo-dipolo o iónicas, etc. en la región polar e interacciones de dispersión, π-π, π-catión, etc. en la región apolar)

-Múltiples centros enlazantes, debido a la gran concentración que hay de anfifilo en el SUPRAS (en torno a 0.1-1 mg/μL). Como resultado, se pueden extraer analitos utilizando bajos volúmenes de SUPRAS en relación al volumen o cantidad de muestra, lo que facilita la obtención de altos factores de concentración y, en la mayoría de los casos, se evita la evaporación de los extractos, lo que supone un ahorro de tiempo y costes. Los factores de concentración alcanzados pueden llegar hasta 800 y dependen fundamentalmente de la estructura y concentración del anfifilo y la concentración/magnitud del agente coacervante, aunque aditivos como los electrolitos pueden también afectar la composición del SUPRAS y consecuentemente el volumen de disolvente producido.

-Elevada área superficial, debido a que las gotas de coacervado permanecen como entidades individuales en el SUPRAS (Figura 9). De esta forma, las microextracciones con SUPRAS son siempre dispersivas sin la necesidad de adicionar un agente dispersante. Ya que la transferencia de masa es muy rápida, los equilibrios de extracción se alcanzan rápidamente.

-Posibilidad de modelar sus propiedades a través de la selección de la estructura del anfifilo o del tipo/concentración de agente coacervante utilizado. Esta característica posibilita el desarrollo de tratamiento de muestras innovadores, como es la integración de la etapa de extracción de los analitos y la eliminación de interferencias utilizando SUPRAS con propiedades de acceso restringido (SUPRAS-RAM) [74]. Estas propiedades han permitido el desarrollo de métodos independientes de la matriz de la muestra que posibilitan la aplicación del mismo método a matrices de características muy diferentes (ej. determinación de anfetaminas en suero, orina, leche, pelo, uña, saliva y sudor [77,87-88].

-Compatibilidad con operaciones analíticas convencionales. Los SUPRAS permiten el desarrollo de protocolos convencionales de extracción ya que operacionalmente se comportan como los disolventes orgánicos convencionales. Por lo tanto, pueden aplicarse en cualquier laboratorio, sin la necesidad de equipos o personal especializado. Así, los SUPRAS son compatibles con cromatografía de líquidos y de gases acopladas a diferentes sistemas de detección, incluyendo espectrometría de masas, y pueden utilizarse en los diferentes formatos de extracción con disolventes, incluyendo LLE y SLE.

- Facilidad de síntesis. La formación del SUPRAS se realiza a través de procesos espontáneos e instantáneos que sólo requieren el establecimiento de las condiciones de operación necesarias para que se produzca la coacervación.

3.5. Formatos de extracción

3.5.1. SUPRAS en LLE

Los SUPRAS son fases líquidas en las que las moléculas anfifílicas que las integran están unidas mediante enlaces no covalentes y en equilibrio con la disolución coloidal a partir de la que se formó, la cual contendrá el anfifilo a la cac.

Esto implica que si el SUPRAS se añade a una muestra líquida, se reestablecerá el equilibrio entre el SUPRAS y la muestra y dado que la relación de volúmenes SUPRAS/muestra es muy elevada, los agregados que constituyen el SUPRAS se disolverán en la muestra hasta alcanzar la cac.

Por tanto, la forma más conveniente para operar en LLE es formar el SUPRAS en el seno de la muestra. Para ello se añade el compuesto anfifílico a la muestra líquida (agua, orina, suero, bebidas, etc.) y se establecen en la misma las condiciones de coacervación (ej. adición de sales, cambios de pH, etc.). Generalmente, el SUPRAS se produce de forma espontánea e instantánea y se lleva a cabo la extracción mediante agitación y posterior centrifugación de la muestra. En la Figura 17 se muestra un esquema general de las etapas implicadas en LLE con SUPRAS.

Figura 17. Esquema general de las etapas implicadas en LLE con SUPRAS.

El SUPRAS puede tener mayor o menor densidad que la muestra. Cuando el SUPRAS es menos denso, éste se recoge con una microjeringa o micropipeta para su posterior análisis. Por el contrario, si el SUPRAS es más denso, la muestra puede desecharse por decantación. También se puede aumentar la viscosidad del SUPRAS bajando su temperatura y así recogerlo más fácilmente. Esto último no siempre es posible y hay que estudiar previamente las características del sistema.

El volumen de SUPRAS formado en la muestra debe conocerse y en este sentido, para muchos de los SUPRAS desarrollados en los últimos años se han derivado ecuaciones que permiten su estimación directa a partir de la cantidad de compuesto anfifílico usado y de la concentración/magnitud del agente coacervante utilizado [83]. Por ejemplo, en la siguiente ecuación se muestra la dependencia del volumen de SUPRAS que se generará en una muestra líquida en función de la concentración de ramnolípido y el agente coacervante cloruro sódico [84].

$$V_{SUPRAS} = \frac{Ramnolípido}{(0.0216\pm0.0005)\cdot NaCl - (0.0134\pm0.0007)} - \frac{(411\pm18)}{NaCl} + (179\pm11)$$

En el caso de que no se hayan derivado estas ecuaciones para un SUPRAS específico, el volumen del mismo formado en la muestra se ha calculado tradicionalmente midiendo la altura (h) que ocupa el SUPRAS en un tubo cilíndrico con un caliper digital y aplicando la ecuación del volumen de un cilindro ($\pi r^2 h$), donde r es el radio del tubo. Cuando el SUPRAS es menos denso que la muestra, la estimación del volumen se realiza enfriando el tubo, decantando la muestra y midiendo el mismo con una microjeringa o calculándolo mediante pesada.

En los últimos años, ha existido interés en desarrollar SUPRAS en los que los agregados, una vez formados, se mantengan estables, lo que es especialmente relevante para su aplicación a la extracción de grandes volúmenes de agua o al tratamiento de aguas residuales [89]. Para ello, se han utilizado agregados con elevada estabilidad cinética, los cuales se mantienen prácticamente estables durante el tiempo de aplicación en el proceso de extracción. Con este fin, se han utilizado SUPRAS formados a partir de vesículas de ácidos carboxílicos utilizando una sal de tetraalquilamonio como agente coacervante y se han aplicado a la eliminación de contaminantes en aguas ambientales [89]. El tiempo de residencia de las moléculas anfifílicas en las vesículas es generalmente de horas o días, lo cual es considerablemente mayor que el correspondiente a micelas ($\sim 10^{-8}$ s).

Los SUPRASs también son adaptables a los formatos miniaturizados de la extracción líquido-líquido. Se han aplicado con éxito a las técnicas de microextracción en gota [90], microextracción en fase líquida con fibra hueca [91] y microextracción en fase líquida con solidificación de la gota flotante [92]. También se han usado los SUPRAS con alta estabilidad cinética mencionados previamente [89], ya que el disolvente debe mantener su estabilidad al entrar en contacto con la muestra líquida.

Otro formato atractivo es la combinación de SUPRAS con nanopartículas magnéticas [93, 94]. Las nanopartículas se pueden incorporar al SUPRAS después de realizar la extracción, o bien, se puede añadir a la muestra el ferrofluido junto al disolvente y las nanopartículas magnéticas para extraer los componentes de interés. Con el uso de este formato, el extracto se puede separar mediante un potente imán y el resto de las sustancias se desechan por decantación. Finalmente, el SUPRAS se solubiliza en una pequeña cantidad de disolvente orgánico y se analiza mediante la técnica seleccionada.

También es importante la automatización del proceso de extracción. Fang et al. [95] incorporaron por primera vez la extracción con SUPRASs al análisis por inyección en flujo mediante el desarrollo de un método para la determinación de coproporfirina en orina. Desde entonces, se han desarrollado diferentes configuraciones y se han aplicado a la determinación de diferentes compuestos [96], aunque sigue siendo un campo poco estudiado. El procedimiento general empieza con la inyección y mezcla de la muestra y los diferentes componentes del SUPRAS. Se han empleado principalmente disolventes formados a partir de la aplicación de temperatura y adición de sales. Una vez que se produce la coacervación, el SUPRAS con los compuestos de interés se retiene en una columna y, seguidamente, se eluye con un disolvente adecuado y se conduce hacia el detector.

3.5.2. SUPRAS en SLE

La aplicación de SUPRAS en SLE generalmente implica el uso de cantidades de muestras entre 100 y 500 mg y volúmenes de SUPRAS comprendidos en el intervalo 200-500 mL. En la actualidad, el procedimiento más utilizado consiste en preparar el volumen de SUPRAS requerido para el tratamiento de un número de muestras determinado, separar la fase SUPRAS de la disolución de equilibrio, y almacenar ambas fases hasta su uso.

La extracción puede llevarse a cabo utilizando dos estrategias. En la primera se adiciona un volumen determinado de fase de equilibrio junto con el SUPRAS. La fase de equilibrio tiene como principal objetivo la humidificación de la muestra y la eliminación de sustancias polares que pueden interferir en la determinación de los compuestos de interés. Esta opción no sería por tanto aconsejable cuando se requiere la extracción de multicomponentes en un amplio intervalo de polaridad. En la segunda estrategia se añade directamente el SUPRAS a la muestra y, por lo tanto, parte del mismo se consumirá en la humidificación de la misma. Sin embargo, esta es la técnica que más se utiliza para la extracción de multicomponentes o de sustancias polares. En este caso, la disolución de equilibrio puede utilizarse para la síntesis de nuevos SUPRAS previa adición del compuesto anfifílico y, si es necesario, la cantidad de agente coacervante que se ha incorporado al SUPRAS. En la Figura 18 se muestra de forma esquemática las principales etapas de un proceso de SLE usando SUPRAS.

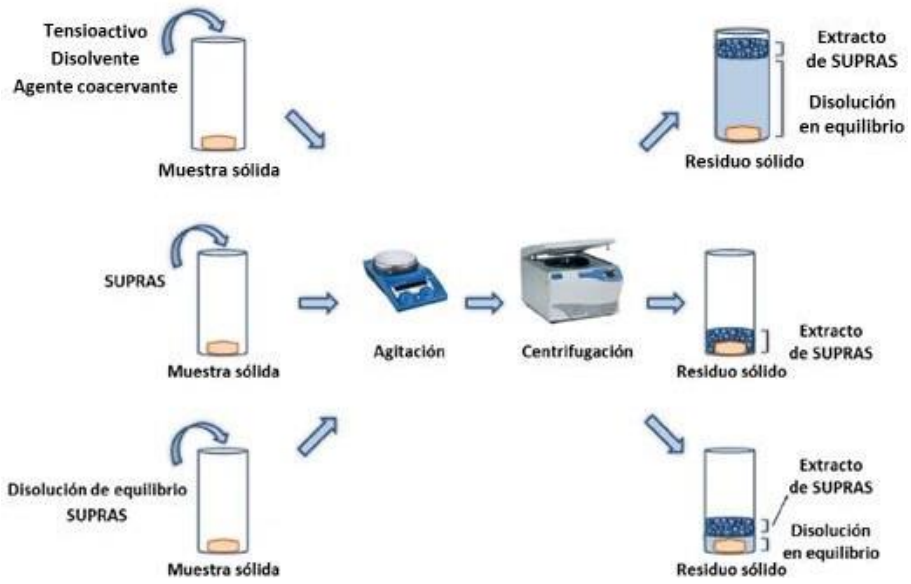


Figura 18. Esquema de las principales etapas de un proceso SLE usando SUPRAS.

3.6. Compatibilidad de los SUPRAS con cromatografía de líquidos-espectrometría de masas

Los extractos de SUPRAS siempre se han inyectado directamente en el sistema de LC. Sin embargo, cuando éstos son muy viscosos, se diluyen antes con un disolvente orgánico (como el metanol o el acetonitrilo) [85]. La mayoría de las aplicaciones utilizan la cromatografía en fase reversa. Por ello, cuando el SUPRAS entra al sistema cromatográfico, las nanoestructuras se disgregan en la fase móvil acuoso-orgánica y dan lugar a monómeros de anfifilo, que normalmente no afectan al comportamiento cromatográfico de los analitos (Figura 11). Por el contrario, en el caso de fases móviles con un alto contenido acuoso, los agregados supramoleculares se disgregan poco a poco en el sistema cromatográfico, dando lugar a micelas, vesículas, etc. (Figura 11) que ofrecen a los analitos una pseudofase a través de la que distribuirse.

La introducción de un proceso de retención adicional supone, en ocasiones, una ventaja en la separación de los compuestos. Sin embargo, muchas veces disminuye la resolución y el extracto se tiene que diluir con un disolvente orgánico antes de ser inyectado en el sistema cromatográfico. Los SUPRAS se pueden aplicar en otros modos cromatográficos como la cromatografía líquida micelar [97] o la cromatografía líquida quiral [98].

En cuanto a los sistemas de detección, los SUPRAS se han usado en LC acoplada a ultravioleta (UV)-visible, fluorescencia y espectrometría de masas. No obstante, el desarrollo más notorio en este campo ha sido el uso combinado de los SUPRAS con la LC acoplada a espectrometría de masas [85,88]. Como norma general, los monómeros de tensioactivo se envían directamente a desecho tras la separación cromatográfica, para evitar efectos negativos como la contaminación de la fuente iónica y/o pérdida de la eficiencia de la ionización y la sensibilidad del detector. A este respecto, los anfifilos que componen el SUPRAS se tratan igual que los componentes de la matriz.

En el caso de que las moléculas anfifílicas coeluyan con los analitos y no puedan enviarse a desecho, lo cual es frecuente en análisis de multicomponentes, deben seleccionarse anfifilos difícilmente ionizables con la fuente de ionización seleccionada para espectrometría de masas. Un ejemplo es el uso de anfifilos con grupos polares alcoholes para determinaciones llevadas a cabo con LC-ESI-MS [19, 85].

3.7. Aplicabilidad de los SUPRAS en el análisis de multicomponentes

Los SUPRAS se han aplicado a la extracción de muy diferentes tipos de analitos. Entre las aplicaciones desarrolladas, destacan la preconcentración de metales [98] y, más recientemente, de nanopartículas metálicas [99], previamente a su determinación mediante espectrometría atómica. Asimismo es interesante el uso de SUPRAS para la separación y purificación de proteínas hidrofóbicas e hidrofílicas, previa a su separación cromatográfica [100]. Recientemente, se ha propuesto el uso de SUPRAS para el enriquecimiento selectivo y extracción de proteínas hidrofóbicas para posterior análisis proteómico.

En relación al uso de SUPRAS para la extracción de compuestos orgánicos, que constituye el objeto de esta Tesis, no se ha explotado de forma generalizada la principal ventaja de los SUPRAS comparada con los disolventes orgánicos: la capacidad que tienen de extraer simultáneamente compuestos con muy diferente polaridad, lo cual es esencial en el análisis de multicomponentes [85]. Así, las principales aplicaciones de los SUPRAS hasta el momento han estado relacionadas con la extracción de hidrocarburos policíclicos aromáticos (PAHs), compuestos policlorados (ej. bifenilos policlorados, PCBs; dibenzofuranos policlorados, PCDFs; dibenzo-p-dioxinas policloradas, PCDDs), tensioactivos, plaguicidas, compuestos bioactivos (ej. vitaminas, drogas, fármacos), alteradores endocrinos, colorantes, micotoxinas, aminas aromáticas, fenoles, etc.

Las áreas de mayor interés han sido medioambiente (principalmente muestras de agua naturales y residuales, suelo, sedimento, lodo, etc.), agroalimentación (incluyendo verduras, carne, pescado, cereales, bebidas enlatadas, frutas, etc.) y epidemiología/diagnósticos clínicos (muestras de orina, plasma, saliva, etc.). Una visión general de estas aplicaciones puede encontrarse en diferentes artículos de revisión publicados en las dos últimas décadas [74, 101-103]. En general, la mayoría de estas aplicaciones se limitan a la extracción de un número reducido de analitos (generalmente de 1 a 5).

La Tabla 3 muestra las aplicaciones más relevantes reportadas hasta la fecha sobre el uso de SUPRAS para la extracción de al menos 10 analitos [73]. Como se muestra en la tabla, las principales aplicaciones han estado relacionadas con la extracción de PAHs, los cuales son en su mayoría hidrófobos y se solubilizan en las regiones hidrocarbonadas de los SUPRAS [104]. La capacidad de los mismos para extraer simultáneamente compuestos polares y apolares se ha demostrado mediante la extracción de 14 fenoles (log K_{ow} 1.5-5.18) a partir de agua de mar y residual [105] y la extracción de bisfenoles y sus diglicidil ésteres de bifenilos (log K_{ow} 2.05-4.34) en una variedad de matrices para evaluar la exposición humana a los mismos, incluyendo alimentos [106] polvo atmosférico [107] y muestras biológicas [101]. El uso de SUPRAS para la extracción de compuestos anfifílicos como tensioactivos es especialmente eficiente debido a la formación de agregados mixtos con el anfifilo que constituye el SUPRAS. Un ejemplo es la extracción de tensioactivos catiónicos a partir de lodo residual con SUPRAS formados por anfifilos aniónicos, obteniéndose recuperaciones en el intervalo 91-100% a pesar de la fuerte retención de los tensioactivos catiónicos por el lodo residual [108]. Por otro lado, la aplicación de los SUPRAS a la extracción de disolventes residuales con puntos de ebullición que varían en un amplio intervalo (32-285 ºC) a partir de fármacos, es un ejemplo de la capacidad de los mismos para el tratamiento de muestras previo a cromatografía de gases [109].

La mayoría de las aplicaciones de los SUPRAS a la extracción de multicomponentes ha utilizado cromatografía de líquidos acoplada a detección fluorescente, y sólo recientemente se han usado en combinación con LC-MS. Por tanto, el gran potencial que tienen los SUPRAS para la extracción de un gran número de sustancias estructuralmente diferentes que cubran un amplio intervalo de polaridad en combinación con las técnicas habitualmente utilizadas (ej. LC acoplada a alta y baja resolución) no se ha demostrado hasta la fecha.

Tabla 3. Aplicaciones de los disolventes supramoleculares en la extracción simultánea de multicomponentes (≥ 10 compuestos).

Compuestos	Muestra	SUPRAS basados en anfifilos	Agente coacervante	Sistema de separación/ detección	Recuperaciones (%R)	Ref.
Hidrocarburos policíclicos aromáticos (PAHs)						
14 PAHs de la lista prioritaria de la EPA (acenafteno y acenaftileno excluídos)	Agua de mar	Polioxoetileno-10-lauril éter acuoso	Temperatura (90 ºC)	LC-FL	38-104	[110]
15 PAHs de la lista prioritaria de la EPA (acenaftileno excluído)	Agua embotellada, agua del río	Triton X-114 acuoso	Temperatura (40 ºC)	LC-FL	30-109	[111]
15 PAHs de la lista prioritaria de la EPA (acenaftileno excluido)	Suero humano	Triton X-100 acuoso	Temperatura (60 ºC)	LC-UV	~ 100	[17]
13 PAHs de la lista prioritaria de la EPA (acenaftileno, acenafteno e indeno[1,2,3,cd]pireno excluídos)	Agua de mar	Polioxoetileno lauril éter y Brij 30 acuosos	Temperatura (78 ºC)	LC-FL	72-99	[112]
16 PAHs de la lista prioritaria de la EPA	Agua natural	Sulfonato de dodecano acuoso	4 M HCl	LC-FL	62-106	[113]
10 PAHs de la lista prioritaria de la EPA: Acenaftileno, Antraceno, Benzo[a]antraceno, Benzo[a]pireno, Criseno, Dibenzo[a,h]antraceno, Fenantreno, Pireno, Fluoreno, Fluoranteno.	Agua del grifo	Sulfato de dodecyl acuoso	6 M HCl	LC-FL	67-95	[114]
14 PAHs de la lista prioritaria de la EPA (Naftaleno y acenaftileno excluídos)	Musgos	Ácido decanoico en THF	0.01 M HCl	LC-FL	70-110	[115]

Fenoles						
Fenol, 2,4-Nitrofenol, 2,4-Dinitrofenol, p-Cresol, 2-Nitrofenol, 2-Clorofenol, 2,4-Dimetilfenol, 4,6-Dinitro-orto-cresol, 4-Cloro-meta-cresol, 2,4,6-Trimetilfenol, 2,4-Diclorofenol, 2,4-Cloro-3,5-dimetilfenol, 2,4,6-Triclorofenol, Pentaclorofenol.	Agua de mar, agua residual	Genapol X acuoso	Temperatura (85ºC)	LC-UV	95-114	[105]
Surfactantes						
10 homologos de surfactantes de dialquildimetil, alquilbencildimetil y alquiltrimetil amonio	Lodos residuales	Sulfonato de dodecano acuoso	3M HCl	LC-ESI-MS (trampa iónica)	91-100	[108]
Disruptores endocrinos						
12 bisfenoles, éteres de diacilglicil bisfenol y derivados	Comidas enlatadas (verduras, legumbres, frutas, pescado y marisco, productos cárnicos y granos.	Tetradecanol en THF	Agua	LC-FL	80-110	[106]
14 bisfenoles	Saliva	Hexanol en THF	Saliva	LC-ESI-MS/MS	95-105.6	[116]
21 bisfenoles y derivados	Bebidas enlatadas, orina, suero, comida enlatada, polvo	Hexanol en THF	Agua	LC-ESI-MS/MS	70-114	[117]
21 bisfenoles y derivados	Polvo de invernadero	Hexanol en THF	Agua	LC-MS/MS	93-109	[118]

Disolventes residuales						
37 disolventes residuales	Fórmulas farmacéuticas (tablas, cápsulas, polvo, supositorios, gránulos, suspensiones, aerosoles, microsferas, inyecciones, emulsiones).	Ácido poli-undecelénico en tetraglima.	Agua	HS-GC-MS	70-120	[109]
Benzodiacepinas						
11 benzodiacepinas	Orina	Hexanol en THF	Orina	LC-ESI-MS/MS	84-105	[119]
Drogas						
12 drogas de abuso	Agua de grifo de distintos países europeos.	Hexanol en THF	Agua	LC-ESI-MS/MS	93-111	[103]
13 drogas de abuso	Pelo humano.	Hexanol en THF	Agua	LC-ESI-MS/MS	93-107	[120]
23 drogas de asalto sexual	Orina	1,2-hexanediol en disolución acuosa de Na_2SO_4.	Sal (Na_2SO_4 1M)	LC-MS (trampa iónica)	79-119	[121]

[a] Ratio volumen de muestra volumen de SUPRAS.

3.8. Cumplimiento de los SUPRAS con los criterios establecidos para los disolventes verdes

En el estado actual de desarrollo, muchos de los SUPRAS utilizados en procesos de microextracción analítica cumplen plenamente o parcialmente la mayoría de los criterios establecidos para determinar si un disolvente puede o no etiquetarse como verde [122].

Así, para la mayoría de los anfifilos y agentes de coacervación usados, la síntesis de los SUPRAS se realiza a través de procesos que requieren nula o baja energía (la coacervación es espontánea a temperatura ambiente) y son eficientes atómicamente (la mayoría del anfifilo se incorpora al SUPRAS). Además las características de los SUPRAS en procesos de microextracción superan a las características de los disolventes convencionales en términos de ámbito de aplicación, eficiencia y posibilidad de modelado, lo que permite su aplicación en procesos de extracción innovadores.

Por otro lado, existen una serie de criterios (toxicidad, biodegradabilidad, estabilidad, e inflamabilidad) para los que las características del SUPRAS serán semejantes a la de los componentes que los constituyen. Por lo tanto, la tendencia actual es el uso de anfifilos (ej. ácidos grasos o ramnolípidos) y de agentes coacervantes (ej. NaCl) cuya inocuidad se haya demostrado.

Con respecto a los criterios relacionados con el mercado (disponible en varios grados de pureza, coste competitivo y disponibilidad a nivel mundial) el foco debe ponerse en los compuestos anfifílicos puesto que son el principal ingrediente del SUPRAS. En estos criterios, los anfifilos sintéticos derivados del petróleo superan a los anfifilos procedentes de fuentes renovables ya que los anfifilos producidos a partir de biomasa mediante procesos de fermentación, tratamientos termo-químicos, etc. son en la actualidad mucho menos competitivos, aunque muestran un creciente auge a nivel mundial. Especialmente interesante es la posibilidad de usar anfifilos de bajo grado de pureza obtenidos a partir de fuentes renovables, lo cual disminuiría considerablemente el coste de producción del SUPRAS, como recientemente se ha demostrado para la producción de SUPRAS a partir de ramnolípidos del 90% de pureza [84].

Todos los SUPRAS pueden transportarse y almacenarse de forma segura, aunque hasta la fecha, dada la simplicidad de su síntesis esta se ha realizado en el laboratorio.

Por último, dado el pequeño volumen de SUPRAS utilizado en procesos de microextracción (del orden de μL), no se ha prestado atención a la reutilización de los mismos. Si bien es cierto que se ha propuesto el uso de las fases de equilibrio para la síntesis de nuevas alícuotas de SUPRAS [84].

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BLOCK A

Development of innovative sample treatment platforms in environmental and food analysis.

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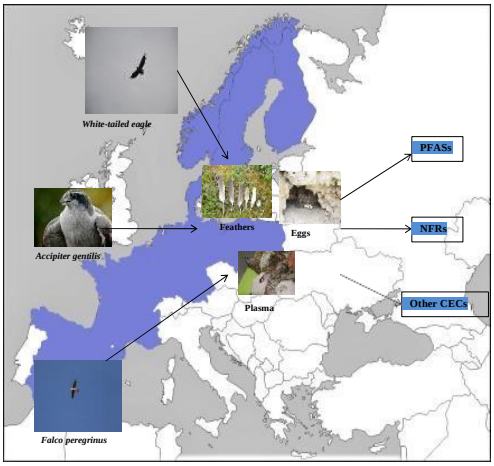
A review on contaminants of emerging concern in European raptors (2002–2020)

Soledad González-Rubio[1,2,3,*], Ana Ballesteros-Gómez[1], Alexandros G. Asimakopoulos[2], Veerle L. B. Jaspers[3**].

a Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, Marie Curie Annex Building, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain
b Department of Chemistry, Norwegian University of Science and Technology (NTNU), NO-7491Trondheim, Norway
c Department of Biology, Norwegian University of Science and Technology (NTNU), NO-7491 Trondheim, Norway.

* Correspondence to: S. G.-Rubio, Department of Analytical Chemistry, Institute of Fine Chemistryand Nanochemistry,Marie Curie Annex Building, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain.
** Corresponding author. E-mail addresses: q02gorus@uco.es (S. González-Rubio), veerle.jaspers@ntnu.no (V.L.B. Jaspers).

Graphical abstract



Highlights

37 studies on CECs in raptors in Europe (2002–2020) were reviewed. PFASs and NFRs were the most studied CECs in eggs, feathers and plasma.

Less studied CECs: bisphenols, UV filters, neonicotinoids, paraffins, parabens

Species: white-tailed Eagle, peregrine falcon and northern goshawk (WesternEU)

Concentrations of PFASs and NFRs generally surpassed those of legacy PBDEs.

Keywords

Birds of prey, Emerging organic contaminants, Perfluoroalkyl substances, Novel flame retardants, Bisphenols, Neonicotinoids

Abstract

Raptors (birds of prey and owls) have been widely used as suitable bioindicators of environmental pollution. They occupy the highest trophic positions in their food chains and are documented to bioaccumulate high concentrations of persistent pollutants such as toxic metals and legacy persistent organic pollutants (POPs). Whereas raptors played a critical role in developing awareness of and policy for chemical pollution, they have thus far played a much smaller role in current research on contaminants of emerging concern (CECs). Given the critical knowledge obtained from monitoring legacy contaminants in raptors, more information on the levels and effects of CECs on raptors is urgently needed. This study critically reviews studies on raptors from Europe reporting the occurrence of CECs with focus on the investigated species, the sampled matrices, and the bioanalytical methods applied. Based on this,we aimed to identify future needs for monitoring CECs in Europe. Perfluoroalkyl substances (PFASs), novel flame retardants (NFRs), and to a lesser extent UV-filters, neonicotinoids, chlorinated

paraffins, parabens and bisphenols have been reported in European raptors. White-tailed Eagle (Haliaeetus albicilla), Peregrine falcon (Falco peregrinus) and Northern goshawk (Accipiter gentilis) were the most frequently studied raptor species. Among matrices, eggs, feathers and plasma were the most widely employed, although the potential role of the preen gland as an excretory organ for CECs has recently been proposed. This review highlights the following research priorities for pollution research on raptors in Europe: 1) studies covering all the main classes of CECs; 2) research in other European regions (mainly East Europe); 3) identification of the most suitable matrices and species for the analysis of different CECs; and 4) the application of alternative sample treatment strategies (e.g. QuEChERS or pressurized liquid extraction) is still limited and conventional solvent extraction is the preferred choice.

1. Introduction

Biomonitoring practices are very important for studying the environmental impact of chemical pollution (Woodruff, 2011). The direct analysis of contaminants in organisms provides information on bioavailability but also information about the intake by biota or potential exposure pathways for humans. Among all the organisms that can be considered sentinels of environmental contamination, raptors (members of Falconiformes, Strigiformes or Accipitriformes) are especially suitable for monitoring persistent, bio-accumulative and toxic (PBT) substances (Movalli et al., 2019; Espín et al., 2016; Sergio et al., 2005, 2006). As top predators, they are likely to have particularly high levels of environmental contaminants and therefore individual- and population-level effects are most likely measurable in raptors prior to other taxa (Kovács et al., 2008; Helander et al., 2008). Furthermore, they are widespread and long-lived organisms, thus allowing the assessment of spatial and temporal trends of PBT

chemicals and their associated effects on populations (Furness, 1993). Another advantage of using these avian species for monitoring is that non-destructive sampling approaches can be applied (e.g. blood, feather, preen gland oil) (Espín et al., 2016; EURAPMON, 2014). Indeed, raptors have been used as bioindicators in many studies worldwide (Elliot et al., 2009, Grove et al., 2009, Behrooz et al., 2014, Martínez-López et al., 2015, Elliot et al., 2015, Rattner et al., 2018, Manning et al., 2008, Hong et al., 2019, Aver et al., 2020). In Europe, there has been a focus with several biomonitoring programmes on raptors to track the success of mitigation strategies for reducing the exposure to contaminants (Gómez-Ramírez et al., 2014). However, these schemes have mainly been performed in western European countries and have hitherto focused on the monitoring of toxic elements, such as mercury (Hg) and lead (Pb), and of legacy persistent organic pollutants (POPs), mainly organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), dioxins, furans and anticoagulant rodenticides (Gómez-Ramírez et al., 2014). Among the POPs, OCPs, PCBs and PBDEs are widespread in wildlife (Blanco et al., 2018; Monclús et al., 2018) and their use is currently regulated by the Stockholm Convention (United Nations Evironment Programme (UNEP, 1972). Although a great effort is currently being made to coordinate and integrate these multiple independent monitoring programmes on raptors in Europe (Gómez-Ramírez et al., 2014; European Raptor Biomonitoring Facility-ERBFacility, 2017; Badry et al., 2020), there is still a greater need to identify current trends at the broader European spatial scale (Espín et al., 2016; Movalli et al., 2019). The current European Raptor Biomonitoring Facility (ERBFacility, COST Action CA16224) is active in this respect, working towards coordinated Europe-wide monitoring of contaminants in raptors, with a view to support the implementation of EU chemicals regulations and reducing chemical risks to the environment and to human health (https://erbfacility.eu/). Further, the LIFE APEX project is working to demonstrate the use of apex predators, including raptors, for the monitoring of contaminants of emerging concern, with a view to better chemicals management in Europe (www.lifeapex.eu). New challenges are

emerging due to the restrictions and bans on the use of POPs, which has led to the emergence of other chemical classes of contaminants in recent years, also known as "contaminants of emerging concern" (CECs) (Thomaidis et al., 2012; Sauvé and Desrosiers, 2014). These chemicals, for which currently no or very limited regulations are established, have received considerable attention due to their widespread occurrence in the environment and their potential toxicity (Blum et al., 2015). According to the network of reference laboratories for monitoring of emerging environmental contaminants (NORMAN), CECs can be defined as substances that have been detected in the environment, but which are currently not included in routine monitoring programmes and whose fate, behaviour and (eco) toxicological effects are not well understood. More than 700 emerging contaminants have been identified in water bodies up to now, such as novel flame retardants (NFRs), perfluoroalkyl substances (PFASs), bisphenols, ultraviolet-filters (UV filters), chlorinated paraffins and parabens (NORMAN List of Emerging Substances, 2016). Despite their current concern, other chemical categories like rodenticides or PBDEs are not considered CECs and are not included in this review. It is essential to improve the effectiveness of evaluation, risk assessment and early warning in relation to the regulation of CECs (Cousins et al., 2020). Until now, only limited biomonitoring schemes for PFASs in raptors are established in Europe at a national scale even though they have been identified as Persistent Bioaccumulative and Toxic (PBT) compounds (Espín et al., 2016). For the remaining CECs, there is still limited data regarding their bioaccumulation in biota. However, asmentioned above, efforts are currently underway through ERBFacility and LIFE APEX to assess occurrence in raptors (Movalli et al., 2019; ERBFacility, 2017; LIFE APEX, 2020). With this as background, the main aim of the present study was to 1) review current studies of CECs in raptors to determine what species, matrices and analytical methods are used and their frequency, and 2) to determine any knowledge gaps and areas for future research highlighted by the literature review.

2. Method of literature review

Only international peer-reviewed studies related to the analysis of CECs in raptors from Europe were collected by the search engines of Web of Science, Science Direct and Google Scholar. Grey or other unpublished studies (such as theses, conference proceedings, book chapters or reports) were not included. Initially, a total of 1742 articles related to the analysis of contaminants in birds were found when using "contaminants" and "birds" as keywords in Web of Science. This search was then filtered by area (Europe), species (raptors) and type of contaminants (PFASs, NFRs and other CECs). For that purpose, we used different combinations of contaminant names (PFASs, NFRs, bisphenols, paraffins, neonicotinoids, benzophenones, UV-filters, parabens) and European countries as keywords along with "Europe", "raptors", "birds of prey", "owls" "contamination", "predators" "scavengers" and species (white tailed eagle, Northern gowshak, peregrine falcon, Eurasian sparrowhawk (Accipiter nisus), common kestrel (Falco tinnunculus), red kite (Milvus milvus), long-eared owl (Asio otus), barn owl (Tyto alba), tawny owl (Strix aluco), common buzzard (Buteo buteo), and others) and/or matrices names (eggs, fat, feathers, blood, plasma, liver, internal tissues, bones). References from the collected publications were also reviewed to ensure that all relevant studies were included. Although a limited period was not initially set, all the peer reviewed studies of our interest were published in the period 2002–2020, which was finally considered. Finally, 37 studies were included in the review. For each study, concentrations of contaminants, analytical methods employed, sample types and discussion on temporal trends and comparison of levels with legacy compounds were collected. Contaminants were classified in three major classes: perfluoroalkyl substances (PFASs), novel flame retardants (NFRs) and other miscellaneous CECs, namely, bisphenols, UV-filters, neonicotinoids, paraffins and parabens. Concentrations in the text are provided as ranges, means or medians of

single contaminants or contaminant classes (total sum of the concentrations of all the contaminants within a class) and expressed in ng g^{-1} or ng mL^{-1}. Contaminant abbreviations used throughout the manuscript are listed in Table 1.

Table 1. Contaminant abbreviations used and ordered alphabetically.

Abbreviation	Contaminant
ACE	Acetamiprid
AllylBZT	2-(2H-benzotriazole-2-yl)-4-methyl-6-(2-propyl)-phenol
ATE	Allyl-2,4,6-tribromophenyl ether
BATE	2-Bromoallyl-2,4,6-tribromophenyl ether
BnP	Benzyl paraben
BP	Bisphenol
BPA	Bisphenol A
BPAF	Bisphenol AF
BPAP	Bisphenol AP
BPB	Bisphenol B
BPF	Bisphenol F
BPM	Bisphenol M
BPP	Bisphenol P
BPS	Bisphenol S
BPZ	Bisphenol Z
Br-PFOS	Branchedperfluorooctanesulfonate
BTBPE	1,2 Bis(2,4,6-tribromophenoxy)ethane
BuP	Butyl paraben
BzP	Benzophenone
BzP-1	Benzophenone-1
BzP-2	Benzophenone-2
BzP-3	Benzophenone-3
BzP-8	Benzophenone-8
DBDPE	Decabromodiphenylethan
Dec	Dechlorane
3,4-DHB	3,4-dihydroxybenzoic acid
4-DHB	4,4-Dihydroxybenzophenone
DP	Dechlorane Plus
DPTE	2,3-Dibromopropyl-2,4,6-tribromophenyl ether
EHDPP	2-Ethylhexyl diphenylphosphate
EtP	Ethylparaben
4-HB	4-Hydroxy benzoate
HBB	Hexabromobenzene
HBCD	Hexabromocyclododecane
HeP	Heptylparaben
IMC	Imidacloprid
LCCP	Long chainchlorinatedparaffin
Lin-PFOS	Linear perfluorooctanesulfonate
MCCP	Medium chainchlorinatedparaffin
MeP	Methylparaben
MPFOA	Perfluoro-n-(1, 2, 3, 4-13C4) octanoicacid
MPFOS	Perfluoro-1-(1, 2, 3, 4-13C4) octanesulfonicacid
NBFRs	Novel brominated flame retardants

NFRs	Novel flame retardants
NP	4-nonylphenol
OC	2-ethylhexyl 2-cyano-3,3-diphenyl acrylate
ODPABA	2-ethylhexyl 4-(dimethylamino) benzoate
4-OH-BzP	4-Hydroxybenzophenone
OH-EtP	Ethylprotocatechuate
OH-MeP	Methylprotocatechuate
OP	4-tert-octylphenol
OOPFRs	Organophosphorus flame retardants
PBEB	Pentabromoethylbenzene
PeBT	Pentabromotoluene
PCNs	Polychlorinated naphthalenes
PeP	Pentylparaben
PFASs	Per- and Polyfluoroalkyl substances
PFBA	Perfluorobutanoicacid
PFBS	Perfluorobutanesulfonate
PFCAs	Perfluoroalkyl carboxylic acids
PFDcA	Perfluorodecanoanoicacid
PFDcS	Perfluorodecanesulfonate
PFDoA	Perfluorododecanoicacid
PFHpA	Perfluoroheptanoicacid
PFHpS	Perfluoroheptanesulfonate
PFHxA	Perfluorohexanoicacid
PFHxS	Perfluorohexanesulfonate
PFNA	Perfluorononanoicacid
PFNS	Perfluorononanesulfonate
PFOA	Perfluorooctanoicacid
PFOS	Perfluorooctanesulfonate
PFOSA	Perfluorooctanesulfonamide
PFPA	Perfluoropentanoicacid
PFPeA	Pefluoropentadecanoicacid
PFPS	Perfluoropentanesulfonate
PFTeA	Perfluorotetradecanoicacid
PFTriA	Perfluorotridecanoicacid
PFUnA	Perfluoroundecanoicacid
PFSAs	Perfluoroalkyl sulfonates
PhP	Phenylparaben
PrP	Propylparaben
SCCP	Short chainchlorinatedparaffin
2-4-6-TBA	2-4-6-Tribromoanisole
TBB	2-Ethylhexyl-2,3,4,5-tetrabromobenzoate
TBOEP	Tri-(2-butoxyethyl)-phosphate
2-4-6 TBP	2,4,6-Tribromophenol
TBPH	3,4,5,6-Tetrabromophthalate
TCEP	Tris(chloroethyl)phosphate
TCiPP	tris (1-chloro-2-propyl) phosphate
TCPP	tris(2-chloroisopropyl) phosphate
TDCiPP	Tris-(2,3-dichloropropyl)-phosphate
THC	Thiacloprid
TPhP	Tris(phenyl)phosphate

3. Results and discussion

To provide a general overview, two tables (Tables 2 and 3) were constructed with the collected data. Table 2 contains data related to the raptor species, including number of analyzed individuals (sample population) and year of collection, countries where the studies were performed, analyzed matrices and measured concentrations. Sample treatment and analytical technique/s employed in each case are presented in Table 3. The most relevant results derived from the studies collected in Tables 2 and 3 are presented below.

Table 2. Raptor species and number of analyzed individuals (sample population), countries where the studies were performed, matrices and concentrations of CECs in European raptors (2002-2020). The studies are ordered by year of publication, starting with the most recent ones.

Species (sample size)	**Country/ sampling year**	**Contaminants**	**Matrix**	**Concentration range (ng g^{-1} or ng mL^{-1})**	**Concentration median or mean (ng g^{-1} or ng mL^{-1})**	**Reference**
White-tailed eagle *(Haliaeetus albicilla)* (n=16) Eurasian sparrowhawk *(Accipiter nisus)* (n=2) Long-eared owl *(Asiootus)* (n=2)	Greenland and France/ 1997-2011	BPs: BPA, BPAF, BPAP, BPF, BPB, BPM, BPP, BPS. BzPs: BzP-1, BzP-2, BzP-3, BzP-8, 4-OH-BzP.	Preen gland	BPs: 0.2-149 (w.w.) BzPs: 0.3-7 (w.w.)	BPs median range: 0.05-70.9 (w.w.) BzPs median range: 0.3-47 (w.w.)	González-Rubio et al. (2020)
			Liver	BPs: 0.05-5 (w.w.) BzPs:0.9-44 (w.w.)		
			Kidney	BPs: 0.02-7 (w.w.) BzPs: 0.7-8 (w.w.)		
			Muscle	BPs: 0.1-3.5 (w.w.) BzPs: 0.3-47 (w.w.)		
Northern goshawk (*Accipiter gentilis*) (n=61)	Norway/ 2015-2016	PFASs: -PFCAs: PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTriA, PFTeA, PFHxDcA -PFSAs: PFBS, PFPS, PFHxS, PFHpS, PFOS, PFDcS -PFOSA NFRs: -DP -NBFRs: BTBPE, TBB, TBPH -OPFRs: TCEP, TBOEP, TPHP, EHDPHP, TCIPP, TDCIPP	Feathers	∑PFASs: 1.9-12.5 (Trøndelag, 2016); 0.3-9.6 (Troms, 2016); (w.w.) ∑NFRs: 17-203(Trøndelag, 2015); 2.6-41.1 (Trøndelag, 2016); 135-314 (Troms, 2015); 8-72 (Troms, 2016); (w.w.)	∑PFASs: mean (median): 5.7 (6.2) (Trøndelag, 2016); 1.8 (1.8) (Troms, 2016); (w.w.) ∑NFRs mean (median): 46.1 (47.7) (Trøndelag, 2015); 19.4 (22.2) (Trøndelag, 2016); 204 (206) (Troms, 2015); 27.5 (25.5) (Troms, 2016); (w.w.)	Briels et al. (2019)

	Plasma	∑PFASs: 2.-36(Trøndelag, 2015); 1.4-23.8 (Trøndelag, 2016); 1.4-28.4 (Troms, 2015); 2-16 (Troms, 2016) ∑NFRs: n.d.		∑PFASs: 14.6 (17.5) (Trøndelag, 2015); 6.3 (6.6) (Trøndelag, 2016); 8.8 (9.2) (Troms, 2015); 6.3 (5.0) (Troms, 2016) ∑NFRs: n.d.		
Western marsh harrier (*Circus aeruginosus*) (n=1)	Spain/ 2010	NFRs: Dec, DP	Eggs	NFRs:-	∑NFRs: 10 (l.w.)	Eljarrat et al. (2019)
White-tailed eagle (*Haliaeetus albicilla*) (n=70)	Norway/ 2015-2016	PFASs: -PFCAs: PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDcA PFUnA, PFDoA, PFTrA, PFTeA, - PFSAs: PFBS, PFPS, PFHxS, PFHpS, Lin-PFOS, Br-PFOS, PFNS -PFOSA NFRs: -NBFRs: TBB, TBPH, BTBPE -OPFRs: TCEP, TBOEP, EHDPHP, TPHP, TCPP, TCIPP, TDCIPP -DPs	Plasma	PFUndA: 2.4-4.4 (Smøla, 2015); 0.7-2.1 (Smøla, 2016); 2.3-5.1 (Steigen, 2015); 0.9-2.2 (Steigen, 2016) ; (w.w.) NFRs: 0.0002-0.3 (Smøla, 2015); 0.002-0.1 (Smøla, 2016); 0.01-1.4 (Steigen, 2015); 0.01-0.08 (Steigen, 2016); (w.w.)	PFUndA (median): 3.59 (Smøla, 2015); 1.15 (Smøla, 2016); 3.36 (Steigen, 2015); 1.40 (Steigen, 2016); (w.w.) NFRs median range: 0.01-0.2 (Smøla, 2015); 0.002-0.1 (Smøla, 2016); 0.01-0.55 (Steigen, 2015); 0.01-0.08 (Steigen, 2016); (w.w.)	Løseth et al. (2019a)
			Feathers	PFUndA: 0.05-0.6 (Smøla, 2015); 0.07-1 (Smøla, 2016); 0.1-0.8 (Steigen, 2015); 0.3-1.1 (Steigen, 2016); (w.w.) NFRs: 0.1-1229 (Smøla, 2015); 0.1-168 (Smøla, 2016); 0.0001-63 (Steigen, 2015); 0.1-68.4 (Steigen, 2016); (w.w.)	PFUndA (median): 0.15 (Smøla, 2015); 0.58 (Smøla, 2016); 0.38 (Steigen, 2015); 0.28 (Steigen, 2016); (w.w.) NFRs median range: 0.1-21.6 (Smøla, 2015); 0.1-27 (Smøla, 2016); 0.1-14 (Steigen, 2015); 0.2-21 (Steigen, 2016); (w.w.)	
White-tailed eagle (*Haliaeetus albicilla*) (n=70)	Norway/ 2015-2016	PFASs: -PFCAs: PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTrA, PFTeA PFSAs: PFBS, PFPS, PFHxS, PFHpS, Lin-PFOS, Br-PFOS, PFNS - PFOSA	Plasma	∑PFASs: 10-47 (Smøla, 2015); 5-13 (Smøla, 2016); 18-53 (Steigen, 2015); 7-33 (Steigen, 2016)	∑PFASs median: 26 (Smøla, 2015); 9 (Smøla, 2016); 32 (Steigen, 2015); 13 (Steigen, 2016)	Løseth et al. (2019b)

Cinereousvulture (*Aegypius monachus*) (n=16)	Spain/ 2016	NFRs: - OPFRs: TCEP, TCIPP, TPHP, TDCIPP	Feathers	∑NFRs: 1-65; (d.w.)	∑NFRs: 18 (13); (d.w.)	Monclús et al. (2019)
Egyptian vulture (*Neophron percnocterus*) (n=77)	Spain/ 2012-2016	PFASs: -PFCAs: PFBA, PFOA, PFNA, PFDA, PFUnA, PFDoA ,PFTrA, PFTeA -PFSAs: PFHxS, PFOS -MPFOA, MPFOS	Plasma	-	PFASs: 0.03-2.3; (w.w.)	Ortiz-Santaliestra (2019)
White tailed-eagle (*Haliaeetus albicilla*) (n=147)	Norway, Sweden and Greenland/ 1968-2015	PFASs: -PFCAs: PFHxA, PFHpA, PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTrA,,PFTeA -PFSAs: PFBS, PFHxS, PFHpS, PFOS, PFDS -PFOSA	Feathers	-	∑PFCAs mean (median) range: 1.8-6.5 (2-6.1) (Norway); 0.2-8.7 (0.1-9) (Sweden); 3.1-9 (3.1-8.2) (Greenland) PFOS: 2.5-11.6 (1.6-9.5) (Norway); 3-22.3 (3-20.1) (Sweden);2.7-4.1 (2.6-4) (Greenland) FOSA: 0.7-3.6 (0.7-3.5) (Norway); 0.4-1.6 (0.3-1.5) (Sweden); 1.3-8.3 (1.1-8.3) (Greenland)	Sun et al. (2019)
Peregrine falcon (*Falco peregrinus*) (n=26)	Greenland/ 1986-2014	PFASs: -PFCAs: PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA ,PFTrA, PFTeA -PFSAs: PFBS, PFHxS, PFHpS, PFOS, PFDS -PFOSA PCNs: CN 42-52/60, 53, 54, 63, 66/67, 68/64, 69	Eggs	∑PFSAs: 113-3900; (d.w.) ∑PFCAs: 29-410; (d.w.) ∑PCNs: 7-493; (d.w.)	∑PFSAs mean (median): 474 (303); (d.w.) ∑PFCAs: 138 (100); (d.w.) ∑PCNs: 40 (21); (d.w.)	Vorkamp et al. (2019)

White-tailed eagle (*Haliaaetus Albicilla)* (n=4) Eagle owl (*Bubo bubo*) (n=10) Golden eagle (*Aquila chrysaetos*) (n=10) Peregrine falcon (*Falco peregrinus)* (n=10) Common kestrel (*Falco tinnunculus*) (n=3) Tawny owl (*Strix aluco)* (n=4)	Sweden, Denmark and the Baltic Sea (Poland)/ 2012-2017	Chlorinated paraffins: SCCPs, MCCPs, LCCPs.	Muscle	-	SCCPs: median range: 540-730; (l.w MCCPs: 360-720; (l.w.) LCCPs: 210-1200; (l.w.)	Yuan et al. (2019)
			Eggs	-	SCCP: median range: 85-220; (l.w.) MCCPs: 85-250; (l.w.) LCCPs: 34-51; (l.w.)	
Black kite (*Milvus migrans*) (Madrid, n=28; Doñana, n=23)	Spain/ 2007-2016	NFRs: -Dec:Dec 602, 603, 604 -DP:*syn*-DP, *anti*-DP	Eggs	NFRs: n.d.-33 (Doñana); n.d.-2452 (Madrid); (l.w.)	NFRs: n.d.-11 (Doñana); n.d.-353 (Madrid); (l.w.)	Blanco et al. (2018)
European honey buzzard (*Pernis apivorus*) (n=10)	Finland/ 2013	Neonicotinoids: ACE, IMC, THC	Blood	ACE: <LOD-0.008 IMC: <LOD-0.04 THC: <LOD-0.03	-	Byholm et al. (2018)
Cinereousvulture (*Aegypius monachus*) (n=16)	Spain/ 2016	NFRs: - OPFRs: TCEP, TCiPP, TPhP, TDCiPP	Contour feathers	∑NFRs: 0.4-24.6; (d.w.)	∑NFRs: 0.4-10.5 (d.w.)	Monclús et al. (2018)
			Down feathers	∑NFRs: 0.4-49 ; (d.w.)	∑NFRs: 0.4-13.6 ; (d.w.)	
Northern goshawk (*Accipiter gentilis)* (n=11) White-tailed Eagle (*Haliaeetus albicilla)* (n=14)	Norway/ 2014	PFASs: -PFCAs: PFBA, PFPA, PFHxA, PFHpA, PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTriA, PFTeA, -	Plasma	∑PFASs: 5-42 (*Accipitergentilis*); 28-79 (*Haliaeetusalbicilla*)	∑PFASs mean (median): 19 (17) (*Accipitergentilis*); 45 (46) (*Haliaeetusalbicilla*)	Gómez-Ramírez et al. (2017)

Common kestrel (*Falco tinnunculus*) (n=10)	Spain/ 2010-2012	UV-filters: BP1, BP3, 4HB, 4DHB, OC, ODPABA, AllylBZT	Eggs	BP1: 28-54; (d.w.) BP3: 19-35; (d.w.) 4HB: 20-1200; (d.w.) 4DHB: <MLOQ-132; (d.w.) AllylBZT: 0.4-3; (d.w.)	BP1 mean: 39 (d.w.) BP3: 25 (d.w.) 4HB: 210 (d.w.) 4DHB: 132 (d.w.) AllylBZT: 1.5 (d.w.)	Molins-Delgado et al. (2017)
European herring gull (*Larus argentatus*) (non raptor species) (n=26)	Poland/ 2010-2012	BPA OP NP	Feathers	BPA: 29-512; (d.w.) OP: 28-564; (d.w.) NP: 5-151; (d.w.)	BPAmean: 145 (d.w.) OP: 162 (d.w.) NP: 38 (d.w.)	Nehring et al. (2017)
Eurasian eagle-owl (*Bubo bubo*) (n=30)	Spain/ 2004-2009	Neonicotinoids: ACE, IMC, THC, Clothianidin, Dinotefuran, Nitenpyram, Thiamethoxam	Blood	IMC: 3.3 Other neonicotinoids: n.d.	-	Taliansky-Chamudis et al. (2017)
Peregrine falcon (*Falco peregrinus)* (n=11) Eurasian eagle-owl (*Bubo bubo*) (n=3) White-tailed Eagle (*Haliaeetus albicilla)* (n=1) Osprey (*Pandion haliaetus*) (n=2)	Germany/ 2014	NFRs: - NBFRs: DPTE, BATE, ATE, HBCD, HBB, 2-4-6-TBA, 2-4-6 TBP, PBEB, PBT	Eggs	NFRs: 0.03-66 (*Falco peregrinus*); 0.6-81 (*Bubo bubo)*; 2-11 (*Haliaeetusalbicilla*); 1-9 (*Pandionhaliaetus*); (l.w.)	NFRs mean range: 1-33 (*Falco peregrinus*); 2-24 (*Bubo bubo)*; 5 (*Haliaeetusalbicilla*); 3-5 (*Pandionhaliaetus*); (l.w.)	Vetter et al. (2017)
Peregrine falcon (*Falco peregrinus)* (n=10)	Greenland/ 1986-2014	NFRs: TBB, BTBPE, DPTE, DBDPE, DP	Eggs	∑NFRs: 0.2-54; (l.w.)	NFRs: 0.5-4.7 (l.w.)	Vorkamp et al. (2017)

Osprey (*Pandion haliaetus*) (n=30) Tawny owl (*Strix aluco)* (n=10) Common kestrel (*Falco tinnunculus*) (n=40)	Sweden/ 1997-2014	PFASs: -PFCAs: PFBA, PFHxA, PFOA, PFNA, PFDcA, PFUnA, PFDoA, -PFSAs: PFHxS, PFOS, -PFOSA	Eggs	PFASs: 0.08-22 (*Falco tinnunculus*, 2014); 0.08-35 (*Strixaluco*, 2014); 0.08-224 (*Pandionhaliaetus*, 2013); 0.3-337 (*Pandionhaliaetus*, 2008-2009); 0.08-667 (*Pandionhaliaetus*, 1997-2001); (w.w.)	PFASs median range: 0.3-4 (*Falco tinnunculus*, 2014); 0.3-8 (*Strixaluco*, 2014); 1-70 (*Pandionhaliaetus*, 2013); 0.5-64 (*Pandionhaliaetus*, 2008-2009); 0.15-103 (*Pandionhaliaetus*, 1997-2001); (w.w.)	Eriksson et al. (2016)
White tailed eagle (*Haliaeetus albicilla*) (n= -)	Sweden/ 1966-2010	PFAS: -PFCAs:PFHxA, PFOA, PFNA, PFDcA, PFUnA, PFDoA,PFTrA, PFTeA, and PFPeA -PFSAs: PFBS, PFHxS, PFOS, PFDS -PFOSA	Eggs	-	PFASs geometric mean range: 0.005-1513 (Location 1 from 1969 to 2010); 0.009-837 (Location 2 from 1966 to 2010); 0.007-370 (Location 3 from 1986-2010); 0.006-174 (Location 4 from 1966-2009); (w.w.)	Faxneld et al. (2016)
White-tailed eagle (*Haliaeetus albicilla*) (n=35)	Norway/ 2011-2012	PFASs: -PFCAs: PFBA, PFPA, PFHxA, PFHpA, PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTriA, PFTeA, -PFSAs: PFBS, PFHxS, Lin-PFOS, Br-PFOS	Plasma	∑PFASs: 1-14	∑PFASs mean (median): 50 (42)	Sletten et al. (2016)
White-tailed eagle (*Haliaeetus albicilla*) (n=20)	Germany and Polish coastal areas of the Baltic Sea/ 1979-1999	Parabens: MeP, EtP, PrP, BuP, BnP, HepP, OH-MeP, OH-EtP, 3,4-DHB, 4-HB	Liver	MeP:<8.-657; (w.w.) 4-HB: 545-68600 Others: n.d.	MeP: mean: 112 4-HB: 11500 Others: n.d	Xue&Kannan (2016)
Black kite (*Milvus migrans*) (n=10)	Spain/ 1999-2013	NFRs: -Dec:Dec 602, 603, 604 -DP	Eggs	∑NFRs: 1-76 (1999); 5-74 (2011); (l.w.)	∑NFRs median: 17 (1999); 19 (2011); (l.w.)	Barón et al. (2015)

Black kite (*Milvus migrans*) (n=22) Red kite (*Milvus milvus*) (n=2) Western marsh harrier (*Circus aeruginosus*) (n=1) Booted eagle (*Aquila pennata*) (n=6) Common kestrel (*Falco tinnunculus*) (n=13) Black-winged kite (*Elanus caeruleus*) (n=1) Barn owl (*Tyto alba*) (n=1)	Spain/ 2010-2012	NFRs: Dec:Dec 602, 603, 604 DP:*anti*-DP, *syn*-DP	Eggs	ΣNFRsorNFRs(l.w.): 7.5-74.4 (*Milvusmigrans*);43.2-48.7 (*Milvusmilvus*); 0.69-88.9 (*Circusaeruginosus*); 3.6-83.8 (*Aquila pennata*); 1-19.6 (*Falco tinnunculus*); 0.6-20.9 (*Elanuscaeruleus*); 0.4-5.4 (*Tyto alba*) .	ΣNFRs mean (l.w.): 30.9 (*Milvusmigrans*);46 (*Milvusmilvus*); 161 (*Circusaeruginosus*); 29.9 (*Aquila pennata*); 8.8 (*Falco tinnunculus*); 22.6 (*Elanuscaeruleus*); 7.3 (*Tyto alba*) .	Barón et al. (2014)
Tawny owl (*Strix aluco*) (n=107)	Norway/ 1986-2009	PFASs: -PFCAs: PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTriA, PFTeA- PFSAs: PFHxS, PFHpS, PFOS, PFDcS -PFOSA	Eggs	∑PFASs: 0.06-6 (1986-2009); 0.06-6 (1986-1995); 0.06-5 (2001-2009); (w.w.)	∑PFASs mean (median): 2 (1) (1986-2009); 1 (0.9) (1986-1995); 2 (1.8) (2001-2009); (w.w.)	Bustnes et al. (2014)
thern goshawk (*Accipiter gentilis*) (n=16) White-tailed eagle (*Haliaeetus albicilla*) (n=24)	Norway/ 2008-2010	PFASs: PFOS	Plasma	PFOS: (*Accipitergentilis)*: 0.3-36 (w.w.); (*Haliaeetusalbicilla)*: 0.2-34 (w.w.)	PFOS: mean (median): (*Accipitergentilis)*: 7.5 (6.2) (w.w.); (*Haliaeetusalbicilla)*: 16 (13) (w.w.)	Bustnes et al. (2013)
White-tailed eagle (*Haliaeetus albicilla*) (n=21)	Norway/ 2011	NFRs: OPFRs: TPHP, TCIPP, TDCPP, TCEP, TBOEP, EHDPP	Plasma	NFRs: 0.1-0.7	NFRs:<LOQ-0.22	Eulaers et al. (2014)

Barn owl (*Tyto alba*) (n=15)	Belgium/ 2008-2009	PFASs: -PFCAs: PFBA, PFPA, PFHxA, PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTriA, PFTeA -PFSAs: PFHxS, PFOS, PFDcS -PFOSA	Muscle	PFASs: 11-477; (w.w.)	PFASs median range: n.d.-135; (w.w.)	Jaspers et al. (2013)
			Liver	PFASs: 6-116; (w.w.) PFOS: 42-992; (w.w.)	PFASs: 16-21; (w.w.) PFOS: 304 (w.w.)	
			Preen oil	PFASs: 2-1208; (w.w.)	PFASs: 22-431; (w.w.)	
			Fat	PFASs: 0.6-609; (w.w.)	PFASs: 0.6-203; (w.w.)	
			Feathers	PFASs: 2-670; (w.w.)	PFASs: 2-37; (w.w.)	
Northern goshawk (*Accipiter gentilis*) (n=56) White-tailed eagle (*Haliaeetus albicilla*) (n=36) Golden eagle (*Aquila chrysaetos*) (n=12)	Norway/ 2008-2010	PFASs: -PFCAs: PFPA, PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTriA -PFSAs: PFHpS, PFOS, PFDcS	Plasma	∑PFASs (*Accipitergentilis)*: 9-99 (2008); 6-608 (2009) ∑PFASs (*Haliaeetusalbicilla*): 33-71 (2008); 16-197 (2009); 10-60 (2010) ∑PFASs (*Aquila chrysaetos)*: 0.9-1 (2008); 3-30 (2010)	∑PFASs: (*Accipitergentilis)*: 21 (2008); 151 (2009) ∑PFASs: (*Haliaeetusalbicilla*): 55 (2008); 57 (2009); 32 (2010) ∑PFASs: (*Aquila chrysaetos)*: - (2008); 12 (2010)	Sonne et al. (2012)
White-tailed eagle (*Haliaeetus albicilla)* (n=5) Golden eagle (*Aquila chrysaetos*) (n=2) Northern goshawk (*Accipiter gentilis*) (n=16)	Norway/ 2008-2009	PFASs: -PFCAs: PFPA, PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTriA -PFSAs: PFHpS, PFOS, PFDcS	Plasma	∑PFASs: 0.9-1 (*Aquila chrysaetos*); 9-99 (*Accipitergentilis*); 33-70 (*Haliaeetusalbicilla*)	∑PFASs: - (*Aquila chrysaetos*); 21 (*Accipitergentilis*); 55 (*Haliaeetusalbicilla*)	Sonne et al. (2010)
Tawny owl (*Strix aluco*) (n=107)	Norway/ 1986-2009	PFASs: -PFCAs: PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTriA, PFTeA -PFSAs: PFHxS, PFHpS, PFOS, PFDcS -PFOSA	Eggs	PFASs (1986-2009): 0.04-12; (w.w.)	PFASs geometric mean range (1986-2009): 0.05-10; (w.w.)	Ahrens et al. (2011)

Peregrine falcon (*Falco peregrinus*) (n=13)	Spain/ 2003-2006	NFRs: -Dec: 602, 603, 604 -DP	Eggs	Dec: n.d.-25; (l.w.) ∑DP: 0.3-17; (l.w.)	Dec geometric mean range: 0.2-8; (l.w.) ∑DP: 2; (l.w.)	Guerra et al. (2011)
Peregrine falcon (*Falco peregrinus*) (n=10)	Sweden/ 1974-2007	PFASs: -PFCAs: PFHxA, PFHpA, PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTriA, PFTeA, PFPeA -PFSAs: PFBS, PFHxS, PFOS, PFDcS -PFOSA	Eggs	PFASs: 0.3-220; (w.w.)	PFASs arithmetic mean range: 0.6-83; (w.w.)	Holmström et al. (2010)
Eurasian sparrowhawk (*Accipiter nisus*) (n=10)	Belgium/ -	PFASs: -PFCAs: PFOA, PFNA -PFSAs: PFHxS, PFOS	Liver	PFOSs: 47.6-775; (w.w.) Other PFASs: 3.2-40.6; (w.w.)	PFOS mean: 236; (w.w.)	Meyer et al. (2009)
			Feathers	-	PFOS mean: 102; (d.w.)	
White-tailed eagle (*Haliaeetus albicilla*) (n=-)	Germany and Poland/ 1979-1999	PFASs: -PFCAs: PFOA -PFSAs: PFOS -PFOSA	Liver	PFOS: 3.9-127;(w.w.) Other: n.d.	-	Kannan et al. (2002)

n.d.: non detected;**w.w.:** wet weight; **d.w.:** dry weight;**l.w.:** lipid weight

Table 3. Sample treatment strategies and analytical techniques and quantification limits for the determination of CECs in European raptors (2002-2020). The studies are ordered by year of publication, starting with the most recent ones.

Sample treatment technique(s) (extraction and clean-up)	Analytical technique(s)/column/mobile phase	LOQs (ng mL^{-1} ; ng g^{-1})	References
BPs and BzP UV-filters: (0.1-0.2 g tissue) Solvent extraction with ethyl acetate.	LC-MS-MS / C_{18} (2.1× 50 mm, 1.3µm)/(A) 0.1% v/v ammonium hydroxide in water and (B) methanol	0.01-6.5	González-Rubio et al. (2020)
NFRs: Solvent extraction with n-hexane:DCM (4:1, v/v) (~160 mg feathers and 1mL plasma) or DCM (preen oil) and clean-up with SPE (Supelclean ENVI Florisil; only for preen oil). PFASs: Solvent extraction with MeOH (0.2 mL plasma) or NaOH (200 Mm) in MeOH(~160 mg feathers) and clean-up with d-SPE (Supelclean ENVI-Carb and acetic acid)	NFRs: GC-MS/ DB-5 (30 m x 0.25 mm, 0.25 µm) for PBDEs and NBFRs or a HT-8 (25 x 0.22 mm, 0.25 µm) for OPFRs. PFASs: LC-MS-MS/ HSS-3T (2.1 x 100 mm, 1.8 µm); HSS-T3 guard column (2.1 x 5 mm, 1.8 µm)/)/ 2 mM NH_4OAc in 90:10 MeOH:water and 2 mM NH4OAc in MeOH	0.1-0.4	Briels et al. (2019)
NFRs: (1 g egg) PLE with hexane:DCM (1:1) and clean-up with SPE (alumina cartridge).	GC-(NCI)-MS-MS/ DB-5 (15 m x 0.25 mm, 0.1 µm)	0.007-32.2	Eljarrat et al. (2019)
NFRs: Solvent extraction with n-hexane (0.013-0.04 g preen oil) orn-hexane:DCM (4:1 v/v) (1 mL plasma and 0.2 g feathers) PFASs: Solvent extraction with MeOH (200-300 µL plasma) or 2M HCl in MeOH (0.2 g feathers) and clean-up with d-SPE (Supelclean ENVI-Carb)	NFRs: GC-MS/ DB-5 (30 m x 0.25 mm, 0.25 µm)/- PFASs: LC-MS-MS/ C_{18} (2.1× 50 mm, 5 µm)/ 2 mM NH_4OAc in 90:10 MeOH:water and 2 mM NH4OAc in MeOH	NFRs: 0.002-4 PFASs: 0.05-0.4	Løseth et al. (2019a)

PFASs (200-300 µL plasma): Solvent extraction with MeOH and clean-up with d-SPE (Supelclean ENVI-Carb, 50 µL of glacial acetic acid)	LC-MS-MS/ C_{18} (2.1× 50 mm, 5 µm)/ 2 mM NH4OAc in 90:10 MeOH:water and 2 mM NH4OAc in MeOH	0.05-0.1	Løseth et al. (2019b)
NFRs (0.14-0.22 g feathers): Solvent extraction with n-hexane-DCM (4:1, v:v) and clean-up with SPE (Supelclean ENVI Florisil topped with anhydrous Na_2SO_4)	GC-MS/HT-8 (25 m x 0.22 mm x 0.25 µm)/-	1	Monclús et al. (2019)
PFASs (0.2 mL plasma): Solvent extraction with ACN and clean-up with d-SPE with activated carbon and glacial acetic acid.	LC-MS-MS/ C_{18}(4.6 x 50 mm, 3.5 µm)/ water with 10 mM ammonium acetate/MeOH (80:20, v/v) and CAN with 10 mM ammonium acetate	0.01-0.1 (LOD)	Ortiz-Santaliestra (2019)
PFASs (~0.18 g feathers): Solvent extraction with MeOH and clean-up with SPE (ENVI-Carb with acetic acid column)	HPLC-QTRAP-MS-MS/-	0.006-0.15 (MDL)	Sun et al. (2019)
PFASs (1 g eggs): Solvent extraction with ACN and clean-up with d-SPE (Supelclean ENVI-Carb) PCNs (1 g eggs): SE withn-hexane:acetone (4:1 v/v) and clean-up with a multi-layer column of aluminium oxide, silica and acid-treated silica	PFASs: LC-QTRAP-MS-MS/C_{18} (2.1 x 150 mm)/ 2mM ammonium acetate:MeOH (90:10, v/v) and 2 mM ammonium acetate:MeOH (10:90, v/v) PCNs: GC-MS/DB-5 (0.25mm, 0.25 µm)/-	PFASs: 0.004-0.2 PCNs: 0.003-0.02	Vorkamp et al. (2019)
Paraffins (2-3 g muscle): Accelerated solvent extraction with DCM:n-hexane (1:1) and clean-up with SPE (multilayered column of Florisil, silica gel, acid silica gel and anhydrous sodium sulfate). Paraffins: (10 g eggs): Solvent extraction with isopropanol and n-hexane:diethyl ether (3:1, v:v) and clean-up with a multilayer SPE column.	APCI-QTOF-MS	0.4-41	Yuan et al. (2019)

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NFRs (1.5 g eggs): Pressurized liquid extraction (PLE) with n-hexane-DCM (1:1 v/v) and clean-up SPE (neutral alumina)	GC-(NCI)-MS-MS/ DB-5 (15 m × 0.1 mm, 0.1 µm)/-	0.007-0.07	Blanco et al. (2018)
Neonicotinoids (100 µL blood): Modified QuEChERS method with ACN extraction and clean-up with d-SPE (Supel QuE PSA-C18-GCB)	LC-MS-MS/C_{18} (1.7 µm, 2.1 mm × 100 mm); C_{18} pre-column (130 Å, 1.7 µm, 2.1 mm × 5 mm)/95 % water, 5 % ACN, 5 mM ammonium formate, 0.1 % formic acid and 95 % ACN, 5 % water, 5 mM ammonium formate, 0.1 % formic acid	0.0006-0.03	Byholm et al. (2018)
NFRs (0.14-0.22 g feathers): Solvent extraction with n-hexane-DCM (4:1, v:v) and clean-up with SPE (Supelclean ENVI Florisil topped with anhydrous Na_2SO_4)	GC-MS/HT-8 (25 m x 0.22 mm x 0.25 µm)/-	1	Monclús et al. (2018)
PFASs (200 µL plasma and <0.1 g feathers): Solvent extraction with MeOH and clean-up with d-SPE (graphitized carbon, 50 µL of glacial acetic acid)	LC-MS-MS/ C_{18} (2.1 x 50 mm, 5 µm)/ 2 mM NH_4OAc in 90:10 MeOH: water and 2 mM methanolic NH_4OAc	-	Gómez-Ramírez et al. (2017)
UV-filters (0.1 g eggs): PLE with EthylAcetate:DCM (1:1 v/v) and clean-up SPE(C_{18})	LC-QTRAP-MS-MS/ C_{18} (50mm × 2.0 mm, 5 µm); Guard column of the same packaging material/ Water and ACN, both with 0.15% formic acid	0.0005-0.02	Molins-Delgado et al. (2017)
BPA, OP, NP (0.1 g feathers): Solvent extraction with methanol, 0.01 M ammonium acetate and chloric acid (VII) and clean-up with SPE (Oasis HLB)	HPLC-Fluorescence detector/C_{18} PAH (250 x 4.6 mm; 5 µm)/ACN and water	0.5-2	Nehring et al. (2017)
Neonicotinoids (500 µL blood): modified QuEChERS extraction with ACN (acetic acid 1% v/v) and clean-up with d-SPE (magnesium sulphate, PSA and polymerically bonded trifunctional C_{18} silica sorbent)	LC-TOF-MS/ C_{18} (250 mm × 4.6, 25 µm)/ 0.1% formic acid in water/ammonium formiate 5 mM and 0.1% formic acid in acetonitrile	2-10	Taliansky-Chamudis et al. (2017)

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NFRs(0.1 g eggs): Solvent extraction with n-hexane and iso-octane and clean-up with deactivated silica gel (30% water, w/w)	GC-MS/HP-5 (30 m x 0.25 mm, 0.25 mm)/-	1.6-17	Vetter et al. (2017)
NFRs (- g eggs): SE with hexane:acetone (4:1) and clean-up with SPE (column with alumimium oxide, silica (with and without H_2SO_4) and Na_2SO_4.	GC-MS/DB-1701 (60 m) and DB-1 (15 m) for DBDPE and BEH-TEBP		Vorkamp et al. (2017)
PFASs (0.25 g eggs): Solvent extraction with ACN and clean-up with d-SPE (ENVI-carb, 100 µL glacial acetic acid)	LC-MS-MS/C_{18} (2.1 mm, 1.7 µm)/2 mM ammonium acetate	-	Eriksson et al. (2016)
PFASs (0.5 g eggs): Solvent extraction with ACN and clean up with d-SPE(graphitized carbon and acetic acid)	LC-MS-MS/C_{18} (50 x 2.1 mm, 1.7 µm)/2 mM ammonium acetate in MeOH and water.	0.01-0.3	Faxneld et al. (2016)
PFASs (200 µL plasma): Solvent extraction with MeOH and clean-up with d-SPE (ENVI-carb,100 µL glacial acetic acid)	LC-MS-MS/C_{18} (2.1 × 50 mm,5 µm)/2 mM NH_4OAc in 90:10 MeOH:water and 2 mM NH_4OAc in MeOH	-	Sletten et al. (2016)
Parabens (0.2-0.3 g liver): Solvent extraction with MeOH:ACN (1:1, v/v).	HPLC-MS-MS/ Zorbax SB-Aq (2.1 x 150mm, 3.5 µm); Guard column C_{18} (2.1 x 20mm, 5 µm)/ MeOH and water with 0.4% (v/v) acetic acid.	0.1-0.5	Xue&Kannan (2016)

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NFRs (1 g eggs): PLE with n-hexane:DCM (1:1 v/v) and clean-up with SPE (alumina)	GC-MS-MS/DB-5 (15 m × 0.1 mm, 0.1 μm)/-	0.05-0.8	Barón et al. (2015)
NFRs (1.5 g eggs): PLE with hexane: DCM (1:1 v/v) and clean-up with SPE (Alumina)	GC-MS-MS/(15 m × 0.1 mm, 0.1 μm)/-	0.03-5	Barón et al. (2014)
PFASs (1 g eggs): Solvent extraction with ACN and clean-up with d-SPE (ENVI-Carb, glacial acetic acid)	LC-QTOF-MS/C_{18} column (150 x 2.1mm, 3 μm)/ MeOH and water (both with 2 mM NH_4OAc)	LODs: 0.06	Bustnes et al. (2014)
PFASs (0.2 g plasma): Solvent extraction with ACN and clean up with d-SPE (ENVI-Carb)	LC-QTOF-MS/ C_{18} (150 x 2.1mm, 3 μm)/ MeOH:water (both with 2 mM NH4OAc)	-	Bustnes et al. (2013)
NFRs (1 mL plasma and 0.2 g feathers): Solvent extraction with hexane:DCM (4/3:1, v:v) (NFRs) or hexane (PBDEs) and clean-up with SPE (Supelclean ENVI-Florisil topped with anhydrous Na_2SO_4)	GC-MS/ DB-5 (30 m x 0.25 mm, 0.25 μm) for PBDEs; HT-8 (25 m x 0.22 mm, 0.25 μm) for NFRs/-	0.02-5	Eulaers et al. (2014)
PFASs (1 g tissues and 0.3 g preen oil): Solvent extraction with MeOH and clean-up with d-SPE (ENVI-Carb, glacial acetic acid)	LC-TOF-MS/C_{18} (150 × 2.1 mm, 3 μm); Guard column (5 × 2.1 mm, 3 μm)/MeOH and water (both containing 2 mM NH_4OAc)	LODs: 0.5-60	Jaspers et al. (2013)

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PFASs(0.2 g plasma): Solvent extraction with ACN and clean-up with d-SPE (ENVI-Carb).	LC-QTOF-MS/ C_{18} (150 x 2.1mm, 3 μm)/ MeOH:water (both with 2 mM NH4OAc)	-	Sonne et al. (2012)
PFASs (0.2 g plasma): Solvent extraction with ACN and clean up with d-SPE (ENVI-Carb)	LC-TOF-MS/ C_{18} (150 x 2.1mm, 3 μm)/ MeOH:water (both with 2 mM NH4OAc)	-	Sonne et al. (2010)
PFASs (1 g eggs): Solvent extraction with ACN and clean up with d-SPE (ENVI-Carb)	LC-QTOF-MS/ C_{18} (150 x 2.1mm, 3 μm)/ MeOH:water (both with 2 mM NH4OAc)	LODs: 0.01-0.05	Ahrens et al. (2011)
NFRs (3-5 g eggs): SE with DCM and clean-up with GPC (Bio Beads SX-3 column) and a silica gel column	GC-MS/ DB-5HT (0.25 mm, 0.10 μm)/-	0.01-1	Guerra et al. (2011)
PFASs (0.2-0.3 eggs): Solvent extraction with ACN and clean-up with d-SPE (Supelclean ENVI-Carb)	LC-MS-MS/ C_{18} (150 × 2.1 mm, 3 μm)/ (2 mM ammonium acetate) MeOH and Milli-Q water	LODs: 0.04-2	Holmström et al. (2010)
PFASs Solvent extraction with ACN and clean-up with d-SPE (activated carbon, glacial acetic acid) (1 g liver) or SPE (Oasis Plus) (1 g feathers)	LC-MS-MS/Fluophase PFP column (50mm×1 mm); C_{18} pre-column (10 mm×1 mm)/ 2 mM NH_4OAc and ACN	LOD: 3.2	Meyer et al. (2009)

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PFASs (1 g liver): Solvent extraction with methyl tert-butyl ether	LC-MS-MS/C_{18} (50 x 2mm, 5 µm)/2 mM NH_4OAc and MeOH		1-72	Kannan et al. (2002)

PSA: Primary secondary amine; **GCB:**Gratiphized carbon black; **PLE:**Pressurized liquid extraction; **SE:** Soxhlet extraction; **Na_2SO_4**: sodium sulfate; **SPE:** solid phase extraction; **GPC:** gel permeation chromatography; **d-SPE:**dispersive solid phase extraction; **GC-MS:** Gas chromatography coupled to mass spectrometry; **LC-MS-MS:**Liquid chromatography coupled to a triple quadrupole spectrometer; **LC-QTOF-MS**: Liquid chromatography coupled to a quadrupole-time of flight mass spectrometer; **LOD:** Limit of detection; **MDL:** Method detection limit; **PFP:** Pentafluorophenyl

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3.1. Occurrence of PFASs, NBRs and other CECs in raptors across Europe

Most of the studies on raptors from Europe focus on POPs; we only found 19 studies on PFASs, 12 on NFRs and 6 on other miscellaneous CECs. An overview of the European countries in which the different classes of contaminants have been assessed in the respective studies is presented in Fig. 1.

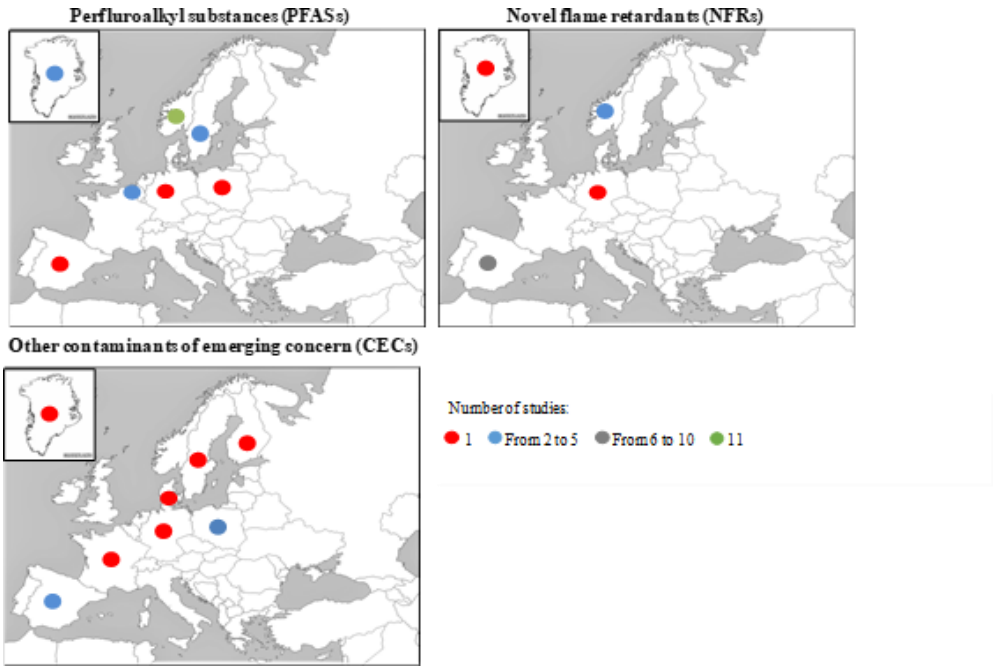

Fig. 1. Maps showing the locations of the studies performed on PFASs, NFRs and other CECs on raptors (birds of prey and owls) in Europe (2002-2020).

As it can be observed, most of the analyses were performed in Western Europe, mainly in Norway and Spain. Raptors from Norway (11 out of 19 studies) were the most collected for the analysis of PFASs and those from Spain for NFRs (7 studies out 12). The compounds that were most frequently determined within each group were carboxylates (PFCAs) and sulfonates (PFSAs), especially

perfluorooctane sulfonic acid (PFOS), for PFASs, and dechloranes (Dec), dechlorane plus (DP; derivative of dechlorane class), and phosphorus flame retardants (PFRs) for NFRs. Regarding the analysis of the other miscellaneous CECs, only six studies were found in the literature with samples from different European countries: two on neonicotinoids in Finland (Byholm et al., 2018) and in Spain (Taliansky-Chamudis et al., 2017), one on UV filters in Spain (Molins-Delgado et al., 2017), one on parabens in Germany and Polish coastal areas of the Baltic Sea (Xue and Kannan, 2016), one on paraffins in Sweden and Denmark (Yuan et al., 2019) and one on bisphenols and benzophenone-type UV-filters in Greenland and France (González-Rubio et al., 2020).

3.1.1. Perfluoroalkyl substances (PFASs)

PFASs are a group of chemicals of anthropogenic origin that have been in use since the 1940s in many consumer products like cookware, food packaging, and stain repellants (Giesy and Kannan, 2001; Begley et al., 2005; Gewurtz et al., 2009). Most of them exhibit similar structures consisting of a partially or fully fluorinated alkyl chain with a particular functional head group leading to compounds with unique physicochemical properties. PFASs are ubiquitous environmental contaminants and, although they exhibit favorable properties in manufacturing, they have proved to be toxic, very resistant to degradation and long-term bioaccumulative (Cousins et al., 2019; Hekster et al., 2003). However, so far, only perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) have been restricted in production and use from 2009 and 2020, respectively, according to Annex B and A of the Stockholm Convention on Persistent Organic Pollutants (UNEP, 2009a; UNEP, 2020). Others have been only recommended for voluntary phase-out, or still unrestricted in their use. The concern related to the toxicity of PFASs has considerably increased, the number of studies related to these compounds over the past decade (Ghisi et al.,

2019, Galatius et al., 2013). Among PFASs, PFCAs and PFSAs are the most studied and bioaccumulate in marine food chains, with highest concentrations found in organisms at higher trophic levels (Kannan et al., 2005). While the toxic effects of legacy compounds have frequently been studied in birds of prey (Bustnes et al., 2011; Mora et al., 2011; Crosse et al., 2013; Elliot et al., 2015), the knowledge about the potential effects of emerging compounds such as several PFASs is scarce. In this section, the most relevant results from studies on PFAS from raptor samples collected in Europe have been reported.

3.1.1.1. PFASs concentrations in liver samples.

An increasing interest has emerged on the presence of PFASs in raptors in Europe since in 2002. Kannan et al. reported for the first time the occurrence of PFOS and related PFASs in liver samples of white-tailed eagles collected from 1979 to 1999 in Germany and Polish coastal areas of the Baltic Sea. They only found quantificable concentrations of PFOS, ranging from <3.9 to 127 ng g–1 wet weigh (ww). Higher concentrations of PFOS were detected in further studies in liver samples collected from Belgium: a concentration range from47.6 to 775 ng g–1(ww)was reported in Eurasian sparrowhawks (Meyer et al., 2009) and from 42 to 992 ng g–1 (ww) in Barn owls (Jaspers et al., 2013). These higher concentrations were potentially due to that individuals from both studies were sampled from an area in the close vicinity of the city of Antwerp (Belgium), where a major PFAS chemical plant is located. Other PFASs were detected in both studies but at lower concentrations (3.2–40.6 ng g–1 (ww) in Meyer et al., 2009 and of 6–116 ng g–1 (ww) in Jaspers et al., 2013.

3.1.1.2. PFASs concentrations and temporal trends in eggs.

Many studies determined PFASs in eggs (6 out of 19). Eggs were found suitable to study temporal trends of PFASs concentrations (Ahrens et al., 2011; Bustnes et al., 2014; Faxneld et al., 2016). However, it is difficult to compare the results of different studies due to the fact that concentrations (although reported as ng g–1) were expressed in different ways (in wet, dry or lipid weight, in arithmetic or geometric means or medians). This highlights the need to harmonize the units for reporting levels in environmental analysis and to have additional data with the publications on wet weight and lipid percentage regardless of the units used in the publication. Although a decline of PFOS should be expected as a result of the phase-out and regulatory interventions, most of them have reported it as the most abundant PFAS being present at high concentrations that kept constant during the whole examined time period. This may be due to the high persistence and long half live of this compound or due to a short course of time between the phase-out and the sample analysis. In 2010, Holmström et al. established for the first-time temporal trends for PFASs in terrestrial biota with the analysis of Swedish peregrine falcon (Falco peregrinus) eggs collected from 1974 to 2007. Authors showed that the overall PFAS profile was dominated by PFOS (83 ng g–1wwmean concentration in samples from 2006) and changes in concentrations were not significant between 1984 and 2006 (mean concentrations: 80–90 ng g–1 (ww)). In contrast, increasing temporal trends of long-chain PFCAs were recorded in many studies. PFCA bioaccumulation potential increases with increasing chain length and they seem to accumulate to a higher extent in lipid-rich tissue, including eggs, than PFASs with shorter chains (Braune and Letcher, 2013). Holmström et al. (2010) reported that PFCA concentrations increased exponentially over the studied time period. Similar results were observed by Eriksson et al. (2016) and Vorkamp et al. (2019). Significant differences in PFOS concentrations in Swedish osprey (Pandion haliaetus) eggs within the periods 1997–2001 (103 ng g–1 ww), 2008–2009 (64 ng g–1 ww) and 2013 (70 ng g–1ww)were determined in Eriksson et al. (2016). Furthermore, a significant increase was observed for long chain PFCAs (C10-C14) in years 1997–2001 and

2008–2009. Concentrations of PFASs in the eggs of peregrine falcons from Greenland collected between 1986 and 2014 (100–303 ng g–1 dw; ΣPFAS median range) were also reported in Vorkamp et al. (2019). In this case, PFOS accounted for 94% on average of all PFSAs, but did not show a significant time trend. The long-chain PFCAs C9, C12 and C14 showed increasing concentrations, with an annual increase of 0.8%, 2.9% and 5.6%, respectively (Vorkamp et al., 2019). A longer term study reported temporal and spatial trends of PFASs in eggs from white-tailed sea eagles from two fresh water and two marine areas in Sweden collected from 1966 to 2010 (Faxneld et al., 2016). Most PFASs showed a general increase (from the 1960s/1980s to 2010) and significant decreasing concentrations were not observed during the most recent years, including PFOS, which is in the same line with the above-mentioned recent studies. Some studies have shown decreasing concentrations of PFOS, for example in tawny owl (Strix aluco) eggs from Norway (1986–2009) with an annual decrease of 1.6% (Ahrens et al., 2011). However, C10-C13 PFCA concentrations increased significantly with an annual increase of 4.2–12%, as observed by other authors (Eriksson et al., 2016; Holmström et al., 2010). The results of Ahrens et al. (2011) suggested that individual traits had very little impact on the concentrations of PFASs in the eggs while there was an important influence of annual variation in environmental conditions such as the North Atlantic Oscillation, temperature, snow or food availability.

3.1.1.3. PFASs concentrations in plasma.

PFASs have also frequently been determined in blood plasma (9 out of 19 studies). In fact, half of the studies analyzing PFASs in raptors have used plasma as preferred matrix due to their protein affinity. Thus, these compounds are primarily retained in blood plasma and internal organs due to their physicochemical properties. The negative impact of the presence of organohalogen compounds in the clinical-chemical parameters of blood plasma was first investigated in 2010 by

Sonne et al. in chicks of three raptor species from Norway (2008–2009). Furthermore, PFASs concentrations in this study (21–55 ng mL–1 ww ;ΣPFAS, median) were higher than those reported in further ones (median, ng mL–1 ww): 42 (Sletten et al., 2016), 17–46 (Gómez-Ramírez et al., 2017), 9–32 (Løseth et al., 2019b), 5–17.5 (Briels et al., 2019). In 2012, Sonne et al. carried out a larger study (Norwegian raptor nestlings, 2008–2010). Concentrations in this study (12–151 ng mL–1 ww; ΣPFAS mean range) were the highest reported in plasma from European raptors so far. Other studies in plasma reported concentrations of a single PFAS either because authors focused the investigation on one of them (PFOS median range: 6.2–13 ng mL–1 ww, Bustnes et al., 2013) or because only one compound was detected (PFUnA median range: 1.15–3.9 ng mL–1 ww, Løseth et al., 2019a, 2019b).

3.1.1.4. PFASs concentrations and temporal trends in feathers.

Sun et al. (2019) reported long-term (1968–2015) spatio-temporal trends of PFAS by using archived body feathers of white tailed-eagles from the West Greenland, Norwegian and Central Swedish Baltic coasts. They observed a significant decline of PFOS since the mid-1990s to 2000 in the Greenland and Norwegian subpopulations, consistent with the phaseout of PFOS-based compounds. However, increasing PFOS trends were observed in the Swedish subpopulation, which is probably due to the high remaining contamination in the Baltic Sea. PFOS concentrations were significantly higher in Sweden (median range: 3–20.1 ng g–1 ww) than in Norway (median range: 1.6–9.5 ng g–1 ww) and Greenland (median range: 1.6–9.5 ng g–1 ww) populations. FOSA (median ranges in ng g–1 ww; Sweden: 0.3–1.5, Norway: 0.7–3.5 Greenland: 1.1–8.3) and ΣPFCA concentrations (median ranges in ng g–1 ww; Sweden: 0.1–9, Norway: 2–6.1 Greenland: 3.1–8.2) were similar among the subpopulations. ΣPFCA concentrations significantly increased in all three subpopulations throughout the study periods. These results indicated that spatial differences in exposure may

influence time trends. Some studies investigated the associations between PFASs in blood plasma and body feathers to find out if feathers could be a non invasive strategy to monitor PFASs exposure in raptors. Gómez-Ramírez et al., 2017 determined PFAS concentrations in blood plasma (45 ng mL–1 ww;ΣPFASs mean) and body feathers (13 ng g–1 ww; ΣPFASs mean) from white-tailed eagle nestlings and found significant correlations between them. However, in Løseth et al., 2019a, only PFUnA was quantified in over 50% of both plasma (1.15–3.59 ng mL–1; PFUnA median range) and feather (0.15–0.58 ng g–1; PFUnA median range) samples, yet their correlation was poor and not significant. Therefore, the authors concluded that the use of feathers for monitoring PFASs may be limited, which is also supported by Jaspers et al. (2019). Correlations between plasma (4.7–17.5 ng mL–1 ww;ΣPFASs median range) and feathers (1.8–6.2 ng g–1 ww;ΣPFASs median range) were also under discussion in another study, since strong and significant associations were found for some PFAS compounds and no correlation was found for others (Briels et al., 2019). Finally, it is also worth mentioning that the lack of correlations between plasma and feathers in some cases may be due to the fact that the latter ones reflect atmospheric contamination and/or different exposure during their time of growth, which may or may not coincide with plasma collection.

3.1.1.5. PFASs concentrations in other matrices.

Other matrices have also sporadically been analyzed to determine PFASs such as muscle, preen oil and fat of barn owls from Belgium (Jaspers et al., 2013). Concentrations reported in this latter study were especially high in the preen oil (22–431 ng g–1 ww; PFAS median range). Furthermore, a recent study (González-Rubio et al., 2020) highlighted the potential role of the preen glad from white-tailed eagles from Greenland as a major excretory organ for a group of CECs (bisphenols and benzophenones), which may be also worth to investigate for PFASs. Therefore, the investigation of PFASs concentrations in excretory matrices (such as preen gland and oil) in future research it is highly recommended. Finally,

on the basis of the studies collected on PFASs in raptors in Europe, eggs and blood are the most used matrices. Furthermore, in plasma, PFASs present the higher detection frequencies for all the compounds in the higher concentrations (Briels et al., 2019; Løseth et al., 2019a) which suggest that the birds are recently and continuously exposed to these emerging contaminants, likely through dietary input. For the study of temporal trends, eggs are the most used matrix (Ahrens et al., 2011; Faxneld et al., 2016). Thus, depending on the type of study that is going to be carried out, either plasma or eggs are recommended. Feathers may not be that suitable, as suggested in Briels et al. (2019) and Løseth et al. (2019a), and other matrices need to be further investigated, with particular attention to excretory ones.

3.1.1.6. Comparison of PFASs and PBDEs.

Several studies simultaneously reported concentrations of PFASs and PBDEs (legacy organic contaminant group) in raptors. Table S1 in the Supporting Information provides a comparison between them. PBDEs are organobromine compounds widely used as flame retardants in building materials, electronics, plastics, textiles, and other materials (McGrath et al., 2017). Because of their toxicity and persistence, some PBDEs were phased-out in 2009 under the Stockholm Convention on persistent organic pollutants (UNEP, 2009b, 2009c). Thus, lower concentrations of these compounds are expected to be bioaccumulated compared to PFAS ones, for which only PFOS and PFOA have been restricted so far too. Table S1 shows PFAS and PBDE concentrations in plasma and feathers from six different studies. In two studies, plasma and feathers were collected from northern goshawks and white-tailed eagles from Norway between 2015 and 2016 (Briels et al., 2019, Løseth et al., 2019a). Other three studies analyzed the plasma of the same species and of golden eagle (Aquila chrysaetos) individuals from Norway in the period 2008–2010 (Sonne et al., 2010; Sonne et al., 2012; Løseth et al., 2019b). The other study analyzed these contaminants in plasma from Egyptian vultures (Neophron percnopterus) from Spain from 2010 to 2016 (Ortiz-

Santaliestra et al., 2019). PFASs concentrations in these studies in plasma were higher than those of PBDEs, as expected. However, when comparing PFASs and PBDEs concentrations in feathers (Table S1) differences were less consistent than those measured for plasma. For example, in Løseth et al. (2019a), the median range of BDE-47 (0.3–1.73 ng g–1) in feathers Norwegian white-tailed eagles was higher than the median range of PFUnA (0.15–0.58 ng g–1). This could be due to a lower PFAS detection frequency rate in feathers, which has been demonstrated in previous studies (Gómez-Ramírez et al., 2017). The rest of studies reported greater concentrations of PFASs than of PBDEs.

3.1.2. Novel flame retardants (NFRs)

Restrictions on the use of legacy flame retardants, such as PBDEs or hexabromocyclododecanes (HBCDs), have resulted in an increased use of novel flame retardants (NFRs) in recent years as alternative to meet flammability standards. Consequently, environmental concentrations of NFRs have been on the rise worldwide. Some of them have already been detected in humans (Ma et al., 2017; Pirard and Charlier, 2018), as well as in a few captive and wild birds (Gentes et al., 2012; Guigueno and Fernie, 2017), marine mammals (Lewis et al., 2020), and fish species (Sapozhnikova and Lehotay, 2013). Similar to PBDEs, NFRs are highly hydrophobic and display relatively low volatility. However, differences in their molecular structure result in specific differences in physicochemical properties and hence, in diverse behaviour in environmental and biological systems. The most common NFRs replacing PBDEs are organophosphorus flame retardants (OPFRs), dechlorane plus (DPs) and “novel” brominated flame retardants (NBFRs) such as decabromodiphenyl ethane (DBDPE), 1,2-bis(2,4,6-tribromophenoxy) ethane (BTBPE), 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB or EHTBB) and bis(2-ethylhexyl)-3,4,5,6-tetrabromo-phthalate (TBPH or BEHTBP). Interest regarding the analysis of NFRs in European raptors has grown in recent years. Furthermore, further investigation of these contaminants in raptors is needed

since these species seemto bemore sensitive to flame retardant toxicity than domesticated species and songbirds (Guigueno and Fernie, 2017). The first study to report the accumulation of dechlorane plus (DP) and dechloranes (Dec) in European biota was carried out by Guerra et al. (2011) by the analysis of eggs in peregrine falcons from Spain. ΣDec means ranged from 0.2 to 8 ng g–1 lipid weight (lw) and a ΣDP mean value of 2 ng g–1 (lw) was reported. In 2014, higher concentrations of Dec were detected in bird eggs of several raptor and non-raptor species from Doñana (a national natural park located in South-western Spain) as well as its surroundings, where small urban areas with intensive agriculture could be found (Barón et al., 2014). ΣDec means ranged from 7 to 46 ng g–1 (lw). In 2015, a temporal trend study was performed in the same geographical area (Barón et al., 2015), which reported Σ(Dec, DP) of 17–19 ng g–1 (lw, median range) in eggs of black kites (Milvus migrans) between 1993 and 2013. However, clear temporal trends were not observed for DP, and the authors highlighted the importance of continuous ecotoxicological monitoring of local species. Recently, Eljarrat et al., 2019 determined Σ(Dec, DP) in one egg of a western marsh harrier individual from central Spain at a mean concentration of 10 ng g–1lw. Regarding NBFRs, Vetter et al. (2017) found concentrations of several NBFRs (HBDC; 2,3-Dibromopropyl-2,4,6-tribromophenyl ether (DPTE); 2-Bromoallyl-2,4,6-tribromophenyl ether, (BATE); Allyl-2,4,6-tribromophenyl ether, (ATE); Hexabromo benzene, (HBB); 2-4-6-Tribromoanisole, (2-4-6-TBA); 2,4,6-Tribromophenol, (2-4-6 TBP); Pentabromoethyl benzene, (PBEB); Pentabromotoluene, (PeBT); mean range: 1–33 ng g–1lw) in the eggs of peregrine falcons collected in 2014 from Southern Germany. Higher NBFR concentrations (mean range: n.d-353 ng g–1 lw) were reported in eggs of black kites collected from an area in Madrid (Spain) that was considered as highly contaminated (Blanco et al., 2018). In 2017, Vorkamp et al. studied temporal trends of legacy and NFRs (TBB, BTBPE, DPTE, DBDPE, DP) in eggs of peregrine falcons from Greenland collected between 1986 and 2014. NBFRs had low median concentrations (ranging from 0.5–4.7 ng g–1 lw) and, as reported by Barón et al. (2015), DP did not show a significant increase. OPFRs (Tris (phenyl)

phosphate (TPhP); tris (1-chloro-2-propyl) phosphate, (TCiPP); Tris-(2-chloroisopropyl) phosphate, (TCPP); Tris (chloroethyl) phosphate, (TCEP); Tri-(2-butoxyethyl)-phosphate (TBOEP); 2-Ethylhexyl diphenyl phosphate, (EHDPP))were determined in feathers and plasma of white-tailed eagle nestlings from Norway (Eulaers et al., 2014). Concentrations in feathers (4–110 ng g–1 ww, median ranges) were much higher than those detected in plasma (<LOQ-0.22 ng mL–1) and were not significantly correlated. The authors pointed to atmospheric depositions of OPFRs on the feathers as a potential source of contamination (Eulaers et al., 2014). In another study, OPFRs (TCEP, TCiPP, TPhP, Tris-(2,3-dichloropropyl)-phosphate (TDCiPP) were determined in down and contour feathers of Spanish cinereous vulture (Aegypius monachus) nestlings and contaminants concentrations were compared between both types of feathers from the same individual (Monclús et al., 2018). They found that concentrations in contour (ΣOPFRs median range: 0.4–10.5 ng g–1 dry weight dw) and down feathers (ΣOPFRs median range: 0.4–13.6 ng g–1dw) were similar. On the basis of these results, a further study on the relationship between corticosterone and OPFR concentrations was assessed and no associations were found (Monclús et al., 2019). Briels et al. (2019) determined concentrations of legacy and NFRs (NBFR: BTBPE, TBB, TBPH; OPFRs: TCEP, TBOEP, TPHP, EHDPHP, TCiPP, TDCiPP; DP) in three non-destructive samples (preen oil, feathers and plasma) of northern goshawks. From all the NFRs analyzed in this study, OPFRs (25.5–206 ng g–1 ww; ΣOPFRs median range) were the dominant compounds in feathers and were thought to originate mainly from external deposition, as they were not detected in the other two matrices. Other NFRs (NBFRs and DPs) were generally not detected in the nestlings, suggesting low presence of these emerging contaminants in their environment and/or low absorption

3.1.2.1. Comparison of NFRs and PBDEs.

Table S2 shows the concentrations of NFRs and PBDEs derived from different studies. Most studies of egg samples collected before 2010, showed higher concentrations of PBDEs than of NFRs. For example, Vorkamp et al. (2017) reported ΣPBDEs andΣNFRs median ranges of 6–1900 ng g–1 and of 0.5–4.7 ng g–1, respectively, in the eggs of peregrine falcons collected from 1986 to 2004 in Greenland. Likewise, Barón et al. (2015) reported median ranges of ΣPBDEs and ΣNFRs of 20–55 and 17–19 ng g–1, respectively) in the eggs of black kites from Spain (1999–2013). In contrast, most studies based on samples collected after 2010 reported greater concentrations of NFRs than of PBDEs, this being in agreement with the increasing phase out and restrictions imposed on PBDEs (Briels et al., 2019; Løseth et al., 2019a, 2019b; Blanco et al., 2018; Monclús et al., 2018; Eulaers et al., 2014; Barón et al., 2014). However, due to their high persistence and bioaccumulation capacity, high PBDE concentrations are still detected in some European regions. For instance, Eljarrat et al. (2019) reportedΣNFRs andΣPBDEs means of 10 and 645 ng g–1 lw, respectively, from an unhatched western marsh harrier egg from central Spain. Although only one egg was determined, a high number of samples from other non-raptor species (collected from 2010 to 2017) were analyzed and PBDEs were detected in all of them at high concentration values. Nevertheless, around 50% decline in concentrations of PBDEs was determined (Eljarrat et al., 2019). High concentrations of PBDEs were also reported by Vetter et al. (2017) in the eggs of peregrine falcons from Germany collected in 2014 (ΣPBDEs mean range: 145–320 ng g–1). These concentrations were higher than the NFRs ones (ΣNFR mean 1–33 ng g–1).

3.1.3. Other contaminants of emerging concern

Due to the low number of studies (n=6) dealing with other emerging contaminant classes in raptors, a thorough analysis of their presence and occurrence trends in Europe was not feasible. Nevertheless, most of these articles were published in the last five years, suggesting a growing interest in their analysis and effects in raptors. Studies of these compounds on non-raptors species are also included for discussion. Neonicotinoids are insecticides that are widely used for pest control and concerns about negative impacts on non-target organisms have raised in the last years (Thompson et al., 2020). In 2018, the European Commission banned the outdoor use of clothianidin (Commission Implementing Regulation (EU), 2018a), imidacloprid (IMC) (Commission Implementing Regulation (EU), 2018b), and thiamethoxam (Commission Implementing Regulation (EU), 2018c) but no regulation have been established for the others yet. Although many studies have reported neonicotinoid bioaccumulation in birds from Europe (Addy-Orduna et al., 2019; Humann-Guilleminot et al., 2019; Lennon et al., 2019), little is known about the accumulation of these contaminants in raptors. In fact, no studies on analysis of neonicotinoids in these species were found beyond Europe. The first evaluation of the exposure of neonicotinoids in raptors was carried out in Taliansky-Chamudis et al. (2017). IMC was the only chemical found in the blood from Eurasian eagle owls (Bubo bubo) from Spain at a concentration of 3.3 ng mL–1. In a recent study, concentrations of three neonicotinoids were determined in the blood of European honey buzzards (Pernis apivorus), but they were found at relatively low concentrations (0.008–0.04 ng mL–1) (Byholm et al., 2018). This low bioaccumulation potential may be related to the fact that raptors are non-insectivorous species and may therefore be less exposed to neonicotinoids. For example, a recent large-scale study on neonicotinoids on avian species in the

United States, found a major decline in diversity of insectivore birds feeding in agricultural areas in comparison with non-grassland and non-insectivorous birds (Li et al., 2020). The decline found in this study indicates that birds more exposed to neonicotinoids (as songbirds) might die and therefore, raptors feeding on birds could be feeding on the less contaminated ones. A rapid metabolism or excretion of neonicotinoids in raptors can be another question to consider. However, although insectivorous birds are more exposed to pesticides, another study (Mineau and Whiteside, 2013) highlighted the possible impact of pesticides on raptors in North America and linked population decline of grassland birds (including some owls and raptors) with lethality of pesticides used. UV-filters, including benzophenones (BzPs), are a class of CECs that are frequently used in sunscreen products and personal care products (Asimakopoulos et al., 2014, 2016). They are PBT compounds and can contaminate drinking water and migrate from food packaging into food (Mao et al., 2019). Furthermore, they are linked to cancer, endocrine disruption, and organ system toxicity (Kinnberg et al., 2015; Watanabe et al., 2015). As a result of this, the use of benzophenone 3 (BzP-3), which is the most represented BzP analogue, is restricted in Europe in cosmetics since 2017 (Commission Regulation (EU), 2017). Many studies have analyzed BzPs in soil, water, sediments and food (Tarazona et al., 2010; Sánchez-Brunete et al., 2011; Li et al., 2012). However, studies on adverse effects and bioaccumulation of these compounds in wildlife are still scarce. The first study on UV-filters in European raptors was reported by Molins-Delgado et al. (2017). Seven UV-filters (Benzophenone-1 (BzP-1); BzP-3; 4-Hydroxybenzophenone (4-OH-BzP); 4,4'-dihydroxybenzophenone (4-DHB); octocrylene (OC); 2-ethylhexyl 4-(dimethylamino) benzoate (ODPABA); 2-(2Hbenzotriazole-2-yl)-4-methyl-6-(2-propyl)-phenol (AllylBZP)) were determined in unhatched eggs from Western marsh harriers (Circus aeruginosus) and common kestrels from a Spanish preserved area. Mean concentrations ranged between 1.5 and 210 ng g–1 d.w. for common kestrels and between 2.7 and 895 ng g–1 d.w for Western marsh harriers. Furthermore, high detection rates from 90 to 100% were reported for all

contaminants, thus indicating bioaccumulation. The rank order of mean concentrations for benzophenone-type UV-filters found in the eggs from the assessed raptor species was 4-OH-BzP > BzP-1 >BzP-3, which was in agreement to the rank found in a recent study on raptor tissues (González-Rubio et al., 2020). In the latter study, concentrations on benzophenone-type UV-filters and bisphenols in tissues (kidney, liver, preen gland, brain, muscle and fat) from white tailed eagles, Eurasian sparrowhawks and long-eared owls from Greenland and France were reported for the first time. Bisphenols (BPs) are another class of CECs that are used in the manufacture of plastic articles (Asimakopoulos et al., 2016). Among BPs, bisphenol A (BPA) is one of the highest production volume chemicals worldwide. However, its production and usage has been regulated due to a raised concern over widespread human exposure and associated health effects (Bonefeld-Jørgensen et al., 2007). The European Union prohibited its use in infant feeding bottles since 2011 (Commission Regulation (EU), 2011). As a result of this, several BP analogues (such as bisphenol F (BPF); bisphenols S (BPS); and bisphenol AF, (BPAF)) are nowadays introduced onto the market as substitutes (Chen et al., 2016). The occurrence and/or effects of BPs in wildlife have been reported (Flint et al., 2012; Bhandari et al., 2015). Regarding birds, many studies have analyzed these contaminants in domestic (particularly broiler chickens) avian species (Singh et al., 2016; Shilov et al., 2020) but few ones have investigated their accumulation on wild birds or raptors. A recent paper is the only peer-reviewed study to document BPA in raptors in Europe and multiresidues of other seven BPs in tissues from white-tailed eagles, long-eared owls and Eurasian sparrowhawks (González-Rubio et al., 2020). BPA and BPF were the dominant contaminant analogues based on the concentrations found in tissues. On the contrary, BPAF and bisphenol Z (BPZ) were not detected in any sample. BPA concentrations have been previously reported in raptor species from outside Europe (Elliott et al., 2019) and in non-raptor species (the herring gull, Larus argentatus) in Europe (Staniszewska et al., 2014; Nehring et al., 2017). Staniszewska et al. (2014) reported mean concentrations of BPA in the muscle, liver and guano of herring gulls from The

Gulf of Gdansk (Southern Baltic) of 62.9, 11 and 996 ng g–1 d.w., respectively. Nehring et al. (2017) also reported BPA concentrations in feathers from the herring gull (ranging on average from 132 to 153 ng g–1 d.w.). Regarding analyses carried out in raptors, Elliott et al. (2019) reported a median BPA concentration of 1.05 ng mL–1 in bald eagle (Haliaeetus leucocephalus) nestling plasma samples (n = 381) from the U.S. In Europe, BPA was detected in preen gland of raptors at concentrations ranging 64.1–149 ng g–1 w.w. (González-Rubio et al., 2020). Parabens are a group of substances of emerging concern commonly employed as preservatives, mainly in personal care products, pharmaceuticals, and food (Błędzka et al., 2014). Parabens are believed to exert endocrine disrupting properties (Vitku et al., 2018). They have been linked to breast cancer, skin cancer, and decreased sperm count (Charles and Darbre, 2013). Because of these risks, five different parabens (propylparaben (PrP); butylparaben (BuP); phenylparaben (PhP); benzylparaben, (BnP) and pentylparaben (PeP)) were banned in cosmetic products in the European Union in 2014 (Commission Regulation (EU), 2014), while others are strictly regulated. Methylparaben (MeP) and PrP are the most commonly used parabens and are often used in combination. Although several studies have reported the ubiquitous occurrence of parabens in humans and the environment (Jiménez-Díaz et al., 2016, Asimakopoulos et al., 2014), little is known about the accumulation of these chemicals in birds. The only study on parabens that has been carried out in raptors found MeP, ethylparaben (EtP), BuP and heptylparaben (HeP) concentrations in 20 liver samples of white-tailed sea eagles from the Baltic Sea coast (Xue and Kannan, 2016). MeP was the most abundant compound, as expected, with a detection rate of 75%, at concentrations that ranged from <8.01 to 657 ng g–1. Furthermore, a very high concentration of 4-hydroxybenzoate (4-HB; 68,600 ng g–1 ww) was reported in the liver of one individual and was detected in 91% of the tissue samples (Xue and Kannan, 2016). A major bioaccumulation of 4-HB might be expected, as it is considered to be the final metabolite of parabens in biota (Aubert et al., 2012). Chlorinated paraffins (CPs) are other industrial chemicals of concern with an extensive global use as

metal-cutting fluids, flame retardants, plasticizers, and sealants (Bayen et al., 2006). They are highly lipophilic and bioaccumulative in humans and wildlife. In 2012, short carbon chain paraffins (SCCPs) were banned in EU but no regulations for medium carbon chain paraffins (MCCPs) or long carbon chain paraffins (LCCPs) are yet established (Commission Regulation (EU), 2012). Regarding their accumulation, very little has been studied in raptors so far. A recent study reported concentrations of CPs in the muscle and eggs of white-tailed eagles, eagle owls, golden eagles (Aquila chrysaetos), peregrine falcons, common kestrels, and tawny owls from Sweden, Denmark and the Baltic Sea (Yuan et al., 2019). LCCPs concentrations were highest and predominated (55% of total CPs) in the muscle of peregrine falcons (LCCP median range: 210–1200 ng g–1 lw). Another recent study outside Europe (Fernie et al., 2020), described for first time the exposure related toxicity and potential effects of SCCPs in American kestrels (Falco sparverius). Authors observed changes in thyroid function. The potential impact of these changes on thyroid-mediated growth and survival in wild birds requires further investigation. These results highlight the lack of data on CECs other than NFRs and PFASs. Due to the high concentrations reported for some of these CEC classes and their potential toxicity, further studies are urgently needed to assess contamination concentrations and evaluate the environmental threat they may pose.

3.2. Suitability of sample matrices for the determination of CECs in raptors

As reviewed above, different sample matrices (e.g., eggs, feathers, preen oil, blood, plasma, serum, and tissues) have been widely collected from different raptor species across Europe to analyze and monitor different types of contaminants. There is not an optimal all-purpose tissue for monitoring contaminants and the scientific relevance of each matrix depends on the aims and objectives of each study and the compound(s) of interest ("fit-for-purpose").

Previous surveys have attempted to show the most suitable matrices for determining major contaminant classes. Espín et al. (2016) published a study in which 249 papers were collected to provide information on sample types most widely used in Europe for the determination of certain contaminant groups, such as POPs, PFASs, metals and anticoagulant rodenticides. They concluded that there is no optimal all-purpose tissue and that the scientific relevance of each sample type depends on the aims and objectives of any study and the compound (s) of interest. Although Espín et al. (2016) provided useful information regarding the matrices that can be used for POPs, data on raptor matrices used to determine CECs have not been reported yet. Fig. 2 shows the matrices that were predominantly used to measure PFASs, NFRs and other CECs in raptors during the period 2002–2020. Data on other miscellaneous CECs (neonicotinoids, parabens, paraffins, bisphenols and UV-filters) was not representative due to the low number of reported studies (n = 6).

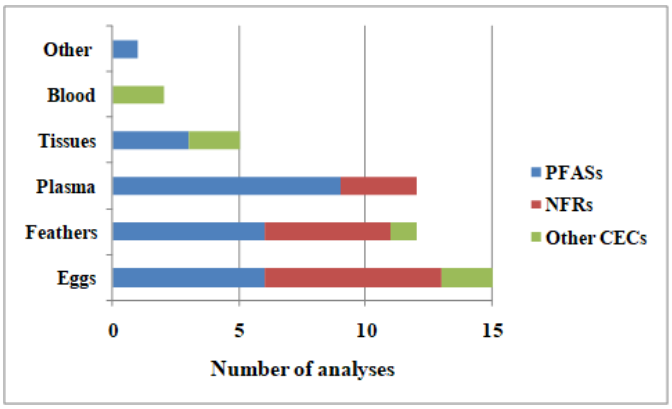

Fig. 2. Number of studies on PFASs, NFRs and other CECs on different raptor matrices in Europe (2002-2020). Preen oil and adipose tissue were referred to as "other".

Plasma, feathers and eggs were the most common sample types used for determining PFASs and NFRs in raptors, as can be observed. These results are in accordance with previous studies performed on raptors (Gómez-Ramírez et al., 2014; Espín et al., 2016) and suggest that eggs and feathers are still the most popular

matrices for pollution biomonitoring since their collection and storage effort is relatively easy (Espín et al., 2020). In addition, their use in biomonitoring offers several advantages as will be discussed below.

3.2.1. Eggs

Many organic contaminants are sequestered in eggs during their formation (Espín et al., 2016). For instance, critical concentrations of PFASs have been found in different studies in the last years (Vorkamp et al., 2019; Holmström et al., 2010). PFASs, NFRs and other organic contaminants are transferred to the yolk depending on the amount of lipids invested in a clutch of eggs, which is different depending on the species (Herzke et al., 2005). Another important aspect is that contamination concentrations in eggs are directly related to the concentrations in female raptors and reflect the exposure in this segment of the population. Furthermore, eggs are useful for the assessment of contaminants concentrations over time and space, since they can accumulate contaminants that were assimilated in previous time periods of variable duration by breeding females (Bourgeon et al., 2013).

3.2.2. Feathers

Feathers are non-destructive samples that have typically been used for monitoring heavy metal concentrations (Burger, 1993). During the last decades their use for biomonitoring organic contaminants is also increasing and has been successfully validated for monitoring internal concentrations of legacy POPs (Jaspers et al., 2006, 2019). Nevertheless, a recent study (Løseth et al., 2019a) indicated that the use of feathers for monitoring CECs may be limited. The study of Løseth et al. (2019a) was the first that presented a wide investigation of the concentrations of both legacy and CECs (specifically PFRs, NBFRs, PFASs and

DPs) in feathers and preen oil in relation to plasma of raptor species from Norway. The authors found good correlations for the legacy POPs but not for PFASs, NFRs and DPs. It is also worth mentioning that Eulaers et al. (2014) found that atmospheric depositions of NFRs on the feathers could cause external contamination. As indicated by Jaspers et al., 2019, no studies have been carried out thus far regarding the suitability of feathers for the analysis of other CECs in raptors, such as BPs or UV-filters. However, previous studies have reported concentrations of BPA and alkylphenols in the feathers of a non-raptor species (the herring gull) from the Baltic Sea (Staniszewska et al., 2014; Nehring et al., 2017). Feathers were selected as the matrix of study because, although BPs and alkyl phenols are generally accumulated by living beings through the dietary intake (Xiao et al., 2006; American Chemistry Council, 2016), their corresponding elimination from the organisms can be achieved through their incorporation into feathers and also laying eggs (Grajewska et al., 2015). The results showed that the contamination in the herring gull feathers depended on the inhabited area and, consequently on the diet of this species. A diet rich in fish manifested elevated BPA concentrations in covert feathers (29–512 ng g–1).

3.2.3. Blood

There is a limited number of articles that use whole blood to determine the target contaminants (Byholm et al., 2018; Taliansky-Chamudis et al., 2017). Although blood is a non-lethal sampling matrix, sample volume is sometimes limited by body size. Furthermore, contaminant half-lives are relatively short in blood and contaminant concentrations are generally lower and more variable than in other matrices. For lipophilic substances, such as POPs, low concentrations are expected in blood since the lipid content in this biological fluid is relatively low compared to other commonly sampled tissues such as eggs. Studies in which analysis were carried out in whole blood were only found for neonicotinoids, which are polar insecticides, but not for PFASs or NFRs (Fig. 2). In all cases, small

volumes of bird blood were used (~100–500 μL) and low limits of quantification were obtained (at the pg ml–1 range). Most of the studies analyzing PFASs in raptors have used plasma as preferred matrix due to their protein affinity. Concentrations of studied PFASs in whole blood are approximately half those in plasma (Ehresman et al., 2007). This difference is attributed to volume displacement by red blood cells (and that fluorochemicals are potentially not present intracellularly (Ehresman et al., 2007)). Apart from PFASs, plasma has also been used for the determination of NFRs (Løseth et al., 2019a; Eulaers et al., 2011), because most lipid soluble contaminants are attached to the triglycerides present in this matrix. Another thing to consider is that although lipophilic contaminants do not build up in blood, they still circulate in the blood, and these concentrations may be more representative of recent exposure, either via diet or recent mobilization from lipid stores if a bird is in a fasted state for any reason.

3.2.4. Internal tissues

Studies on internal organs and tissues are less common and limited to PFASs and other CECs analysis (see Fig. 2), since most raptors are protected species. Thus, the collection of these matrices for biomonitoring purposes is based mainly on carcasses. Liver and fat were the most analyzed matrices since non-polar compounds are mainly accumulated in matrices that contain a high proportion of triglycerides (Jaspers et al., 2013; Eulaers et al., 2014). González-Rubio et al. (2020) reported that the liver, kidney and preen gland tissues from raptors were suitable for the biomonitoring of BPs and BzP contaminants. Furthermore, a greater bioaccumulation of these chemicals in preen gland was found and a need for further investigation of the role of this matrix as a potential excretory organ for CECs was highlighted.

3.3. Selection of raptor species for biomonitoring CECs

Most studies on CECs in raptors in Europe have been performed on eagles, owls and Accipiters which are at the top of the food chain and therefore, may be most susceptible to effects. Depending on the species, raptors may be migratory and therefore it is often difficult to know where they incorporated contaminants during the annual cycle (Badry et al., 2020). Furthermore, the choice of species and associated traits (such as foraging in terrestrial or freshwater habitats) need to be matched to the fate pathways of the compounds of interest. Thus, the accumulation of different POPs and CECs in different raptor species may vary. The selection of species for previous biomonitoring studies was mainly performed based on the abundance, geographical distribution and conservation status. Recently Badry et al. (2020) proposed a selection for raptor species for monitoring priority pollutants in Europe (mercury, lead, anticoagulant rodenticides, pesticides and medicinal products) based on information on the distribution and key ecological traits (food web, foraging trait, diet, preferred habitat, and migratory behaviour). They identified the common buzzard (Buteo buteo) and tawny owl (Strix aluco) as good biomonitoring species for these pollutant classes. Other factors affect this selection, as the difficulty to capture them alive for blood sampling or the restriction on handling protected raptors (for which only addled eggs or those found dead can be used). Studies are usually based on samples that have been previously collected for other purposes. The raptor species collected for analyzing PFASs, NFRs and other CECs are demonstrated in Fig. 3.

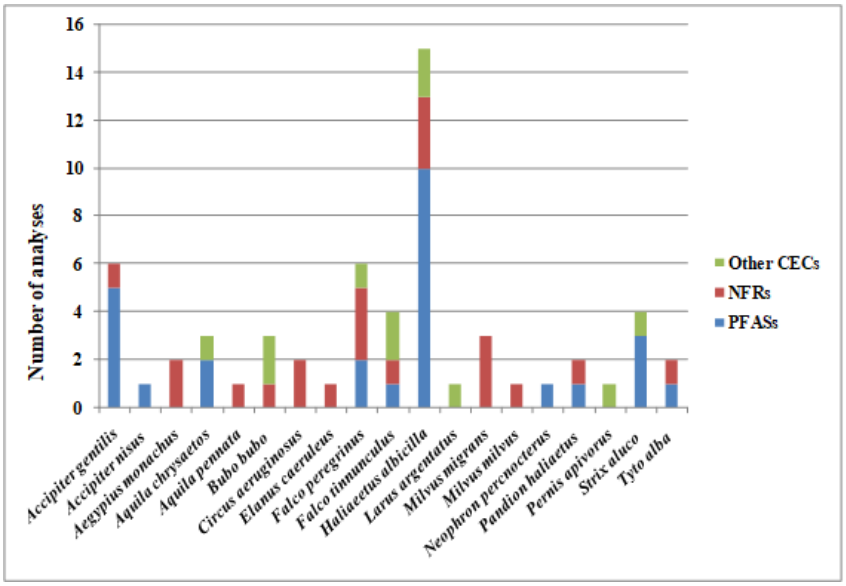

Fig. 3. Number of studies on PFASs, NFRs and other CECs on different raptorspecies in Europe (2002-2020).

Among raptors, the white-tailed eagle, the northern goshawk and the peregrine falcon, were the most frequently studied. The white tailed eagle was also the most analyzed species for PFASs determination. Among owls, the tawny owl was the most studied for the determination of PFASs and other CECs. No data on common buzzard are yet available for CECs in Europe, and therefore it would be interesting to investigate the suitability of this species as it was identified as a good European wide biomonitor for the priority compounds proposed by Badry et al (2020).

3.4. Preferred bioanalytical methods for the analysis of CECs in raptors

The development of efficient and sensitive analytical methods is crucial for correctly assessing the exposure to organic contaminants in wildlife. The analytical methodologies for the determination of emerging contaminants in the reviewed studies are mainly based on sample preparation step (extraction and

clean-up with specific solvents or adsorbents) and subsequent analysis commonly based on liquid (LC) or gas chromatography (GC) coupled to mass spectrometry (MS) of low and high resolution. The frequency of use of different sample pretreatments (extraction and/or clean-up) per contaminant class per matrix, respectively, is presented in Figs. 4 and 5.

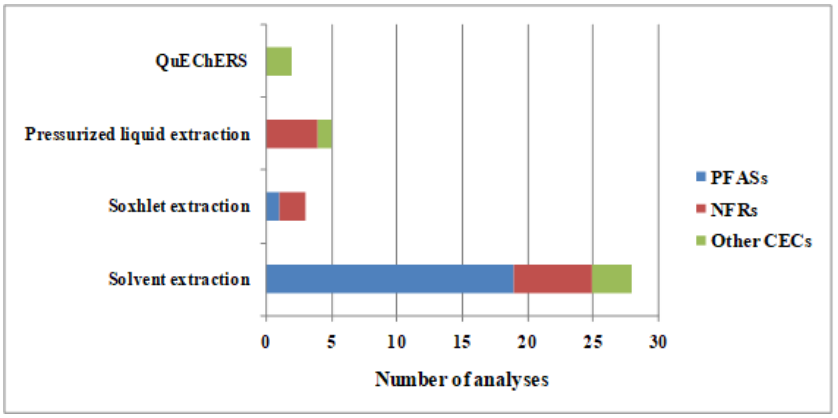

Fig. 4. Number of studies on PFASs, NFRs and other CECs on raptors in Europe (2002-2020) according to the sample treatment strategy.

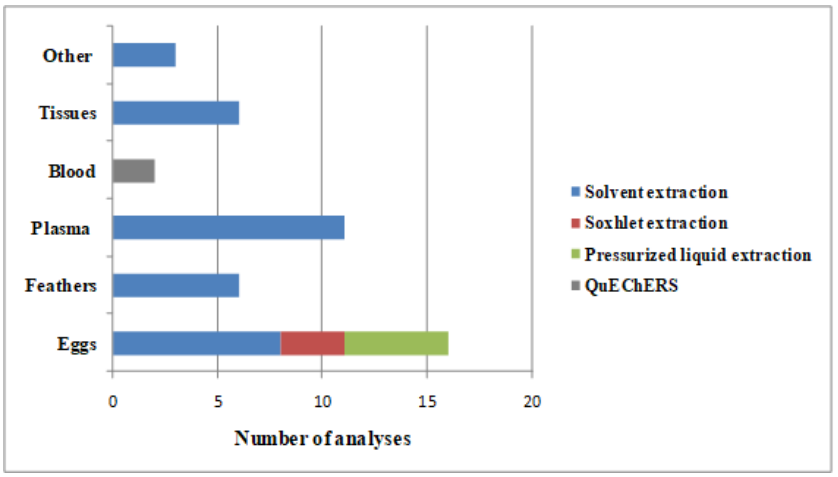

Fig. 5. Number of studies on PFASs, NFRs and other CECs on raptors in Europe (2002-2020) using different sample treatment strategies according to the sample matrix. Preen oil and adipose tissue were referred to as "other".

Conventional solvent extraction (thus including liquid-liquid and solid-liquid extraction, depending on the sample analyzed) was preferred for the determination of NFRs and was the only method employed for PFASs. Due to the non-polar nature of NFRs, the most common organic solvents were mixtures of hexane and dichloromethane (Briels et al., 2019; Monclús et al., 2018). For PFASs (more polar substances), methanol or acetonitrile were the preferred choice (Løseth et al., 2019a; Eriksson et al., 2016). Conventional solvent extraction was predominantly applied to eggs and it was the only method applied in plasma, feathers, tissues and other matrices such as fat or preen oil. Other extraction techniques were also reported namely pressurized liquid extraction (PLE) (Barón et al., 2014), Soxhlet extraction (Vorkamp et al., 2017) and QuEChERS (salting-out extraction and simultaneous dispersive SPE clean-up) for the determination of neonicotinoids in blood samples (Byholm et al., 2018; Taliansky-Chamudis et al., 2017). As it can be observed in Table 3, the application of classical extraction techniques for the determination of other CECs in eggs, such as Soxhlet, has declined since 2012 in favor of more modern approaches, e.g. PLE, since lower solvent consumption and rapid and simpler procedures are obtained. In general, Soxhlet was applied using dichloromethane (Guerra et al., 2011) and hexane:acetone (4:1, v/v) (Vorkamp et al., 2019; Vorkamp et al., 2017) as extraction solvents, while PLE was carried out with hexane:dichloromethane (1:1, v/v) (Blanco et al., 2018). QuEChERS extraction step, which is usually based on a salting out process with acetonitrile and that is widely employed for enhancing the extraction of polar and medium polar compounds, was applied to determine neonicotinoids in blood (Taliansky-Chamudis et al., 2017; Byholm et al., 2018). The amount of extracted sample was highly variable and depended on the method and the matrix. For solid samples, these amounts varied from 0.1 g (dw) (Vetter et al., 2017) to 10 g (lw) of egg media (Yuan et al., 2019) and, for liquid samples from 0.1 mL of blood (Byholm et al., 2018) to 1 mL of plasma (Eulaers et al., 2014). Further clean-up after extraction was usually necessary. For PFASs, clean-up consisted always of dispersive-SPE using acidified ENVI-Carb as sorbent. On the

contrary, different clean-up procedures were developed for the determination of PBDEs and NFRs, such as SPE using ENVI-Florisil (especially in recent years) or acidified alumina cartridges (Monclús et al., 2019; Blanco et al., 2018). One study also proposed gel permeation chromatography on a Bio Beads SX-3 (packed with neutral, porous styrene divinyl benzene copolymer beads) (Guerra et al., 2011). Regarding other CECs, dispersive-SPE clean-up (by using magnesium sulphate, primary secondary amine (PSA) and polymerically bonded trifunctional C18 silica sorbent) was applied for neonicotinoids while a QuEChERS method (based on a mixture of PSA, C18 and graphitized carbon black (GCB)) and SPE (C18 or Oasis HLB cartridges) was used for UV filters and bisphenols. PSA can eliminate organic and fatty acids, C18 removes fats, sterols and other nonpolar interferences and GCB eliminates pigments and sterols. Regarding the instrumental analysis for NFRs, GC–MS on a DB-5 capillary column was the most reported technique, with limits of quantification (LOQs) ranging from 0.002 to 32.2 ng g–1 (or mL–1). Regarding the analysis of PFASs, LC-MS-MS and LC-time-of-flight (TOF)-high resolution MS with a C18 column were the most applied techniques, with LOQs ranging from 0.004 to 0.4 ng g–1 or ng ml–1. LOQs were very low in the case of UV-filters (0.5–20 pg g–1) (Molins-Delgado et al., 2017) and neonicotinoids (0.6–30 pg mL–1) (Byholm et al., 2018), potentially due to the combination of selective and sensitive sample treatment and analysis, namely PLE coupled to LC-QTRAP-MS-MS or QuEChERS coupled to LC-MS-MS, respectively. The development of new analytical methods for CECs is desirable to decrease the limits of detection and quantification since they are usually found in trace concentrations, and for expanding the applicability in a variety of matrices.

4. Conclusions

In this study, a comprehensive and critical review concerning the presence and analysis of PFASs, NFRs and other CECs in European raptors (birds of prey and owls) was presented. It was concluded that in Europe, most of the studies on CECs were performed in Western Europe, mainly in Norway and Spain. Most temporal trend studies on PFASs in European raptor eggs reported increasing concentrations of PFCAs over time and inconclusive results for PFOS. Furthermore, concentrations of PFASs in plasma and feathers were greater than for legacy PBDEs in most cases. Regarding the occurrence of NFRs, most studies in which raptor samples (eggs, feathers and plasma) were collected after 2010 reported greater concentrations than PBDEs, this being in accordance with the phase out and restrictions imposed on these persistent organic pollutants. Likewise, the detection of other miscellaneous CECs (including neonicotinoids, UV-filters, chlorinated paraffins and bisphenols) in raptors and the low data available in the literature suggests an urgent need to collect more information on these compounds in order to evaluate the environmental threat they may pose. White-tailed Eagle, Peregrine falcon, Northern goshawk and tawny owl were the most frequently studied raptor species in Europe. However, studies on CECs in common buzzard are currently lacking and could be recommended for investigation as this species, along with tawny owl, has also been proposed as a European wide biomonitoring for priority contaminants in a recent study (Badry et al., 2020). Regarding the matrices, eggs, and blood plasma can be recommended for PFASs analysis in raptors, while studies on different matrices for other CECs are limited. There is not an optimal all-purpose tissue for monitoring contaminants in raptors and the selection of the matrix depends on the objectives of each study, the conservation status of the species regarding sampling permits and the compound(s) of interest. Feathers are a popular non-destructive sampling matrix for raptors, but as highlighted by recent studies feathers may not be suitable for

some CECs and specific attention is needed to potential external contamination on the feather surface. A recent study highlighted the potential role of the preen gland as a major excretory organ for CECs, which can be further investigated as a potential matrix for contaminants in raptors. Regarding sample treatment and analysis, conventional solvent extraction prior to both GC and LC coupled to mass spectrometry were the most used sample treatment and instrumental technique(s), respectively. Alternative sample treatments (e.g. QuECheRS or pressurized liquid extraction) have been only scarcely investigated in this field. Further analytical developments are expected in the future to increase the scope of the analysis and to improve the analytical features of the methods, such as extraction efficiency and clean-up, key factors in these complex and diverse matrices. The use of high-resolution MS will probably also be an increasing trend, since it opens the possibility of non target screening and the identification of unknown substances or untargeted species by retrospective screening. The results of this study highlight the need for further research on CECs in other European regions (mainly Eastern Europe), with the aim to improve the assessment of temporal and spatial trends. It is also advisable to further examine suitable matrices and other species and to develop new and sensitive analytical strategies for their analysis (especially for CECs) in raptors. Finally, the toxicological effects and ecological impact of the exposure to CECs on birds of prey and owls in Europe have not received much attention thus far and this would be essential to guide which CECs should be investigated in future research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPORTING INFORMATION

A review on contaminants of emerging concern in European raptors (2002-2020)

Soledad González-Rubio[1,2,3*], Ana Ballesteros-Gómez[1], Alexandros G. Asimakopoulos[2], Veerle L. B. Jaspers[3*].

[1]Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, Marie CurieAnnex Building, Campus of Rabanales, University of Córdoba, 14071, Córdoba, Spain.

[2]Department of Chemistry, Norwegian University of Science and Technology (NTNU), NO-7491,Trondheim, Norway

[3]Department of Biology, Norwegian University of Science and Technology (NTNU), NO-7491, Trondheim, Norway *Corresponding authors: Soledad González-Rubio: q02gorus@uco.es, Veerle L.
B. Jaspers:veerle.jaspers@ntnu.no

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Table S1. Concentrations of PFASs reported from different studies on feathers and plasma in European raptors. Concentrations of PBDEs are shown for comparison.

Country	Sampling Year	Matrix	Concentration (ng g^{-1} or ng mL^{-1}, ww or dw)	Reference
Norway	2015-2016	Feathers	**∑PBDEs** median range: 0.6-1.3 **∑PFAS** median range: 1.8-6.2	**Briels et al. (2019)**
		Plasma	**∑PBDEs** median range: 0.1-0.2 **∑PFAS** median range: 5-17.5	
Norway	2015-2016	Feathers	**BDE-47** median range: 0.3-1.73 **PFUnDA**median range: 0.15-0.58	**Løseth et al. (2019a)**
		Plasma	**BDE-47** median range: 0.06-0.19 **PFUnDA**median range: 1.15-3.59	
Norway	2015-2016	Plasma	**∑PBDEs** median range: 0.1-0.3 **∑PFAS** median range: 9-32	**Løseth et al. (2019b)**
Spain	2012-2016	Plasma	**PBDEs** geometric mean range: 0.01-0.03 **PFAS** geometric mean range: 0.03-2.3	**Ortiz-Santaliestra et. al (2019)**
Norway	2008-2010	Plasma	**∑PBDEs** median range: 0.7-2 **∑PFAS** median range: 21-55	**Sonne et al. (2012)**
Norway	2008-2009	Plasma	**∑PBDEs** mean range: 0.7-9 **∑PFAS** mean range: 12-151	**Sonne et al. (2010)**

Table S2. Concentrations of PBDEs and NFRs reported from different studies on European raptors.

Country	Sampling Year	Matrix	Concentration (ng g^{-1} or ng mL^{-1})	Reference
Norway	2015-2016	Feathers	∑**PBDEs** median range: 0.2-5 ∑**NFRs** median range: 2.6-314	**Briels et al. (2019)**
Spain	2010	Eggs	∑**PBDEs** mean: 645 ∑**NFRs** mean: 10	**Eljarrat et al. (2019)**
Norway	2015-2016	Plasma Feathers	**BDE-47** median range: 0.06-0.2 **NFRs** median range: 0.002-0.5 **BDE-47** median range: 0.3-1.7 **NFRs** median range: 0.1-27	**Loseth et al. (2019)**
Spain	2007-2016	Eggs	**PBDEs** mean range: 0.6-84 **NFRs** mean range: 4-353	**Blanco et al. (2018)**
Spain	2016	Feathers	∑**PBDEs** median range: 0.01-1.08 ∑**NFRs** median range: 0.4-49	**Monclús et al. (2018)**
Germany	2014	Eggs	∑**PBDEs** mean range: 145-320 ∑**NFRs** mean range: 1-33	**Vetter et al. (2017)**
Greenland	1986-2004	Eggs	∑**PBDEs** median: 6-1900 ∑**NFRs** median range: 0.5-4.7	**Vorkamp et al. (2017)**
Spain	1999-2013	Eggs	∑**PBDEs** median range: 20-55 ∑**NFRs** median range: 17-19	**Barón et al. (2015)**
Spain	2010-2012	Eggs	∑**PBDEs** mean range: 2-23 ∑**NFRs** mean range: 7-46	**Barón et al. (2014)**
Norway	2011	Plasma Feathers	**PBDEs** median range: <LOQ-0.17 **NFRs** median range: <LOQ-0.22 ∑**PBDEs** median range: 0.05-0.6 ∑**NFRs** median range: 4-110	**Eulaers et al. (2014)**

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Bioaccumulation potential of bisphenols and benzophenone UV filters: A multiresidue approach in raptor tissues

Soledad González-Rubio [a,b,c], Kristine Vike-Jonas [b], Susana V. Gonzalez [b], Ana Ballesteros-Gómez[a], Christian Sonne[d], Rune Dietz[d], David Boertmann[d], Lars Maltha Rasmussen[e], Veerle L.B. Jaspers [c,**], Alexandros G. Asimakopoulos [b,*].

a Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, Marie CurieAnnex Building, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain.
b Department of Chemistry, Norwegian University of Science and Technology (NTNU), NO-7491Trondheim, Norway.
c Department of Biology, Norwegian University of Science and Technology (NTNU), NO-7491 Trondheim, Norway.
d Department of Bioscience, Aarhus University, Arctic Research Centre (ARC), Frederiksborgvej399, PO Box 358, DK-4000 Roskilde, Denmark
e Greenland Institute of Natural Resources, Kivioq 2, Nuuk 3900, Greenland.

* Correspondence to: A G. Asimakopoulos, Department of Chemistry, Norwegian University ofScience and Technology (NTNU), 7491 Trondheim, Norway.
** Correspondence to: V L. B. Jaspers, Department of Biology, Norwegian University of Science andTechnology (NTNU), 7491 Trondheim, Norway.
E-mail addresses: veerle.jaspers@ntnu.no (V.L.B. Jaspers), alexandros.asimakopoulos@ntnu.no (A.G.Asimakopoulos).

Graphical abstract

Highlights

Occurrence of eight bisphenols and five benzophenone UV filters in raptor tissues.
Bisphenol A, bisphenol F, benzophenone-8 and 4-hydroxybenzophenone were predominant.
Potential bioaccumulation of contaminants in specific raptor tissues was indicated.
The potential role of the preen gland as a major excretory organ was suggested.

Keywords

Bisphenols, Benzophenone UV filters, Birds of prey, Owls, Europe, Greenland

Abstract

Environmental exposure to bisphenols and benzophenone UV filters has received considerable attention due to the ubiquitous occurrence of these contaminants in the environment and their potential adverse health effects. The occurrence of bisphenols and benzophenone UV filters is well established in human populations, but data is scarce for wildlife, and especially for raptors (birds of prey, falcons and owls). In this study, concentrations of eight bisphenols and five benzophenone UV filters were determined in six raptor tissues, including muscle, kidney, liver, brain,

preen gland (uropygial gland) and adipose. The tissue samples (n=44) were taken from dead raptor species (1997–2011), including Eurasian sparrowhawks (Accipiter nisus, n=2) and long-eared owls (Asio otus, n=2), both from France, and white-tailed eagles (Haliaeetus albicilla, n=16) from Greenland. Overall, six bisphenols and four benzophenone UV filters were found in the samples. Bisphenol A (BPA), bisphenol F (BPF), benzophenone-8 (BzP-8) and 4-hydroxybenzophenone (4-OH-BzP) were the most abundant contaminants, accounting for median concentrations of 67.5, 3.01, 27.1 and 9.70 ng/g wet weight (w.w.), respectively. The potential role of the preen gland as a major excretory organ for bisphenols and benzophenone UV filters was suggested since the median sum concentration of the two contaminant classes in the white-tailed eagle tissues showed higher bioaccumulation potential in the preen gland (5.86 ng/g w.w.) than the liver (2.92) and kidney (0.71). The concentrations of these contaminants in the tissues of the three raptor species indicated a pattern of increasing detection rates and median concentrations with an increase of the species size and their expected trophic.

1. Introduction

Emerging contaminants (ECs) are currently found widespread throughout the different environmental compartments and have impacted the natural ecosystems worldwide (Thomaidis et al., 2012). Bisphenols (BPs) and benzophenone UV filters (BzPs) are high-volume produced ECs that are used in a multitude of consumer products, including packaging materials (e.g., foodstuffs), personal care products, and pharmaceuticals (Asimakopoulos et al., 2016a; Caballero-Casero et al., 2016; Karthikraj and Kannan, 2018; Lu et al., 2018), while

both chemical classes are documented as cooccurring in plastic products (Asimakopoulos et al., 2016a). They exhibit endocrine-disrupting properties and are associated to adverse health effects in humans such as obesity, thyroid related diseases, diabetes, and reproductive dysfunction among others (Joensen et al., 2018; Magueresse-Battistoni et al., 2017; Philips et al., 2020). BPs and BzPs have recently attracted increased scientific attention through biomonitoring studies published on humans, aquatic and marine ecosystems (Asimakopoulos et al., 2014, 2016b; Rocha et al., 2018; Romera-García et al., 2019; Vela-Soria et al., 2014; Yu and Wu, 2014). BPs are a class of chemicals with two phenolic functional groups in their structure, which include several analogues mainly used in the production of polycarbonate plastics and epoxy resins (Asimakopoulos et al., 2016a). Until recently, bisphenol A (BPA) was the most used and studied BP, but due to increased concerns of its toxicity, several BP analogues are nowadays introduced into the global markets as substitutes, e.g., bisphenol F (BPF) and bisphenol S (BPS) (Chen et al., 2016; Qui et al., 2019). However, these chemical substitutes are also currently raising concerns of toxicity and widespread environmental occurrence (Lehmler et al., 2018; Xue et al., 2017). BzPs are another class of chemicals that obtain a diarylketone scaffold molecular structure in which different substitute groups are attached (Asimakopoulos et al., 2014, 2016a). They are frequently used as UV filters in sunscreen products and personal care products (Asimakopoulos et al., 2014, 2016a; Karthikraj and Kannan, 2018), while benzophenone-3 (BzP-3) is the most represented BzP analogue (Asimakopoulos et al., 2014; Li and Kannan, 2018). Among the wildlife species, raptors [Accipitriformes (hawks, eagles, ospreys) and Strigiformes (owls)] are important sentinels of environmental contamination and exposures (Espín et al., 2016). They are particularly suitable for monitoring persistent, bioaccumulative and toxic (PBT) contaminants because: (a) they are apex predators, and therefore particularly sensitive "biotracers" of environmental contaminants (Helander et al., 2008; Kovács et al., 2008); (b) they are found widespread in the ecosystems and are relatively long-lived organisms, thus allowing the assessment of spatial and temporal trends of contaminant

concentrations (Furness, 1993; Sun et al., 2019, 2020); and (c) specific biological samples, e.g., eggs, feathers and preen oil, can be collected non-invasively without sacrificing or harming the raptors (Eulaers et al., 2011). Numerous studies are published on the determination of contaminants in raptors (Ahrens et al., 2011; Barón et al., 2014; Bustnes et al., 2015; Jaspers et al., 2011, 2013). However, so far most of them are focused on persistent organic pollutants (POPs), while data on the bioaccumulation of ECs in raptors is limited (Briels et al., 2019; Løseth et al., 2019). With this background, the present study aims to investigate the occurrence profiles of BPs and BzPs in selected raptor tissues and assess their bioaccumulation potential. To our knowledge, this is the first peer-reviewed study on the occurrence of eight BPs and five BzPs in raptor tissues.

2. Experimental section

2.1. Chemicals and materials

Methanol of LC-MS grade was purchased from VWR Chemicals (Trondheim, Norway). Ethylacetate, hydrochloric acid (HCl), ammonium hydroxide and ammonium acetate were purchased from Sigma-Aldrich (Steinheim, Germany). Waterwas purified with aMilli-Qgrade water purification system (Q-option, Elga Labwater, Veolia WaterSystems LTD, U.K.). β-Glucuronidase from Helix pomatia (type HP-2, aqueous solution, ≥100,000 units/mL) was purchased from Sigma-Aldrich (Steinheim, Germany). Standards of BPA (≥99%), bisphenol AF (BPAF, ≥99%), bisphenol AP (BPAP, ≥99%), bisphenol B (BPB, ≥98%), BPF (≥98%), bisphenol M (BPM, ≥99%), bisphenol P (BPP, ≥99%), BPS (≥98%), bisphenol Z (BPZ, ≥99%), benzophenone-1 (BzP-1, ≥99%), benzophenone-2 (BzP-2, ≥97%), benzophenone-3 (BzP-3, ≥98%), benzophenone-8 (BzP-8, ≥98%) and 4-hydroxybenzophenone (4-OH BzP, ≥98%) were purchased

from Sigma-Aldrich (Steinheim, Germany). Internal standards (ISs) were purchased from Cambridge Isotope Laboratories (Andover MA, USA): 13C isotope of BPA (BPA-13C12; ≥99%), BPAF (BPAF-13C12; ≥99%), BPB (BPB-13C12; ≥ 99%), BPF (BPF-13C12; ≥99%), and BPS (BPS-13C12; ≥98%). A Branson Model 3510-DTH Ultrasonic Cleaner (Branson, Danbury CT, U.S.) was used for the ultrasonication of samples. A Biotage TurboVap Classic LV Concentration Evaporator Workstation (Biotage, Charlotte, NC, U.S.) was utilized for concentrating the extracts using a heated water bath under a nitrogen gas stream. An Innova 44 Incubator Shaker (J&M Scientific, Woburn, Massachusetts, U.S.) was used for the incubation of samples, and a mechanical shaker (KS501 digital, IKA, U.K.) was used for shaking the samples.

2.2. Study population and sample collection

Three different species, including white-tailed eagles (Haliaeetus albicilla), long-eared owls (Asio otus) and Eurasian sparrowhawks (Accipiter nisus), were sampled during 1997–2011 in Greenland and France. Depending on sample availability, a selection was performed from the tissues of muscle, kidney, liver, brain, preen gland (uropygial gland) and adipose. The white-tailed eagles (n= 16) were found dead or dying in West Greenland between March 1997 and January 2009. The Eurasian sparrowhawks (n= 2) and long-eared owls (n = 2) were found dead by the Association of CHENE (Centre d'Hébergement et d'Etudes sur laNature et l'Environnement) inNormandy, France, between 2009 and 2011. Relevant information on raptors analyzed in this study (Raptor ID, sampling area and year of collection) is provided in Table S1. Further details on the white-tailed eagles (i.e., date and place found, estimated age, plumage, sex, cause of death, weight, etc.) can be found in Jaspers et al. (2013), and for the Eurasian sparrowhawks and long-eared owls in Table S2. Tissues were carefully dissected using stainless steel tools rinsed thoroughly between tissues and individuals. All samples were properly labelled and stored in the darkness at −80 °C.

2.3. Sample preparation

Tissue samples of 0.1–0.2 g were weighted into 15 mL polypropylene (PP) tubes. The sample preparation protocol was adapted from Asimakopoulos et al. (2016b) with minor modifications. Briefly, 1 mL of 1.0 M ammonium acetate aqueous buffer was added to the samples and ultrasonication followed for 45 min. Another 1mL of 1.0Mammoniumacetate (aqueous buffer) that contained 44 units of β-glucuronidase (prepared by spiking 100 μL of β-glucuronidase into 100 mL of 1.0 M ammonium acetate solution) was added to the samples and were incubated at 37 °C for 12 h to establish total concentrations (free and conjugated chemical species). Thereafter, the samples were extracted 3 consecutive times with 4 mL ethyl acetate (3 × 4). For each extraction, the mixture was shaken for 60 min and then centrifuged at 4500g for 10 min. The supernatants were combined, and 2 mL of Milli-Q water were added. The derived mixtures were further shaken for 5 min, centrifuged at 4500g for 10min, and the collected supernatants were eventually transferred into a PP tube and concentrated to ≈250 μL using the water bath set at 30 °C under a gentle nitrogen stream. Finally, methanol was added up to a total volume of 1 mL, vortex mixed, and transferred for UPLC–MS/MS analysis.

2.4. UPLC-MS/MS analysis

The chromatographic separation was carried out using an Acquity UPLC I-Class system (Waters, Milford, U.S.) coupled to a triple quadrupole mass analyser (QqQ; Xevo TQ-S)with a ZSpray ESI ion source (Waters, Milford,U.S.). The LC

column usedwas a Kinetex C18 (50 × 2.1mm, 1.3 μm) connected to a Phenomenex C18 guard column (2.0 × 2.1mm). The column temperaturewas set at 30 °C. Themobile phase consisted of solvent (A) 0.1% v/v ammonium hydroxide in Milli-Q water and (B) methanol. The flowratewas 300 μL min−1 and the injection volume was 4 μL. The gradient elution initiated with 75% A, held for 10 s, decreased to 25% A within 3.4 min, then further decreased to 1% A, held for 30 s, and reverted to 75% A that was held for 20 s, for a total time run of 4 min. The mass spectrometer was operated in multiple reaction monitoring(MRM) mode. Electrospray ionization was performed under negative ionization mode (ESI−). Optimal source settings were the following: source gas temperature 150 °C, capillary voltage −1500 V and nebulizer gas pressure 7.0 bar. Precursor and product ions and relevant detection MS/MS parameters of each target analyte are shown in Table S3. Quantification of the target analytes was accomplished based on the internal standard method and with matrix-matched calibration standards (Asimakopoulos et al., 2014, 2016b).

2.5. Method performance characteristics

The analytical method was validated by analyzing non-spiked, preextraction spiked and post-extraction spiked triplicates in pool samples of liver (n = 6) and preen gland (n = 13). The isomers, BPM and BPP, were quantified in samples as a single 1:1 mixture. Contamination during sample preparation was evaluated by analyzing reagent blanks that followed the whole procedure as actual samples. The performance of the developed method was described based on correlation coefficients, relative recoveries, precision, method detection (MDLs) and method quantification (MQLs) limits, ion ratios (IRs%; when applicable), retention and relative retention times (RTs, RRTs), and matrix effects (MEs %). The MDLs and MQLs were estimated and presented here for a typical sample weight of 0.2 g as 3 and 10 times the signal-to-noise ratio in 0.5 mL solvent matrix,

respectively. Estimation of MEs (%) during analysis was performed for each target analyte using Eq. (1):

$$ME\ (\%) = (MF - 1) \times 100$$

where MF is the matrix factor, which is estimated by the ratio of the peak area of the target analyte in the post-extraction fortified matrix to that in standard solvent (both at the amount of 20 ng). The IR (%) for a target analyte relates the area of the peak of the qualifier with that of the quantifier ion.

2.6. Data analysis and statistical treatment

UPLC-MS/MS data was acquired with the MassLynx v4.1 software, while quantification processing was performed with TargetLynx (Waters, Milford, U.S.). Excel (Microsoft, 2018) was used for general descriptive statistics. Data analysis, including descriptive statistics, did not include data <MQLs. A substitution or a non-parametric method (e.g., Kaplan-Meier) was not applied for the censored values (<MQLs) due to their large proportion in the concentration data set (Asimakopoulos et al., 2016b). Concentrations were reported as ng/g wet weight (w.w.).

3. Results and discussion

3.1. UPLC-MS/MS bioanalytical method performance

Correlation coefficients were assessed by running internal calibration curves prepared in methanol (fortified with target BPs and BzPs at 0.1, 0.2, 0.5, 1,

2, 5, 10, 20 and 50 ng/mL and ISs at 20 ng/mL). The correlation coefficients in all cases were above 0.991. The IS used, IR (%), RT and RRT for each target analyte are presented in Table S4. The MDLs and MQLs were in the range from0.004 to 2.00 and from0.01 to 6.50 ng/g w.w., respectively (Table S5). The MQLs allowed for the quantification of BPs and BzPs with optimal precision at the low concentrations that were present in the actual biological samples. The precision of the UPLC-MS/MS method was evaluated in terms of repeatability (n = 3 for each matrix, liver and preen gland; the fortified amounts of the target analytes and ISs were 20 and 10 ng, respectively) using the internal standard method and expressed as relative standard deviation (RSD%) (Table S6). The precision for most target analytes was <15% and overall ranged from 3.22 (BzP-8) to 26.3 (BPM/BPP)% and from 3.12 (4-OHBzP) to 24.1 (BPB)% in preen gland and liver matrix, respectively. Trueness of the method was assessed through relative recoveries (adjusted by ISs) in preen gland and liver matrix by fortification of the target analytes (n=3 for eachmatrix; the fortified amounts of target analytes and ISs were 20 and 10 ng, respectively); the mean recoveries for BPs and BzPs are presented in Table 1.

Table 1. Mean relative recoveries (%; n=3 for each matrix) for the target analytes.

Contaminants	**BPA**	**BPF**	**BPB**	**BPS**	**BPZ**	**BPAP**	**BPAF**
Preen Gland	85.6	117	98.5	111	70.0	97	104
Liver	125	109	137	107	70.0	101	103
Contaminants	**BPM/BPP**	**BzP-8**	**BzP-3**	**BzP-2**	**BzP-1**	**4-OH-BzP**	
Preen Gland	86.8	107	92.3	78.1	98.0	115	
Liver	116	77.4	29.8	71.0	57.6	70.0	

The recoveries for most target analytes were found to be ≥70%. However, in liver, BP-3 and BP-1 presented recoveries <60%, while BPA and BPB presented recoveries >120%, and both findings were attributed to the higher metabolic activity of the specific matrix; the liver contains excess concentrations of endogenous enzymes responsible for biotransformation reactions of contaminants, e.g., cytochrome P450 (Ashrap et al., 2017; Dudda and Kürzel, 2006). The MFs and MEs (%) for the target analytes are shown in Tables S7-S8. All target analytes

demonstrated ionization suppression with the highest observed for BzP-3, BPZ and BPAP. The bioanalytical method protocol involved a deconjugation step with β-glucuronidase to account for the glucuronide and sulfate conjugated species of contaminants since their occurrence is documented in other instances [e.g., for polycyclic aromatic hydrocarbons (PAHs)] in raptor tissues (Watanabe et al., 2010).

3.2. Bioaccumulation in raptor tissues

A total of 44 biological samples were analyzed (Tables 2–4); and most BPs and BzPs were detected, except for BPAF, BPZ and BzP-2.

Table 2. BPs and BzPs in different tissues from white-tailed eagles (*Haliaeetus albicilla*); concentrations expressed in ng g^{-1} w.w.

Matrix	Raptor ID	BPA	BPAP	BPB	BPF	BPM/BPP	BPS	**ΣBPs**	BzP-1	BzP-3	BzP-8	4-OH-BzP	**ΣBzPs**	ΣAll
Preen gland (n=8)	39640	64.1	-	-	-	-	-	**64.1**	-	-	-	-	-	64.1
	39644	-	-	-	-	-	-	-	-	-	7.42	-	**7.42**	7.42
	39645	-	-	-	-	-	-	-	0.89	-	-	-	**0.89**	0.89
	39649	-	-	-	-	0.49	-	**0.49**	-	-	-	-	-	0.49
	39651	70.9	-	-	-	-	-	**70.9**	-	-	-	-	-	70.9
	39652	-	-	-	-	-	-	-	4.30	-	-	-	**4.30**	4.30
	39653	149	-	1.81	1.22	-	-	**152**	-	-	-	-	-	152
	39654	-	-	-	-	-	-	-	-	0.31	-	-	**0.31**	0.31
Liver (n=4)	39652	-	-	-	2.53	0.05	-	**2.58**	-	-	-	-	-	2.58
	39653	-	-	0.72	2.21	0.12	0.21	**3.26**	-	-	-	-	-	3.26
	39654	-	-	-	-	0.11	-	**0.11**	1.43	-	-	9.70	**11.1**	11.2
	39657	-	-	-	-	-	-	-	0.95	-	-	-	**0.95**	0.95
Kidney (n=3)	39652	-	-	-	-	0.05	-	**0.05**	-	-	-	-	-	0.05
	39653	-	-	-	-	-	-	-	0.71	-	-	-	**0.71**	0.71
	39654	-	0.81	-	-	0.02	-	**0.83**	-	-	-	8.21	**8.21**	9.04
Muscle (n=3)	39652	-	-	-	3.50	-	-	**3.50**	-	-	-	-	-	3.50
	39653	-	-	-	-	-	-	-	-	-	-	-	-	-
	39654	-	-	-	-	-	-	-	-	-	-	-	-	-
Adipose (n=2)	39652	-	-	-	-	-	-	-	-	-	-	-	-	-
	39653	-	-	-	-	-	-	-	-	-	-	-	-	-
Detection Rate		3/20	1/20	2/20	4/20	6/20	1/20	**10/20**	5/20	1/20	1/20	2/20	**8/20**	16/20
Median		70.9	0.81	1.26	2.37	0.08	0.21	**2.92**	0.95	0.31	7.42	8.95	**2.62**	3.38
Mean		94.6	0.81	1.26	2.36	0.14	0.21	**29.7**	1.65	0.31	7.42	8.95	**4.24**	20.7
Min.		64.1	0.81	0.72	1.22	0.02	0.21	**0.05**	0.71	0.31	7.42	8.21	**0.31**	0.05
Max.		149	0.81	1.81	3.50	0.49	0.21	**152**	4.30	0.31	7.42	9.70	**11.1**	152

Table 3. BPs and BzPs in different tissues from long-eared owls (*Asio otus*); concentrations expressed in ng g^{-1} w.w.

Matrix	Raptor ID	BPA	BPAP	BPB	BPF	BPM/BPP	BPS	**ΣBPs**	BzP-1	BzP-3	BzP-8	4-OH-BzP	**ΣBzPs**	ΣAll
Preen gland	117R2010	-	-	-	-	-	-	**-**	-	0.47	-	-	**0.47**	0.47
(n=2)	76R2011	6.23	-	-	-	-	-	**6.23**	-	0.68	-	-	**0.68**	6.91
Liver	117R2010	-	0.76	-	-	-	1.10	**1.86**	-	-	-	-	-	1.86
(n=2)	76R2011	-	-	-	-	0.05	-	**0.05**	-	-	-	-	-	0.05
Kidney	117R2010	-	0.96	0.42	7.32	0.03	0.20	**8.93**	-	-	-	-	**-**	8.93
(n=2)	76R2011	-	-	-	-	-	-	**-**	-	-	-	-	-	-
Muscle	117R2010	-	-	-	-	0.13	2.06	**2.19**	-	8.21	46.9	-	**55.1**	57.3
(n=2)	76R2011	-	-	1.84	-	-	-	**1.84**	-	0.32	-	-	**0.32**	2.16
Adipose	117R2010	-	-	-	6.24	-	-	**6.24**	-	-	-	-	-	6.24
(n=2)	76R2011	-	-	-	-	-	-	-	-	-	-	-	-	-
Brain	117R2010	-	-	-	-	-	-	-	-	-	-	-	-	-
(n=2)	76R2011	-	-	-	2.48	-	-	**2.48**	-	-	-	-	-	2.48
Detection Rate		1/12	2/12	2/12	3/12	3/12	3/12	**8/12**	0/12	3/12	1/12	0/12	**4/12**	9/12
Median		6.23	0.86	1.13	6.24	0.05	1.10	**2.33**	-	0.57	46.9	-	**0.57**	2.48
Mean		6.23	0.86	1.13	5.35	0.07	1.12	**3.72**	-	2.42	46.9	-	**14.1**	9.60
Min.		6.23	0.76	0.42	2.48	0.03	0.20	**0.05**	-	0.32	46.9	-	**0.32**	0.05
Max.		6.23	0.96	1.84	7.32	0.13	2.06	**8.93**	-	8.21	46.9	-	**55.1**	57.3

Table 4. BPs and BzPs in different tissues from Eurasian sparrowhawks (*Accipiter nisus*); concentrations expressed in ng g^{-1} w.w.

Matrix	Raptor ID	BPA	BPAP	BPB	BPF	BPM/BPP	BPS	**ΣBPs**	BzP-1	BzP-3	BzP-8	4-OH-BzP	**ΣBzPs**	ΣAll
Preen gland	113R2009	-	-	-	-	0.17	-	**0.17**	-	-	-	-	-	0.17
(n=2)	143R2010	-	-	0.74	6.02	-	0.21	**6.97**	-	-	-	-	-	6.97
Liver	113R2009	-	-	-	-	-	-	-	-	-	-	-	-	
(n=2)	143R2010	-	-	-	-	-	5.23	**5.23**	-	-	-	44.3	**44.3**	49.53
Kidney	113R2009	-	-	-	-	-	-	-	-	-	-	-	-	-
(n=2)	143R2010	-	-	1.60	-	-	-	**1.6**	-	-	-	-	-	1.6
Muscle	113R2009	-	-	-	-	-	-	-	-	-	-	-	-	-
(n=2)	143R2010	-	-	-	-	-	-	-	-	-	-	-	-	-
Adipose	113R2009	-	-	-	-	-	-	-	-	-	-	-	-	-
(n=2)	143R2010	-	-	-	-	-	-	-	-	-	-	-	-	-
Brain	113R2009	-	-	-	-	-	-	-	-	-	-	-	-	-
(n=2)	143R2010	-	-	1.69	-	-	-	**1.69**	-	-	-	-	-	1.69
Detection Rate		0/12	0/12	3/12	1/12	1/12	2/12	**5/12**	0/12	0/12	0/12	1/12	**1/12**	5/12
Median		-	-	1.60	6.02	0.17	2.72	**1.69**	-	-	-	44.3	**44.3**	1.69
Mean		-	-	1.34	6.02	0.17	2.72	**3.13**	-	-	-	44.3	**44.3**	11.9
Min.		-	-	0.74	6.02	0.17	0.21	**0.17**	-	-	-	44.3	**44.3**	0.17
Max.		-	-	1.69	6.02	0.17	5.23	**6.97**	-	-	-	44.3	**44.3**	49.5

The DRs rank order for BPs was: BPM/BPP (10/44; 10 out of 44) N BPF (8/44) > BPB (7/44) > BPS (6/44) > BPA (4/44) > BPAP (3/44), while that for BzPs was: BzP-1 (5/44) > BzP-3 (4/44) > 4-OH-BzP (3/44) > BzP-8 (2/44). The maximum DR was 22.7% (10/44) for BPM/BPP, while most of the analogues demonstrated similar DRs that ranged b15% (Table S9). The DRs rank order for ΣBPs among the raptor species decreased as: long-eared owl [66.6% (8/12)] > white-tailed eagle [50% (10/20)] > Eurasian sparrowhawk samples [41.6% (5/12)]. The median ΣBPs concentrations were found 2.92, 2.33 and 1.69 ng/g w.w. for white-tailed eagle, long-eared owl and Eurasian sparrowhawk samples, respectively. The DRs rank order for ΣBzPs among the three species was: white-tailed eagle [40% (8/20)] > long-eared owl [33.3% (4/12)] > Eurasian sparrowhawk samples [8.33% (1/12)]. The median ΣBzPs concentrations were 2.62 and 0.57 ng/g w.w. for white-tailed eagle and long eared owl samples, respectively, while only 4-OH-BzP was detected in a single liver sample from a Eurasian sparrowhawk at 44.3 ng/g w.w. In comparison to the Eurasian sparrowhawk, which is a small bird of prey (mean weight male/female: 144/264 g) that feeds on passerine birds, the white-tailed eagle is a very large species of sea eagle (mean weight male/female: 4.0/5.6 kg) feeding on the highest trophic position (including fish and seabirds), while the long-eared owl is a medium sized owl (mean weight male/female: 256/308 g) feeding on small mammals (Bell et al., 2010; Cramp and Simmons, 1980; Cramp, 1985; Ivanovsky, 2010; Olsen and Marks, 2019; Seibold and Helbig, 1996; Sulkava et al., 1997). The occurrence data of ΣBPs and ΣBzPs in the three studied raptor species showed a pattern of increasing DRs and median concentrations of the contaminants with the increase of the raptor species size. Further studies with larger sample sizes should be conducted to confirm this pattern. It is noteworthy that interspecies differences in contaminant metabolism exist even among raptor species (Watanabe et al., 2010). Moreover, the white-tailed eagle, having a considerably longer life-expectancy, has also higher bioaccumulation potential.

The rank order of [ΣAll] (total load of the selected contaminants; [ΣBPs]+[ΣBzPs]) medians in the white-tailed eagle matrices decreased as: preen gland (5.86 ng/gw.w.) N liver (2.92) N kidney (0.71). The muscle matrix demonstrated low DR (1/3), while no target analyte was detected in adipose tissue of the white-tailed eagle. The non detection of these contaminants in adipose tissue indicated that high concentrations were less likely to occur in this matrix of the white-tailed eagle compared to the preen gland and liver matrix. The number of samples per matrix was limited to draw any further conclusions, especially for the long-eared owl and Eurasian sparrowhawk species. However, in both of these species, all preen gland samples demonstrated [ΣAll] that ranged from 0.17 to 6.97 ng/g w.w. Overall, the significant concentrations determined in preen gland indicated that it is a potential excretory organ for BPs and BzPs. This finding is in accordance to previous studies on POPs. It is well-documented that preen gland contains relatively high concentrations of POPs (Jaspers et al., 2013; Solheimet al., 2016), but to our knowledge, this is the first documentation on BPs and BzPs, which are plastic and personal care product related ECs. Further association of BPs and BzPs concentrations with features such as age and sex were tempered in this study by the small sample size and low DRs. In contrast to the raptor species studied here, the DRs of some BPs and BzPs analogues in human biological media, e.g., adipose tissue and urine, especially for BPA and BP-3, can reach up to 100% (Rocha et al., 2018; Wang et al., 2015). Nonetheless, the concentrations demonstrated similarities between raptors and humans on the order of magnitude. The rank order of median concentrations for BPs found here for raptors was: BPA (67.5 ng/g w.w.) >BPF (3.01) >BPB (1.60) >BPAP (0.81) >BPS (0.65) >BPM/BPP (0.08) >BPAF, BPZ (not detected) (Fig. 1). The typical rank order of BPs median concentrations in human urine is: BPA (1.65 ng/mL) > BPF (0.58) >BPS (0.35) >BPAP (0.25) >BPZ ~ BPB (0.17) >BPP (0.16)) >BPAF (not detected) (Philips et al., 2020). In both raptors and humans, the highest concentrations were found for BPA and BPF. For the BzPs class, which additionally demonstrates interrelationships between BP-3, as a precursor analogue, with its documented metabolites (BzP-8, 4-OH-BzP, BzP-

1 and BzP-2), the rank order of median concentrations found here for raptors was: BzP-8 (27.1 ng/g w.w.) >4-OH-BzP (9.70) >BzP-1 (0.95) >BzP-3 (0.47) >BzP-2 (not detected) (Fig. 1).

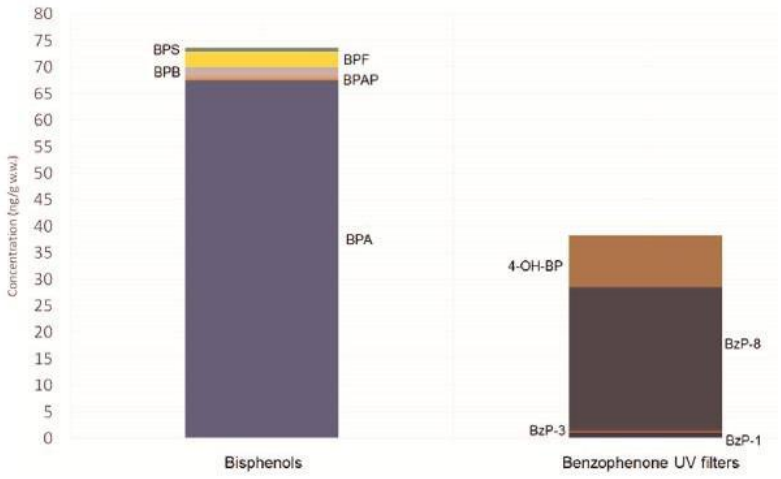

Fig. 1. Concentration profiles of the sum concentrations of bisphenols (∑BzPs) in raptor tissues; the median concentration values (based on values > MQLs were used to generate the chart [note: BPM/BPP was not shown due to the low medium concentration (0.08 ng/g w.w.)].

Consequently, the rank order for BzPs in raptors was found to be reversed when compared to that of the typical median concentrations excreted in human urine: BzP-3 (1.02 ng/mL) >BzP-2 (0.56) >BzP-1 (0.46) >4-OH-BzP (0.30) >BzP-8 (0.06) (Asimakopoulos et al., 2016b). By comparison, BzP-3 and BzP-1 were found in similar concentrations between raptor matrices and human urine, BzP-8 and 4-OH-BzP were found ~2 orders of magnitude higher in raptor matrices, while BzP-2 was not detected in these three raptor species. From the BPs class, BPF was found in an adipose tissue (6.24 ng/g w.w.) and a brain sample (2.48 ng/g w.w.) from the long-eared owls, BPB was found in a brain sample (1.69 ng/g w.w.) from the Eurasian sparrowhawks, while similarly to BPs, BzPs were not found in either adipose tissue or brain matrix. On the contrary, in human adipose tissue, BPA and BP-3 are documented in significant concentrations, similar to those found in

human urine (that is established as the main excretory pathway), which could even reach the order of a few tens and thousands of nanograms per gram (ng/g), respectively (Rocha et al., 2018; Wang et al., 2015). This contradiction on the occurrence profile of the target analytes between humans and raptors suggests differences in contaminant metabolism, toxico kinetics and bioaccumulation potential between the species. Further comparison to other human tissues, e.g., brain and liver, was tempered due to the limited human data availability for only some BPs and BzPs (Geens et al., 2012).

3.3. Comparison to relevant wildlife studies

To the best of our knowledge, this is the first peer-reviewed study to document multiresidues of both contaminant classes, BPs and BzPs, in raptor tissues. From the BPs class, only BPA was studied extensively so far in raptors. Elliott et al. (2019) reported a median BPA concentration of 1.05 ng/mL in bald eagle (Haliaeetus leucocephalus) nestling (eaglet) plasma samples (n = 381) from the U.S. Furthermore, BPA concentrations in feathers from the herring gull (Larus argentatus; which is a large seabird) were reported ranging on average from 132 to 153 ng/g dry weight (d.w.) (Nehring et al., 2017). It is noteworthy that the excretion of several contaminants in raptors is documented also through feathers (epidermal excretory organ) (Jaspers et al., 2019; Solheim et al., 2016), and similarities were indicated when comparing the BPA concentrations in feathers (from the herring gull; Nehring et al., 2017) with those found in our study in preen gland (from the white-tailed eagles; 64.1–149 ng/g w.w.).Mean concentrations of BPA in the muscle (n = 10), liver (n = 10) and guano (n = 7) from herring gulls (Larus argentatus) were reported 62.9, 111 and 996 ng/g d.w., respectively (Staniszewska et al., 2014). In addition, BPA concentrations in the muscle (n = 1) and liver (n = 1) of a great black-backed gull (Larus marinus) were reported 39.8 and 324 ng/g d.w., respectively (Staniszewska et al., 2014). However, in our study, BPA was neither detected in muscle nor liver samples. This difference can be

attributed to the different feeding patterns and habits, but also to the elimination capacity of specific contaminants among species. Staniszewska et al. (2014) noted that herring gulls gather for feeding purposes on landfill sites, which are highly contaminated areas. This feeding behavior of the gulls on industrial and urban wastes is also well-documented in other studies (Barón et al., 2014; Tongue et al., 2019). Concentrations of BzP-1, BzP-3, 4-OH-BzP, and 4,4′-dihydroxybenzophenone (which is an isomer of BzP-1) were reported by Molins-Delgado et al. (2017) in 39 unhatched raptor eggs of 7 wild living species [western marsh harrier (Circus aeruginosus), common kestrel (Falco tinnunculus), white stork (Ciconia ciconia), slender-billed gull (Chroicocephalus genei), black headed gull (Chrococephalus ridibundus), gull-billed tern (Gelochelidon nilotica), and gadwall (Anas strepera)]. Mean concentrations of BP-3, BP-1, 4-OH-BzP and 4,4′-dihydroxybenzophenone were ranging between species from 22.1 to 46.7 ng/g d.w., 38.1 to 433, 88.4 to 895, and 29.0 to 132, respectively, while the DRs in eggs ranged from 95 to 100% for all analogues indicating bioaccumulation. This was reflected in the egg concentrations, in which the rank order of mean BzPs concentrations found in those (for the majority of the assessed species) decreased as: 4-OH-BzP > BP1 > BP-3 (Molins-Delgado et al., 2017). This agreed to the rank order found in the raptor tissues in our study. Similar bioaccumulation and biomagnification potential of BzPs along the food chain was also recently suggested for marine organisms (Cocci et al., 2020).

4. Conclusions

In this study, the occurrence and concentrations of BPs and BzPs were determined in raptor tissues. It was shown that the liver, kidney and preen gland tissues are suitable for the biomonitoring of these contaminants. The occurrence data in the tissues of the three raptor species suggested a pattern of increasing DRs

and median concentrations with the increase of the raptor species size and expected trophic position, although this needs to be further confirmed with larger sample sizes. The potential role of the preen gland as a major excretory organ for BPs and BzPs was suggested. BPA, BPF, BzP-8 and 4-OH-BzP were the dominant contaminant analogues based on the concentrations found in the tissues. The results provided in this study indicate that BPs and BzPs have bioaccumulation potential in specific raptor tissues.

CRediT authorship contribution statement

Soledad González-Rubio: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing-original draft. Kristine Vike-Jonas: Methodology, Resources, Software, Writing - review & editing. Susana V. Gonzalez: Methodology, Resources, Writing - review & editing. Ana Ballesteros-Gómez: Investigation, Methodology, Resources, Supervision, Writing - review & editing. Christian Sonne: Resources, Writing - review & editing. Rune Dietz: Resources. David Boertmann: Resources, Writing - review & editing. Lars Maltha Rasmussen: Resources, Writing - review & editing. Veerle L.B. Jaspers: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing - review & editing. Alexandros G. Asimakopoulos: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPORTING INFORMATION

Bioaccumulation potential of bisphenols and benzophenone UV filters: A multiresidue approach in raptor tissues

Soledad González-Rubio [a,b,c], Kristine Vike-Jonas [b], Susana V. Gonzalez [b], Ana Ballesteros-Gómez[a], Christian Sonne[d], Rune Dietz[d], David Boertmann[d], Lars Maltha Rasmussen[e], Veerle L.B. Jaspers [c,**], Alexandros G. Asimakopoulos [b,*].

a Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, Marie Curie Annex Building, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain.

b Department of Chemistry, Norwegian University of Science and Technology (NTNU), NO-7491 Trondheim, Norway.

c Department of Biology, Norwegian University of Science and Technology (NTNU), NO-7491 Trondheim, Norway.

d Department of Bioscience, Aarhus University, Arctic Research Centre (ARC), Frederiksborgvej 399, PO Box 358, DK-4000 Roskilde, Denmark

e Greenland Institute of Natural Resources, Kivioq 2, Nuuk 3900, Greenland.

* Correspondence to: A G. Asimakopoulos, Department of Chemistry, Norwegian University of Science and Technology (NTNU), 7491 Trondheim, Norway.

** Correspondence to: V L. B. Jaspers, Department of Biology, Norwegian University of Science and Technology (NTNU), 7491 Trondheim, Norway.

E-mail addresses: veerle.jaspers@ntnu.no (V.L.B. Jaspers), alexandros.asimakopoulos@ntnu.no (A.G. Asimakopoulos).

Table S1. Raptor individuals selected for the study (Raptor ID, species, sex, sampling area andyear of collection).

Bird ID	Species	Sex	Sampling area	Year of collection
39640	White-tailed eagle	Male	Greenland	1998
39641	White-tailed eagle	Male	Greenland	1998
39643	White-tailed eagle	Female	Greenland	Unknown
39644	White-tailed eagle	Female	Greenland	1997
39645	White-tailed eagle	Male	Greenland	1998
39646	White-tailed eagle	Male	Greenland	1998
39647	White-tailed eagle	Male	Greenland	1999
39648	White-tailed eagle	Male	Greenland	1999
39649	White-tailed eagle	Female	Greenland	1999
39650	White-tailed eagle	Male	Greenland	1998
39651	White-tailed eagle	Female	Greenland	2000
39652	White-tailed eagle	Female	Greenland	Unknown
39653	White-tailed eagle	Female	Greenland	2001
39654	White-tailed eagle	Female	Greenland	2007
39655	White-tailed eagle	Male	Greenland	1999
39656	White-tailed eagle	Male	Greenland	2009
39657	White-tailed eagle	Female	Greenland	2009
113R2009	Eurasian sparrowhawk	Female	France	2009
143R2010	Eurasian sparrowhawk	Female	France	2010
117R2010	Long-eared owl	Female	France	2010
76R2011	Long-eared owl	Female	France	2011

Table S2. Further details concerning the Eurasian sparrowhawks and long-eared owls.

Bird ID	Area found	Latitude	Longitude	Age
113R2009	Saint-Arnoult	49.52	0.66	+1Y
143R2010	Anneville-Ambourville	49.46	0.88	1Y
117R2010	Martainville-Epreville	49.46	1.29	+1Y
76R2011	Le Havre	49.49	0.11	+1Y

Table S3: Precursor and product ions of the target analytes, their collision energies and cone voltage values for UPLC-MS/MS analysis

Target Analyte	Precursor ion (m/z)	Product ion (s) (m/z)	Cone voltage (V)	Collision energy (eV)	Quantifier (m/z)	Qualifier (m/z)
BPA	227.1	212.1	50.0	18.0	212.1	133.1
		133.1	50.0	24.0		
BPAF	335.0	265.1	40.0	24.0	265.1	177.0
		177.0	40.0	42.0		
BPAP	289.1	274.1	20.0	18.0	274.1	195.1
		195.1	20.0	28.0		
BPB	241.1	212.2	20.0	18.0	212.2	212
BPF	199.1	105.0	46.0	20.0	105.0	93.0
		93.0	46.0	20.0		
BPM/BPP	354.1	330.2	8.0	46.0	330.2	133.0
		133.0	14.0	26.0		
BPS	249.0	156.0	40.0	22.0	108.0	156.0
		108.0	40.0	26.0		
BPZ	267.1	173.1	40.0	24.0	173.1	145.1
		145.1	40.0	36.0		
BzP-1	213.0	135.0	46.0	18.0	135.0	91.0
		91.0	46.0	28.0		
BzP-2	245.0	135.0	40.0	14.0	135.0	109.0
		109.0	40.0	16.0		
BzP-3	227.1	211.1	40.0	20.0	211.1	183.0
		183.0	40.0	34.0		
BzP-8	243.0	123.0	46.0	16.0	123.0	93.0
		93.0	46.0	18.0		
4-OH-BzP	197.0	120.0	60.0	22.0	92.0	120.0
		92.0	60.0	28.0		

Table S4. Internal standard (IS) used for each target analyte, ion ratios (IRs%),retention times (RTs) and relative retention times (RRTs).

Target Analyte	Internal standard	IR% (RSD%)	RT[c] (min) (RSD%)	RRT[c] (min) (RSD%)
BPA	BPA-$^{13}C_{12}$	48.5 (17.1)[a]	2.36 (0.84)	1.01 (0.87)
BPAF	BPAF-$^{13}C_{12}$	7.01(13.4)[a]	2.01 (0.58)	1.00 (0.58)
BPAP	BPB-$^{13}C_{12}$	4.80 (10.2)[a]	2.86 (0.25)	1.06 (0.26)
BPB	BPB-$^{13}C_{12}$	Not applicable	2.71 (0.29)	1.00 (0.36)
BPF	BPF-$^{13}C_{12}$	45.0 (18.1)[a]	1.80 (1.60)	1.00 (1.65)
BPM/BPP	BPB-$^{13}C_{12}$	Not applicable	3.94 (0.09)	1.46 (0.15)
BPS	BPS-$^{13}C_{12}$	30.9 (6.10)[a]	0.40 (2.34)	1.01 (2.34)
BPZ	BPB-$^{13}C_{12}$	56.3 (5.7)[a]	3.11 (0.38)	1.15 (0.43)
BzP-1	BPS-$^{13}C_{12}$	79.6 (3.01)[b]	0.48 (0.74)	1.23 (0.74)
BzP-2	BPS-$^{13}C_{12}$	62.4 (2.81)[a]	0.39 (1.80)	1.01 (1.80)
BzP-3	BPB-$^{13}C_{12}$	4.22 (12.2)[a]	3.21 (0.23)	1.19 (0.29)
BzP-8	BPF-$^{13}C_{12}$	93.3 (8.72)[a]	1.89 (0.19)	1.06 (0.35)
4-OH BzP	BPS-$^{13}C_{12}$	21.5 (12.3)[a]	0.48 (1.48)	1.22 (1.48)

[a] N=4 highest calibration concentrations
[b] N=6 highest calibration concentrations
[c]RTs and RRTs were reported as the mean of the values recorded from the calibration standards

Table S5. Method detection (MDLs) and quantification limits (MQLs) in ng/g w.w.for the target analytes.

Target Analyte	MDLs	MQLs
BPA	1.50	4.90
BPAF	0.004	0.01
BPAP	0.20	0.70
BPB	0.10	0.40
BPF	0.40	1.20
BPM/BPP	0.004	0.01
BPS	0.10	0.20
BPZ	0.30	1.10
BzP-1	0.10	0.30
BzP-2	0.10	0.30
BzP-3	0.10	0.20
BzP-8	0.90	3.10
4-OH BzP	2.00	6.50

Table S6. Intra-day Precision (RSD%; n=3 for each matrix) for the target analytes.

Contaminants	BPA	BPF	BPB	BPS	BPZ	BPAP
Preen Gland	13.9	11.2	13.4	8.22	10.8	15.3
Liver	6.61	22.4	24.1	5.23	3.43	5.43
Contaminants	**BPM/BPP**	**BzP-8**	**BzP-3**	**BzP-2**	**BzP-1**	**4-OH-BzP**
Preen Gland	26.3	3.22	7.71	8.61	7.53	15.8
Liver	5.52	21.0	29.5	3.82	5.12	3.12

Table S7. Matrix factors (MFs; n=3 for each matrix) for the target analytes in preen gland and liver samples.

Contaminants	BPA	BPF	BPB	BPS	BPZ	BPAP	BPAF
Preen Gland	0.5	0.6	0.3	0.4	0.2	0.2	0.6
Liver	0.6	0.7	0.7	0.6	0.2	0.4	0.7
Contaminants	**BPM/BPP**	**BzP-8**	**BzP-3**	**BzP-2**	**BzP-1**	**4-OH-BzP**	
Preen Gland	0.6	0.9	0.2	0.5	0.5	0.5	
Liver	0.9	1.0	0.2	0.6	0.5	0.6	

Table S8. Matrix effects (MEs%) for the target analytes in preen gland and liver samples.

Contaminants	BPA	BPF	BPB	BPS	BPZ	BPAP	BPAF
Preen Gland	-52	-45	-69	-59	-81	-83	-43
Liver	-38	-27	-29	-44	-85	-57	-32
Contaminants	**BPM/BPP**	**BzP-8**	**BzP-3**	**BzP-2**	**BzP-1**	**4-OH-BzP**	
Preen Gland	-39	-8	-81	-52	-51	-45	
Liver	-14	-1	-81	-44	-49	-38	

Table S9. BPs and BzPs in all tissues from all three species; concentrations expressed in ng/g w.w.

n=44	BPA	BPAP	BPB	BPF	BPM/BPP	BPS	**ΣBPs**	BzP-1	BzP-3	BzP-8	4-OH-BzP	**ΣBzPs**	ΣAll
Detection Rate	4/44	3/44	7/44	8/44	10/44	6/44	**23/44**	5/44	4/44	2/44	3/44	**13/44**	30/44
Median	67.5	0.81	1.60	3.01	0.08	0.65	**2.48**	0.95	0.47	27.1	9.70	**0.95**	2.92
Mean	72.5	0.84	1.26	3.94	0.12	1.50	**14.9**	1.65	1.99	27.1	20.7	**10.3**	15.9
Min.	6.23	0.76	0.42	1.22	0.02	0.20	**0.05**	0.71	0.31	7.42	8.21	**0.31**	0.05
Max.	149	0.96	1.84	7.32	0.49	5.23	**152**	4.3	8.21	46.9	44.3	**55.1**	152

BLOCK B

Development of generic sample treatment platforms for the control of contaminants in foods.

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A new sample treatment strategy based on simultaneous supramolecular solvent and dispersive solid-phase extraction for the determination of ionophore coccidiostats in all legislated foodstuffs

S. González-Rubio, D. García-Gómez[1,*], A. Ballesteros-Gómez, S. Rubio

Departamento de Química Analítica, Instituto Universitario de Investigación en Química Fina y Nanoquímica IUIQFN, Universidad de Córdoba, Campus de Rabanales,Edificio Marie Curie (anexo), E-14071 Córdoba, Spain.

∗ Corresponding author.

E-mail address: dgg@usal.es (D. García-Gómez).

1 Current address: Department of Analytical Chemistry, Nutrition and Food Science, University of Salamanca, Spain

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Supramolecular solvent (SUPRAS), Dispersive solid phase extraction (dSPE), LC-MS/MS, Ionophore coccidiostats, Food analysis

Abstract

A single-step sample treatment, for the simultaneous extraction and clean-up for the determination of ionophore coccidiostats in EU legislated foodstuffs, is here proposed. The treatment is based on the combination of: (i) a supramolecular solvent with restricted access properties (SUPRAS-RAM), spontaneously formed by the addition of hexanol, water and THF to the sample; and (ii) dispersive solid phase extraction (dSPE). The SUPRAS-RAM extract was directly compatible with LC-MS/MS and no further re-extraction, evaporation or cleanup procedures were necessary. SUPRAS-RAM efficiently extracted the ionophores (recoveries in milk, eggs, fat, liver, kidney, and chicken and beef muscle were in the range 71−112%) and removed proteins and carbohydrates, whereas dSPE removed fats and other lipophilic compounds. The method was validated following the European Commission Decision 2002/657/EC. Detection limits (0.004−0.07 μg kg−1) were far below the maximum residue limits (1−150 μg kg−1). Method analytical and operational characteristics were suitable for routine determination of ionophores.

1. Introduction

Coccidiosis is an intestinal tract infection of farmed species produced by a protozoa of the phylum Apicomplexa (Shirley & Lillehoj, 2012; Mitchell, Smith, & Ellis-Iversen, 2012). One of the most effective ways of treating and preventing coccidiosis is the use of coccidiostats, which are drugs used worldwide as feed

additives and administered to animals for almost their entire life (Clarke et al., 2014). Coccidiostats can be categorized as synthetic coccidiostasts or as naturally occurring ionophores, such as monensin (MON), narasin (NAR), lasalocid (LAS), salinomycin (SAL), maduramicin (MAD) and semduramicin (SEM). The latter group is characterized by multiple tetrahydrofuran rings connected together in the form of spiroketal moieties (Riddell, 2002) and its determination is the focus of this research. Ionophores are potent drugs and, when residues occur in food, they may exacerbate certain coronary disease conditions (Dorne et al., 2013). Thus, the European Commission Regulation (mainly EC124/2009a; EC 37/2010; EC86/2012; EU 1277/2014), together with the Commission Directive 2009b/8/EC, established maximum residue limits (MRLs) for food of animal origin (i.e., eggs, milk, liver, kidney, muscle and fat). This restrictive legislative framework has led to the need of developing effective and sensitive analytical methods. Although ionophores can be detected by LC-fluorescence and LC-UV after post column derivatization, today the predominant technique for ionophore screening and confirmation is LC-MS/MS (Clarke et al., 2014). Table 1 shows the most relevant methods reported in the last decade for ionophore determination by LC-MS/MS.

Table 1
Analytical methods based on LC-MS/MS reported for the determination of ionophores in the legislated matrices in the last decade[1] of NAR).

Ionophore	Legislated matrix	Sample treatment	Chemicals involved in sample treatment	Separation/detection	Relative Recoveries (%)	Analytical limits (μg kg^{-1})	References
LAS MON NAR MAD SAL SEM	Muscle, eggs	• Solvent extraction • Dispersive SPE • Solvent evaporation and reconstitution	• ACN (10 mL) • $MgSO_4$ (0.6 g); C_{18} sorbent (0.125 g)	LC (HILIC)-MS/MS (matrix- matched calibration)	Muscle: 92–114 Eggs: 102–107	LOD: 0.021–0.13	(Dasenaki and Thomaidis, 2019)
LAS MON NAR MAD SAL	Muscle	• Solvent extraction (twice) • Solvent evaporation • Defatting (twice) • SPE (C_{18}) • Solvent evaporation and reconstitution	• H_2O (2 mL), AcH (0.16 mL), ACN (4.7 mL), ethyl acetate (3.1 mL) • ACN (5 mL), hexane (1 mL) • H_2O (20 mL) for sample loading; ethyl acetate (15 mL) for analyte elution	HPLC-MS/MS (matrix-matched calibration)	71.6–87.6	LOQ: 0.58–1.92	(Zhao et al., 2018)
LAS MON NAR MAD SAL SEM	Milk	• QuEChERS method • Solvent evaporation and reconstitution	• ACN (4 mL ×2), NaCl (1 g), $MgSO_4$ (0.8 g), anhydrous sodium acetate (b0.2 g)	HPLC-MS/MS (matrix-matched internal standard calibration)	94–110	LOQ: 0.07–0.9	(Pereira et al., 2016)
LAS MON NAR MAD SAL SEM	Eggs	• Solvent extraction • SPE (Hybrid-SPE) • Solvent evaporation and reconstitution	• 0.1% formic acid in (8:2) ACN:water (8 mL), 0.1 M EDTA (500 μL). • Elution with 1 mL of 0.1% formic acid in ACN	LC-MS/MS (matrix-matched calibration)	75–108	CCβ: 2.7–169	(Piatkowska et al., 2016)
LAS MON NAR MAD SAL	Milk, muscle, eggs, liver	• Solvent extraction (twice) • Solvent evaporation (up to 10 mL ACN) • SPE (ENVI-Carb) • Solvent evaporation and reconstitution	• ACN (20 mL), anhydrous sodium sulphate (5 g) • ACN (10 mL) for analyte elution	LC-MS/MS (matrix-matched calibration)	Milk: 79.4–113.8 Muscle: 76.2–96.7 Liver: 68.2–80.5 Eggs: 71–102	LOQ: 0.4–1	(Jing et al., 2016)
LAS MON NAR MAD SAL SEM	Muscle, liver, kidney	• Solvent extraction • Matrix dilution	• ACN (3 mL), isopropanol (2 mL), anhydrous sodium acetate (0.87 g), anhydrous magnesium sulphate (0.55 g) • 1:1 with water	LC-MS/MS (matrix-matched calibration)	Liver: 80–96	LOQ: 0.6–11	(Matus & Boison, 2016)
LAS MON NAR MAD SAL SEM	Milk, muscle	• Milk: QuEChERS method • Solvent evaporation and reconstitution • Muscle: Solvent extraction • Solvent evaporation and reconstitution	• NaOH 1 M (600 μL), ACN (10 mL), NaCl (1 g), $MgSO_4$ (4 g) • ACN (20 mL)	UHPLC-MS/MS (matrix-matched internal standard calibration)	Muscle: 80–125 Milk: 84–120	LOQ: 0.13–0.42 mg kg^{-1}	(Clarke et al., 2013)
LAS MON NAR MAD SAL SEM	Milk	• Solvent extraction • SPE (Oasis HLB) • Solvent evaporation and reconstitution	• ACN (2 mL) • Loading: supernatant (3 mL) and H_2O (6 mL); elution: ACN (3 mL)	LC-MS/MS (matrix-matched calibration)	77–118	LOQ: 0.1–0.5	(Nász & Károlyné, 2012)
LAS MON NAR MAD SAL SEM	Muscle, eggs	• Solvent extraction • Solvent evaporation and reconstitution	• ACN (20 mL)	UHPLC-MS/MS (matrix-matched calibration)	Muscle: 68–81 Eggs: 55–82	CCβ: 2.44–209	(Moloney et al., 2012)
LAS MON NAR MAD SAL SEM	Eggs	• Solvent extraction (twice) • Defatting (twice) • SPE (Silica) • Solvent evaporation and reconstitution	• ACN (6 mL), anhydrous sodium sulphate (4 g) • hexane (5 mL) • Loading with ACN (12 mL), elution with ACN (2 mL)	LC-MS/MS (matrix-matched calibration)	83–115	CCβ: 2.4–201	(Galarini et al., 2011)
LAS MON NAR SAL	Milk	• Protein precipitation	• ACN (5 mL)	LC-MS/MS (matrix-matched calibration)	60.7–118	LOQ: 0.5–1	(Thompson et al., 2011)

Table 1 (*continued*)

Ionophore	Legislated matrix	Sample treatment	Chemicals involved in sample treatment	Separation/detection	Relative Recoveries (%)	Analytical limits (μg kg^{-1})	References
LAS MON NAR MAD SAL SEM	Eggs	• Solvent extraction (twice) • Solvent evaporation and reconstitution	• ACN (4 mL)	LC-MS/MS (matrix-matched calibration)		LOQ: 0.14–3.4	(Spisso et al., 2010)
LAS MON MAD SAL	Muscle, eggs	• Solvent extraction (twice) • Solvent evaporation and reconstitution	• ACN (10 mL), anhydrous sodium sulphate (10–15 g)	LC-MS/MS (matrix-matched calibration)	Muscle: 82.4–114.2 Eggs: 79.5–125.2	LOQ: 0.1–0.2	(Shao et al., 2009)

Only methods involving at least four of the six legislated ionophores have been considered. As it is shown in the table, most of the reported methods were only applied to one or two of the six legislated foodstuffs, preferably muscle, eggs and/or milk. Sample treatment was essentially matrix dependent (see Table 1). Thus, treatment of muscle often involved repetitive solvent extraction with acetonitrile or solvent mixtures (e.g. ACN-ethyl acetate, ACN-isopropanol), followed by intensive cleanup with SPE or dispersive SPE, sometimes with additional steps as defatting, and intermediate and/or final solvent evaporation (Dasenaki and Thomaidis, 2019; Zhao et al., 2018; Jing, Ge, Lian-feng, & Jian-chen, 2016). Sample treatment reported for milk varied from simple deproteinization (Thompson, Noot, & Kendall, 2011) to the use of the QuEChERS method (Clarke, Moloney, O'Mahony, O'Kennedy, & Danaher, 2013; Pereira et al., 2016) or more complex treatments (Jing et al., 2016; Nász & Károlyné, 2012). Sample treatment for eggs has been quite similar to that reported for muscle (Dasenaki & Thomaidis, 2019; Piatkowska, Jedziniak, & Zmudzki, 2016; Jing et al., 2016; Galarini, Fioroni, Moretti, Pettinacci, & Dusi, 2011). Because of the high complexity of the matrices, neither of the methods was interference-free and matrix-matched calibration, sometimes combined with internal standard calibration, was invariably required to obtain accurate results (Table 1). Nevertheless, relative recoveries of ionophores in some matrices, as shown in Table 1, are still low. For example, relative recoveries were below 70% for some compounds for liver in Jing et al., and Moloney et al. reported recoveries<60% for two coccidiostats in eggs and<70% for LAS in muscle. The relative recovery of LAS reported by Thompson et al. was also

low for milk (60.7%). To the best of our knowledge, no universal, quick, and green sample treatment methodology for the determination of ionophores in all the legislated foodstuffs has been reported so far in the literature. Such a comprehensive, cost-effective, and environment-friendly methodology would be of great interest to simplify ionophore's routine control. In this work, we have tried to meet this goal by developing a single-step sample treatment based on the combination of supramolecular solvents (SUPRAS) and dispersive SPE. The working hypothesis is that this combination has the capability to provide a simple, quick, and cost-effective sample treatment for ionophores in all legislated foodstuffs. SUPRAS are nanostructured liquids generated spontaneously from colloidal suspensions of amphiphiles via self-assembly processes (Caballo, Sicilia, & Rubio, 2017). They have been shown to be very attractive for the development of efficient sample treatments for analytes covering a wide polarity range (Caballo et al., 2017; Ballesteros-Gómez, Rubio, & Sicilia, 2009). Furthermore, SUPRAS properties can be tuned by proper selection of amphiphiles and the environment for their self-assembly (Ballesteros-Gómez, Sicilia, & Rubio, 2010). Some of them are synthesized from colloidal solutions made up of alkanols or of carboxylic acids in tetrahydrofuran and have restricted-access properties (SUPRAS-RAM). This allows the extraction of a huge variety of low molecular weight solutes while excluding macromolecules through precipitation or size-exclusion mechanisms. Reported uses of SUPRAS RAM to extract contaminants from different matrices can be found in the literature (Salatti-Dorado, Caballero-Casero, Sicilia, Lunar, & Rubio, 2017; Caballero-Casero, Çabuk, Martínez-Sagarra, Devesa, & Rubio, 2015; Ballesteros-Gómez & Rubio, 2012; García-fonseca & Rubio, 2016; Accioni, García-Gómez, Girela, & Rubio, 2018). Therefore, SUPRAS RAM have the capability to efficiently extract ionophores and also to clean up the sample regarding proteins and carbohydrates. However, because of their amphiphilic character, the extraction of lipids will be facilitated by formation of mixed aggregates with the amphiphile making up the SUPRAS and this might cause matrix interferences. Lipids can be efficiently removed by evaporation of SUPRAS extracts (Salatti-

Dorado et al., 2017). However, introducing an evaporation step in sample treatment hinders high sample throughput, an attribute highly desirable in the control of food quality. Accordingly, in order to prevent/reduce interferences from lipids in the determination of ionophores by LC-MS/MS, we investigated here a new strategy based on the use of dispersive SPE, that has proved to be a good alternative for sample cleanup (Islas, Ibarra, Hernandez, Miranda, & Cepeda, 2017). In this work we present the development of a new sample treatment combining SUPRAS-RAM and dSPE, capable for efficient extraction of the analytes and for the removal of matrix interferences and that can be applied to all the legislated foodstuffs.

2. Experimental section

2.1. Reagents and standards

All solvents were LC-grade and were used as supplied. 1-Hexanol was purchased from Merck (Darmstadt, Germany) and tetrahydrofuran (THF) and methanol (MeOH) were supplied by VWR-Prolabo Chemicals (Bois, France). Formic acid was a Sigma-Aldrich reagent (St. Louis, MO, USA). Ultra-high-quality water was generated from a Milli-Q water purification system (Millipore-Sigma, Madrid, Spain). Magnesium sulfate (MgSO4) and Primary-Secondary Amine (PSA), investigated for matrix cleaning, were obtained from Sigma-Aldrich and Supelco (Bellefonte, PA, USA), respectively. Narasin A (NAR), salinomycin A monosodium salt hydrate (SAL), lasalocid A sodium salt (LAS), maduramicin ammonium salt (MAD) and monensin A sodium salt (MON) were also Sigma-Aldrich chemicals. Sodium semduramicin (SEM) was kindly donated by the Federal Office of Consumer Protection and Food Safety EU (Berlin, Germany). Stock solutions for individual coccidiostats (1000 μg mL−1) were prepared in

MeOH and stored at −20 °C. They were stable for at least 6 months. Intermediate and working solutions of coccidiostat mixtures were prepared by appropriate dilution in Milli-Q water and stored at 4 °C for at least one month.

2.2. Apparatus

The analysis was performed using an Agilent Technologies 1200 series LC (Waldbronn, Germany) coupled to a 6420 triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI) (Waldbronn, Germany). Separation was carried out on a C18 Phenomenex Luna analytical column, (100 mm × 2 mm, particle size: 3 μm). Raw data were processed using Agilent MassHunter Software® (version B.07.00). Some selectivity studies were carried out using a LC ThermoQuest spectra system (San Jose, CA, USA) equipped with a SCM 1000 vacuum membrane degasser, a P2000 binary pump, an AS3000 autosampler and a Spectra System UV6000 LP detector. The volume of SUPRAS was measured using a digital caliper (0−150 mm/0−6″). Other equipment included a Heathrow Scientific vortex (Illinois, USA), a 36×2.2/1.5 mL angle rotor high speed brushless centrifuge MPW350R from MPW Med-Instruments (Warschaw, Poland) and a homogenizer disperser Ultra-Turrax T25 Basic from Ika (Werke, Germany).

2.3. Determination of ionophore coccidiostats in foodstuffs

2.3.1. Sample collection and pretreatment

All samples were purchased in local supermarkets in Córdoba (Spain). Meat samples (kidney, fat, liver, chicken muscle and beef muscle) were cut and stored at −20 °C in plastic bags, while milk and eggs were directly stored at 4 °C. Before extraction with SUPRAS, all samples (except milk) were homogenized by use of a high-speed Ultra-Turrax.

2.3.2. SUPRAS-RAM/dSPE-based microextraction and cleanup

Aliquots of 130 mg of meat or eggs, or 65 mg of fat were dispersed in 1275 μL of Milli-Q water in 2 mL microtubes. For milk, 1275 μL aliquots were directly used. Then, the following reagents were sequentially added to the microtubes: for SUPRAS-RAM, 184 μL of hexanol and 84 μL of THF; and for dSPE, 150 mg of MgSO4 and 25 mg of PSA. Microtubes were then vortexed for 2 min at 3000 rpm, followed by centrifugation for 10 min at 15,000 g for meat and egg samples or for 20 min at 20,000 g for milk samples. The SUPRAS phase standing at the top of the solution was withdrawn with a syringe and transferred to a chromatographic vial for analysis. Fig. 1 shows a schematic of the SUPRAS + dSPE sample treatment for the legislated foodstuffs.

2.3.3. Quantification by LC-MS/MS

All ionophore coccidiostats were separated and quantified by LC MS/MS. The mobile phase consisted of solvent (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The flow rate was 250 μL min−1 and the injection volume was 2 μL. The elution program was as follows: linear gradients from 50% to 75% of B in 15 min and from 75% to 95% of B in the next 5 min and isocratic conditions at 95% of B for the last 8 min. Finally, initial conditions were re-equilibrated for 7 min. For sample analysis, a post run of 3 min with 50% THF and 50% methanol was necessary for column cleaning. Mass spectrometric conditions for coccidiostats detection were optimized by direct infusion of 10 μg mL−1 of individual coccidiostat standards in Milli-Q water (in triplicate). Positive ionization mode was used for all the target compounds. The most abundant fragment was used as quantifier ion while the second served as qualifier ion. Products ions and detection parameters of each MS/MS transition are shown in Table S1 in supporting information (SI). Optimal source settings were the

following: source gas temperature 350 °C, capillary voltage +4000 V, nebulizer gas pressure 35 psi.

2.4. Validation

Validation was carried out according to the guidelines established by the Commission Decision 2002/657/EC (2002) for quantitative methods of analysis. This decision provides rules concerning the performance of analytical methods to be used for determining veterinary drug residues in products of animal origin. The following analytical parameters were validated: linearity, sensitivity, trueness, precision, decision limit, selectivity, and ruggedness. Linearity was assessed by running calibrations using blank samples fortified before extraction at five concentrations, below and above the MRL value for each ionophore (see MRLs in Table S2 and concentration ranges used for calibration, expressed as factors of MRLs, in Table S3). Detection limits were calculated from six independent complete analysis of each target foodstuff containing no detectable levels of ionophores, by using a signal-to-noise ratio of 3. Since no certified reference materials were available, trueness was assessed through absolute recovery by spiking blank samples before extraction at 3 concentration levels below and/or above the MRL for each ionophore (Table 2 specifies the spike level for each ionophore and matrix, expressed as a percentage of the MRL value).

Table 2. Absolute recoveries obtained for MON and SAL by extraction with six SUPRASobtained under different synthetic conditions

SUPRAS	[a]SUPRAS synthesis (%, v/v)			SUPRAS Volume ±[b]SD (µL)	Recovery ±[b]SD (%)	
	Hexanol	THF	Water		MON	SAL
1	10	5	85	155±2	96±4	107±6
2	10	25	65	297±3	97±5	105±6
3	5	5	90	75±2	60±3	57±4
4	5	25	70	159±2	97±2	93±5
5	3	5	92	48±2	38±2	34±3
6	3	25	72	92±2	56±4	54±3

[a] Total volume: 1.5 mL; [b] n =3

Precision was evaluated in terms of repeatability and within-laboratory reproducibility. For this purpose, every spiked sample (at the same 3 concentration levels specified in Table 2 for each ionophore) was determined in five replicates in three consecutive days (n = 15). Repeatability, expressed as relative standard deviation (RSD), was calculated as the square root of the average value of the intra-day variances, and within-laboratory reproducibility as the square root of the inter-day variance. Decision limits were calculated by analyzing 20 blank samples for each target foodstuff, fortified with ionophores at the corresponding permitted limit, and it was calculated from the concentration at the permitted limit plus 1.64 times the corresponding standard deviation ($\alpha = 5\%$). The selectivity of the method was checked by analyzing 20 blank samples for each food sample and examining the presence of false positive results due to endogenous matrix interferences. Additionally, matrix effects were assessed by comparing the slopes of the calibration curves obtained from standards in water and matrix-matched standards, prepared by fortification of SUPRAS extracts after extraction of blank

samples. Fortification of SUPRAS extracts was carried out at the same concentration levels specified for each ionophore and matrix in Table S3. The results were analyzed by ANCOVA (critical P-value: 0.05). The ruggedness of the method, related to differences in the analysis arising from the diversity of the matrices, was assessed by comparing absolute recoveries within matrices at the medium concentration level (at MRL values or below) by one-way ANOVA (critical P-value: 0.05).

3. Results and discussion

3.1 Optimization of the supramolecular solvent-based extraction of ionophores

The SUPRAS was formed in situ by the addition to the sample of a colloidal suspension of hexanol in THF and, subsequently, water to induce liquid phase separation (Salatti-Dorado et al., 2017). It was checked that the water content in milk (i.e., 87–88%) was able to directly induce the formation of the SUPRAS. In the case of solid food-stuffs, water was added to the sample to both induce SUPRAS formation and to disperse the sample during extraction. In all cases, the SUPRAS formed spontaneously at the top of the solution and it was in equilibrium with an aqueous-THF solution, containing hexanol at the critical aggregation concentration, and the sample (see Fig. 1).

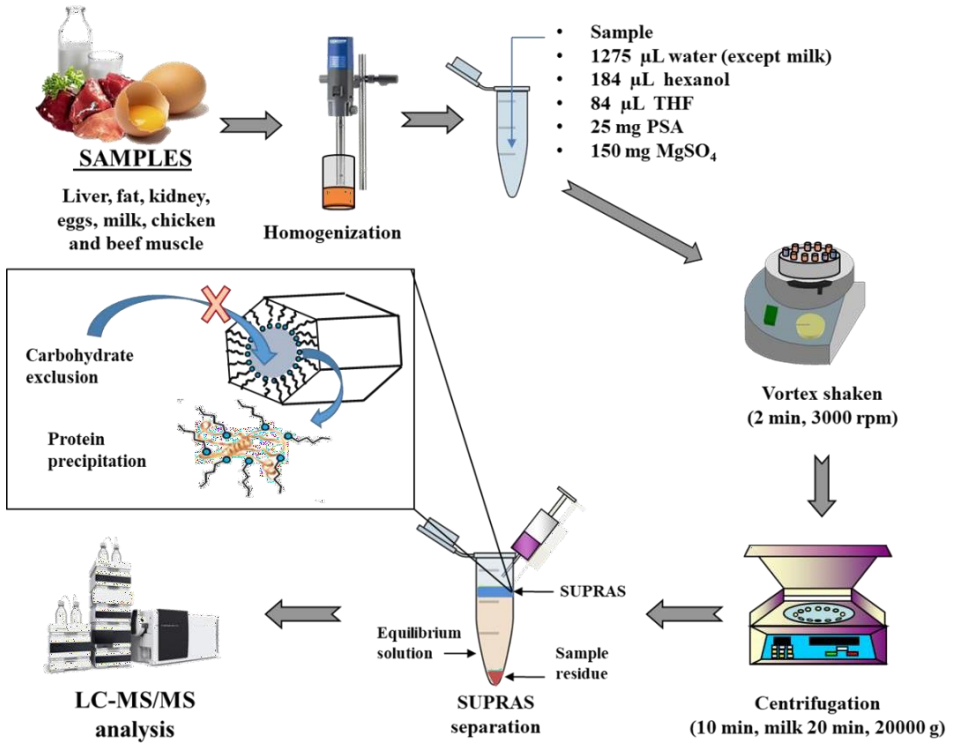

Fig. 1 Scheme of the SUPRAS+dSPE sample treatment for the determinationof ionophores in the legislated foodstuffs.

The inverted hexagonal aggregates in which hexanol molecules arrange in the SUPRAS (Fig. 1) offer different types of interactions for ionophores, namely dispersion interactions in the hydrocarbon chains and hydrogen bond donors and acceptors in the polar groups. Because ionophores have both an alkyl backbone and oxygen-rich functional groups, which can establish dispersion interactions and hydrogen bonds, respectively (Fig. S1), mixed-mode mechanisms could be established for ionophore solubilization in the SUPRAS. The partition equilibrium for ionophores between the SUPRAS and the aqueous-THF solution was firstly investigated. For this purpose, MON and SAL, which have the same number of hydrogen bond donors (viz. 4) and acceptors (viz. 11) and are representative of the structures of the rest of ionophores in terms of THF rings (e.g. 1 for SAL, LAS and NAR and 3 for MON, MAD and SEM, see Fig. S1) were selected as model

compounds. The study involved the production of six types of SUPRAS from different percentages of hexanol, THF and water and the de- termination of absolute recoveries of MON and SAL. Both ionophores were spiked to the aqueous solution at a concentration of 20 ng mL^{-1}. Experiments were done in triplicate. Table 3 provides the composition of the synthetic solutions for the tested SUPRAS (SUPRAS 1–6), the volume of SUPRAS generated in each case and the absolute recoveries of MON and SAL.

Table 3. Absolute recoveries for the determination of ionophores in different food matrices

Sample	[a] Ionophore concentration	(b Mean recovery ± SD) %					
		SEM	MON	LAS	SAL	MAD	NAR
Liver	0.5 MRL	112±3	102±7	96±2	96±8	87±3	89±2
	1 MRL	110±4	95±6	83±5	90±7	89±8	91±2
	1.5 MRL	88±2	100±1	82±7	101±6	100±4	99±2
Milk	0.5 MRL	105±4	108±10	108±8	99±6	110±6	106±5
	1 MRL	97±2	93±5	94±6	99±6	95±5	93±2
	1.5 MRL	99±2	103±2	101±5	101±7	94±1	98±0
Chicken muscle	0.5 MRL	109±1	92±9	100±7	100±4	108±4	80±5
	1 MRL	107±7	105±1	101±5	71±2	110±6	109±10
	1.5 MRL	99±5	100±4	92±3	100±1	100±6	94±3
Beef muscle	0.5 MRL	104±2	108±3	74±4	83±1	79±3	90±4
	1 MRL	102±2	91±3	104±1	102±1	102±12	103±2
	1.5 MRL	99±2	99±6	101±2	100±6	110±3	92±2
Kidney	0.5 MRL	88±6	105±13	104±2	78±6	100±13	100±8
	1 MRL	104±9	86±2	110±3	102±1	105±5	102±5
	1.5 MRL	98±3	101±2	94±2	100±3	100±3	100±1
Egg	0.5 MRL	97±9	88±13	109±4	110±4	108±7	85±1
	1 MRL	102±4	105±5	110±4	92±2	90±3	88±8
	1.5 MRL	100±1	100±1	97±3	101±2	100±0	104±4
Fat	0.5 MRL	103±6	81±9	105±7	93±14	97±10	89±6
	1 MRL	104±7	107±12	95±10	98±4	101±4	97±9
	1.5 MRL	103±2	99±3	96±5	101±1	100±1	93±10

[a] MRLs values in Table S-2; [b] n=3

As it was expected, the volume of SUPRAS was linearly and exponentially dependent on the percentage of hexanol and THF, respectively (Salatti-Dorado et al., 2017). Partition of MON and SAL to the SUPRAS phase was favored at the highest percentages of hexanol and/or THF (SUPRAS 1, 2 and 4 in Table 2), which is an evidence that both hydrogen bonding and dispersion interactions were contributing to ionophore solubilization. The SUPRAS-1, formed from 10% of hexanol and 5% of THF, was selected as optimal due to the low percentage of THF required for its formation. Once the optimal SUPRAS was selected, absolute recoveries were calculated for all the six ionophores at different concentrations levels in water (0.5, 1, 5, 25, 50, 100 and 250 ng mL^{-1}). Average recoveries kept within the ranges 90–104% (NAR), 96–107% (MON), 93–109% (SAL), 92–102% (SEM), 92–103% (MAD) and 98–109% (LAS), this proving the favorable partition coefficient for the target analytes in the extractant phase.

3.2 Optimization of sample cleanup by combination of RAM properties of SUPRAS and dispersive SPE

Water-induced alkanol-based SUPRAS have been previously reported to be environment responsive (Ballesteros-Gómez & Rubio, 2012). Thus, as indicated above, alkanols such as hexanol arrange as inverted hexagonal aggregates where the polar groups surround aqueous cavities and the hydrocarbon chains are dispersed in THF (Fig. 1). The content of water in the SUPRAS and, consequently, the size of the aqueous cavities of the inverted hexagonal aggregates, increase as the percentage of THF in the colloidal suspension does. This means that this SUPRAS has the potential to behave as a restricted access liquid for polar

macromolecules such as polysaccharides that are size excluded from the vacuoles. Moreover, proteins precipitate in the presence of THF and the alkanol. Accordingly, both proteins and carbohydrates will be primarily excluded from extraction into the SUPRAS. On the other hand, dispersive SPE has been widely used for interference removal (Islas et al., 2017). In this work, PSA alone and in combination with C18, were used as sorbents for dSPE. PSA was selected for matrix- cleaning due to its ability to remove organic and fatty acids while C18 was chosen to remove fats, sterols and other nonpolar interferences. It was checked in preliminary experiments that the volume of SUPRAS decreased in the presence of these sorbents, so $MgSO_4$ was added to favor complete liquid phase separation. The capability of SUPRAS-RAM for interference removal was investigated alone and in combination with dSPE by subjecting both fortified and unfortified blank samples to the whole process (section 2.3.2). Fortification was carried out at the five levels specified in Table S3 to check linearity. The concentration of ionophores in the SUPRAS extracts from fortified samples was measured by LC-MS/MS. The absorbance of the SUPRAS extracts obtained from unfortified blank samples was measured by LC-DAD, in the range 200–500 nm, within the elution region for the target compounds (viz. 22–27 min). It was checked that linearity was not obtained in some matrices, such as fat and eggs, when dSPE was carried out with mixtures of PSA and C_{18}, whereas this phenomenon was not observed as experiments were run in the presence of only PSA. This lack of linearity might be due to a strong interaction between C_{18} and ionophore coccidiostats. The C_{18} phase competed with SUPRAS and leaded to significant losses of the target compounds, particularly at low concentrations. Therefore, the combination SUPRAS/dSPE ($MgSO_4$ + PSA) was selected for further studies. Fig. 2 compares chromatograms at 250 nm of SUPRAS extracts from blank foodstuff samples, in the absence (in green) and presence (in blue) of $MgSO_4$ and PSA.

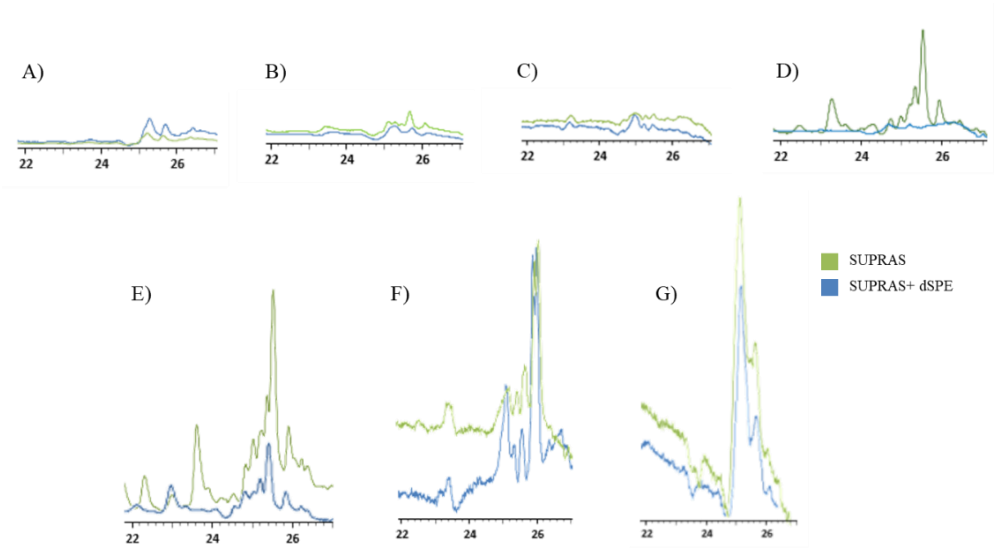

Fig. 2 UV-Vis chromatograms obtained for A) liver, B) milk, C) chicken muscle, D) beef muscle, E) kidney, F) egg and G) fat with either SUPRAS/dSPE (in blue) or SUPRAS (in green) sample treatment (optimal conditions).

Background signals were quite similar for several foodstuffs in the elution region of ionophores (e.g. liver, milk and chicken muscle, Fig. 2A–C), independently of the extraction strategy (SUPRAS or SUPRAS-dSPE). However, an important improvement in background signals was obtained for the rest of matrices (Fig. 2D–G), some of them of special complexity (e.g. eggs and fat), by the action of dSPE. Less intense background signals were also observed in the presence of PSA-based dSPE out the elution region for analytes (data not shown) for all the matrices investigated. Therefore, since clean-up is advisable to ensure the good performance and maintenance of the system when analyzing a wide variety of complex food matrices, the combination of SUPRAS-dSPE ($MgSO_4$ + PSA) was selected as optimal sample treatment for the analysis of ionophore coccidiostats in foodstuffs.

3.3 Validation

3.3.1 Linearity and sensitivity

Calibration curves of ionophores in the legislated foodstuffs were linear in the tested concentration range. Table S3 lists the concentration ranges, calibration slopes, correlation coefficients, and standard deviations of residuals for each ionophore and sample calculated by least- square regression. Correlation coefficients ranged from 0.991 to 0.9996. In all cases, method detection limits were far below the MRLs and ranged from 0.004 μg kg^{-1} for SEM in beef muscle to 0.07 μg kg^{-1} for LAS in eggs.

3.3.2 Trueness

Absolute recoveries were all in the interval 71–112% (Table 3) and they were in good agreement with guidelines by the Commission Decision 2002/657/EC (2002) (i.e., recoveries in the ranges 50–120%, 70–110% and 80–110% for concentrations ≤1 μg kg^{-1}, 1–10 μg kg^{-1} and ≥10 μg kg^{-1}, respectively). Consequently, ionophores can be determined accurately with the proposed method.

3.3.3 Selectivity

To assess possible interferences from matrix components, two approaches were implemented. The first one consisted in analyzing 20 blank liver, milk, chicken and beef muscle, kidney, egg, and fat samples, and checking for false positives at the retention times of the analytes. No peaks were detected in the regions of interest by measuring the quantifier and qualifier ions specified in Table S1. The second approach involved the comparison of the slopes of the calibration curves from standards in water and matrix-matched standards (see section 2.4).

The volume of SUPRAS that was produced in water and in the presence of beef muscle and liver matrices was similar (around 120 μL). However, it decreased in the presence of milk (94 μL) and kidney (100 μL) and increased in the presence of egg (130 μL), chicken muscle (150 μL) and fat (195 μL) samples. These volumes kept constant for the different types of matrices when different samples were analyzed. So, for comparison of the slopes of the calibration curves in food matrices and in water, the first ones were corrected to consider the variation in SUPRAS volume. Table S4 shows the results of the Bonferroni and Sidak post-hoc tests to make pairwise comparisons (ionophore/matrix). Matrix effects for around 60% of tests (green tick) were acceptable and in the range 87–109%. According to these results, external calibration could be applied to chicken muscle. However, since significant differences were found for the rest of tests (red cross), matrix-matched calibration is proposed for the determination of ionophores in the legislated foodstuffs by applying the generalized sample treatment here developed.

3.3.4 Precision

The repeatability and reproducibility values for ionophores in fortified food samples at three concentration levels are summarized on Table S5. Relative standard deviations were in the ranges 1–10% and 5–17% for within- and between-run experiments, respectively, which was consistent with the Commission Decision 2002/657/EC (2002) criteria.

3.3.5 Decision limits

The decision limits (viz. the limits at and above which it can be concluded with an error probability of α that a sample is not compliant) were calculated for all the ionophores and food matrices (Table S6). Decision limits were in the ranges 2.03–2.11 μg kg^{-1}, 5.08–5.25 μg kg^{-1}, 30.05–30.21 μg kg^{-1} and 50.8–50.9 μg kg^{-1} for ionophores with MRLs of 2, 5, 30 and 50 μg kg^{-1}, respectively.

Decision limits were 30.21 and 101.5 μg kg^{-1} for MON and LAS in liver, 1.06 μg kg^{-1} for NAR in milk, 8.06 and 60.75 μg kg^{-1} for MON and LAS in chicken muscle, 10.05 and 22 μg kg^{-1} for LAS in beef muscle and kidney and 155 and 12.09 μg kg^{-1} for LAS and MAD in eggs (see MRLs in Table S2). All these values mean that the method is fit-for- purpose according to Commission Decision 2002/657/EC (2002) criteria.

Ruggedness

Regarding ruggedness, the major change to be investigated involves differences arising from the diversity of studied matrices. Recoveries (Table 2) at MRL values or below (medium level) were statistically compared by one-way ANOVA (see Table S7). A P-value of 0.6117 was obtained, indicating that the recoveries in the 7 matrices were statistically identical and, therefore, the performance of the method in these matrices is similar.

3.3.6 Analysis of foodstuff samples

The suitability of the proposed method for determining ionophore coccidiostats in legislated foodstuff was assessed by analyzing fourteen non-fortified samples of chicken and beef muscle, fat, egg, milk, kidney and liver, all purchased in local supermarkets in Córdoba (South Spain). Analysis were carried out in triplicate. Some ionophores (e.g. SEM, MON, SAL and NAR) were found in three samples (fat, egg and kidney) (see Table S8). Concentrations of MON and SAL in fat, NAR in egg and SAL in kidney surpassed the corresponding MRL value (i.e.2 μg kg^{-1}) and they were above the corresponding decision limits. For the rest of analyzed samples, ionophores were undetected (data not shown) or they were below the detection limits (e.g. SEM and NAR for fat, Table S8). Positive samples

were fortified with ionophores at the corresponding MRL values and analyzed by triplicate. Absolute recoveries were all in the range 85–106%. Fig. 3 shows the extracted ion chromatograms of the quantifier and qualifier transitions of the ionophores that were found in the samples at concentrations above their detection limit (see Table S8).

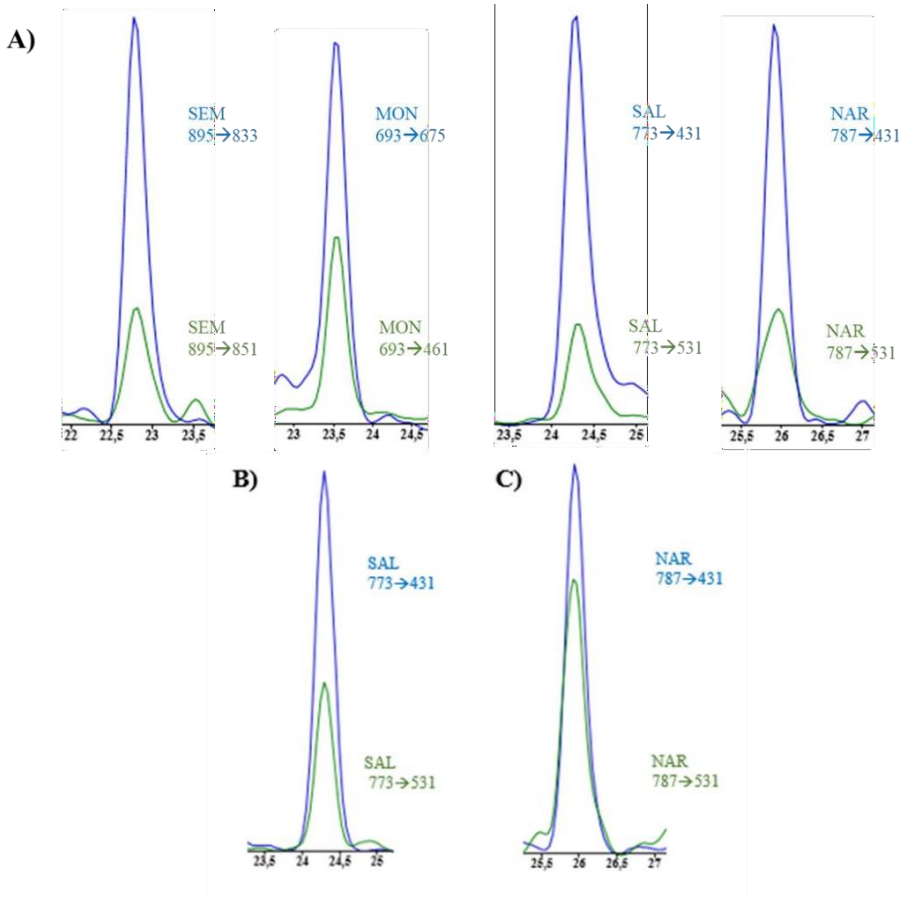

Fig. 3 MRM chromatograms of the qualifier (in green)and quantifier (in blue) ions for the detected ionophores in samples subjected to SUPRAS/dSPE sample treatment. A) fat (0.90±0.06 μg kg^{-1} of SEM, 3.4±0.2 μg kg^{-1} of MON, 3.3±0.2 μg kg^{-1} of SAL and 3.7±0.1 μg kg^{-1} of NAR); B) kidney (4.7±0.3 μg kg^{-1} of SAL) and C) egg (5.6±0.4 μg kg^{-1} of NAR).

4 Conclusions

The combination of SUPRAS-RAM and dSPE provided a new sample treatment strategy for ionophores that was applicable to all the legislated foodstuffs. The ability of supramolecular solvents to behave as restricted access materials simultaneously allowed the extraction of ionophores and the removal of interferences with a simplified sample treatment. In addition, the combined use of dispersive SPE provided cleaner extracts in such complex matrices. No further clean-up or evaporation/reconstitution steps were required. This strategy constitutes a good alternative to the conventional methods and we hypothesize that its further application to the extraction of other contaminants in other foodstuffs may allow its use for generic sample treatment. To the best of our knowledge, this is the first time that a method combining SUPRAS + dSPE has been developed and successfully applied in food analysis.

CRediT authorship contribution statement

S. González-Rubio: Investigation, Writing - original draft, Visualization. D. García-Gómez: Conceptualization, Formal analysis, Writing - review & editing, Supervision. A. Ballesteros-Gómez: Conceptualization, Formal analysis, Writing - review & editing, Supervision. S. Rubio: Conceptualization, Formal analysis, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPORTING INFORMATION

A new sample treatment strategy based on simultaneous supramolecular solvent and dispersive solid-phase extraction for the determination of ionophore coccidiostats in all legislated foodstuffs.

S. González-Rubio[1], D. García-Gómez[1,2*], A. Ballesteros-Gómez[1], S. Rubio[1]

1. Departamento de Química Analítica, Instituto Universitario de Investigación en Química Fina y Nanoquímica IUIQFN, Universidad de Córdoba, Campus de Rabanales, Edificio Marie Curie (anexo), E-14071 Córdoba, España.

2. Current address: Department of Analytical Chemistry, Nutrition and Food Science, University of Salamanca, Spain. *Corresponding author, email: dgg@usal.es

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Table S-1. Compound specific chromatographic and MS/MS parameters for each ionophore

Ionophore	Precursor ion $[M+Na]^+$ (m/z)	Product ions (m/z)		Fragmentor (DP) (V)	Collission energy (CE) (eV)	t_R (min)
		Quantifier	Qualifier			
SEM	895.5	833.4	851.4	220	30	22.84
MON	693.4	675.2	461.1	190	60	23.55
LAS	613.3	377.1	577.2	190	44	24.14
SAL	773.4	431.1	531.2	220	60	24.44
MAD	939.5	877.4	895.4	190	36	24.45
NAR	787.5	431.1	531.2	250	56	26.02

Table S-2. Maximum Residue Limits (expressed in µg kg-1) set for ionophores in the legislated matrices.

Sample	**Animal origin**	**SEM**	**MON**	**LAS**	**SAL**	**MAD**	**NAR**
Liver	Bovine	2[f]	30[d]	100[a]	5[f]	2[b]	50[f]
Milk	Bovine	2[f]	2[d]	n.a.[a*]	2[f]	2[b]	1[f]
Muscle	Poultry	n.l.	8[h1,h2]	60[a]	5[g]	30[c]	50[e]
Muscle	Bovine	2[f]	2[d]	10[a]	2[f]	2[b]	5[f]
Kidney	Bovine	2[f]	2[d]	20[a]	2[f]	2[b]	5[f]
Egg	Poultry	2[f]	2[f]	150[a]	3[f]	12[b]	2[f]
Fat	Porcine	2[f]	2[f]	5[f]	2[f]	2[b]	5[f]

(a): Commission Regulation 1277/2014/EC, (b): Commission Regulation 610/2012/EC (c): Commission Regulation 388/2011/EC, (d): Commission Regulation 37/2010/EC, (e) Commission Regulation 885/2010/EC, (f) Commission Regulation 124/2009/EC, (g) Commission Regulation 167/2008/EC, (h1): Commission Regulation 1096/2008/EC (h2): Commission Regulation 109/2007/EC; * n.a.= non-authorized . n.l.: non-legislated.

Table S-3. Calibration curve parameters for ionophores in different food matrices

Ionophore		Liver	Milk	Chicken muscle	Beef muscle	Kidney	Egg	Fat
SEM	[a]Concentration range	0.25-5.0	0.25-5.0	n.l.	0.25-5.0	0.25-5.0	0.25-5.0	0.25-5.0
	[b](Slope ± SD)x10^{-2}	11.9±0.3	13.15±0.09		13.2±0.3	12.4±0.1	10.9±0.4	4.9±0.2
	[c]R	0.995	0.9990		0.9990	0.9993	0.9992	0.996
	[d]S_{yx} x10^{-2}	2.5	1.2		3.2	0.1	0.4	0.09
	[e]MDL	0.08	0.02		0.004	0.01	0.03	0.02
MON	[a]Concentration range	0.07-1.33	0.25-5.0	0.06-1.25	0.25-5.0	0.25-5.0	0.25-5.0	0.25-5.0
	(Slope ± SD)x10^{-2}	152±2	30.8±0.4	58±2	31.1±0.4	14.2±0.3	11.7±0.2	7.3±0.2
	R	0.9990	0.9992	0.998	0.9991	0.998	0.997	0.998
	S_{yx} x10^{-2}	0.3	3	0.5	0.5	0.4	0.2	0.1
	MDL	0.07	0.05	0.03	0.01	0.02	0.03	0.01
LAS	[a]Concentration range	0.12-2.5	n.a.	0.08-1.7	0.12-2.5	0.62-12.5	0.25-5.0	0.25-5.0
	(Slope ± SD)x10^{-2}	7.4±0.1		40.2±0.6	19±0.2	3.89±0.03	7.0±0.2	7.5±0.1
	R	0.994		0.9993	0.998	0.9990	0.991	0.998
	S_{yx} x10^{-2}	1.8		3	4.5	3	5.7	0.2
	MDL	0.07		0.05	0.05	0.03	0.07	0.01
SAL	[a]Concentration range	0.25-5.0	0.25-5.0	0.1-2.0	0.25-5.0	0.25-5.0	0.25-5.0	0.25-5.0
	(Slope ± SD)x10^{-2}	8.7±0.2	37.2±0.4	94±2	21.1±0.9	17.6±0.5	17.7±0.3	8.2±0.2
	R	0.995	0.9993	0.996	0.995	0.996	0.9990	0.998
	S_{yx} x10^{-2}	0.5	2.6	1.2	0.9	0.6	0.5	0.1
	MDL	0.07	0.05	0.04	0.05	0.01	0.03	0.01
MAD	[a]Concentration range	0.25-5.0	0.25-5.0	0.017-0.33	0.25-5.0	0.25-5.0	0.04-0.8	0.25-5.0
	(Slope ± SD)x10^{-2}	16.4±0.6	20.6±0.2	213±6	11.4±0.2	15.8±0.2	135±2	8.4±0.2
	R	0.996	0.9991	0.997	0.9994	0.994	0.9994	0.998
	S_{yx} x10^{-2}	0.7	3.8	0.4	0.2	3	3.2	0.1
	MDL	0.03	0.02	0.05	0.04	0.06	0.01	0.01
NAR	[a]Concentration range	0.25-5.0	0.25-5.0	0.02-0.5	0.25-5.0	0.25-5.0	0.25-5.0	0.25-5.0
	(Slope ± SD)x10^{-2}	5.98±0.08	12.8±0.2	160±3	8.58±0.09	12.3±0.8	8.3±0.1	5.80±0.07
	R	0.997	0.997	0.998	0.9992	0.9996	0.998	0.998
	S_{yx} x10^{-2}	2.8	2	0.9	3.2	0.3	0.1	0.1
	MDL	0.05	0.06	0.03	0.01	0.02	0.01	0.03

[a] Where a value of 1 corresponds to the specific MRL
[b] Units are in absorbance units MRL^{-1}
[c] Coefficient of correlation
[d] Standard deviation of residuals
[e] Limit of detection of the method (in µg kg^{-1})
n.a.: non-authorized. n.l.: non-legislated

Table S-4. Pairwise comparison results obtained by applying Bonferroni and Sidak Post- Hoc tests (same results) between external (SUPRAS-dSPE) and each foodstuff calibration curves.

	SEM	MON	LAS	SAL	MAD	NAR
Liver	✓	X	X	X	✓	X
Milk	✓	X	n.a.	✓	X	✓
Chicken muscle	n.l.	✓	✓	✓	✓	✓
Beef muscle	✓	X	✓	X	✓	X
Kidney	✓	✓	✓	X	✓	✓
Eggs	✓	✓	X	X	X	X
Fat	X	✓	✓	X	✓	X

Table S-5. Repeatability and reproducibility (in RSD%) for the determination of ionophores in different food matrices

Sample	[a]Repeatability ([b]RSD %)						[a]Reproducibility ([c]RSD %)					
	SEM	MON	LAS	SAL	MAD	NAR	SEM	MON	LAS	SAL	MAD	NAR
Liver	7.0	9.3	7.7	3.1	6.0	5.5	12	14	13	8	13	10
	9.5	3.3	4.3	7.1	4.9	4.1	14	7.6	8.5	11	12	8.8
	10	8.4	4.2	8.3	7.3	4.4	15	13	10	10	11	8.8
Milk	5.4	3.2	n.a.	5.0	3.5	5.7	9.3	8.6	11	12	9.7	10
	4.1	2.5		4.2	3.1	4.9	10	5.7	7.6	6.1	5.8	8.0
	7.7	4.9		3.6	2.8	4.2	10	7.2	5.2	5.8	5.8	6.0
Chicken muscle	n.l.	9.4	8.6	8.9	5.2	8.8	12	13	14	13	8.5	9.8
		4.1	3.7	6.8	3.2	5.1	14	13	8.6	10	7.7	10
		8.4	7.4	9.7	5.2	10	16	14	10	16	9.7	15
Beef muscle	7.2	5.9	3.7	5.7	5.7	5.3	12	9.9	5.2	10	11	7.2
	5.8	6.9	3.1	6.0	5.8	5.7	7.8	15	7.2	12	6.2	8.6
	4.4	6.8	3.7	5.2	5.8	5.7	7.0	9.9	7.6	11	11	9.0
Kidney	10	1.5	4.7	10	5.4	3.5	17	5.2	6.7	15	7.8	7.9
	6.5	4.9	3.7	9.6	2.6	6.9	10	8.6	8.7	15	8.6	12
	9.7	5.1	4.5	6.7	5.7	6.3	9.0	6.7	5.4	11	6.1	8.0
Egg	4.3	8.9	3.3	3.0	5.2	6.5	9.6	11	5.3	10	11	13
	7.5	9.8	3.7	9.6	6.5	10	10	13	5.6	13	12	16
	4.8	5.9	2.5	6.6	5.3	4.7	12	13	4.5	13	6.4	6.5
Fat	10	4.5	4.7	4.8	5.2	6.0	16	7.3	9.5	6.5	5.7	7.0
	9.1	3.3	3.8	3.4	5.1	5.5	14	11	8.3	11	6.9	10
	9.2	5.7	3.4	4.0	3.4	4.6	12	11	8.1	9.1	7.9	9.6

[a] Same concentration levels as stated in Table 2. MRLs values in Table S-2; [b] n=5 for each concentration; [c] five replicates in three consecutive days (n=15) for each concentration.

n.a: non-authorized. n.l.: non-legislated

Table S-6. [a]Decision limits for the determination of ionophores in food matrices

Ionophore	Liver	Milk	Chicken muscle	Beef muscle	Kidney	Egg	Fat
SEM	2.09	2.06	n.l.	2.08	2.09	2.11	2.05
MON	30.21	2.03	8.06	2.09	2.06	2.10	2.05
LAS	101.5	n.a.	60.75	10.05	22	155	5.25
SAL	5.11	2.06	5.09	2.09	2.05	3.15	2.05
MAD	2.09	2.05	30.05	2.08	2.03	12.09	2.08
NAR	50.9	1.06	50.08	5.08	5.09	2.06	5.18

[a] Calculated from 20 blank samples fortified at the corresponding permitted limit (see Table S-2). n.a.: non-authorized. n.l.: non-legislated

Table S7. One-way ANOVA of mean absolute recoveries (medium level, see Table 2) in different matrices.

ANOVA

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Liver	6.0	558.0	93.0	84.4
Milk	5.0	477.0	95.4	6.8
Chicken muscle	5.0	496.0	99.2	261.2
Beef muscle	6.0	604.0	100.7	23.1
Kidney	6.0	609.0	101.5	66.3
Egg	6.0	587.0	97.8	81.8
Fat	6.0	602.0	100.3	20.7

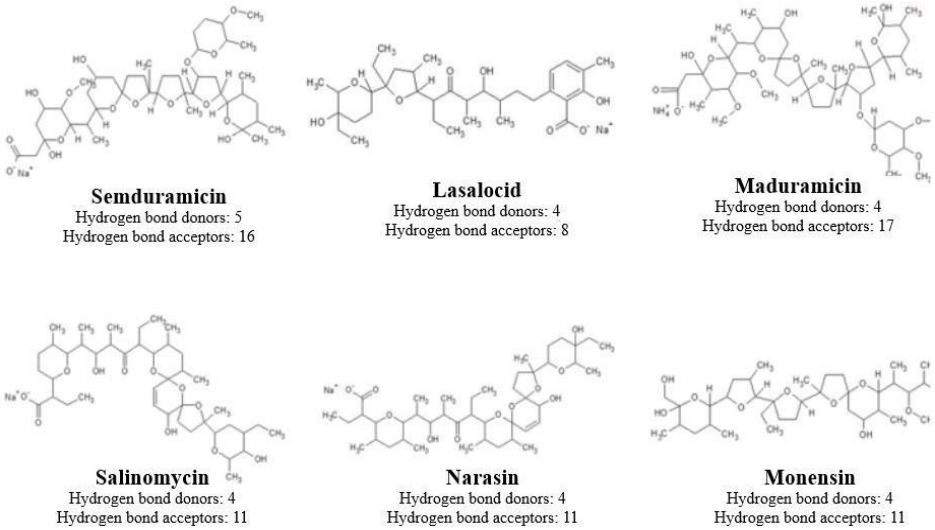
Source of variation	*d.f.*	*Sum of squares*	*Variance*	*F-Value*	*P-Value*
Between groups	6	335.77	55.96	0.752	**0.611**
Within groups	33	2453	74.33		
Total	39	2788.77			

Significance level $\alpha = 0.05$; F_{crit} 2.389

Table S-8. Concentrations (expressed as µg kg^{-1}) and standard deviations (SD) found for ionophores in positive foodstuff samples analyzed by the proposed method

	SEM±SD	MON±SD	LAS±SD	SAL±SD	MAD±SD	NAR±SD
Fat	0.90±0.06	3.4±0.2	<LOD	3.3±0.2	<LOD	3.7±0.1
Egg	<LOD	<LOD	<LOD	<LOD	<LOD	5.6±0.4
Kidney	<LOD	<LOD	<LOD	4.7±0.3	<LOD	<LOD

Figure S-1. Molecular structures of ionophores and their corresponding number ofhydrogen bond donors and acceptors.



BLOCK C

Development of sample treatment platforms for the environmental monitoring of contaminants in a wide polarity range.

Talanta

239 (2022) 123108

Double-headed amphiphile-based sponge droplets: synthesis, characterization and potential for the extraction of compounds over a wide polarity range

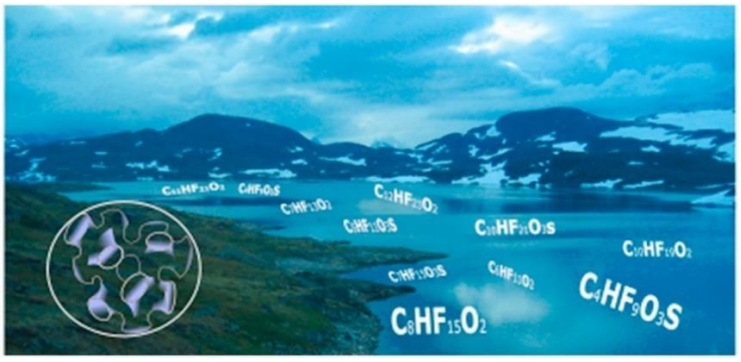
S. González-Rubio[a], A. Ballesteros-Gómez [a,*], D. García-Gómez[b], S. Rubio[a]

a Department of Analytical Chemistry. Institute of Fine Chemistry and Nanochemistry. Universidad de Córdoba, Campus de Rabanales, Edificio Marie Curie (anexo), E- 14071, Córdoba, Spain

b Department of Analytical Chemistry, Nutrition and Food Science, University of Salamanca, Spain

* Corresponding author. E-mail address: ana.ballesteros@uco.es (A. Ballesteros-Gómez).

Graphical abstract



Keywords: Supramolecular solvent (SUPRAS); Sponge phases; Double- headed amphiphile; Liquid-liquid extraction; Liquid chromatography-mass spectrometry; perfluorinated compounds.

Abstract

Supramolecular solvents (SUPRASs) are gaining momentum in the multi- residue analysis of liquid samples thanks to the delimited hydrophilic and hydrophobic microenvironments in their nanostructures. In this work, SUPRASs with increased hydrophilicity were synthesized with the aim of enhancing the recoveries of polar compounds. For this purpose, a double- headed amphiphile, 1,2-decanediol, was self-assembled in hydro-organic media in the presence and absence of sodium chloride. The SUPRASs formed, characterized by scanning electron microscopy, consisted of sponge droplets made up of a highly convoluted three-dimensional (3D) network of amphiphile. The network contained interconnected bilayers that were intersected by similarly interconnected aqueous channels with high and nearly constant water content (~30%, w/w). Both the inherently open structure of the sponge morphology and the increased hydrophilic-hydrophobic balance of the amphiphile, provided highly hydrophilic microenvironments into the aggregates that rendered in increased recoveries for 15 perfluorinated compounds (PFCs, C_4-C_{18}, log P_{ow} values from 0.4 to 11.6) in natural waters. Extraction took 15 min without further clean-up or evaporation of extracts which were readily compatible with LC-MS/MS quantitation. Absolute recoveries for PFCs, at the level of a few ng L^{-1}, were in the range 70-120%,

except for perfluoropentanoic acid (40%) and perfluorobutane sulfonic acid (51%). Detection limits for PFCs in water were in the range 0.005-0.01 ng L^{-1}, which allowed their determination in slightly polluted waters (0.07-2.33 ng L^{-1}). This work proves that hydrophilicity in SUPRASs can be tailored through the amphiphile and the morphology of their aggregates, and that this characteristic improves recoveries in multi-residue analysis.

1. Introduction

The design and production of tailored solvents for analytical extractions has become a fruitful field in recent years because of the possibility they offer to improve yields, selectivity, sustainability and costs [1]. Innovative approaches based on tailored ionic liquids (ILs), deep eutectic solvents (DESs) or supramolecular solvents (SUPRASs) have enabled the development of unique sample treatment platforms for determination of organic compounds in a variety of food, environmental or biological matrices [2-4]. Thus, the development of new tailor-made solvents for their further application has experienced fast grown in the last few years [1].

Supramolecular solvents are nanostructured liquids made up of coacervate droplets that are produced from colloidal suspensions of amphiphiles by establishing environmental conditions that lead to their spontaneous self-organization into a new liquid phase (see Figure S1 in Supplementary Information, SI) [5]. The ordered structures in SUPRASs can be tailored by

proper design of the environment (e.g., selection of the driving force leading to coacervation) and/or the components (e.g., selection of the head group and hydrocarbon chain of the amphiphile) [6,7].

Considerable progress has been made in SUPRAS-based analytical extractions in the last two decades by just selecting the proper amphihile for each application [5,8]. Thus, the use of non-aromatic anionic amphiphiles overcame the main drawbacks of the traditional temperature-induced SUPRASs based on Triton X and PONPE surfactant series, such as high signal backgrounds in LC-UV/fluorescence detection, unacceptable chromatographic peak widths and decomposition of thermally labile compounds [9]. Further advances in tailored SUPRASs have been related to the use of alkanols, which have allowed the production of densely packed water-induced SUPRASs that provides a huge number of binding sites for solute solubilization (up to 1 mg μL^{-1}) [10]. These SUPRASs also feature restricted access properties, thus allowing the integration of analyte extraction and interference removal in a single step, and have negligible signal in ESI-MS [11], which make them suitable for mass detection [12]. Likewise, some SUPRASs have been reported to provide novel solubilization mechanisms (e.g. halogen bonding [13], polar hydrophobicity [14]) that have been successfully exploited for developing innovative applications. Also, engineered SUPRASs have permitted to expand their scope of application to GC-MS [15,16], proteomic [17], wastewater treatment [18], biomass valorization [19], etc. However, despite all these advances, the production of SUPRASs with tailored properties is still in its

infancy and there is a long way to go to exploit its full potential in analytical extractions.

In this work, progress in this area is intended by designing and producing engineered SUPRASs made up of double-headed amphiphile sponge dropletswith the aim of rising the efficiency in the liquid-liquid extraction of compounds that feature a wide range of polarities, prior to their screening/quantification by LC-MS/MS. This is a demanding challenge in many fields where multi-component determination is routine (e.g., environmental, agri-food, anti-doping, etc.) and at which conventional solventsdo not provide a suitable solution.

Sponge droplets (also called L3 phases) consist of a highly convoluted and interconnected three-dimensional network of amphiphile bilayers intersected by similarly interconnected nanometer-sized aqueous channels [20-22]. These bicontinuous phases offer enormous amphiphilic surface areas and highly hydrophilic, in addition to hydrophobic, environments [22]. So they have the potential to solubilize compounds in a wide polarity range. The formation of sponge phases has been reported for a wide variety of amphiphilic systems but frequently they only exist within a narrow range of amphiphile concentration, pH, and temperature, and are highly sensitive to additives [20,23,24]. To the best of our knowledge, sponge phases have not been used for analytical purposes.

On the other hand, amphiphiles possessing more than one head group present unique aggregation behavior and important biological functions [25]. Thus, micellar sizes and aggregation numbers decrease as the number of head

groups rises owing to the enhanced electrostatic repulsion and/or steric hindrance among the amphiphilic molecules. The hydrocarbon chains remain highly folded in the resulting micelles and this renders them wetter than their single-head group counterparts [26]. So, both the larger amphiphilic head group and the wetter micellar environment should result in more hydrophilic SUPRASs, thus facilitating the extraction of very polar compounds.

Here we investigate the production of water-induced SUPRASs with sponge morphology from the double-headed amphiphile 1,2-decanediol. Our working hypothesis was that combining both strategies (i.e., larger amphiphilic head groups and wetter SUPRAS nanostructures), the recoveries for very polar compounds should increase while keeping good extraction rates for very non-polar compounds, thus facilitating multi-component extraction. These SUPRASs were characterized and their extraction potential for compounds covering a wide polarity range was tested by extracting perfluorinated compounds (PFCs) from environmental waters as model compounds prior to their determination by LC-MS/MS. The selected PFCs consisted in sulfate and carboxylate head groups and alkyl chain lengths in the range C4-C18. Results were compared to those provided by decanol, a single-head amphiphile SUPRAS consisting of inverted hexagonal aggregates [10]. To the best of our knowledge, this is the first SUPRAS produced from the coacervation of alkanediols.

2. Materials and methods

2.1. Chemicals

All solvents were LC-grade and were used as supplied. Tetrahydrofuran (THF) and methanol (MeOH) were purchased from VWR- Prolabo Chemicals (Bois, France) while isopropanol was obtained from Panreac (Barcelona, Spain). Ultra-high-quality water was generated from a Milli-Q water purification system (Millipore-Sigma, Madrid, Spain). 1- Decanol, 1,2-decanediol and sodium chloride were supplied by Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate was a Fluka reagent (Buchs, Stwitzerland). Certified native perfluorinated compounds (PFCs) and isotope- labeled internal standards (IS), all of them prepared in methanol at a concentration of 2 μg mL^{-1}, were purchased as custom solution mixtures from Wellington Laboratories (Guelph, ON, Canada). The target PFCs included three perfluoroalkane sulfonic acids (PFSAs) and twelve perfluoroalkyl carboxylic acids (PFCAs). Table S1 in Supplementary Information shows thenames and acronyms for the native PCFs and IS selected in this study. Two stock solutions of a mix of PFCs and a mix of ISs (both at a concentration of 0.2 μg mL^{-1} each), as well as intermediate and working solutions, were prepared in MeOH and stored at -20°C.

2.2. SUPRAS formation and characterization

The formation of water-induced 1,2-decanediol-based SUPRASs was investigated by constructing phase diagrams for the ternary mixtures THF:water:1,2-decanediol, THF: aqueous NaCl (1M):1,2-decanediol and isopropanol:water:1,2-decanediol. For this purpose, the components of each ternary mixture were added at different percentages (w/w %) in centrifuge tubes, magnetically stirred (1000 rpm, 30 min) and centrifuged (3000 g, 5 min). Region boundaries in the phase diagrams for SUPRAS formation were assigned by visual inspection (i.e. existence of an isotropic solution, two liquid phases or solid-liquid phases).

The chemical composition of the SUPRASs was determined as follows. The water content was measured in a coloumetric Karl Fischer titrator from Metrohm (Herisaus, Switzerland). The percentage of 1,2-decanediol was calculated by weighting a SUPRAS aliquot of 200 μL before and after evaporation until obtaining a dry residue that corresponded to the amount of amphiphile. Finally, the THF or isopropanol content in the SUPRAS was calculated by difference.

The SUPRAS volumes obtained as a function of the composition of the ternary mixture of the bulk synthesis solution were measured in centrifuge tubes with narrow necks designed by authors. Pobel S.A. (Madrid, Spain, web page: www.pobel.com) constructed them from commercial heavy-duty glass cylindrical centrifuge tubes with round-bottom but reducing the diameter from a specified height. The measures were as follows: bottom: 34 mm of outside diameter and 57 mm height, neck: 8 mm of internal diameter and 35 mm height. Their capacity was between 41 and 42 mL. The volumes of SUPRASs

were calculated by measuring their height in the cylindrical neck (π·r2·h) of the centrifuge tubes with a digital caliper. Non-linear regression was used to fit a model for the prediction of the volume of SUPRAS within the SUPRAS region of analytical interest. The design of the model was carried out by usingthe statistical program Statgraphics Centurion XV.All experiments were performed in duplicate.

The presence of coacervate droplets in the SUPRAS was investigated with a light microscope (Leica model DME; Wetzlar, Germany) equipped with an automatic photocamera, using the bright field.

Investigation of the nanostructures in the SUPRAS was undertaken by scanning electron microscopy (SEM). For this purpose, the SUPRAS (˜10 μL)was fixed with glutaraldehyde and then it was embedded with a 6% aqueous agarose solution. After that, the sample was washed three times with sodium cacodylate, and stained with OsO4 (1%) for contrast enhancement. Samples were then dehydrated with a graded series of acetone (30, 50, 70, 80, 90, 100
%) and then they were dried using the critical point drying. Finally, samples were coated with gold and observed under SEM. The accelerating voltage wasset at 10 kV.

2.3. Determination of PFCs in environmental waters

2.3.1. Samples

Water samples (reservoir, rivers and well) were collected in Córdoba province (South of Spain) in March 2021. The reservoir water was taken in La Breña, located in Almodóvar del Río village. River waters were collected in

the Guadalquivir, flowing by Córdoba city, and Guadiato, located in a protected natural area close to Trassierra village (Baños de Popea), which has frequent hiking activity. The well water was also located in the latter area. All samples were collected in plastic bottles, and filtered through 0.45μm cellulose acetate filters (Millipore HNWP, Bedford, MA, USA), to remove suspended particles. Then, they were stored at 4°C until analysis.

2.3.2. SUPRAS-based microextraction of PFCs

Water aliquots (36 mL) containing 1 M NaCl were transferred to specially designed centrifugation tubes with narrow necks (i.d. 8 mm, 42 mL volume) which contained 175 mg of 1,2-decanediol dissolved in 4 mL of THF.After sealing the tubes with parafilm to avoid THF evaporation, the mixtures were magnetically stirred for 15 min at 1,000 rpm, and then they werecentrifuged for 5 min at 3,000 g to speed up the separation of SUPRAS from the aqueous solution. The supramolecular extracts (about 230μL) were standing at the top of the solutions in the narrow necks of the tubes. An aliquotof 200 μL of each extract was withdrawn with a microsyringe, and it was transferred to a sealed glass vial with insert for subsequent LC-MS/MS analysis. Figure S2 in SI shows a schematic of the SUPRAS-based extraction procedure. All experiments were done in triplicate.

2.3.3. Quantification of PFCs by LC(ESI-)MS/MS

All PFCs were separated and quantified using a liquid chromatograph (Waters, Acquity H-Class, Milford, MA, USA) coupled to a hybrid triple

quadrupole/linear ion trap (Applied Biosystems MSD Sciex, 5500QTRAP, Four Valley, ON, Canada) equipped with a TurboIonSpray (TIS) interface. Separation was carried out on a phenyl-hexyl analytical column (100mm x 2mm, particle size: 3μm) from Phenomenex Luna (USA). An additional LC column (Agilent Eclipse Plus C8, 5 μm, 4.6 mm × 50 mm) was inserted between the pump and the injector in order to trap possible PFCs released fromthe instrument. All data were controlled and processed using the Analyst 1.6.2 Software. The mobile phase consisted of a mixture of (A) 70% water and 30% methanol containing 2 mM ammonium acetate and (B) 2 mM ammonium acetate in methanol. The flow rate was 250 μL min^{-1} and the injection volume was 5 μL. The column temperature was set at 30ºC. The elution program was as follows: isocratic conditions at 100% of A for the first minute and linear gradient from 0% to 100% of B in 12 min. These isocratic conditions were maintained during 7 min. Finally, initial conditions were re-equilibrated for 10 min. Negative ionization mode was used for all PFCs. The most abundant fragment was used as quantifier ion while the second served as qualifier ion. Products ions and detection parameters of each MS/MS transition are shown in Table **S1**. Optimal source settings were the following: source gas temperature 400°C, capillary voltage -4500 V, nebulizer gas pressure 50 psi and curtain gas pressure 40 psi.

3. Results and discussion

3.1. Synthesis and characterization of 1,2-decanediol-based SUPRASs

Among double headed alcohol-based amphiphiles, 1,2-decanediol was selected for SUPRAS formation because the length of its hydrocarbon chain is at an intermediate point in the range of the target PFCs (i.e. C_4-C_{18}). Colloidal suspensions of this amphiphile were obtained in both a protic (isopropanol) and an aprotic (THF) solvent and coacervation was investigated by the addition of water, a poor solvent for 1,2-decanediol.

3.1.1. Phase diagrams

Ternary phase diagrams for the mixtures 1,2-decanediol-THF-water and 1,2-decanediol-isopropanol-water are depicted in Fig. 1A and B, respectively. Both ternary plots exhibited three different domains that were set by visual inspection: (*i*) the SUPRAS region, (*ii*) the isotropic solution domain at low and medium content of water, and (*iii*) the amphiphile precipitation region at low organic solvent content. SUPRASs in THF:water formed in a broader region than those prepared in isopropanol:water and, in addition, the minimum percentage of THF required for coacervation (i.e. 8.5%, w/w) was lower than that of isopropanol (i.e. 10.1%, w/w). This behavior was similar to that occurring in the coacervation of alkylcarboxylic acids in organic solvent:water media and it has been attributed to the different solvation ability of solvents for the amphiphile which can be guided by their Hildebrand solubility parameter (δ) [27]. Thus, solvents having lower δ value (δ_{THF} = 9.5 $cal^{1/2}cm^{-3/2}$ < $\delta_{Isopropanol}$ = 11.5$cal^{1/2}cm^{-3/2}$) will have higher solvency for the

amphiphile, and consequently, the coacervation will occur at less percentage of organic solvent. On the other hand, the minimum concentration of amphiphile required to observe coacervation was 0.25 % (w/w) in both THF:water and isopropanol:water media.

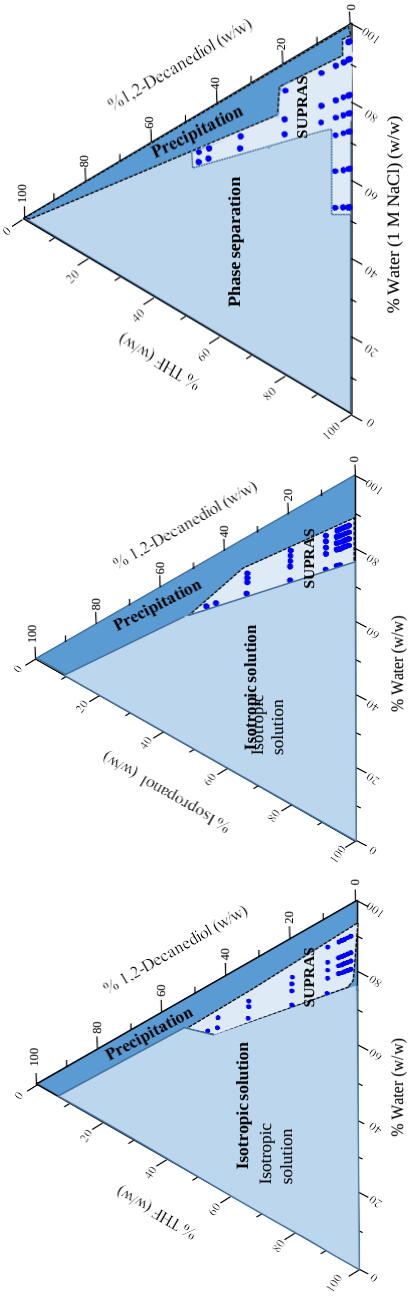


Figure 1. Ternary phase diagrams for (A) 1,2-decanediol:water:THF, (B) 1,2-decanediol:water:isopropanol and (C) 1,2-decanediol:water (1MNaCl):THF mixtures. All ingredient concentrations are expressed as percentages (w/w).

The influence of salts on the coacervation of 1,2-decanediol in THF:water was further investigated. Salts have long been known to influence the temperature-induced coacervation of non-ionic surfactants in water solutions, their effects being concentration-dependent [28]. Thus, salts destruct the hydration layer of amphiphile head groups at low concentrations (e.g. around 0.1-0.4 M), which causes decrease in the effective area per molecule at the interface and the corresponding increase in surfactant monomer packing and aggregate growth. At high salt concentration (e.g. 1-2 M), most of the water is captured at the ion hydration spheres and *salting out* occurs [29].

Figure 1C shows the phase diagram obtained for the ternary system 1,2-decanediol:THF: aqueous NaCl (1M). Addition of salt caused the broadening of the SUPRAS region and the solvent formed in a wider range of THF:water compositions, which suggests that salting out was an additional mechanism helping to 1,2-decanediol coacervation. The most valuable asset obtained by the addition of salt was the decrease in the minimum concentration required for coacervation of both amphiphile (0.125%, w/w) and THF (4.4%, w/w). This decrease is beneficial not only from an analytical point of view (i.e.,higher analyte concentration factors can be achieved), but also with a view towards the environmental sustainability of the sample treatment process. It is worth mentioning that unlike the phase diagram for 1,2-decanediol in

THF:water (Fig. 1A), there was not an isotropic region in the presence of salt,but a phase separation of THF owing to a salting-out effect, a phenomenon previously reported by our research group [30].

Given that SUPRAS formation in THF, both in the absence and presence of salt, was more favorable compared to isopropanol, we decided to further investigate the two first ones for determining their characteristics for PFC extraction.

The volume of SUPRAS produced in the colloidal system was a function of both the concentration of amphiphile and the organic solvent. *SUPRAS volume*section can be found at the Supplementary Information.

3.1.2. SUPRAS chemical composition

The chemical composition of SUPRASs produced at increasing percentages of THF is shown in Table 1.

Table 1. Chemical composition and percentage of incorporation of 1,2-decanediol into SUPRASs at representative experimental conditions

Synthetic mixture, (%, w/w)			[a]SUPRAS composition, (%, w/w)			% 1,2-decanediol in SUPRAS
1,2-decanediol	THF	Water	1,2-decanediol	THF	Water	
3.0	8.6	88.4	54.5±0.2	14±2	31±4	99.1±0.4
3.0	13.0	84.0	44.7±0.7	21±2	34±3	93.2±0.5
3.0	17.5	79.5	36.3±0.1	31±1	33±1	84.3±0.1
3.0	22.0	75.0	29.2±0.4	38±1	32.5±0.9	82±1
1,2-decanediol	THF	Water (NaCl 1M)	1,2-decanediol	THF	Water (NaCl 1M)	
4.9	8.6	86.5	59±1	6.0±0.5	35±2	99±2
4.9	17.3	77.8	38±1	32±1	30±1	96±3
4.9	21.8	73.3	30.6±0.7	39±4	30.1±0.2	100±4
4.9	26.3	68.8	23.4±0.3	49±2	27.61±0.01	99±5

[a]Mean values of three replicates ± standard deviation.

In all cases, water contents were around 30% (w/w) and kept almost invariable while changing the THF/water ratio in the synthetic solution. This was a differential feature of decanediol-based SUPRASs compared to those based on 1-decanol, where the water content was lower and dependent on the THF:water ratio in the synthetic mixture (i.e., water content ~4-21 % w/w) [10]. This differential feature seems to be related to the higher hydration degree of the head of the surfactant as consequence of the presence of an extra –OH group. Furthermore, aggregates made up of double-headed surfactants are expected to be more open, as described in the Introduction, thus favoring the interactions with water molecules.

As can be seen in Table 1, the content of THF in SUPRAS increased (14-38% w/w) as the organic solvent increased in the synthetic solution. When NaCl

was present, values for THF varied in a wider interval (6-49% w/w). The concentration of the amphiphile also changed accordingly. This means that SUPRAS composition was environment dependent and that it could be tuned for obtaining SUPRAS with different solubility properties to maximize recoveries for each analytical application.

The amphiphile was almost fully incorporated into the SUPRAS phasein a wide range of compositions, being the incorporation more favourable in NaCl-containing aqueous solutions (Table 1). This behaviour is logical considering the salting out effect for the amphiphile and the consequent reduction in the *cac* value. So, under salty conditions, and at low THF/water ratios in the absence of salt, the synthesis occurred through an energy-saving process (spontaneous coacervation at room temperature) that had a high-atom economy (1,2-decanediol incorporated entirely into the SUPRAS).

3.1.3. SUPRAS structure

Figure 2A shows a representative optical micrograph obtained from the synthesized SUPRASs. Optical microscopy studies clearly revealed that the liquid phases here reported were not continuous but made of coacervate droplets within the interval 4-16 μm (mean value 14 μm). So, as expected, these liquid phases were produced through self-assembly and coacervation. The morphology of SUPRAS aggregates was investigated with SEM according to the procedure specified in section 2.2. Figure 2B shows arepresentative image for SUPRASs formed in THF-water-salt. Similar images were obtained for the SUPRASs in the absence of salt (data not shown). This

figure clearly shows that the SUPRAS lacked any well-ordered internal structure and consisted in a random 3D amphiphile bilayer network separatingwater pores, which is consistent with the characteristics of a sponge phase [20-24].

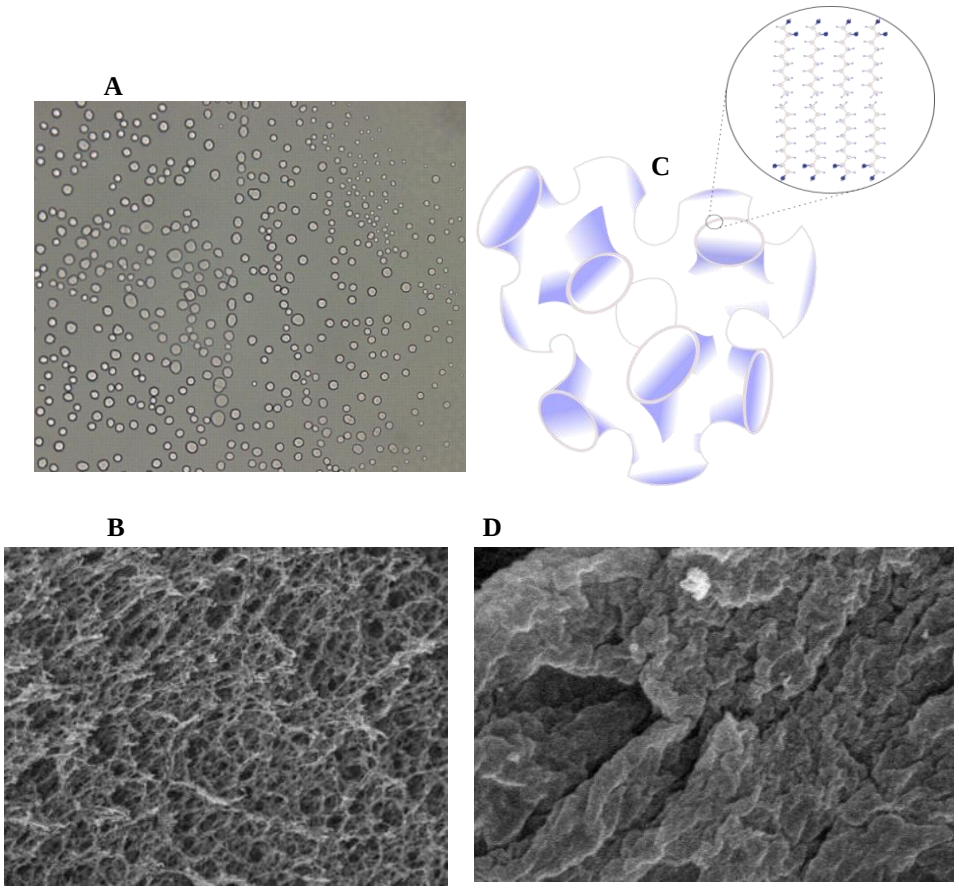


Figure 2. (A) Micrograph of a SUPRAS using light microscopy in the bright field (magnification: 20X). (B) SEM micrograph of a SUPRAS at the magnification 400X. (C) Illustration of the sponge morphology and magnification of the 1,2-decanediol bilayer. (D) SEM micrograph of a SUPRAS at the magnification 4.97 KX. SUPRAS were obtained from 1,2-decanediol in 10% THF (v/v) and 1M NaCl.

An illustration of the porous and bilayer-rich nanostructure is given in Figure 2C. Magnification of the SEM micrographs (Figure 2D) showed typical features of the sponge morphology [31]; ellipsoidal structures nearly flat and surrounded by a network of curved areas with a smooth appearance. Indifferent sections of this micrograph it was possible to follow the bilayer over large distances (several micrometers range), which is an indication that the bilayer was indeed continuous [31]. In concordance with the previously reported sponge coacervates, which were made up of ternary systems made upamphiphile-organic solvent-water (with and without salt) [20,31,32], it is probable that THF is incorporated at the SUPRAS hydrophobic region while water flows through the pores. This assumption is in good agreement with thenearly constant percentage of water into the SUPRASs (Table 1). The high water content, along with the double-headed groups of 1,2-decanediol provide a broad hydrophilic region for solubilization of polar compounds.

3.2. Extraction of PFCs from water by sponge droplets of 1,2-decanediol

In order to check our working hypothesis (*the recoveries of compounds in a wide polarity range should improve with the use of SUPRASs featuring increased hydrophilicity*), we compared the results obtained for the extraction of perfluoroalkyl sulfonates (C4-C10) and perfluoroalkyl carboxylates (C5-C18),with log Pow values from 0.4 to 11.6, with SUPRASs made up of 1,2-decanediol and 1-decanol. As described in previous sections, the first one consists of a double-headed amphiphile arranged in sponge droplets, while the

second one is made up of a single-head amphiphile arranged in inversehexagonal aggregates [10]. Both SUPRASs were synthesized in THF-water media under the same experimental conditions (150 mg of amphiphile, 4 mL of THF and 36 mL of Milli-Q water spiked with 9 ng L^{-1} of each PFC).

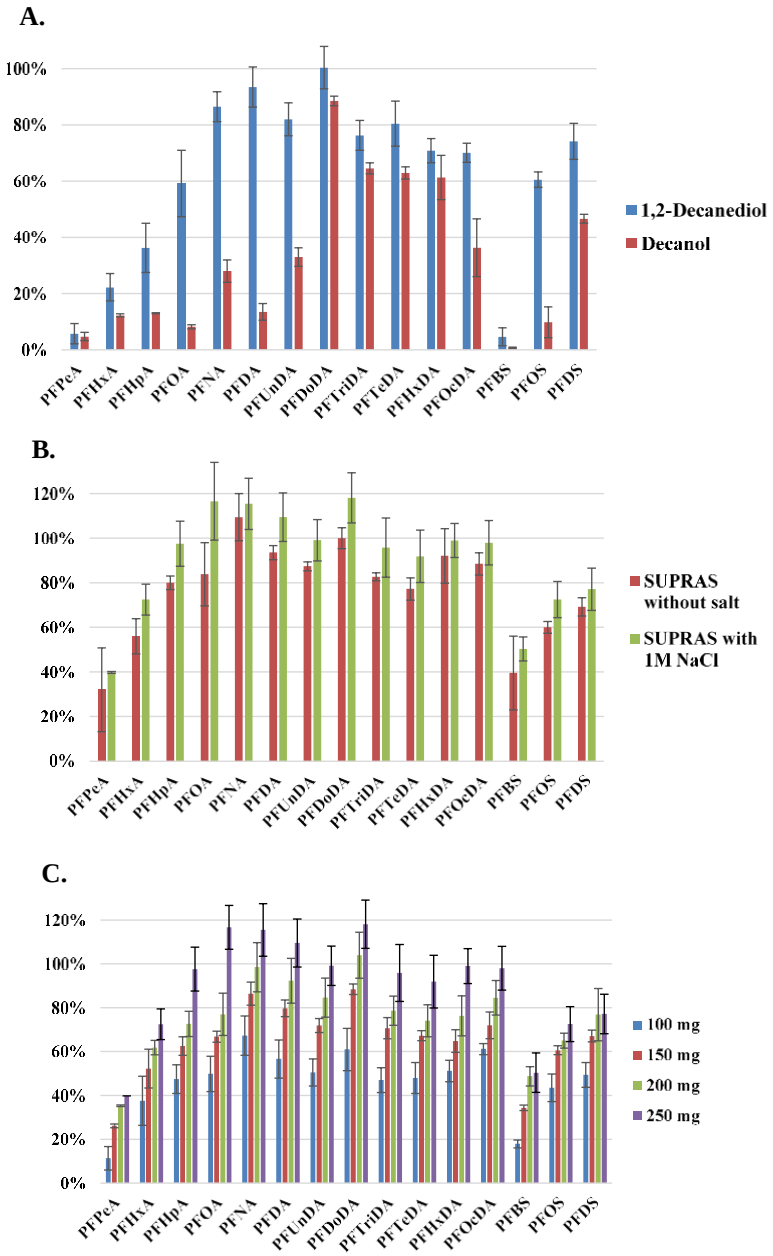


Figure 3. Recoveries ± SD (in %) of PFCs extracted with SUPRAS formed from (A)1- decanol and 1,2-decanediol in THF-water; (B) 1,2-decanediol in THF-water and THF- aqueous NaCl (1M); and (C) 1,2-decanediol in THF-aqueous NaCl. Water sample: 36 mL spiked with 9 ng L^{-1} of each PFC. Amount of amphiphile: (A) 150 mg; (B) 250 mg. THF: 10%.

Figure 3A shows that the recoveries for PFCs greatly improved for 1,2-decanediol compared to 1-decanol. Thus, they were above 60% for PFSAs and PFCAs in the range C_8-C_{10} and C_6-C_{18}, respectively.

Under the same conditions, the applicability of 1-decanol-based SUPRAS was much more limited for both most polar and most apolar PFCs, i.e., recoveries above 60% were found only for PFCAs with carbon atoms in the range 12-16 and were always more than 10% lower than those obtained for 1,2-decanediol-based SUPRAS.

The recoveries of PFCs with the use of sponge droplets synthesized from 1,2-decanediol-THF-water and 1,2-decanediol-THF-water (1M NaCl) was also investigated. SUPRASs were synthesized by adding 36 mL of Milli- Q water spiked with 9 ng L^{-1} of each PFC, both in the absence and presence ofNaCl to 4 mL of THF containing 250 mg of 1,2-decanediol. The results are shown in Figure 3B. Recoveries for PFCs improved in around 10-20% for SUPRASs that were synthesized in the presence of NaCl due to the salting-out effect. Under these conditions, recoveries were above 70% for all the compounds except for the most polar PFPeA (39±1%) and PFBS (50±5%).

The influence of the amount of amphiphile employed for the synthesisof the sponge droplets on the recoveries for PFCs was investigated by synthesizing SUPRAS from 100, 150, 200 and 250 mg of 1,2-decanediol. Forthis purpose, the amphiphile was dissolved in 4 mL of THF and it was added to spiked Milli-Q water samples (36 mL, 9 ng L^{-1}) containing 1 M NaCl. Giventhe low concentration of PFCs in most of natural waters (at the low ng L^{-1}

level), selection of the optimal conditions was guided by enrichment factors for PFCs, in addition to recoveries. Figure 3C shows the results. The SUPRASvolumes generated for the tested amounts of amphiphile were 106±3, 190±4, 275±4 and 360±7 μL for 100, 150, 200 and 250 mg of 1,2-decanediol, respectively, which gives theoretical concentration factors of 340, 189, 131 and 100, respectively. As expected, absolute recoveries of PFCs progressively increased as the amount of 1,2-decanediol did. Thus, only 2 out of 15 PFCs were out the range of what are generally considered acceptable mean recoveries (70-120%) with 250 mg of 1,2-decanediol. Finally, we proposed the use of 175 mg of 1,2-decanediol for the extraction of PFCs, as a compromise between absolute recoveries, which were below 60% only for three of them (i.e. PFPeA 31.1±0.9, PFHxA 57±1 and PFBS 41±1), and enrichment factors,which were between 54 and 174 without the need for evaporation steps.

3.3. Analytical performance

Calibration curves were prepared by extracting 36 mL of Milli-Q water, fortified with PFCs in the range 0.05-10 ng L^{-1}, according to the procedure specified in the section materials and methods. Deuterated internal standards (10 ng L^{-1}, Table S1) were added to the SUPRAS extracts before measurement.Table 2 shows the results.

Correlation coefficients indicated good fit to linearity in all cases. Method detection (MDL) and quantification (MQL) limits were calculated considering a signal to noise ratio of 3 and 10 and were in the range 0.005-0.01 and 0.02-0.03 ng L^{-1}, respectively.

Table 2. Analytical performance of the proposed method for the determination of PFCs in natural waters

Calibration parameters	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTriDA	PFTeDA	PFHxDA	PFOcDA	PFBS	PFOS	PFDS
Intercept ± SD /10^2	3.4±1.0	41±29	6.3±6.6	22±43	10±36	11±13	31±48	0.04±0.09	7.6±22	2.6±3.2	0.9±1	1.5±0.4	3.2±1.4	-3.3±1.8	-0.5±0.3
Slope ± SD ($ng^{-1}L$) /10^2	90±4	434±45	1914±256	518±83	720±58	257±21	743±78	2.6±0.2	350±35	71±5	44±2	6.6±0.1	14.9±0.3	21.4±0.4	2.36±0.08
Correlation coefficient	0.994	0.970	0.996	0.927	0.975	0.975	0.958	0.977	0.962	0.980	0.990	0.996	0.993	0.995	0.984
Repeatability (RSD %)															
Guadalquivir river	5	5	13	13	8	6	17	3	3	3	3	3	8	5	5
La Breña reservoir	2	2	10	10	5	4	7	5	5	5	5	5	5	8	8
Guadiato river	10	10	15	17	5	7	16	7	7	7	7	7	1	8	8
Well	10	10	17	19	9	5	8	4	4	4	4	4	5	6	6

The selectivity for the quantification of PFCs was roughly assessed by comparing the recoveries of the corresponding standards extracted from Milli-Q water and four natural water samples spiked at a concentration of 5 ng L^{-1}. The results obtained are shown in Figure S4. Recoveries of all PFCs were quantitative (in the range 90-118%; median 100%; mean 100.9%) thus indicating the absence of matrix effects.

The precision, evaluated in terms of repeatability and expressed as relative standard deviation (RSD), was calculated from the analysis in triplicate of four natural water samples fortified with 5 ng L^{-1} of PFCs, by following the procedure described in the section materials and methods. Results are also shown in Table 2. In all cases, RSD values were between 1 and 19% (median 6%, mean 7%).

Table 3 compares different extraction and quantification features of representative LC-MS/MS methods reported for PFCs in natural waters in the last three years with those obtained for the method based on sponge droplets. The extraction of PFCs from waters is mainly done with SPE.

Table 3. Comparison of the method developed in this work with LC/MS-based methods reported in the literature in the determination of PFCs in natural waters

Compounds	Sample type and Volume	Sample Treatment	Organic Solvents and Solutions Involved in Sample Treatment	Separation, Detection, Calibration	[a]Extraction time per sample	Linearity ng L^{-1}	LOD ng L^{-1}
7 PFCAs (C_4-C_{10}) and 3 PFSAs (C_4, C_6, C_8)	River water (1000 mL)	• SPE (Oasis HLB) • Evaporation Reconstitution	• Methanol (15 mL)	UPLC-MS/MS (Isotopically labelled IS)	5.6 h	0.025-2	0.01-0.1
11 PFCAs (C_4-C_{14}) and 5 PFSAs (C_4, C_6,C_7,C_8,C_{10})	Seawater (1000 mL)	• SPE (Oasis HLB) • Evaporation • Reconstitution	• Methanol (27 mL)	LC-MS/MS (Isotopically labelled IS)	8.5 h	0-25000	0.0046-0.16
8 PFCAs (C_4-C_6, C_8, C_{13}) and 3 PFSAs (C_4, C_6, C_8)	River water (1000 mL)	• SPE (Oasis WAX) • Evaporation • Reconstitution	• A solution of NH_4OH/CH_3OH (0.5%, v/v) (4 mL) • Methanol (4 mL)	LC-MS/MS (Isotopically labelled IS)	5.6 h	200-50000	0.03-0.2
10 PFCAs (C_4-C_{13}) and 4 PFSAs (C_4,C_6, C_8, C_{10})	Superficial/ underground water (5 mL)	• Online SPE (Oasis WAX) • Evaporation • Reconstitution	• Water with NH_4OH (0.05%, v/v) (24 mL) • Methanol with NH_4OH (0.05%, v/v) (52.8 mL)	LC-MS/MS (Isotopically labelled IS)	12 min	0.2-250	0.2-5
10 PFCAs (C_4-C_{12}, C_{14}) and 3 PFSAs (C_4, C_6, C_8)	Surface water (2 mL)	• µ-SPE • Evaporation • Reconstitution	• 10 mM NaOH in methanol (250 µL) • Methanol (250 µL) • 1% Acetic acid (250 µL) • 10 mM NaOH in methanol (100 µL)	LC-MS/MS (Isotopically labelled IS)	5 min	10-9000	0.3-6.6
12 PFCAs (C_4-C_{14}) and 6 PFSAs (C_4 - C_{10})	Drinking, tap and river water (20 mL)	• Dodecanol-based SUPRAS microextraction	• Tetrahydrofuran (1 mL) • Dodecanol (250 µL) • NaCl (4 g)	LC-Orbitrap (Matrix-matched calibration)	1 min	500-500.000	10-80
12 PFCAs (C_5-C_{18}) and 3 PFSAs (C_4, C_8, C_{10})	River, reservoir and well water (36 mL)	• Double Head Amphiphile-Based Sponge Droplet microextraction	• Tetrahydrofuran 4 mL	LC-MS/MS (Isotopically labelled IS)	15 min	0.05-10	0.005-0.01

[a]Only the time required for SPE or LLE is considered (evaporation, centrifugation, etc. is not included)

Treatment of 1 L samples is common (e.g., references 1-3 in Table 3), which takes several hours excluding the evaporation steps. The use of lower water volumes with SPE have been also proposed (e.g., references 4 and 5 in Table 3) in detriment of concentration factors and consequently, sensitivity. The use of methods based on water-immiscible organic solvents to carry out liquid-liquid extractions (LLE) is less common for PFCs from waters due to the broad polarity range and amphiphilic character of these compounds that make more difficult their extraction. In this sense, sponge SUPRAS provided a good alternative to conventional LLE approaches with a fast and simple

procedure that provided suitable sensitivity for the monitorization of pollution levels of PFCs in natural waters (LODs in the range 0.005-0.01 ng L^{-1}, Table 3).

3.4. Determination of PFCs in natural waters

Four natural water samples collected in the province of Córdoba, South of Spain (i.e., La Breña reservoir, a well located in Trassierra and Guadalquivir and Guadiato rivers) were analyzed in triplicate in order to validate the applicability of the proposed method. **Table 4** shows the results obtained and **Figure 4** shows the extracted ion chromatograms of the PFCs found in the Guadalquivir river sample.

Table 4. Concentrations ± SD (n=3) of PFCs in natural waters (ng L^{-1})

Sample	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTriDA	PFTeDA	PFHxDA	PFOcDA	PFBS	PFOS	PFDS
Guadalquivir river	0.30±0.03	0.53±0.03	0.17±0.02	0.44±0.04	0.38±0.02	0.34±0.03	0.39±0.01	0.18±0.02	0.45±0.05	0.48±0.02	0.44±0.04	0.15±0.01	2.33±0.02	0.97±0.10	0.41±0.06
La Breña reservoir	0.32±0.05	0.73±0.10	0.11±0.01	0.4±0.04	0.61±0.02	0.60±0.03	0.67±0.07	0.13±0.02	0.74±0.08	0.65±0.07	0.20±0.03	0.19±0.02	0.24±0.03	0.22±0.03	0.38±0.01
Guadiato river	0.07±0.01	0.18±0.02	0.09±0.01	0.16±0.01	0.18±0.03	0.15±0.01	0.17±0.02	0.13±0.02	0.14±0.01	0.15±0.02	0.17±0.02	<LOD	0.48±0.05	0.41±0.04	0.59±0.09
Well	<LOD	<LOD	0.15±0.02	<LOD	<LOD	<LOD	<LOD	0.08±0.01	<LOD	<LOD	<LOD	0.15±0.02	<LOD	0.22±0.06	0.40±0.01

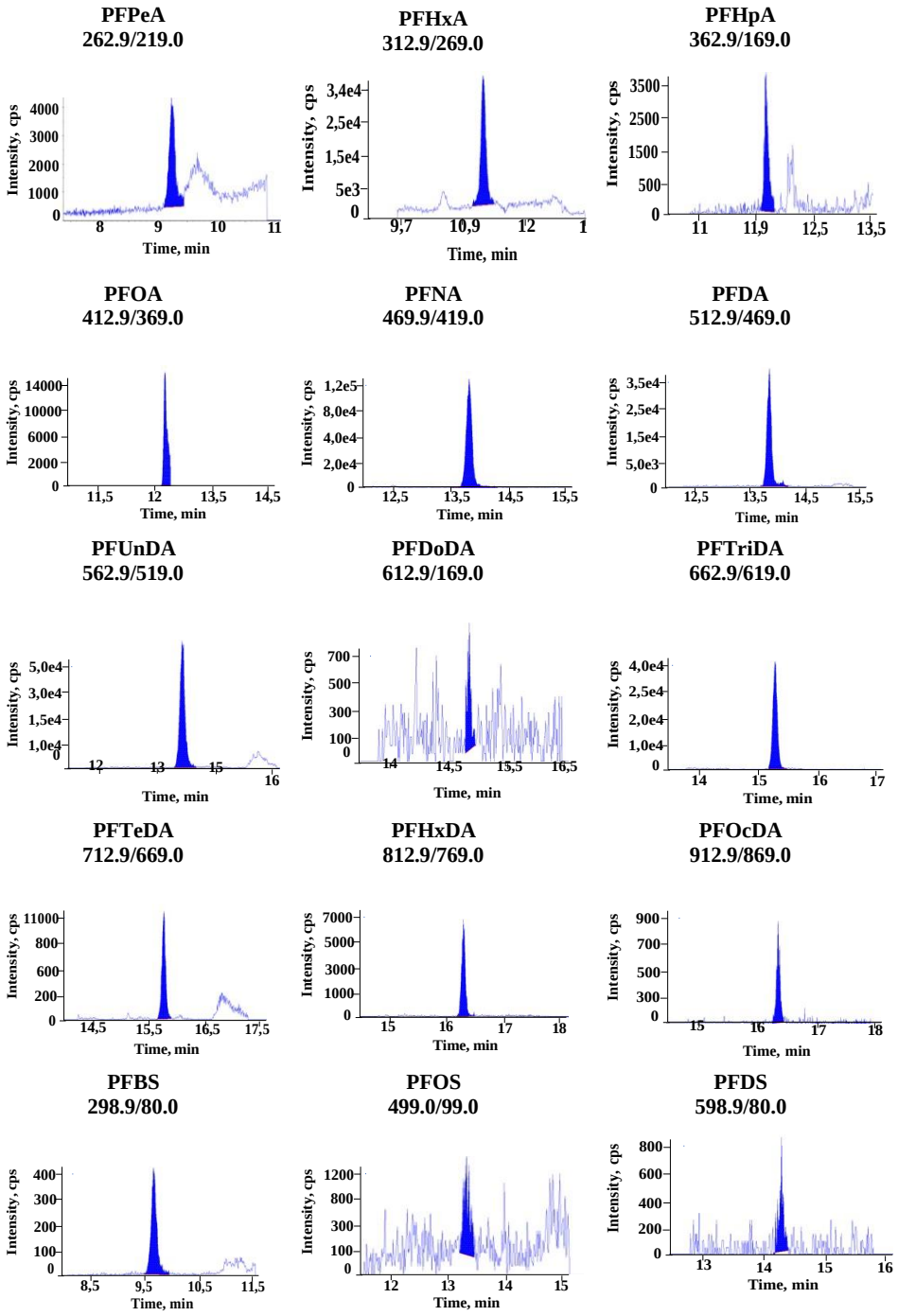


Figure 4. Extracted ion chromatograms of detected PFCs (quantifiers) in the sample forthe river Guadalquivir.

Except for the sample collected in the well, all the target PFCs were foundin the samples at the low ng L^{-1} level. In all cases, excepting for la Breña reservoir, concentrations for PFCAs were lower than those for PFSAs. Valuesfor PFCs were lower in Guadiato river (natural park location) and especially in the well water. On the contrary, higher concentrations of PFCs were found in water collected from Guadalquivir river (0.17-2.33 ng L^{-1}), which flows byCórdoba city and it is more exposed to industrial contamination and in La Breña reservoir (0.11-0.74 ng L^{-1}), in which recreational activities such as fishing and boating are common. Levels were consistent with the values generally found in the literature (0.01-5 ng L^{-1}) [39-41].

Conclusions

By the selection of double-headed amphiphiles and water as coacervation-inducing agent, SUPRASs with extended hydrophilic regions in their nanostructures have been tailored. Combination of both strategies has resultedin high water content sponge droplets that feature increased recoveries for compounds over a wide polarity range, such as PFCs from waters, hereselected as analytes for a proof of concept study. Sponge droplets are synthesized through spontaneous and energy-saving self-assembly processes that have high atom economy (practically all the amphiphile is incorporated into the SUPRAS), thus fitting the green chemistry synthetic principles. Regarding the application developed for PFCs, some valuable analytical and operational characteristics have been obtained compared to previously reported methods. Thus, PFCs are quantified at similar sensitivity that those

methods requiring the treatment of high water volumes (e.g. 1 L) but taking farless time (e.g. 15 min instead of 5.6-8.5 h) and avoiding the evaporation step. This will all result in an increased sample throughput.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reportedin this paper.

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Credit author statement

Soledad González-Rubio: Investigation, Writing – original draft, Writing – review & editing; Ana Ballesteros-Gómez: Supervision, Writing-Review & Editing, Conceptualization; Diego García-Gómez: Supervision, Writing-

Review & Editing, Conceptualization; Soledad Rubio: Conceptualization, Project administration, Funding acquisition.

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SUPPLEMENTARY INFORMATION

Double-Headed Amphiphile-Based Sponge Droplets: Synthesis, Characterization and Potential for the Extraction of Compounds over a Wide Polarity Range

S. González-Rubio[1], A. Ballesteros-Gómez [1*], D. García-Gómez[1,2], S. Rubio[1]

[1]Department of Analytical Chemistry. Institute of Fine Chemistry and Nanochemistry. Universidad de Córdoba, Campus de Rabanales, Edificio Marie Curie (anexo), E-14071 Córdoba, Spain.

[2]Department of Analytical Chemistry, Nutrition and Food Science, University of Salamanca, Spain.

[*]Corresponding author, email: ana.ballesteros@uco.es, Phone 34 957 218643

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Figure S1. General scheme for SUPRAS production.

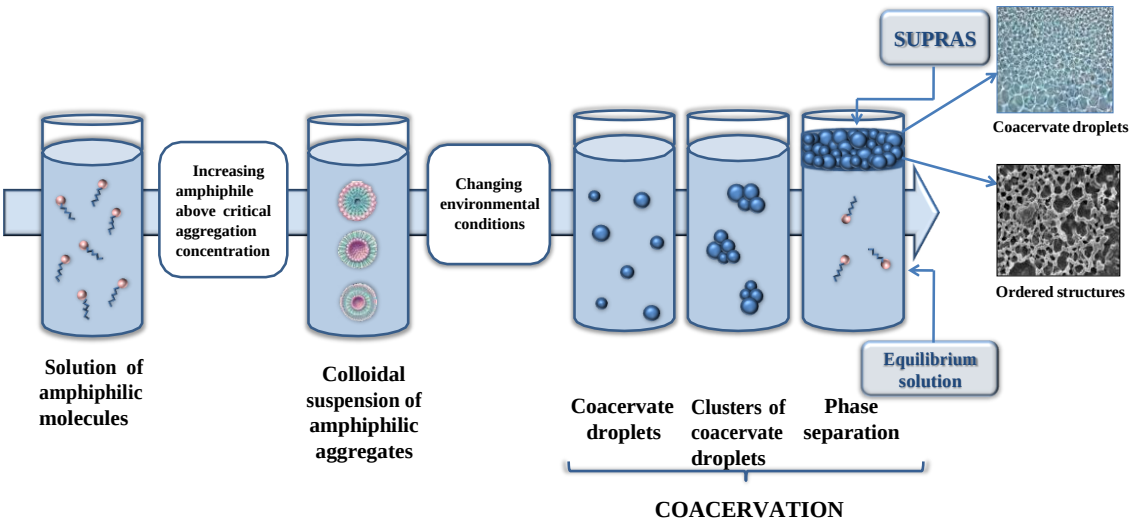


Figure S2. Schematic picture of the proposed SUPRAS extraction method forthe determination of PFCs in natural waters.

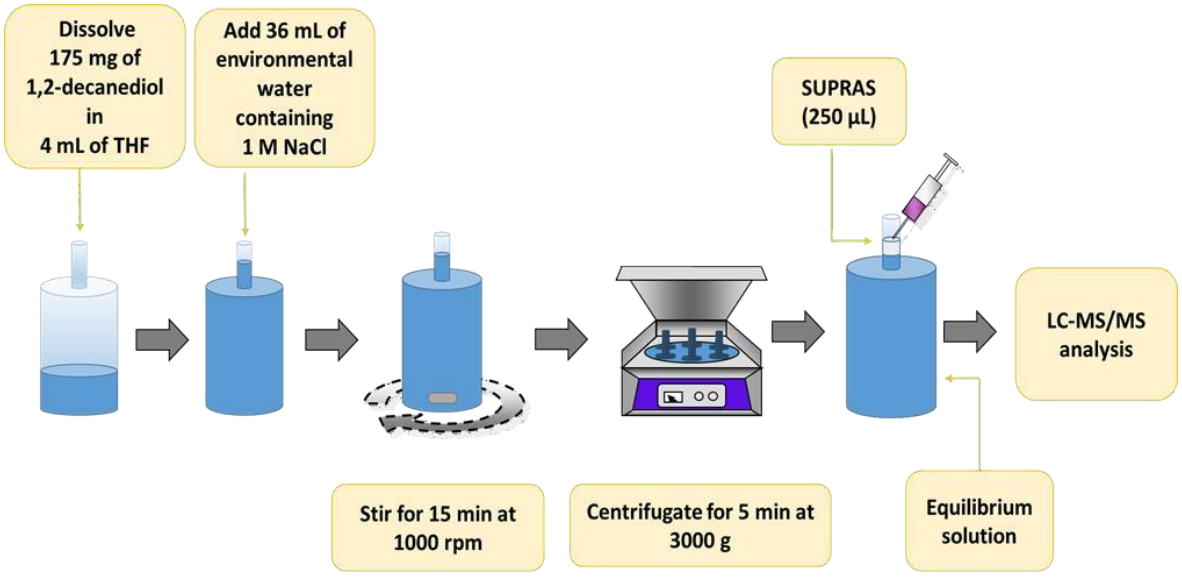


Figure S3.Volume of SUPRAS (µL) as a function of the initial concentration of 1,2-decanediol (mg, A and B) and THF (%, v/v, C and D) in the synthesis solution with the absence of salt (A and C) and presence of 1M NaCl (B and D) in water. Total volume of synthesis solution: 40 mL

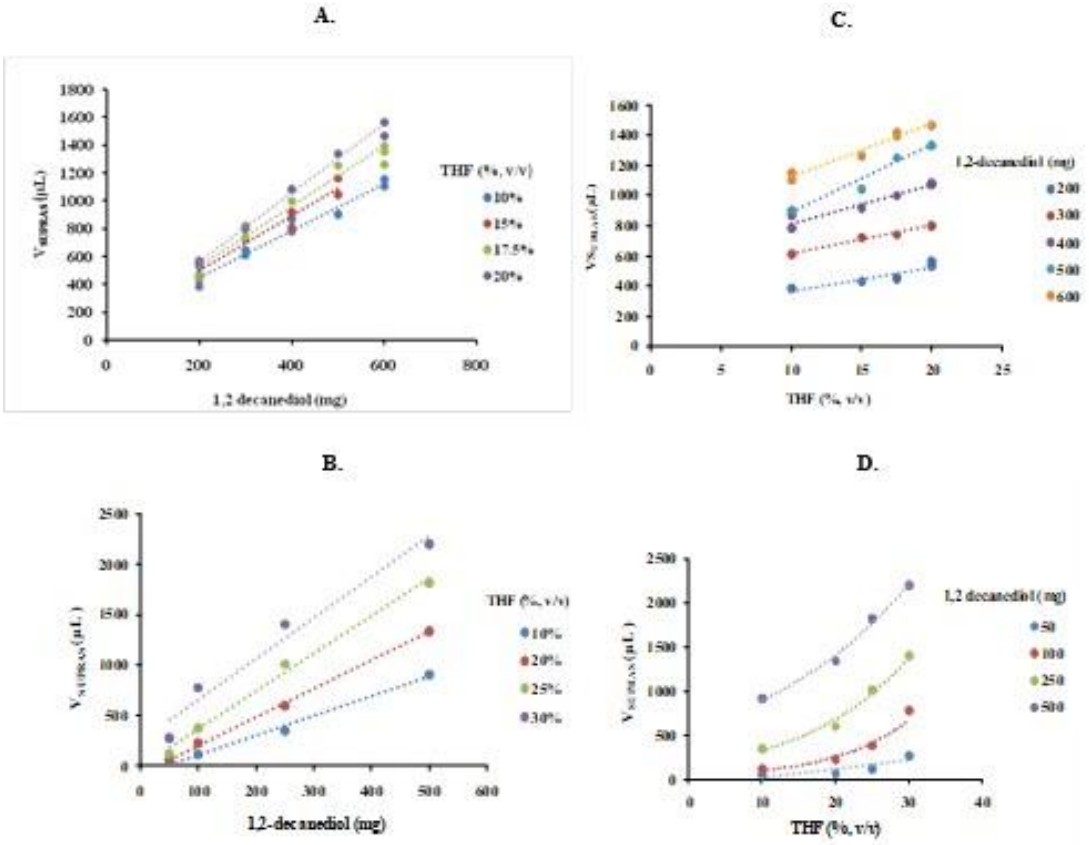
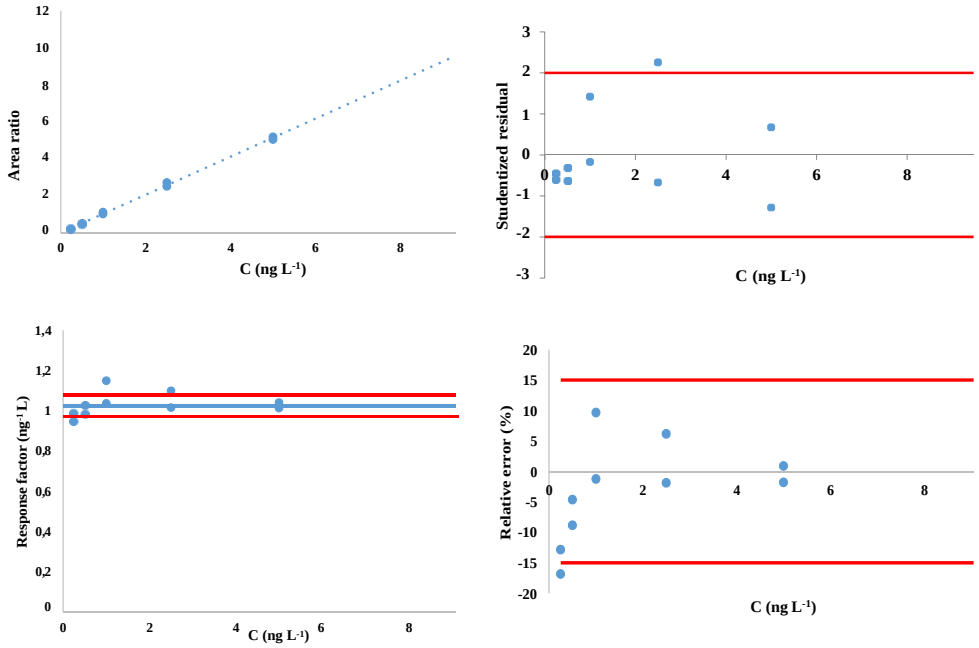
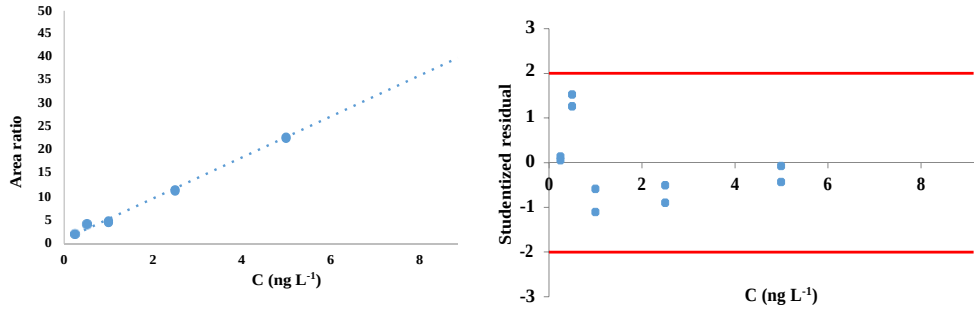


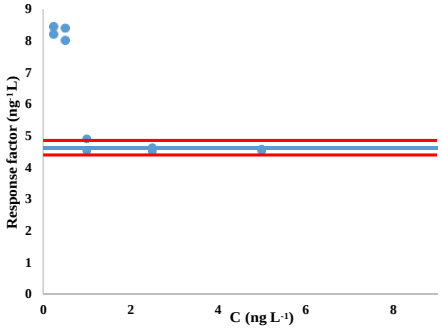
Figure S4. Linearity study for the calibration curves of perfluorinatedcompounds. For each of the investigated substances, the following plots are represented: Calibration plot (area ratio versus standard concentration); Residuals plot (Studentized residual versus standard concentration); Response factor plot (response factor versus standard concentration) and Relative error of back-calculated concentrations (% relative error versus standard concentration).

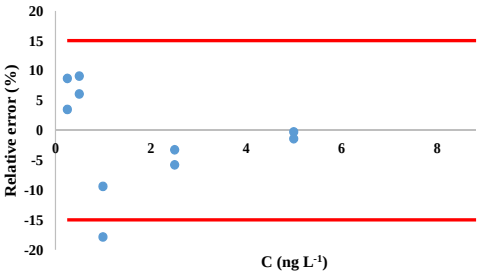
PFPeA



PFHxA




Response factor (ng⁻¹L)
C (ng L⁻¹)


Relative error (%)
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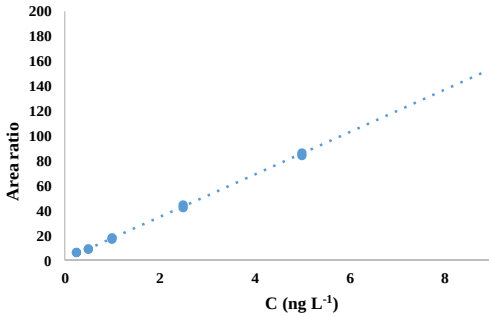
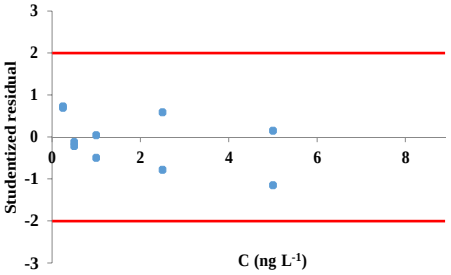
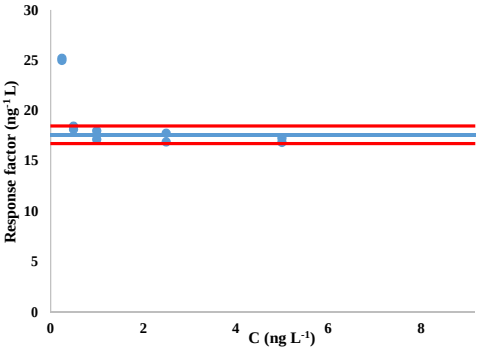
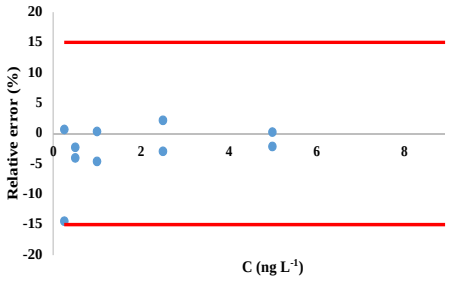
PFHpA


Area ratio
C (ng L⁻¹)


Studentized residual
C (ng L⁻¹)


Response factor (ng⁻¹L)
C (ng L⁻¹)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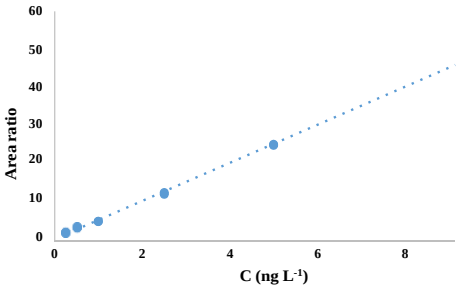
C (ng L⁻¹)

PFOA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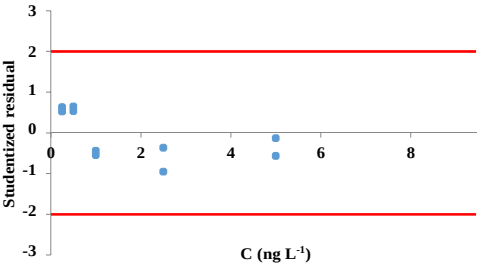
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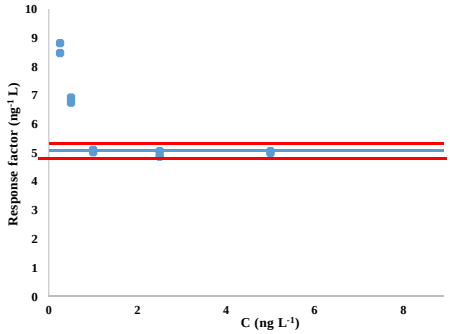
C (ng L⁻¹)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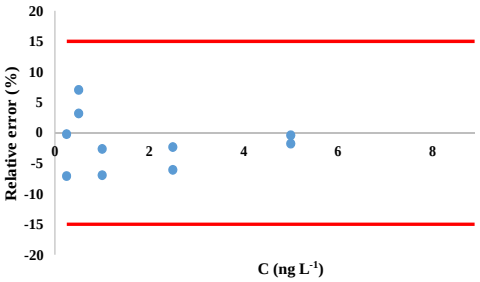
C (ng L-1)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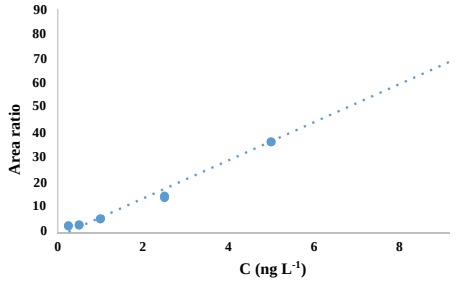
PFNA


Area ratio
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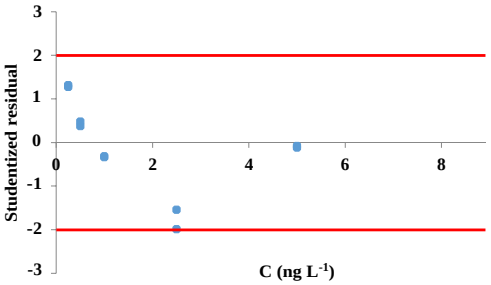
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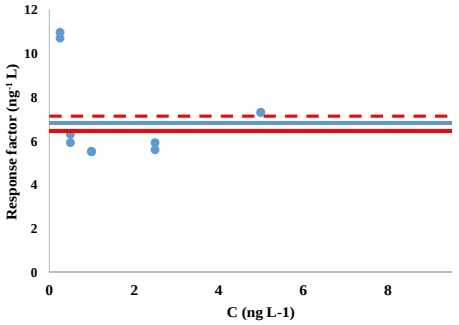
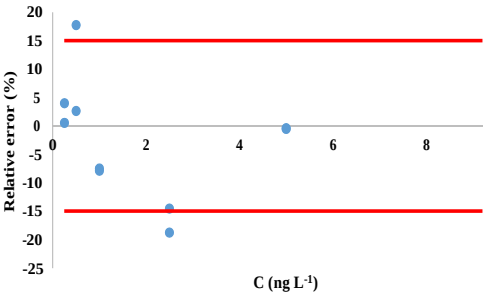
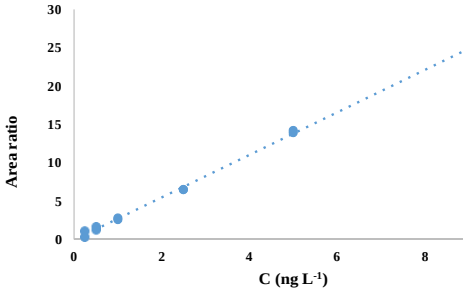
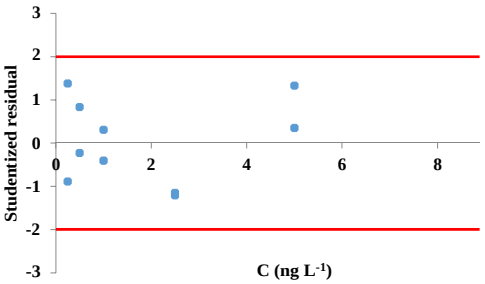
C (ng L-1)

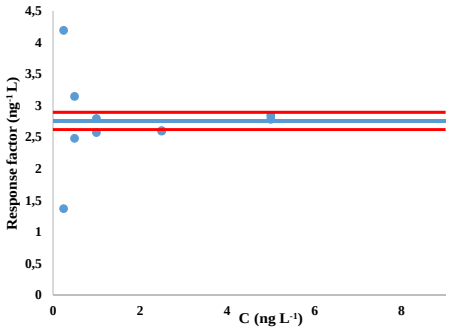

Response factor (ng-1 L)
C (ng L-1)

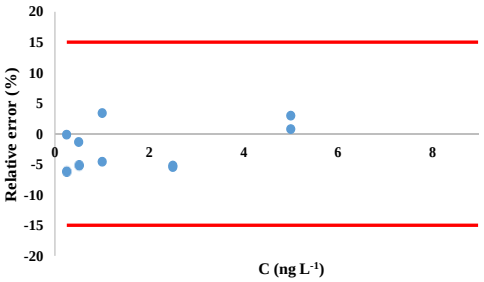

Relative error (%)
C (ng L-1)

PFDA


Area ratio
C (ng L-1)


Studentized residual
C (ng L-1)





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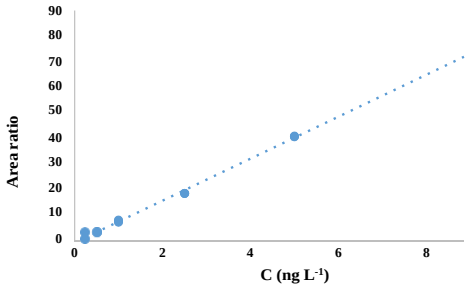

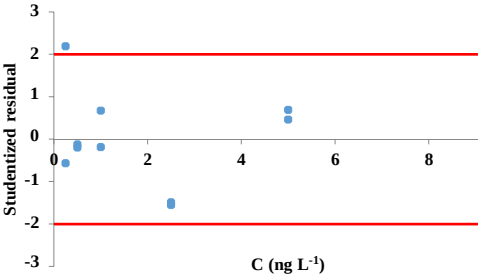

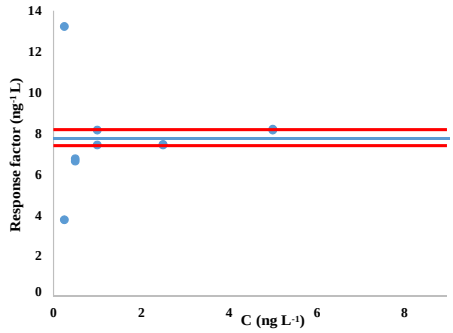

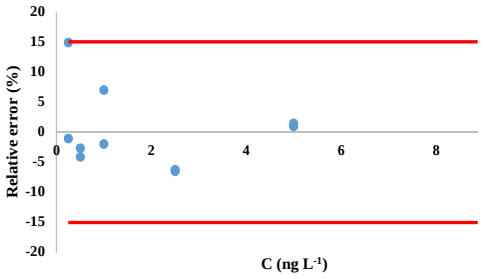

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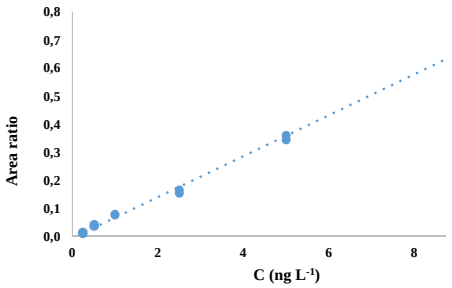

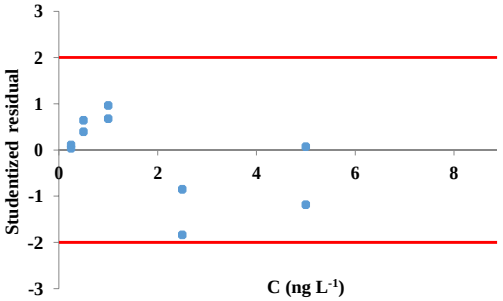

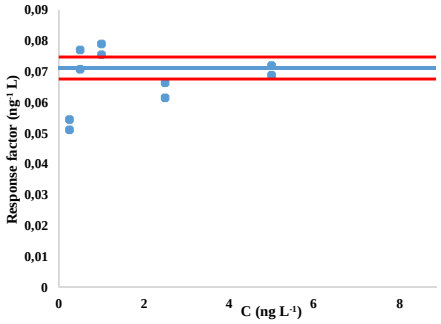

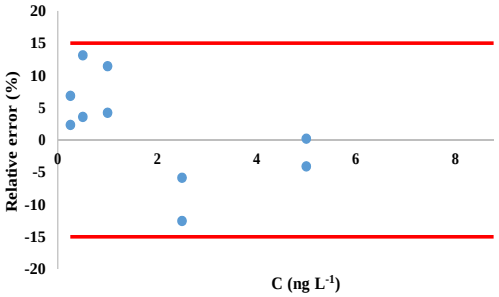

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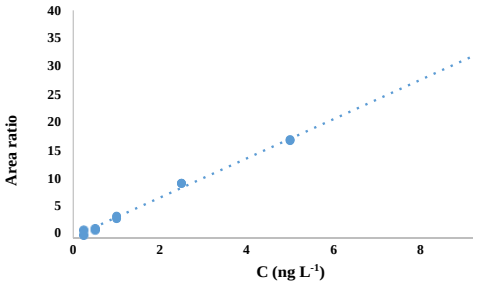

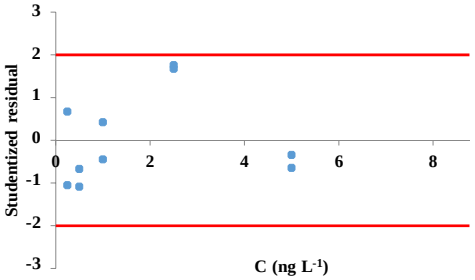

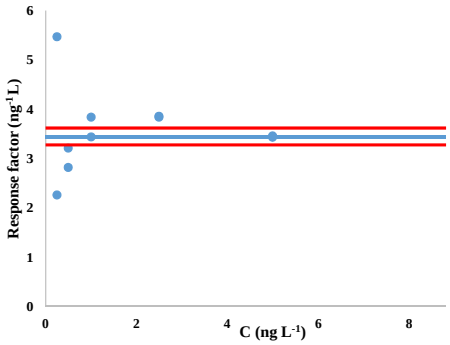

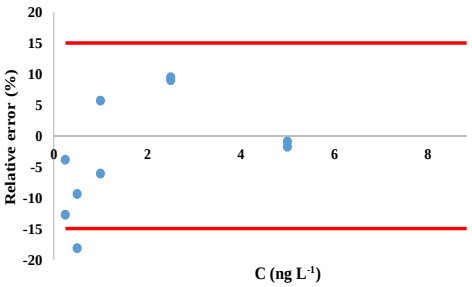

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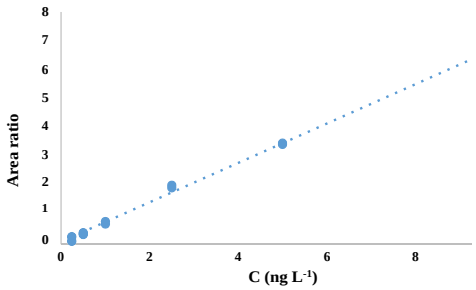

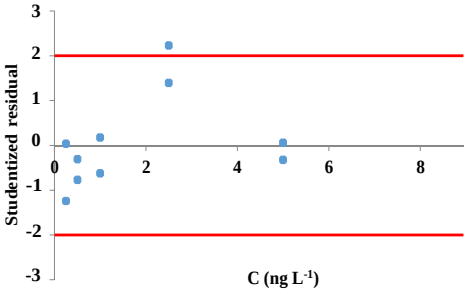

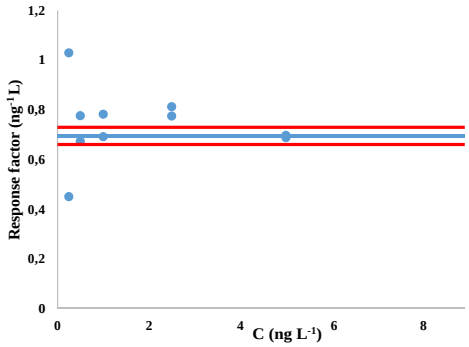

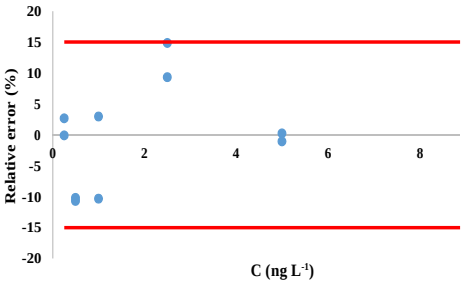

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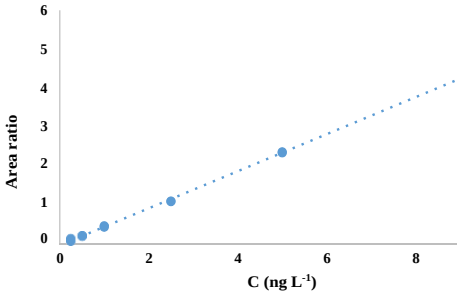

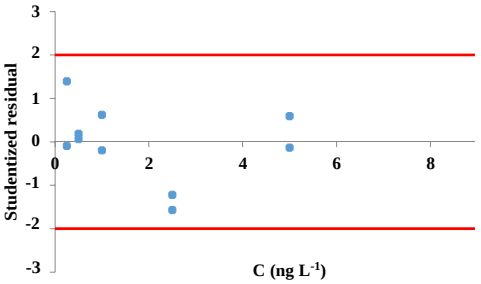

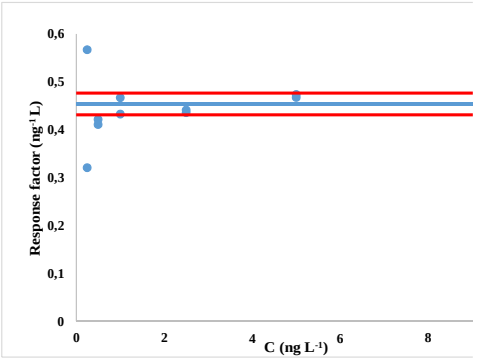

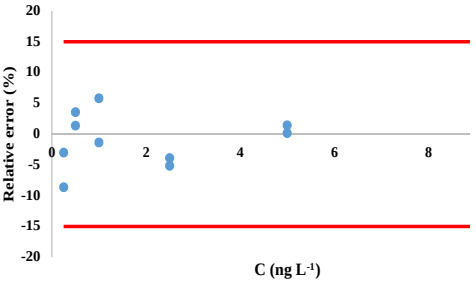

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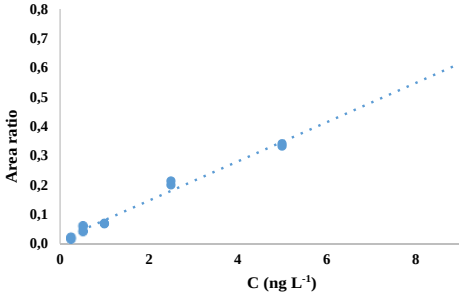
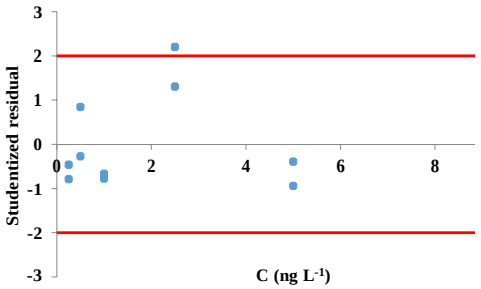
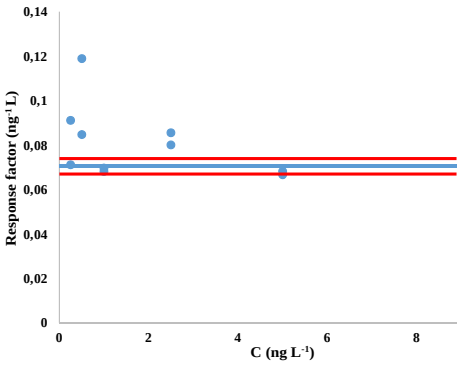
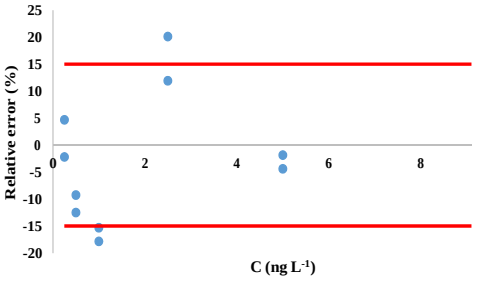
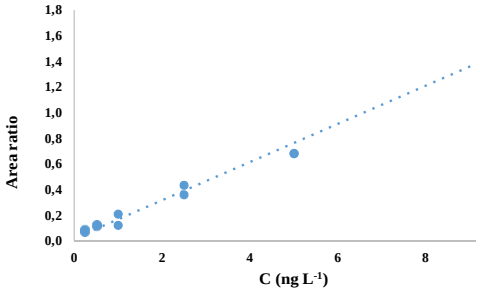


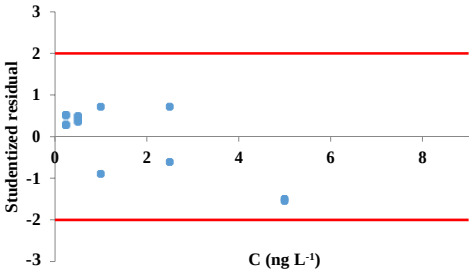


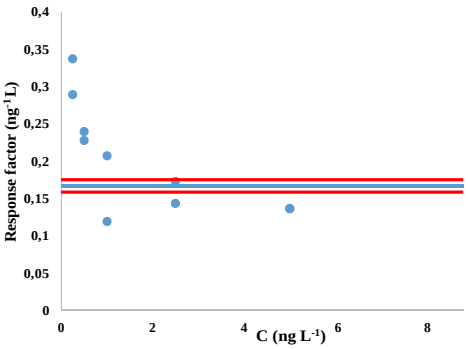

Response factor (ng⁻¹ L)
C (ng L⁻¹)

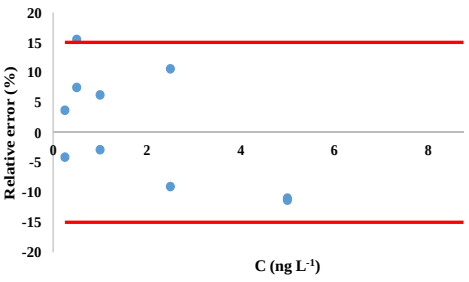

Relative error (%)
C (ng L⁻¹)

PFBS

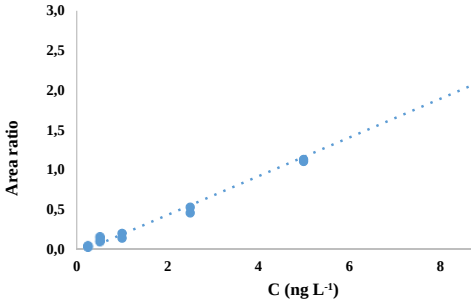

Area ratio
C (ng L⁻¹)

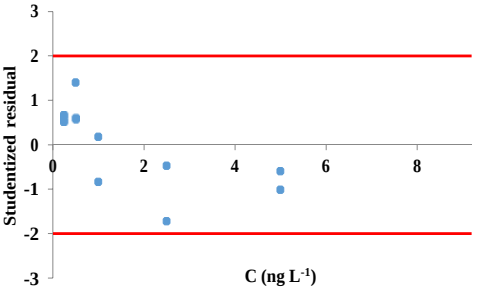

Studentized residual
C (ng L⁻¹)

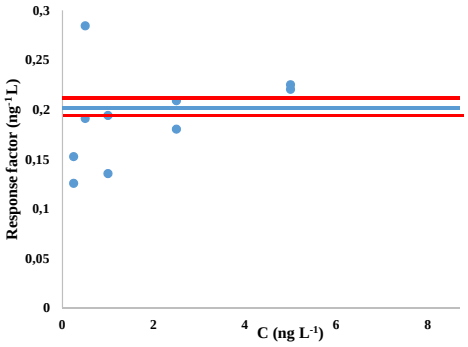

Response factor (ng⁻¹ L)
C (ng L⁻¹)

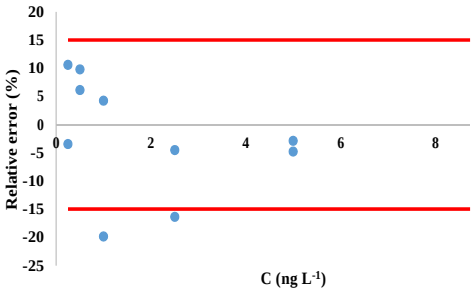

Relative error (%)
C (ng L⁻¹)

PFOS

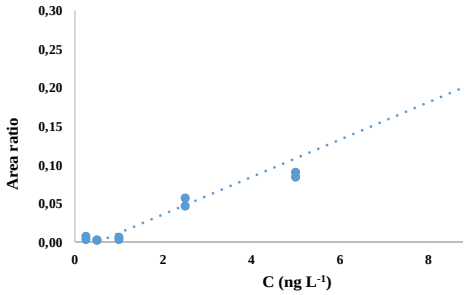

Area ratio
C (ng L⁻¹)

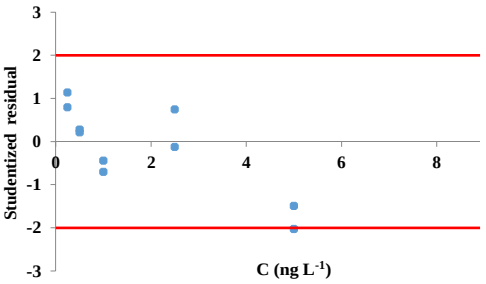

Studentized residual
C (ng L⁻¹)

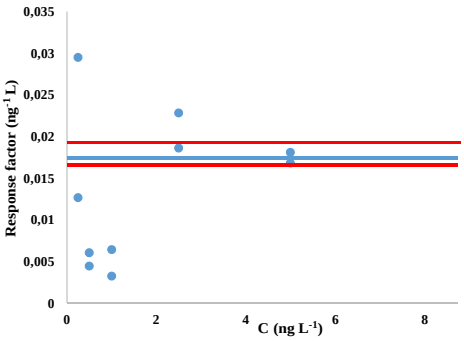

Response factor (ng-1 L)
C (ng L-1)


Relative error (%)
C (ng L-1)

PFDS


Area ratio
C (ng L-1)


Studentized residual
C (ng L-1)


Response factor (ng-1 L)
C (ng L-1)

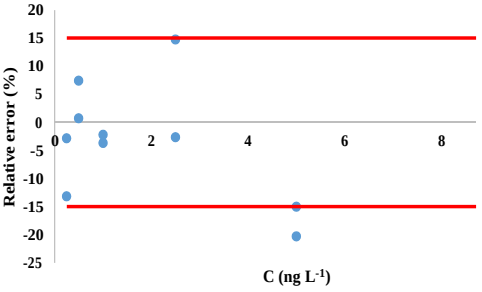
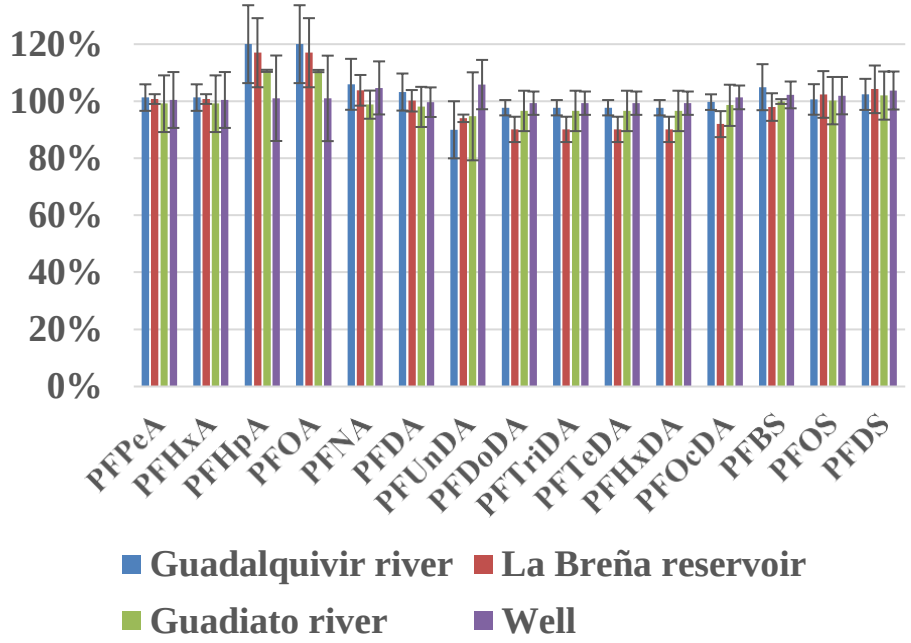

Relative error (%)
C (ng L-1)

Figure S5. Recoveries ± SD (%) of PFCs in natural waters spiked at a concentration of 5 ng L^{-1}



VOLUME EQUATIONS

The corresponding equations for THF:water (1) and THF:water:NaCl (2) were as follows:

$$V_{SUPRAS} = [(0.093\ Z + 0.455)\ X - (8.615\ Z - 195{,}79)] \quad (1)$$

$$V_{SUPRAS} = [(0.124\ Z + 0.455)\ X - (-9.065\ Z + 154.13)] \quad (2)$$

The dependent variable, V_{SUPRAS}, is the volume of SUPRAS (μL) generated in 40 mL of colloidal solution, and the independent variables X and Z are the amount of 1,2-decanediol (mg) and THF (v/v) in the synthetic solution, respectively. The good capability of prediction of these equations was proved by their coefficient of determination (98.8 and 96.8 for equations 1 and 2,

respectively). Equation 1 was valid within the ranges 10-20 % (v/v) of THF and 0.25-1.5 % (w/v) of 1,2-decanediol while equation 2 was applicable in theranges 5-30% (v/v) of THF and 0.125-1.25 % (w/v) of amphiphile. Considering that for obtaining high concentration factors, low amounts of amphiphile and of THF are employed, the lowest SUPRAS volumes in the region of analytical interest will be obtained with the presence of salt.

Table S1. Octanol-water constants and mass and chromatographic parameters for the native perfluorinated compounds (PFCs) and isotope-labelled internalstandards (IS) investigated in this study.

Native PCFs and IS	**Acronym**	**[a]Octanol-water partition coefficient (**log P)	**Precursor ions** (*m/z*)	**Product ions** (*m/z*)		**Fragmentor voltage** (V)	**Collision energy** (eV)	**Rt** (min)
				Qualifier	**Quantifier**			
Perfluoropentanoic acid	PFPeA	2.8	262.9	-	219.0	-19	-14	9.36
Perfluorohexanoic acid	PFHxA	3.5	312.9	269.0	119.0	-18	-14	11.21
Perfluoroheptanoic acid	PFHpA	4.2	362.9	319.0	169.0	-23	-15	12.29
Perfluorooctanoic acid	PFOA	4.8	412.9	169.0	84.0	-45	-16	13.04
Perfluorononanoic acid	PFNA	5.5	462.9	419.0	219.0	-40	-16	13.60
Perfluorodecanoic acid	PFDA	6.2	512.9	469.0	219.0	-40	-16	14.10
Perfluoroundecanoic acid	PFUnDA	6.8	562.9	519.0	269.0	-21	-17	14.54
Perfluorododecanoic acid	PFDoDA	7.6	612.9	570.0	169.0	-50	-22	14.96
Perfluorotridecanoic acid	PFTriDA	8.3	662.9	619.0	169.0	-75	-16	15.26
Perfluorotetradecanoic acid	PFTeDA	9.0	712.9	669.0	169.0	-15	-17	15.55
Perfluorohexadecanoic acid	PFHxDA	10.3	812.9	769.0	169.0	-24	-20	16.04
Perfluorooctadecanoic acid	PFOcDA	11.6	912.9	869.0	169.0	-24	-20	16.44
Perfluorobutanesulfonic acid	PFBS	0.4	298.9	80.0	99.0	-60	-50	9.91
Perfluorooctanesulfonic acid	PFOS	2.5	499.0	80.0	99.0	-55	-82	13.55
Perfluorodecanesulfonic acid	PFDS	3.6	598.9	99.0	80.0	-60	-60	14.48
Perfluoro-n-[1,2-$^{13}C_2$]hexanoic acid	$^{13}C_2$PFHxA	-	314.9	-	270.0	-23	-15	12.29
Perfluoro-n-[1,2,3,4-$^{13}C_4$]octanoic acid	$^{13}C_4$PFOA	-	416.9	-	372.0	-45	-16	13.04
Perfluoro-n-[1,2,3,4,5-$^{13}C_5$]nonanoic acid	$^{13}C_5$PFNA	-	467.9	-	423.0	-40	-16	13.60
Perfluoro-n-[1,2-$^{13}C_2$]decanoic acid	$^{13}C_2$PFDA	-	514.9	-	470.0	-40	-16	14.10
Perfluoro-n-[1,2-$^{13}C_2$]undecanoic acid	$^{13}C_2$PFUnDA	-	564.9	-	520.0	-23	-14	14.54
Perfluoro-n-[1,2-$^{13}C_2$]dodecanoic acid	$^{13}C_2$PFDoDA	-	615.0	-	570.0	-15	-19	14.96
Perfluorohexane[$^{18}o_2$]sulfonic acid	$^{18}O_2$PFHxS	-	403.0	-	84.0	-72	-64	12.32
Perfluoro-1-[1,2,3,4 $^{13}C_4$]octanesulfonic acid	$^{13}C_4$PFOS	-	503.0	-	80.0	-55	-82	13.55

BLOCK D

Development of sample treatment platforms for the analysis of multicomponents in antidoping control.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A comprehensive study on the performance of different retention mechanisms in sport drug testing by liquid chromatography tandem mass spectrometry

Soledad González-Rubio[a], Ana Ballesteros-Gómez[a,*], Daniel Carreras[b], Gloria Muñoz[b], Soledad Rubio[a]

[1]Departamento de Química Analítica, Instituto de Química Fina y Nanoquímica, Edificio Anexo Marie Curie, Campus de Rabanales, Universidad de Córdoba, Córdoba, 14071, España.

b Anti-doping Control Laboratory, Spanish Agency of Health Protection in Sport (AEPSAD), Spain.

* Corresponding author. E-mail address: ana.ballesteros@uco.es (A. Ballesteros-Gómez).

Keywords: Anti-doping control; Initial testing procedures; Liquid chromatography; Mass spectrometry; Column comparison

Abstract

Anti-doping substances listed by the World Anti-Doping Agency (WADA) include hundreds of compounds of very different physico-chemical properties. Anti-doping control laboratories need to screen all these substances in the so-called Initial Testing Procedures (ITPs) what is very challenging from an analytical point of view. ITPs are mostly based on reversed-phase (RP) liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using C18 columns, which feature poor retention and peak tailing for polar and basic compounds, respectively. While studies on this field dealing with the comparison of stationary phases are focused on certain chemical classes, this research provides a wide multi-target approach. For this purpose, a representative group of 93 anti-doping agents (log P from -2.4 to 9.2) included in ten different classes of prohibited substances was selected. A comprehensive study on the performance of six columns and four eluents on different separation parameters (retention factors, asymmetry factors, co-elutions, total run times) and matrix effects (signal enhancement or suppression) was performed for LC-MS/MS-based ITPs. Columns working in both RP [C18, C8, phenyl hexyl (PH), pentafluorophenyl (PFP) and mixed-mode hydrophilic/RP (HILIC-RP)) and hydrophilic (HILIC)] modes were investigated. Eluents contained methanol or acetonitrile as organic modifiers, with or without the addition of ammonium acetate. The best column-mobile phase binomial for ITPs was PFP using water-methanol (0.1% formic acid) as eluent, while HILIC was the best option for highly polar non-aromatic anti-doping agents, which were poorly addressed by PFP. Excellent good peak shapes and relative acceptable matrix interferences were obtained for HILIC-RP, which was tested for the first time for the analysis of anti-doping agents, although the number of compounds eluting too fast was too high. On the whole, the alkyl phase C18

showed the worst performance and although C8 and PH were better, their performance did not surpass that of PFP. Possible retention mechanisms underlying separation in the different stationary phases were discussed. This research provides valuable information to anti-doping control labs for improving LC-MS/MS-based ITPs and it proposes PFP as a suitable alternative to the already established C18.

Introduction

The annually published list of the World Anti-Doping Agency (WADA) on prohibited substances in sports encompasses a large number of compounds, classified as S0–S9 and P1 categories. Compounds included in this list cover a wide range of functionalities and physicochemical properties [1]. The WADA´s List is not exhaustive since any substance showing a similar structure or effect to a named substance will be also prohibited. On the other hand, many substances do not have a single analytical target and must be monitored through different metabolites or biomarkers. So, hundreds of substances are of potential interest in anti-doping testing programs.

According to WADA´s rules, drug testing in doping control laboratories involves the use of Initial Testing Procedures (ITPs) for the screening of all the substances covered by the Prohibited List, followed by more specific confirmatory methods for the analysis of presumptive adverse analytical findings [2]. Sensitivity and specificity of ITPs are critical for the control of compliance of athletes with anti- doping regulations since doping agents not spotted in the screening step will be not subjected to confirmatory analysis.

To tackle these challenges, current ITPs are based on technology-driven methodologies that use a quite limited portfolio of methods intended to generate a comprehensive set of data for each doping control sample (basically urine) [3,4]. In this respect, gas chromatography mass spectrometry (GC-MS) and liquid

chromatography mass spectrometry (LC-MS) are the most widely used techniques in current ITPs, although the share of prohibited substances covered by LC-MS is substantially larger than that measured by GC-MS [5,6]. Low resolution MS with a triple quadrupole (QqQ) analyzer is usually preferred over high resolution MS due to its high sensitivity in the single reaction monitoring (SRM) mode, fast polarity switching and short scan times [7]. Three strategies are primarily followed for sample preparation; solid phase extraction (SPE), liquid-liquid extraction (LLE) and matrix dilution (dilute-and-shoot, D&S, methods), each with advantages and drawbacks and specific scope of application [8,9].

Liquid chromatographic separation of multiclass sport drugs for screening purposes is primarily carried out on alkyl-bonded silica columns (typically octadecylsilane, C18) [8,10], although pentafluorophenyl propyl (PFP) phases have gained popularity for drug monitoring due to their improved selectivity [11,12]. The more frequent mobiles phases include formic acid/ACN and formic acid/methanol in the absence or presence of ammonium acetate [10]. Hydrophilic interaction liquid chromatography (HILIC) has been investigated as an alternative to RP-LC for highly polar and charged compounds [13-15]. Some multi-target approaches have been reported with HILIC, such as the mixture of stimulants, narcotics, and beta-adrenergic agents [16] or stimulants, glycerol, AICAR, ethyl glucuronide, morphine-3-glucuronide, and myo-inositol trispyrophosphate [17]. Given the high number of compounds of interest in ITPs and their wide range of physicochemical properties, there is not a single optimal stationary phase for liquid chromatographic separation. Major problems associated to ITPs are peak tailing for basic compounds, poor retention of specific compounds and the lack of specificity, particularly as D&S methods are used for sample preparation [7,8,10]. So, the comparative study of how different stationary and mobile phases influence these parameters (e.g. retention factors, peak shapes and matrix effects) on ITPs could help to acquire a deeper and wider knowledge of the retention mechanisms and chromatographic behavior of the different substance categories of the WADA´s list and to help to select knowledge-based stationary and mobile phases.

Only few studies have been devoted to compare the use of different stationary phases in the chromatographic analysis of prohibited substances in sports and most of them have involved only few compounds. A representative example is the study of Gray et al., who proved the advantages of using HILIC for the quantification of five ephedrines against C_{18} in terms of peak shape, sample loading capacity and ESI sensitivity [18]. Also, Petruczynik et al. compared different RP columns (octadecyl silica, polar octadecyl, phenyl, phenyl-hexyl, polar reversed phase, pentafluorophenyl, and cyanopropyl) for the analysis of fourteen psychotropic drugs [19,20]. Authors observed significant differences in retention times, peaks shape, efficiency and separation selectivity. The best peak shape was obtained with the cyanopropyl column while the highest efficiency was measured for the phenyl-hexyl and the polar RP column (ether-linked phenyl base with polar endcapping).

This research was intended to acquire a deeper knowledge of the performance of different mobile phase-stationary phase combinations in LC-ESI-MS/MS-based ITPs with the aim of improving the analytical performance of multi-target methodologies in anti-doping analysis. The study focused on three parameters of great interest from a practical standpoint, specifically retention factors, asymmetry factors and matrix effects. Ninety-three prohibited substances (or their metabolites) belonging to ten categories of the WADA´s list (S1-S9 plus P1) were selected as target compounds (Table ESI1, in electronic supporting information) [1]. Six stationary phases were selected for this comparative study (Table 1). They included the most frequent stationary phase used in this context (C18) and those with increased popularity (PFP and HILIC). Likewise, stationary phases that could help to better understand their retention mechanisms (C8 vs C18 and PH vs PFP) or not tested yet in ITPs (e.g. mixed mode HILIC-RP) were also considered. Mixed-mode chromatography, which combines more than one interaction mode is becoming increasingly popular in drug analysis since it offers a unique selectivity, especially for polar and charged molecules [21]. To the best of our knowledge, although mixed-mode phases have been used for sample preparation in anti-

doping analysis (multimode solid-phase extraction) [22,23], they have not been applied for chromatographic separation yet. As mobile phases we tested acetonitrile and methanol (organic modifiers) in the presence and absence of ammonium acetate as additive.

Below, the best chromatographic approaches in terms of separation (retention factors and asymmetry factors) and selectivity for ITPs in the analysis of anti- doping substances are discussed.

Materials and methods

2.1 Reagents and standards

All solvents were LC-grade and were used as supplied. Acetonitrile (ACN) and methanol (MeOH) were purchased from PanReac (Darmstadt, Germany). Formic acid and ammonium acetate were Sigma-Aldrich reagents (St Louis, MO, USA). Ultra-high-quality water was generated from a Milli-Q water purification system (Millipore-Sigma, Madrid, Spain). Anti-doping drugs suppliers are shown in Table ESI2. Stock solutions for individual drugs (prepared at 1, 100 or 1000 μg mL^{-1}) were prepared in MeOH and stored at 4 or -20ºC. Intermediate and working solutions of drug mixtures were prepared by appropriate dilution in MeOH and stored at - 20ºC for at least one month.

2.2. LC-ESI-MS/MS studies under different chromatographic conditions

Studies were carried out using an Agilent Technologies 1200 series LC (Palo Alto, CA, USA) coupled to a hybrid triple quadrupole/linear ion trap (Applied Biosystems MSD Sciex, 4000QTRAP, Foster City, CA, USA) equipped with a Turbo

V Ion Source (TIS). All data were acquired and processed using the Analyst 1.5.1 software. Separation of the 93 prohibited substances was carried out under both reversed phase and hydrophilic modes. All the column characteristics and mobile phase composition and gradients are specified in Tables 1 and ESI3, respectively.

Table 1. Relevant information on the stationary phases selected in this study

Column trade name (Supplier)	Stationary phase (acronym)	Structure	Dimensions (internal diameter x length; mm)	Particle size (µm)	Surface area (m^2/g)	Core-shell	Partition mechanism: Primary interactions	Adsorptio onto silica: Secondary interaction
Raptor C18 LC (RESTEK)	Octadecylsilane (C18)		3x100	2.7	130	Yes	Dispersion	Ion exch H-bonding, dipole-dipo ion-dipole
Raptor fluorophenyl LC (RESTEK)	Pentafluorophenylpropyl (PFP)		3x100	3	130	Yes	Dispersion, π-π, dipole-dipole, H-bonding	Ion exch H-bonding, dipole-dipo ion-dipole
Infinity Lab Poroshell 120 EC-C8 (Agilent Technologies)	Octylsilane(C8)		3x100	2.7	130	Yes	Dispersion	Ion exch H-bonding, dipole-dipo ion-dipole
Roc phenyl hexyl LC (RESTEK)	Phenylhexyl (PH)		3x100	3	300	No	Dispersion, π-π	Ion excnan H-bonding, dipole-dipo ion-dipole
Acclaim Mixed-Mode HILIC-1 (Thermo Scientific)	Alkyl diol in RP mode and water layer on diol groups in hydrophilic mode (HILIC-RP)		3x150	3	300	No	Dispersion, dipole-dipole, H-bonding	Ion exchan H-bonding, dipole-dipo ion-dipole
Raptor HILIC-Si LC (RESTEK)	Water layer on silica (HILIC)		3x100	2.7	130	Yes	Dipole-dipole, H-bonding	Ion exchan H-bonding, dipole-dipo ion-dipole

For columns working in RP mode (C8, C18, PFP, PH), four aqueous mobile phases containing MeOH or ACN as organic modifiers, and formic acid (pH 2.7), with or without addition of 2.5 mM ammonium acetate, were investigated. For the HILIC and RP-HILIC columns, only ACN was used as organic modifier. In all cases, the flow rate was 250 μL min^{-1} and the injection volume was 5 μL. Experiments were performed in duplicate. Detection of drugs was performed in both positive and negative ionization modes, using the following operation parameters: source gas temperature 400°C, capillary voltage: 4500 V, nebulizer gas pressure: 50 psi, drying gas pressure: 50 psi, curtain gas pressure 20 psi; declustering potential: 30 V. Products ions obtained for the different drugs and detection parameters of each MS/MS transition are shown in Table ESI2.

2.3. Matrix effect studies in urine

Ten spot urine samples were collected from healthy volunteers and a pooled sample was prepared from equal volumes. Aliquots of 250 μL of pooled urine were fortified with a mixture of the 93 prohibited substances (Table ESI1) in methanol and diluted up to 1 mL with methanol (final concentration of the doping agents: 25 ng mL^{-1}). Experiments were run in triplicate. In order to achieve robust and reproducible chromatographic results in the HILIC mode, the column requires adequate column equilibration and preconditioning [18]. So, to prevent changes in retention times due to matrix effects throughout a series of injections, at least three blank urine samples were injected in HILIC to precondition the column before the first chromatographic run of the sequence, as recommended by Görgenset al. [17].

2.4. Calculation of chromatographic and analytical parameters

Retention factors (k) were calculated as $k = (t_R - t_0) / t_0$, where t_R and t_0 are the retention time of the substances and column hold-up time, respectively. Values for k in the range 1-10 were considered as optimal although, at the option of the chromatographer, it is possible to expand this range between 0.5 and 20 [24]. Asymmetry factors (A_s) were automatically calculated by 10% of peak height (Analyst 1.5.1 software). The range 1.0-1.5 was considered acceptable for A_s values [24]. Because urine was only diluted before analysis without further treatment, matrix effects (ME) were expected and calculated as $\text{Peak area}_{\text{spiked diluted urine}}/\text{Peak area}_{\text{standard solution}} * 100$. Relative matrix effects (ME_{rel}) were subsequently calculated as ME – 100. Positive and negative values indicated signal enhancement and suppression, respectively. Substances with values of ME_{rel} in the range ±20 were considered not to be significantly affected by matrix effects [24].

3. Results and Discussion

3.1. General considerations

The comparison of different chemical-bonded stationary phases can help to identify the column with the best performance for ITPs, to recognize columns of equivalent performance and, therefore interchangeable, or to find out columns ofquite different performance for the development of an orthogonal separation.

Table ESI1 shows the name, chemical structure and molecular formula of the anti- doping compounds, ranked by WADA categories, as well as different parameters of interest for their retention behavior, the decision limits (DL) for the confirmatory quantification of threshold substances [25] and the minimum required performance levels (MRPLs) for detection and identification of non-

threshold substances [26]. The selected compounds covered a great number of functionalities (e.g. alcohol, carboxyl, ether, ester, ketone, primary/secondary/tertiary amines, amides, sulfonyls, etc.) and a wide range of polarity (log P from -2.4 to 9.2). Likewise, they included acids, bases and neutrals, and many of them bore aromatic rings and/or heterocycles.

Columns were compared for a given mobile phase, so that the observed differences were the result of distinct solute-column interactions only. In this respect, the selected columns provided a variety of interactions for solute partition including from single (e.g. C8, C18) to multiple (e.g. PFP, HILIC-RP). Likewise, interaction with silica support was expected to be an important secondary mechanism for retention of solutes in all the selected columns. Silanol groups, with an average pK_a of approximately 7 in modern silicas [27], were expected to be unionized at the working pH (2.7), which should increase the chromatographic performance ofionic basic compounds.

Retention in HILIC is achieved through the establishment of a water-rich layer immobilized onto the surface of the polar stationary phase, which is commonly formed at percentages of water in the mobile phase in the approximate interval 3-50% [13-17]. Solute partition takes place between this water-rich and the mobile phase. Contrarily to the RP mode, water is the strongest solvent in HILIC, followedby methanol, which is too strong for most HILIC separations. So, as usual, we usedACN as the weak solvent. Preliminary experiments were carried out to find the best chromatographic conditions with gradients starting from 3 to 15% of water and finishing from 40 to 60% of water, but the chromatographic profile was quitesimilar as a result of the poor retention of most of the selected compounds by HILIC mechanisms.

Regarding the HILIC-RP stationary phase (Table 1), it can be used in either HILIC mode (in high organic conditions) or RP mode (in high aqueous conditions). Initially, we investigated the operation of this column in both modes. In HILIC mode, the chromatographic behaviour was quite similar to the silica-based HILIC column, without any benefit from the presence of the hydrocarbon chain in the

stationary phase for the retention of nonpolar compounds. So, we decided to work only in RP mode. Only ACN was investigated as the organic modifier in the mobile phase with the aim of facilitating the HILIC mode as an additional operating mechanism during gradient conditions (e.g. below 50% water). In this way, RP and HILIC modes would be predominant at the beginning and end of the chromatographic run, respectively.

3.2. Retention factors

Figure 1 shows some chromatographic parameters (total run time, compounds with k<1 and k>10 and number of coelutions) considering all the selected compounds, columns and mobile phases.

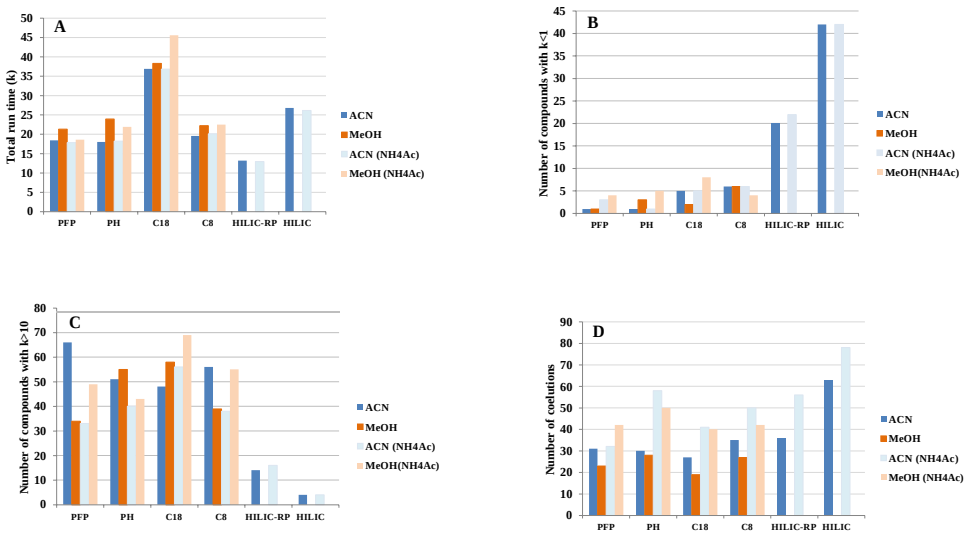

Figure 1. Different chromatographic parameters for the selected 93 anti-doping agents in six stationary phases and four mobile phases. (A) Run times, expressed as retention factor (k), number of anti-doping agents with (B) k < 1 and (C) k > 10, and (D) number of coelutions (k variations in ± 0.1 min) of chromatographic peaks.

The total run time required for elution of the 93 doping agents, expressed as retention factors (k), followed the order HILIC-RP<PFP˜PH˜C8<HILIC<C18 (Figure 1A). Total run times increased for MeOH compared to ACN, as it might be expected from the higher elution power of the latter. In most cases, the addition of ammonium acetate hardly influenced total run times, particularly as ACN was used as organic modifier.

One-way ANOVA (α=0.05) was performed to highlight significant differences in mean retention factors between all the combinations of columns and mobile phases (Table ESI4). Due to the high number of compounds and functionalities, general simple trends could not be established. PH, PFP and C8 with ACN:water(NH_4Ac), PH and PFP with MeOH:water(NH_4Ac) and C8 with ACN:water resulted in the lowest mean retention factors with reversed phases (I, Table ESI4) and were not statistically different. The lowest mean retention factors were obtained for HILIC-RP and HILIC phases (J and K, α=0.05, Table ESI4) without significant differences between mobile phases with and without NH_4Ac. The longest k mean values were obtained for C18 without significant differences for methanolic mobile phases and aqueous ACN (A, Table ESI4).

Doping agents eluting in the optimal k interval (1<k<10) greatly varied for some columns as a function of mobile phase composition (Table ESI5), that highlighting the importance of the selection of both the organic modifier and additives for achieving good chromatographic performance. In this respect, the best results were obtained for HILIC-RP and PFP, while the worst records were obtained for C18. The number of compounds eluting too fast (k<1) was negligible or acceptable for all the columns working in RP mode, except for HILIC-RP (Figure 1B), which was surprising, particularly as compared to C8, since it not only has a longer hydrocarbon chain but it also provides more types of interactions for solute retention (Table 1). The poor retention of HILIC for many of the doping agents selected (Figure 1B) was reflected in the considerable number of compounds eluting too fast. Figure 1C illustrates that, except for HILIC-RP and HILIC, ammonium acetate seemed to be an important factor influencing the retention of

the selected doping agents. Likewise, it illustrates that the number of compounds eluting at k values above 10 was from moderate to high. Nevertheless, all the columns, except C18, gave acceptable k values if the range for this parameter was expanded to 20 [24].

Finally, for estimating the degree of co-elutions we used as indicator the number of compounds with differences of ±0.1 min in k values (Figure 1D). Unexpectedly, the addition of ammonium acetate resulted in a higher number of co-elutions. The use of methanol instead of ACN resulted in a lower number of co-elutions, although differences were not so pronounced as with ammonium acetate. The highest number of co-elutions corresponded to the HILIC column due to its poor retention for the medium and non-polar compounds.

3.3. Retention mechanisms

In order to shed some light on the retention mechanisms underlying the observed k values, we investigated the relationships log P versus k, considering log P as an indicative parameter of the relative solubility of solutes between the stationary and mobile phases and, consequently, being representative of partition mechanisms. The use of log P as a simple retention predictor for identification of solutes by LC-HRMS has been widely investigated [28]. Figure 2 shows the plots for the different columns using ACN:water as the mobile phase.

The k values for all the columns operating under RP conditions showed considerable dispersion. The highly nonpolar compounds (log P > 4) eluted later and there were many compounds with low-medium polarity (log P < 2) eluting throughout the entire chromatogram, this suggesting the occurrence of different interaction mechanisms of solutes with the stationary phase. The same undefined trend was found for all the mobile phases (Figures ESI1-ESI3).

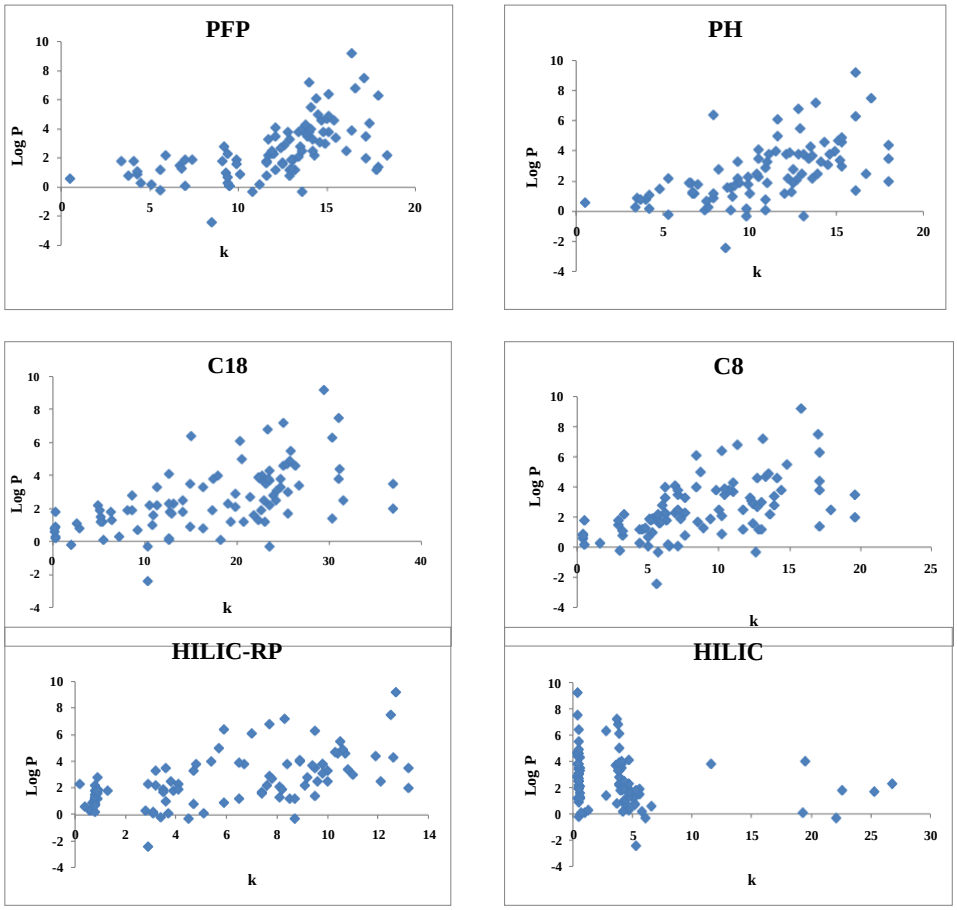

Figure 2. Plots of log P versus k obtained for the 93 anti-doping agents in six stationary phasesand a mobile phase made up of ACN-water and formic acid (0.1%).

As it was expected, the HILIC mode was unable to deal with the separation of a wide polarity range of compounds. HILIC chromatographic runs showed two primary elution windows, a first one where most of compound co-eluted, and a second one providing good separation for a few compounds, which was not relatedto their polarity (log P).

Great efforts have been performed in computational chemistry to predict retention times in LC based on different chemical properties of solutes with the aim of avoiding false positive identification in LC-HRMS [29]. Thus, quantitative structure–retention relationship (QSRR) models have been developed to predict the retention times of 146 endogenous metabolites and banned compounds of interest in doping analysis using PFP and HILIC columns [30]. Figure ESI4 shows the log P versus the experimental retention times obtained for both columns. The results were similar to those obtained in our study; very poor correlation for PFP and two elution windows for HILIC, the first one occurring at retention times below 1 where a high number of compounds coeluted. Although additional descriptors have been used to develop QSRR models for doping substances (e.g. atomic polarizabilities and electronegativities in PFP and folding degree index and toxicities for algae in HILIC), the relative errors for retention time prediction are still high for a great number of compounds [30].

3.3.1. Comparison of stationary phases

In order to elucidate the predominant interactions for different stationary phases, columns were compared for given mobile phases. Thus, the k values (n=93) for two different columns, were plotted each versus other for a given mobile phase. In this way, deviations from a best-fit line through the data can be interpreted as the result of differences in solute-column interaction [31]. The larger the deviations or more scattered the plot, the less similar are the two columns in terms of solute retention. Table 2A shows the determination coefficients (R^2) for each pair of columns working in RP mode at the different mobile phases. Figure ESI5 illustrates, as an example, the dependences obtained for PFP, FP, C18 and C8 with both ACN-water and methanol-water.

In absence of ammonium acetate, the best correlations in ACN-water (R^2 from 0.776 to 0.896 in Table 2A) were found for columns having a moderate-long alkyl chain in the stationary phase (i.e. PH, C18, C8, HILIC-RP), in which dispersion

interactions are important for solute partition. The worst correlations were found for PFP since this stationary phase is less hydrophobic and it offers a wider range of interactions for solute retention (Table 1). Particularly interesting was the decrease in correlations observed for the columns PFP/PH and PH/C18 as MeOH-water was used as the mobile phase instead of ACN-water. This behaviour was in agreement with the findings previously reported that ACN suppresses solute-column π-π and dipole-dipole interactions [31], and supports the relative importance of these interactions in FPF and PH columns, mainly as methanol is used as organic modifier. The addition of ammonium acetate to the mobile phase modified correlation values (Table 2A), that suggesting the influence of this salt on retention mechanisms.

Since the selected compounds widely vary in functionalities and properties, we further analysed the chromatographic behaviour of doping agents by grouping them by acidic and basic properties. The behaviour of very polar compounds (log P ≤ 1) was also comparatively investigated for the different columns.

Table 2. Determination coefficients (R^2) calculated from the linear plots k versus k obtained in chromatographic runs performed with different columns and the same mobile phase and considering (A) all the target, (B) acidic and (C) basic doping agents

Compared columns	(A) All selected doping agents (n=93)				(B) Acidic doping agents (n=11)				(C) Basic doping agents (n=59)			
	ACN-water	ACN-water(NH_4Ac)	MeOH-water	MeOH-water(NH_4Ac)	ACN-water	ACN-water(NH_4Ac)	MeOH-water	MeOH-water(NH_4Ac)	ACN-water	ACN-water(NH_4Ac)	MeOH-water	MeOH-water(NH_4Ac)
PFP/PH	0.765	0.722	0.530	0.651	0.929	0.916	0.677	0.838	0.738	0.778	0.647	0.494
PFP/C18	0.672	0.749	0.679	0.498	0.885	0.867	0.897	0.848	0.695	0.728	0.454	0.409
PFP/C8	0.639	0.760	0.743	0.781	0.847	0.86	0.858	0.897	0.597	0.744	0.689	0.634
PH/C18	0.882	0.746	0.510	0.697	0.946	0.975	0.722	0.945	0.896	0.867	0.594	0.702
PH/C8	0.776	0.846	0.704	0.861	0.891	0.970	0.897	0.960	0.768	0.865	0.806	0.843
C18/C8	0.896	0.829	0.727	0.728	0.975	0.991	0.911	0.988	0.869	0.763	0.672	0.776
PFP/HILIC-RP	0.631	0.595	-	-	0.814	0.683	-	-	0.557	0.734	-	-
PH/HILIC-RP	0.827	0.623	-	-	0.947	0.842	-	-	0.784	0.756	-	-
C18/HILIC-RP	0.827	0.778	-	-	0.916	0.905	-	-	0.856	0.856	-	-
C8/HILIC-RP	0.809	0.736	-	-	0.868	0.879	-	-	0.834	0.770	-	-

3.3.1.1. Acidic doping agents

Regarding acidic doping agents (i.e. those containing carboxylic groups, n= 11, pK_a 0.35 to 4.2, log P -0.3 to 6.3, Table ESI1), a very poor correlation was found for the plots log P versus k (R^2 from 0.152 to 0.655 including all the columns and mobile phases, data not shown). Thus, not only partition to the stationary phase but interaction with the silica support seemed significant for their retention. However, very good correlations (R^2 from 0.814 from 0.975) were found for column comparison using ACN-water as the mobile phase (Table 2B). Since most of the acidic doping agents bore aromatic rings, the suppression of π-π and dipole-dipole interactions by ACN, were considered an important factor for the similar behaviour observed. The use of methanol as organic modifier or ammonium acetate as additive highlighted the differences in retention mechanism among some columns (e.g. PFP/PH and PH/C18 in methanol or PFP/HILIC-RP and PH/HILIC-RP with ammonium) as indicated for the decrease in R^2 values. Excellent correlations were found for C18/C8 independent of the mobile phase composition (Table 2B). In the case of the HILIC column, all the acidic doping agents but two compounds with negative log P values and 11-nor-Δ9- tetrahydrocannabinol-9-carboxylic acid (log P 6.3), eluted at k values below 1. Figure ESI6 shows representative plots for column comparison regarding acidic doping agents.

3.3.1.2. Basic doping agents

Concerning basic doping agents (n=59, pK_a from -4 to 12.5, log P from -2.4 to 7.2, Table ESI1), the study included highly basic and polar compounds, which constitutes a great challenge for their chromatographic separation. Very poor correlation was found for log P as a function of k (R^2 was below 0.500 for all the columns and mobile phases investigated, data not shown), so combination of partition and adsorption onto silica should be considered to explain their retention

mechanisms. The determination coefficients for the plots k versus k for the different column pairs (Table 2C) were quite similar both in magnitude and variation to those obtained for the whole of the target compounds (Table 2A), which is somehow logical considering that basic compounds accounted for the 63% of the total of selected targets.

3.3.1.3. Very polar doping agents

With regard to the chromatographic behaviour of very polar doping agents (n = 19, log P from -2.4 to 1) the alkyl phases C18, C8 and HILIC-RP showed the worstperformance and a number of compounds between 4 and 8 (21-42%) eluted too fast (k < 1). On the contrary, only one compound (ecgonine methyl ester) eluted at k below 1 with phenyl phases (PFP and PH). This good performance could be a consequence of π-π interactions since all these polar compounds except ecgonine methyl ester had one or two aromatic rings. All the polar compounds showing poor retention in any of the RP columns were efficiently retained in HILIC, as it was expected from the hydrophilic partition mechanism. So, HILIC permitted a real orthogonal separation for polar compounds not efficiently separated by phenyl phases.

3.3.2. Comparison of mobile phases

The mobile phases specified in Table ESI3, containing ACN or methanol as organic modifiers and ammonium acetate as additive, were compared for given columns.

3.3.2.1. Influence of organic modifiers

Figure 3 compares k values for mobile phases made up of ACN-water and methanol-water (both containing only formic acid 0.1% v/v), in four columns

working in RP mode (i.e. PFP, PH, C18 and C8). Although total run times were slightly longer for mobile phases containing methanol (as it was shown in Figure 1A), the chromatographic profiles in PFP, PH and C18 clearly illustrated a different behavior of polar and nonpolar compounds (except for some outliers).

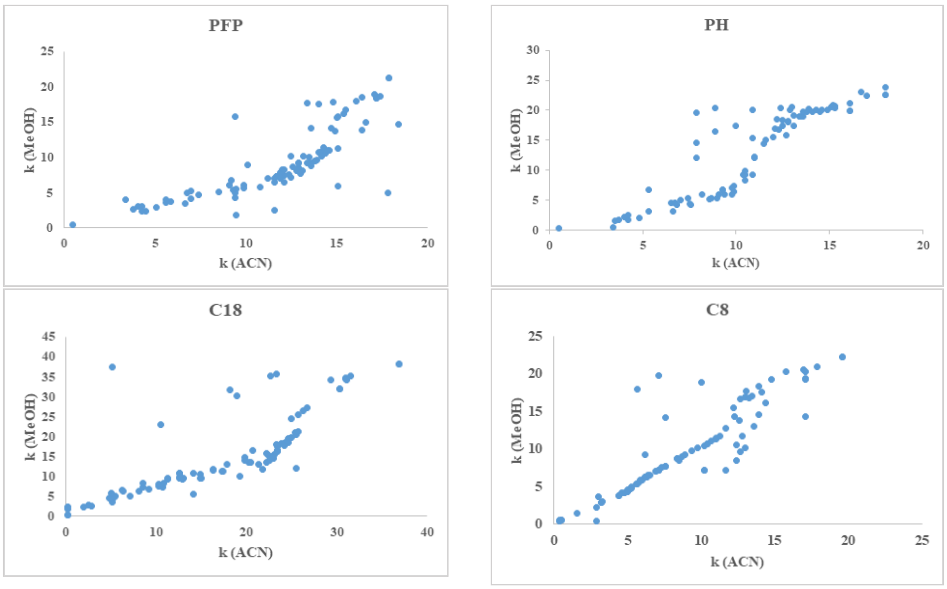

Figure 3. Plots showing the comparison of k values obtained in four stationary phases using eluents made up of ACN-water and methanol-water, both with formic acid 0.1%, for the 93anti-doping agents.

Thus, at the first section of the chromatograms (until around k_{ACN} =10 for PFP and PH, and k_{ACN} =20 for C18), doping agents (mainly highly and medium polar) eluted faster with mobile phases containing methanol compared to ACN. These results suggest that the mixture methanol-water was a better solvent than the mixture ACN-water for these compounds, which is supported by its higher polarity (i.e. δ_{water} 23.5 $cal^{1/2}$ $cm^{-3/2}$, $\delta_{methanol}$ 14.5 $cal^{1/2}$ $cm^{-3/2}$ and δ_{ACN} 11.9 $cal^{1/2}$ $cm^{-3/2}$) [32]. Particularly interesting is the fact that two of the three types of intermolecular interactions that contribute to the Hildebrand solubility parameter, namely dispersion (fd), polar (fp) and hydrogen bonding (fh) forces (Teas or fractional

solubility parameters), are very different for methanol and ACN (i.e. fp and fh). Thus, Teas parameters (%) for methanol and ACN are fd= 30, fp= 22, fh= 48 and fd= 39, fp= 45, fh= 16, respectively [32]. Considering that Teas parameters for water are fd= 18, fp= 28, fh= 54 [32], this means that mixtures methanol-water and ACN-water will provide similar dispersive interactions, while they will greatly differ in hydrogen bonding and polar interactions. Given the high number of hydrogen bond donors and acceptors present in doping agents (Table ESI1), solubility of polar and medium polar doping agents in the mixture methanol-water, with high fh, should be favoured, thus providing faster elution.

At the second section of the chromatograms for PFP, PH and C18 (Figure 3), where the percentage of organic solvent in the mobile phase increases, nonpolar compounds eluted faster in mixtures ACN-water compared to methanol-water, which is supported by the higher solvation properties of ACN for these compounds. A third section, roughly linear and slope near 1, was obtained in PH (Figure 3), which suggests that both polar and hydrogen bonding interactions were similarly effective in eluting the highly retained compounds. The above specifiedsections were only roughly observed for C8 (Figure 3).

3.3.2.2. Influence of ammonium acetate

The addition of ammonium acetate to both methanol-water and ACN-water scarcely influenced the chromatographic profile for the doping agents. Thus, the plots k(ACN) versus k(ACN-NH_4Ac) and k(MeOH) versus k(MeOH-NH_4Ac) fitted linear relationships quite well, with slope values in the ranges 0.930-1.256 and 0.802-1.086 and determination coefficients (R^2) in the intervals 0.774-0.988 and 0.823-0.850, for ACN and methanol, respectively. Figure ESI7 shows, as an example, the plots for the different columns and mobile phases containing ACN inthe absence and presence of ammonium acetate.

However, the effect of the ammonium salt on individual k values was quite different for ACN- and methanol-based mobile phases. Figure 4 shows, for each of

the tested column, the percentage of compounds for which k values increased, kept constant or decreased as ammonium salt was added to mobile phases containing ACN (Fig. 4A) or methanol (Fig. 4 B).

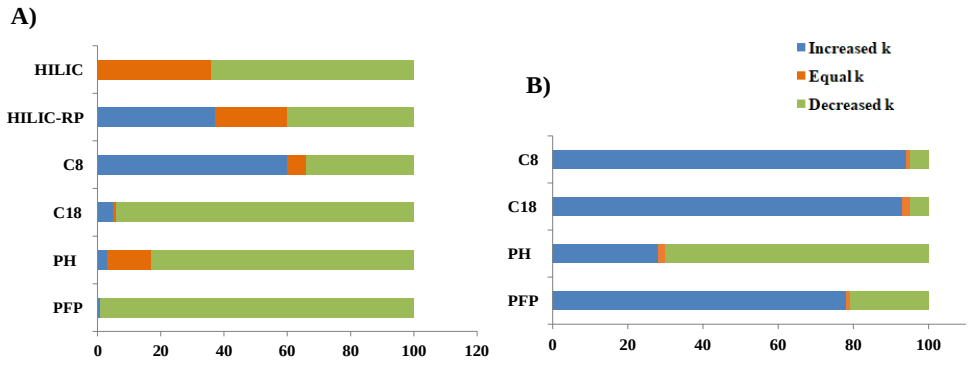

Figure 4. Percentage of anti-doping agents with increased (blue), invariable (orange) or decreased (green) k values when adding ammonium acetate to mobile phases made up of (A) ACN-water and (B) methanol-water, both with formic acid 0.1%, for the selected 93 anti-doping agents and columns.

A higher number of compounds with decreased k values were observed in PFP, PH and C18 columns as ammonium acetate was added to the ACN-based mobile phase (Fig. 4A). The behaviour for C8 was quite different, and around 60% of thecompounds underwent increase in k values. On the other hand, the k values kept constant for a considerable number of compounds in HILIC and HILIC-RP columns, which is logical considering that many of them were poorly retained. Also, regarding the magnitude of k variation, these columns provided the smaller individual variations in the absence and presence of ammonium acetate. The retention behaviour for ACN-based mobile phases in the different columns was substantially similar to that obtained for the doping agents grouped in acids, basesand neutrals (data not shown), which suggests that suppression of ionic interactions among doping agents and silanol groups did not play a role in the increase of the number of compounds with decreased k values, as expected at the working pH [12,33]. On the other hand, except for PH, addition of ammonium salt

to methanol-based mobile phases mainly caused the increase of k values for the doping agents investigated (Figure 4B). The columns giving the higher variations for individual k values in the absence and presence of ammonium were C18 and C8 for both ACN- and methanol-based mobile phases.

Although we can't provide a verifiable justification for the decrease and increase observed in k values under the addition of ammonium acetate, some facts could help to provide some explanation. Thus, the same behaviour obtained for alkyl andphenyl phases (e.g. PFP, PH and C18 in Figure 4A and PFP, C18 and C8 in Figure 4B) suggests that secondary interactions with the silica support (other than ionic) or changes in properties in the mobile phase (e.g. solvation properties causing salting out of solutes to the stationary phase [34-37]), could be the responsible forthe decrease and increase of k values, respectively. Regarding interactions of ammonium acetate with silanol groups, it is worth noting that both ions can establish hydrogen bonds. In the case of ammonium, it has four hydrogen bond donors of the so-called weak hydrogen bond category [38]. In the case of acetate, it has one hydrogen bond acceptor. So, both ions can bind to silanol groups and compete by these sites with doping agents, most of which have donor and/or acceptor hydrogen bonds (see Table ESI1), thus provoking the decrease of k values.On the other hand, salting out effects by the addition of salts to mobile phases have been previously reported [34-37], and their occurrence in our research is justified by the kosmotropic character of acetate ions [39]. Thus, carboxylate head groups in acetate ions are strongly hydrated (hydration number from 5 to 7), which renders these ions excellent salting-out agents [40]. Salting out causes higher retention of solutes in the stationary phase and consequently increments in their k value. The differences observed for the mobile phases ACN-water and methanol-water (Figure 4) may derive from their different solvency properties for acetate; ACN is a hydrogen bond acceptor (the same as acetate) while MeOH, like water, is both hydrogen bond donor and acceptor. The higher the solvency for acetate, the greater the salting-out effect, and consequently increased k values will be preferentially obtained in MeOH compared to ACN.

3.4. Asymmetry factors

Chromatographic peak shapes, evaluated through the asymmetry factor (A_s), varied among stationary phases and eluent systems. Figure 5 shows the percentageof compounds included within each of the four ranges of values set for A_s for the six columns and the four mobile phases (A: ACN-water; B: methanol-water; C: ACN-water(NH_4Ac); D: methanol-water(NH_4Ac).

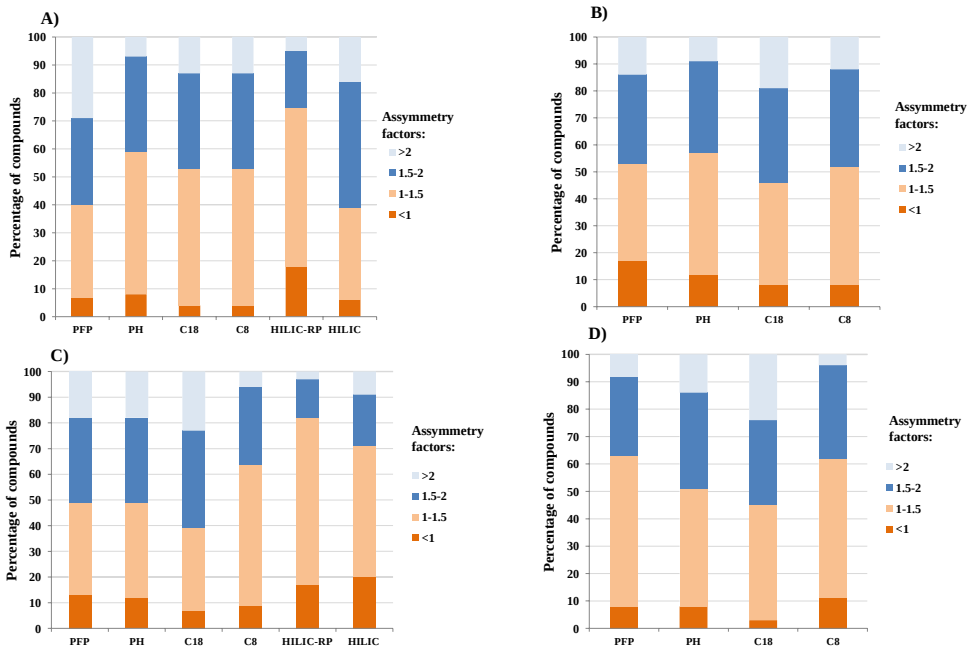

Figure 5. Percentage of anti-doping agents included within each of the four ranges of values set for A_s for six columns and four mobiles phases. (A): ACN-water; (B): methanol-water; (C) ACN-water(NH_4Ac); (D): methanol-water(NH_4Ac). All the mobile phases contained formic acid 0.1%.

HILIC-RP in ACN-water(NH_4Ac) provided the best peak shapes (65% of the compounds within the A_s range 1-1.5) among all the columns and mobile phases combinations. Likewise, considering that all the compounds with A_s values below 1 (fronting peaks) had acceptable peak shapes (i.e. A_s 0.8-0.9) [20], the percentage

of compounds within the acceptable range could be considered up to 82%. It is also worth noting that the percentage of compounds with A_s values above 2 was low (i.e. 5%).

The presence of ammonium acetate also improved considerably peak shapes in HILIC; the percentage of compounds within the range 0.8-1.5 for A_s increased from 39% (Fig. 5A) to 71% (Fig. 5C). It has been previously reported that solutions of formic acid alone in acetonitrile-rich mobile phases (without ammonium salts)have an extremely low ionic strength, due to the increase of the pK_a of the acid on addition of organic solvent, and distorted peaks for ionized compounds may be obtained in HILIC [33].

Regarding the rest of columns (PFP, PH, C18 and C8) the behavior was quite different as a function of the mobile phase composition (organic modifier and additive), but the percentage of compounds within A_s range 0.8-1.5 was at best between 53 and 64%. The worst peak shapes under all the mobile phase conditions were obtained for C18, which is in agreement with previous results obtained for basic psychotropic drugs [19]. Bad peak shapes were also obtained for PFP, except for methanol-water(NH_4Ac), a behavior that has been also reported for basic psychotropic drugs [20]. Better results were obtained for C8 in the presence of ammonium acetate (in both ACN- and methanol-based mobile phases), while for PH, the percentage of compounds within A_s range 0.8-1.5, decreased under addition of this additive.

3.5. Matrix effects

Matrix effects were evaluated from 4-fold diluted urine according to the procedurespecified in section 2.3 [17] and they were expressed as relative matrix effects (ME_{rel}), that were calculated as indicated in section 2.4. For these experiments we selected mobile phases made up of methanol-water (formic acid 0.1% v/v) for PFP,

PH, C18 and C8, and ACN-water(NH_4Ac) (formic acid 0.1% v/v) for HILIC-RP and HILIC. Figure 6 shows the results grouped in four categories, namely compounds not influenced by matrix effects (ME_{rel} ± 20%, in blue), those affected by signal enhancement (ME_{rel} +21 to +100, in green) or suppression (ME_{rel} -21 to -100, in red), and compounds exceeding these limits and therefore significantly influenced by interferences (in grey).

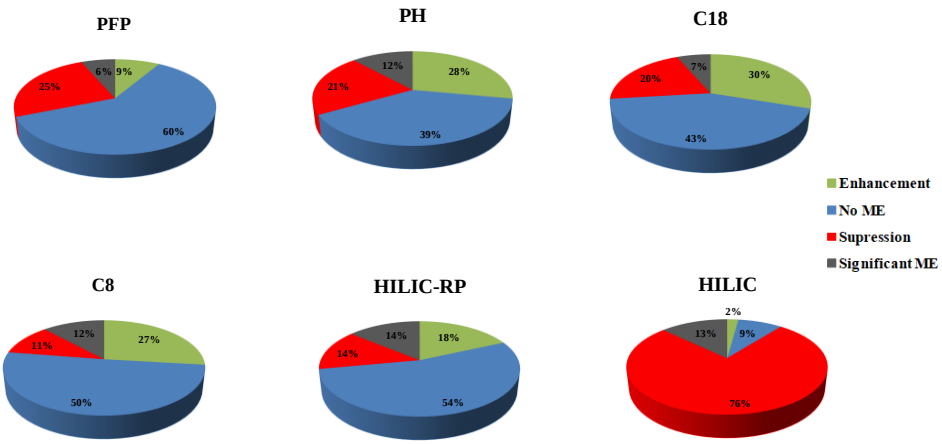

Figure 6. Pie charts for comparison of matrix effects in the different columns. Mobile phase composition: methanol-water (formic acid 0.1%) for PFP, PH, C18 and C8, and ACN- water(NH_4Ac) (formic acid 0.1%) for HILIC-RP and HILIC. The compounds not influenced by matrix effects (ME_{rel} ±20%) are depicted in blue, those affected by signal enhancement (MErel +21 to +100, in green) or suppression (MErel -21 to -100, in red), and compounds exceeding these limits in grey.

Although none of the columns provided acceptable results for the whole of doping agents from diluted urine, the comparison of ME_{rel} values clearly showed that stationary phases influenced selectivity and that the best and worst results regarding ME were obtained for PFP and HILIC, respectively. The percentage of compounds with unacceptable ME_{rel} values was similar in all columns (12-14%)

except for PFP (6%) and C18 (7%). The C8/HILIC-RP and PH/C18 binomials behaved quite similarly in terms of matrix interferences. The stationary phase influenced the type of matrix effect too; ionization suppression was dominant in HILIC while an enhancement of the MS signal was preferably observed for PH, C8 and C18.

With the aim of better understanding the different behavior of columns regardingmatrix effects, ME_{rel} values were plotted as a function of their retention factors (Figure 7).

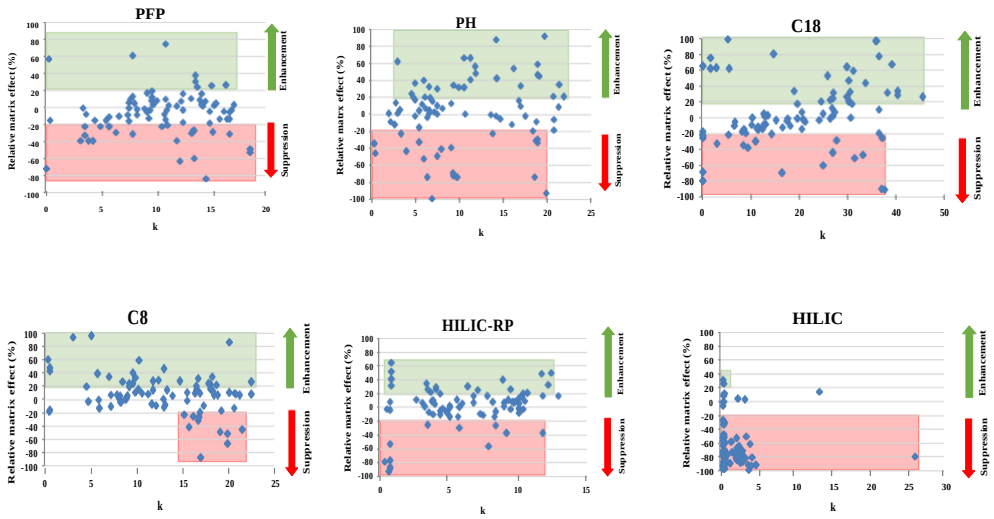

Figure 7. Matrix effects as a function of retention factors for anti-doping agents in the six columns. Mobile phases are the same as in Figure 6. Compounds strongly influenced by matrix effects (in grey in Figure 6) are not represented in this figure.

No clear pattern was observed for the sign and intensity of the matrix effects alongthe chromatogram for any of the columns, despite the fact that the detection of early and late eluted compounds in RP and HILIC mode, respectively, should potentially be more influenced by coelution of salts and polar endogenous compounds present in the sample [41]. In fact, considering individual compounds, a great variability was obtained for the intensity of the matrix effects observed in

the different columns, which along with the variability of urine composition, suggests the difficulty in predicting matrix effects and the need for a more exhaustive sample treatment, particularly for doping agents with low DL or MRPL (Table ESI1).

Figure ESI8 shows, as an example, the extracted ion chromatograms for the selected doping agents using the PFP column from standards in methanol (in red), 4-fold diluted urine spiked with 25 ng mL^{-1} each (in blue), and unfortified urine (in green).

3.6. Comparison of the general performance of the different columns and eluents investigated for sport drug testing

The most representative results related to retention factors, asymmetry factors and relative matrix effects for the selected anti-doping agents in the different columns and eluents were compared. For this purpose, each column-mobile phase binomial was qualitatively ranked in five groups featuring different performance for each of the parameters considered, being the ranking from the best to the worst measured values as follows: green > yellow > blue > grey > red (see Table 3).

As expected, no column-mobile phase binomial gave the best performance for all the considered chromatographic parameters, given the great variety of structurally unrelated compounds involved in ITPs. However, interesting conclusions, having practical implications, can be drawn. Thus, on the whole, the best column-mobile phase binomial for ITPs was PFP using water-methanol (at pH 2.7 fixed with formic acid) as eluent. The variety of interactions provided by PFP for substance partition (Table 1) provided adequate retention for analytes in a wide polarity range. Particularly interesting are the π- π and hydrogen bonding interactions available in PFP, given the frequent presence of aromatic rings and hydrogen bond donors and acceptors in anti-doping agents (Table ESI1). Thus, only one (i.e. ecgonine methyl ester) out of the 93 compounds investigated (log P in the range -2.4 to 9.2) eluted at k below 1. The use of methanol instead of ACN as organic

modifier is crucial for the establishment of π-π interactions since ACN suppresses them [28]. The addition of ammonium acetate to the mobile phase may slightly improve peak shapes, but the rest of parameters compare unfavourably with methanolic aqueous phases, at least for the anti-doping compounds selected in this study.

Concerning the rest of columns working in RP mode, it is worth noting the behaviour of HILIC-RP. Excellent general good peak shapes were obtained as compared with the rest of columns. Also anti-doping agent detection was less affected by matrix interferences. However, the number of compounds eluting too fast was too high. Given that only ACN was used as organic modifier, with the aim of establishing HILIC conditions at the end of the chromatogram, the use of methanol as modifier in the presence of ammonium acetate could increase k values for these compounds. Further studies are necessary to evaluate the potential of this stationary phase in ITPs.

Regarding results from HILIC (Table 3), it is clear its inefficiency for ITPs involving a high number of structurally unrelated anti-doping agents encompassing a wide polarity range. However, HILIC provides today the most efficient separation for highly polar non-aromatic anti-doping agents, which are not affordable with PFP.

Table 3. Qualitative comparison of different chromatographic parameters obtainedfor the anti-doping agents selected in the stationary and mobile phases investigated. Parameter values have been ranked in five qualitative categories from best to worst:green > yellow > blue > grey > red.

Stationary phase	Mobile phase	Run times (k)	Number of compounds 1<k<10	Number of compounds k<1	Number of coelutions	Number of compounds A_s= 0.8-1.5	Number of compounds ME_{rel} = ±20%
PFP	ACN-water						
	ACN-water(NH_4 Ac)						
	MeOH-water						
	MeOH-water(NH_4 Ac)						
PH	ACN-water						
	ACN-water(NH_4 Ac)						
	MeOH-water						
	MeOH-water(NH_4 Ac)						
C18	ACN-water						
	ACN-water(NH_4 Ac)						
	MeOH-water						
	MeOH-water(NH_4 Ac)						
C8	ACN-water						
	ACN-water(NH_4 Ac)						
	MeOH-water						
	MeOH-water(NH_4 Ac)						
HILIC-RP	ACN-water						

	ACN-water(NH_4Ac)						
HILIC	**ACN-water**						
	ACN-water(NH_4Ac)						

On the whole, the alkyl phase C18 showed the worst performance and although C8 and PH were best, their performance did not surpass that of PFP. So, according to the results here obtained, PFP combined with methanol-water (formic acid 1%) constitutes an excellent alternative to C18 in ITPs. Thus, PFP provides faster chromatographic runs (around 40% quicker, Fig. 1A), higher number of compounds eluting in the optimal k interval (58 versus 33, Table ESI5), and much better selectivity (i.e. the percentage of compounds showing no matrix effects was 60% for PFP versus 43% for C18, Fig. 6). On the other hand, the performance of PFP was similar or slightly superior to C18 regarding the rest of parameters investigated; compounds eluting too fast (1-2, Fig. 1B), coelutions (around 20%, Fig. 1D) and asymmetry factors (53 and 46% of compounds with As in the interval 0.8-1.5 for PFP and C18, respectively, Fig. 5B).

4. Conclusions

Although no stationary/mobile phase combination scored the highest for all the evaluated chromatographic and matrix effects parameters, the use of RP and mixed-mode columns with additional retention mechanisms other than dispersion are more effective in improving the multi-target separation of anti-doping substances, thus establishing alternatives to the standard octadecylsilane (C18) phase. The π-π, dipole-dipole and H-bonding interactions provided by the RP pentafluorophenylpropyl (PFP) phase are particularly interesting in ITPs given the huge number of anti-doping substances having aromatic rings and hydrogen bonds. The use of methanol instead of ACN was advantageous in this column by enhancing these interactions for retention with the addition of formic acid as the

only additive. HILIC provided efficient separation for highly polar non-aromatic compounds and therefore an orthogonal separation to PFP. Good results were obtained with the mixed-mode alkyl diol phase (HILIC-RP), so that dipole and H-bonding interactions significantly improved peak shape and reduced matrix effects with respect to the standard C18, however more experimental effort is required regarding this column in order to know its potential in ITPs.

CRediT authorship contribution statement

Soledad González-Rubio: Investigation, Writing-Original Draft, Methodology, Conceptualization, Formal analysis. Ana Ballesteros-Gómez: Conceptualization, Writing-review & editing, Supervision. Daniel Carreras: Supervision of research, revision of the manuscript. Gloria Muñoz: Design of methodology, revision of the manuscript; Soledad Rubio: Conceptualization, Writing-review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPORTING INFORMATION

A comprehensive study on the performance of different retention mechanisms in sport drug testing by liquid chromatography tandem mass spectrometry

Soledad González-Rubio[1], Ana Ballesteros-Gómez[*1], Daniel Carreras[2], Gloria Muñoz[2], Soledad Rubio[1]

[1] Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, Marie Curie Building (Annex), Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

[2]Anti-doping control laboratory. Spanish Agency of Health Protection in Sport (AEPSAD)

[*]Corresponding author: ana.ballesteros@uco.es

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Figure ESI2. Plots of log P versus k obtained for the 93 anti-doping agents selected in four stationary phases in a mobile phase made up of methanol-water and formic acid (0.1%).

Figure ESI3. Plots of log P versus k obtained for the 93 anti-doping agents selected in four stationary phases in a mobile phase made up of methanol-water(NH_4Ac) and formic acid (0.1%).

Figure ESI4. Plots of log P versus retention times obtained for 146 endogenous metabolites and banned substances in (A) PFP using ACN-water (0.1% formic acid) as eluent, and (B) HILIC, using ACN-water(NH4Ac 20 mM) as eluent. Data extracted from reference 30 in the manuscript.

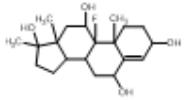
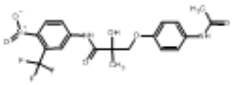
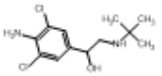
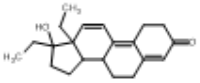
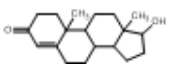
Figure ESI5. Plots showing the comparison of k values obtained in four stationary phases (PFP, PH, C18 and C8) using eluents made up of (A1-A6) ACN-water and (B1-B6) methanol-water, both in formic acid 0.1%, for 93 anti-doping agents.

Figure ESI6. Plots showing the comparison of k values obtained for acidic anti-doping agents in the following stationary and mobile phases: (A) PFP and PH in ACN-water; (B) PFP and PH in methanol-water; (C) C18 and C8 in ACN-water(NH_4Ac); (D) C18 and C8 in methanol-water(NH4Ac); (E) PFP and HILIC-RP in ACN-water and (F) PFP and HILIC-RP in ACN-water(NH4Ac). All mobile phases contained formic acid 0.1%.

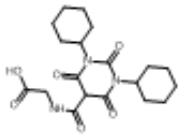
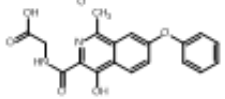
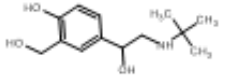
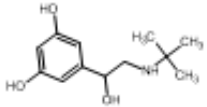
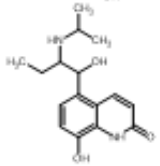
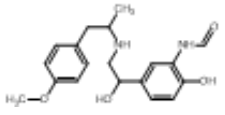
Figure ESI7. Plots showing the comparison of k values obtained in the six stationary phases investigated using eluents made up of ACN-water and ACN-water (NH_4Ac), both in formic acid 0.1%, for the 93 anti-doping agents selected.

Figure ESI8. Extracted ion chromatograms with the PFP column for the 93 anti-doping agents selected at a concentration of 25 ng mL^{-1} each, using as eluent methanol-water, both in formic acid 0.1%. The chromatographic peaks correspond to standards in methanol (red), 4-fold diluted fortified urine (blue), and 4-fold diluted urine (green).

Table ESI1. Chemical structure and molecular formula for the selected WADA´s prohibited substances or metabolites, ranked by WADA categories (S1-S9, P1), as well as different parameters of interest for their retention behaviour and WADA approved decision limits (DL) and minimum required performance level (MRPL).

Selected WADA´s prohibited substances or their metabolites (n=93)	Chemical structure	Molecular formula	[a]Log P	[a,b]pKa (acid)	[a,b]pKa (basic)	[a]H-bond donors	[a]H-bond acceptors	[a]Number of aromatic rings	[c]DL or MRPL (ng mL^{-1})
Anabolic agents (S1)									
9α-fluoro-17α-methyl-4-androsten-3α,6β,11β,17β-tetrol (Metabolite of Fluoxymesterone)		$C_{20}H_{31}FO_4$	1.4	n/a	-	4	5	0	5
Andarine		$C_{19}H_{18}F_3N_3O_6$	2.2	11,2	-4	3	9	2	2
Clenbuterol		$C_{12}H_{18}Cl_2N_2O$	2.2	14,1	9,6	3	3	1	0.2
Tetrahydrogestrinone		$C_{21}H_{28}O_2$	3.0	18,9		1	2	0	5
Testosterone		$C_{19}H_{28}O_2$	3.3	18,5		1	2	0	Non applicable

19-Norandrosterone (Metabolite of Nandrolone)		$C_{18}H_{28}O_2$	3.3	18.3	1	2	0		2
Boldenone		$C_{19}H_{26}O_2$	3.5	18.9	1	2	0		5
4-Androsten-17α-ethynyl-17β-ol-3-one (Metabolite of Danazol)		$C_{21}H_{28}O_2$	3.5	n/a	-	1	2	0	5
Oxandrolone		$C_{19}H_{30}O_3$	3.7	15.1	-	1	3	0	5
1-methylene-5α-androstan-3α-ol-17-one (Metabolite of Metenolone)		$C_{20}H_{30}O_2$	3.8	n/a	-	1	2	0	5
3-OH-stanozolol (Metabolite of Stanozolol)		$C_{21}H_{32}N_2O_2$	4.0	11.5	2,3	3	3	0	2
1α-methyl-5α-androstan-3α-ol-17-one (Metabolite of Mesterolone)		$C_{20}H_{32}O_2$	4.1	n/a	-	1	2	0	5
17α-methyl-5α-androstane-3α,17β-diol (Metabolite of Methyltestosterone)		$C_{20}H_{34}O_2$	4.3	n/a	-	2	2	0	2

Hypoxia-inducible factor (HIF) activating agents (S2)									
Daprodustat		$C_{19}H_{27}N_3O_6$	2.5	0,35	-9,1	2	6	0	2
Roxadustat		$C_{19}H_{16}N_2O_5$	3.4	2,8	3,8	3	6	3	2
Beta-2 Agonists (S3)									
Salbutamol		$C_{13}H_{21}NO_3$	0.3	10,3	9,4	4	4	1	ᶜ1200
Terbutaline		$C_{12}H_{19}NO_3$	0.9	8,9	9,7	4	4	1	20
Procaterol		$C_{16}H_{22}N_2O_3$	1.5	8,5	9,9	4	4	1	20
Formoterol		$C_{19}H_{24}N_2O_4$	1.8	8,6	9,8	4	5	2	ᶜ50

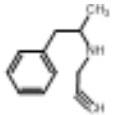
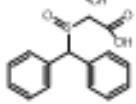
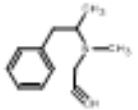
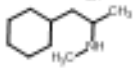
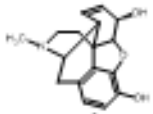
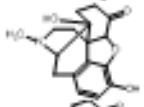
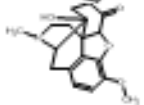
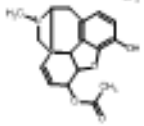
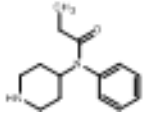
Higenamine		$C_{16}H_{17}NO_3$	2.2	8,7	9,6	4	4	2	20
Tulobuterol		$C_{12}H_{18}ClNO$	2.3	13,9	9,6	2	2	1	20
Vilanterol		$C_{24}H_{33}Cl_2NO_5$	3.8	10,1	9,4	4	6	2	20
Hormone and metabolic modulators (S4)									
Aminoglutethimide		$C_{13}H_{16}N_2O_2$	1.2	11,7	4,3	2	3	1	20
Anastrozole		$C_{17}H_{19}N_5$	2.1	-	2	0	4	2	20
Bis-(4-cyanophenyl) metanol (Metabolite of Letrozole)		$C_{15}H_{10}N_2O$	2.2	12,8	-	1	3	2	20
Exemestane		$C_{20}H_{24}O_2$	3.1	20		0	2	1	20

4-OH-cyclofenil (Metabolite of Cyclofenil)		$C_{19}H_{20}O_3$	3.8	n/a	-	3	3	2	20
SR9009		$C_{20}H_{24}ClN_3O_4S$	4.4	-	6,1	0	6	2	20
GW501516 Sulfone (Metabolite of GW501516)		$C_{21}H_{18}F_3NO_5S_2$	4.6	3	-	1	10	3	20
GW501516 Sulfoxide (Metabolite of GW501516)		$C_{21}H_{18}F_3NO_4S_2$	4.6	3	-	1	10	3	20
~~Raloxifene~~		$C_{28}H_{27}NO_4S$	6.1	8,9	7,9	2	6	3	20
4-OH-toremifene (Metabolite of Toremifene)		$C_{26}H_{28}ClNO_2$	6.8	n/a	n/a	1	3	3	20

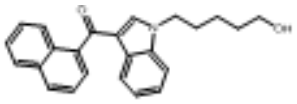
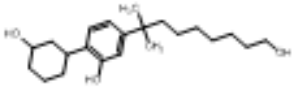
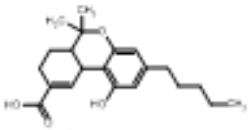
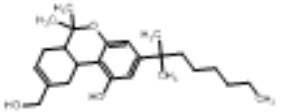
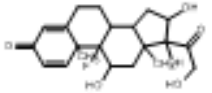
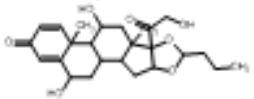
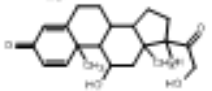
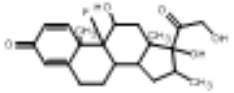
Toremifene		$C_{26}H_{28}ClNO$	7.2	-	8,8	0	2	3	20
17-ketone fulvestrant (Metzbolite of Fulvestrant)		$C_{32}H_{45}F_9O_3S$	7.5	n/a	-	-	-	1	20
Fulvestrant		$C_{32}H_{47}F_9O_3S$	9.2	10,3		2	9	1	20
Diuretic and masking agents (S5)									
Acetazolamide		$C_4H_6N_4O_3S_2$	-0.3	-	7,2	2	7	1	200
Chlorothiazide		$C_7H_6ClN_3O_4S_2$	-0.2	-	6,9	2	6	1	200
Chlortalidone		$C_{14}H_{11}ClN_2O_4S$	0.9	-	9,4	3	5	2	200
Triamterene		$C_{12}H_{11}N_7$	1.0	-	6,2	3	7	2	200
Bendroflumethiazide		$C_{15}H_{14}F_3N_3O_4S_2$	1.2	-	8,5	3	10	1	200
Quinethazone		$C_{10}H_{13}ClN_2O_3S$	1.2	-	9,3	3	5	1	200

Furosemide		$C_{12}H_{11}ClN_2O_5S$	2.0	3,5	-1,5	3	7	2	200
Bumetamide		$C_{17}H_{20}N_2O_5S$	2.8	3,6	7,7	3	7	2	200
Indapamide		$C_{16}H_{16}ClN_3O_3S$	2.9	-	8,8	2	5	2	200
Etacrynicacid		$C_{13}H_{12}Cl_2O_4$	3.8	3,5	-5	1	4	1	200
Stimulants (S6)									
Ritalinic acid (Metabolite of Methylphenidate)		$C_{13}H_{17}NO_2$	-2.4	3,7	10,1	2	3	1	100
Benzoylecgonine (Metabolite of Cocaine)		$C_{16}H_{19}NO_4$	-0.3	3,2	9,5	1	5	1	100
Carphedon		$C_{13}H_{14}N_2O_2$	0.1	-	-2	1	2	1	100
Etilefrine		$C_{10}H_{15}NO_2$	0.1	9,1	9,7	3	3	1	100
Pentetrazol		$C_6H_{10}N_4$	0.1	-	-0,5	0	3	1	100

Methylphenidate		$C_{14}H_{19}NO_2$	0.2	-	9,1	1	3	1	100
Nikethamide		$C_{10}H_{14}N_2O$	0.3	-	3,6	0	2	1	100
Ecgonineméthylester (Metabolite de Cocaine)		$C_{10}H_{17}NO_3$	0.6	14,6	9	1	4	0	100
Cathine		$C_9H_{13}NO$	0.8	13,9	9,4	2	2	1	[c]6000
Cathinone		$C_9H_{11}NO$	1.1	18,6	7,5	1	2	1	100
Methylenbedrine		$C_{11}H_{17}NO$	1.7	13,9	9,3	1	2	1	[c]11000
Methylhexaneamine		$C_7H_{17}N$	1.9	-	10,6	1	1	0	100
Phenylpropanolamine		$C_9H_{13}NO$	1.9	-	7,3	0	2	1	100
Strychnine		$C_{21}H_{22}N_2O_2$	1.9	-	8,3	0	3	1	100
Cocaine		$C_{17}H_{21}NO_4$	2.3	-	8,6	0	5	1	100

N-Desmethylselegiline (Selegiline)		$C_{12}H_{15}N$	2.3	-	n/a	1	1	1	100
Diphenylmethyl (sulfinyl)-acetic acid (Metabolite of Modafinil)		$C_{15}H_{14}O_3S$	2.7	4,1	-	1	4	2	100
Selegiline		$C_{13}H_{17}N$	2.8	-	8,7	0	1	1	100
Propylhexedrine		$C_{10}H_{21}N$	3.5	-	10,6	1	1	0	100
Narcotics (S7)									
Morphine		$C_{17}H_{19}NO_3$	0.8	10,2	8,2	2	4	1	[c]1300
Oxymorphone		$C_{17}H_{19}NO_4$	0.8	10,1	8,2	2	5	1	50
Oxycodone		$C_{18}H_{21}NO_4$	1.2	13,6	8,7	1	5	1	50
6-Acetylmorphine (Metabolite of Diamorphine or heroin)		$C_{19}H_{21}NO_4$	1.3	10,2	9	1	5	0	50
Norfentanyl		$C_{14}H_{20}N_2O$	1.6	-	10	1	2	1	2

Hydromorphone		$C_{17}H_{19}NO_3$	1.8	10,1	8,6	1	4	1	50
Pethidine		$C_{15}H_{21}NO_2$	2.5	-	8,7	0	3	1	50
Pentazocine		$C_{19}H_{27}NO$	3.3	7,6	12,4	1	2	0	50
Norbuprenorphine (Metabolite of Buprenorphine)		$C_{25}H_{35}NO_4$	3.8	9,8	10,5	3	5	0	50
Methadone		$C_{21}H_{27}NO$	3.9	-	9,2	0	2	2	50
Fentanyl		$C_{22}H_{28}N_2O$	4.0	-	9	0	2	2	2
Buprenorphine		$C_{29}H_{41}NO_4$	5.0	7,5	12,5	2	5	0	5
Cannabinoids (S8)									
JWH-018 5-pentanoic acid metabolite (Metabolite of JWH-018)		$C_{26}H_{23}NO_3$	4.7	n/a	n/a	1	3	2	1

JWH-018 N-(5-hydroxy-pentyl) metabolite (Metabolite of JWH-018)		$C_{24}H_{23}NO_2$	4.9	n/a	n/a	1	2	2	1
CP 47, 497-C8-hydroxy, C8-homolog (Metabolite of CP 47, 497-C8-homolog)		$C_{22}H_{36}O_3$	5.5	-	-	3	3	1	1
11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid		$C_{21}H_{28}O_4$	6.3	4,2	-	2	4	1	°180
HU-210		$C_{25}H_{38}O_3$	6.4	9,7	-	2	3	1	1
Glucocorticoids (S9)									
Triamcinolone		$C_{21}H_{27}FO_6$	1.2	13,4	-	4	7	1	30
6-β-OH-budesonide (Metabolite of Budesonide)		$C_{25}H_{34}O_7$	1.3	13,7	-	3	7	1	30
Prednisolone		$C_{21}H_{28}O_5$	1.6	12,6	-	3	5	1	30
Betamethasone		$C_{22}H_{29}FO_5$	1.8	13,4	-	3	6	1	30

20(R)-Hydroxyprednisolone (Metabolite of Prednisolone)		$C_{21}H_{32}O_5$	1.9	11,8	-	4	5	0	30
Budesonide		$C_{25}H_{34}O_6$	2.5	13,7	-	2	6	1	30
Triamcinoloneacetonide		$C_{24}H_{31}FO_6$	2.5	13,4	-	2	7	1	30
Beta-blockers (P1)									
Atenolol		$C_{14}H_{22}N_2O_3$	0.2	14,1	9,7	3	4	1	100
Nadolol		$C_{17}H_{27}NO_4$	0.7	13,6	9,8	4	5	1	100
Acebutolol		$C_{18}H_{28}N_2O_4$	1.7	13,9	9,6	3	5	1	100
Pindolol		$C_{14}H_{20}N_2O_2$	1.8	14,1	9,7	3	3	1	100
Bisoprolol		$C_{18}H_{31}NO_4$	1.9	14,1	9,7	2	5	1	100

a: Values obtained from PubChem; b: Values obtained from DrugBank; c: DL values; n/a: non available values

Table ESI2. Suppliers and MS parameters for the selected prohibited substances.

Selected prohibited substances, metabolites or markers	Supplier	ESI Polarity	[a]Precursor ion	Fragment ion		Collision Energy, V (Quantifier; Qualifier)V)
				Quantifier	Qualifier	
Anabolic agents (S1)						
9α-fluoro-17α-methyl-4-androsten-3α,6β,11β,17β-tetrol	NMI	+	354.0	198.3	-	35
Andarine	TRC	-	440.0	150.0	289.0	40;32
Clenbuterol	Cerilliant	+	277.0	259.2	210.0	10;12
Tetrahydrogestrinone	NMI	+	313.0	241.0	199.0	30;35
Testosterone	LGC	+	289.0	109.0	97.0	27;20
19-Norandrosterone	NMI	+	277.3	259.2	241.2	15;15
Boldenone	LGC	+	287.0	121.0	91.0	32;44
4-Androsten-17α-ethynyl-17β-ol-3-one	Steraloids	+	313.2	109.1	97.1	21;21
Oxandrolone	Dr. Ehrenstorfer	+	307.0	93.0	-	33
1-methylene-5α-androstan-3α-ol-17-one	NMI	+	303.0	83.0	91.0	40;40
3-OH-stanozolol	NMI	+	345.0	97.0	107.0	44;43
1α-methyl-5α-androstan-3α-ol-17-one	NMI	+	305.0	81.7	269.4	30;10
17α-methyl-5α-androstane-3α-17β-diol	NMI	+	307.2	289.4	-	10
Hypoxia-inducible factor (HIF) activating agents (S2)						
Daprodustat	TRC	-	392.0	291.0	223.0	30;60
Roxadustat	TRC	+	353.5	278.3	222.2	20;40

Beta-2 Agonists (S3)							
Salbutamol	LGC	+	240.3	148.0	121.0	25;28	
Terbutaline	LGC	+	226.3	152.0	125.0	21;23	
Procaterol	Sigma-Aldrich	+	291.1	273.2	231.0	19;24	
Formoterol	NMI	+	345.2	149.1	121.1	30;33	
Higenamine	Chromadex	+	272.0	107.1	142.9	31;25	
Tulobuterol	TRC	+	227.9	153.9	117.9	23;27	
Vilanterol	TRC	+	486.1	468.0	450.0	23;23	
Hormone and metabolic modulators (S4)							
Aminoglutethimide	LGC	+	233.0	188.0	146.0	14;20	
Anastrozole	TRC	+	294.1	225.1	210.0	33;33	
Bis-(4-cyanophenyl) methanol	NMI	+	235.2	76.6	90.6	53;50	
Exemestane	TRC	+	297.1	121.1	105.0	25;32	
4-OH-cyclofenil	NMI	+	297.4	279.3	133.1	15;20	
SR9009	CaymanChemicals	+	438.1	125.0	89.2	29;70	
GW501516 Sulfone	TRC	+	485.9	257.0	272.0	40;30	
GW501516 Sulfoxide	TRC	+	470.0	257.0	272.0	20;15	
Raloxifene	LGC	+	474.2	84.0	112.0	40;29	
4-OH-toremifene	TRC	+	422.0	72.0	386.0	35;30	
Toremifene	TRC	+	406.0	72.0	70.0	23;36	
17-ketone fulvestrant	TRC	+	605.4	587.4	491.4	25;30	
Fulvestrant	LGC	+	607.1	589.2	467.5	25;30	
Diuretic and masking agents (S5)							

Acetazolamide	Pharmacopeia USP	+	222.9	176.9	164.0	10;21
Clorothiazide	DrEherestofer	-	294.0	178.9	213.9	66;50
Chlortalidone	Pharrmacopeia USP	-	337.0	190.0	146.0	20;22
Triamterene	LGC	+	254.0	237.0	195.0	35;45
Bendroflumethiazide	LGC	-	419.9	289.0	239.0	34;34
Quinethazone	LGC	-	287.9	242.0	158.9	10;40
Furosemide	LGC	+	331.6	313.6	80.6	15;30
Bumetanide	Pharmacopeia USP	-	362.9	80.0	207.0	44;23
Indapamide	LGC	-	364.1	188.8	215.8	38;24
Etacrynicacid	Pharmacopeia USP	-	301.0	243.0	192.0	20;33
Stimulants (S6)						
Ritalinicacid	LGC	+	201.1	84.2	56.2	30;42
Benzoylecgonine	LGC	+	290.2	168.2	149.9	29;40
Carphedon	NMI	+	219.1	174.1	145.1	15;15
Etilefrine	Pharmacopeia	+	180.9	123.2	-	9
Pentetrazol	LGC	+	138.8	96.0	69.0	13;20
Methylphenidate	Cerilliant	+	234.3	84.3	174.3	30;20
Nikethamide	LGC	+	179.0	108.0	80.0	18;19
Ecgoninemethylester	LGC	+	200.1	182.0	150.0	20;35
Cathine	Cerilliant	+	152.1	117.1	133.9	23;10
Cathinone	Cerilliant	+	149.9	116.9	131.8	36;15
Methylephedrine	LGC	+	180.1	138.8	147.1	10;10
Methylhexaneamine	Sigma	+	115.8	43.0	99.0	40;30

Phendimetrazine	Cerilliant	+	192.2	146.2	115.2	37;37
Strychnine	Sigma	+	335.4	184.0	156.0	63;44
Cocaine	LGC	+	304.2	150.1	77.0	25;48
N-Desmethylselegiline	LGC	+	173.9	54.9	118.9	30;13
Diphenylmethyl (sulfinyl)-aceticacid	TRC	-	273.0	167.0	-	30
Selegiline	Cerilliant	+	188.0	91.0	65.0	21;45
Propylhexedrine	LGC	+	156.1	69.1	82.7	20;25
Narcotics (S7)						
Morphine	LGC	+	286.4	145.0	153.0	30;30
Oxymorphone	LGC	+	302.4	227.1	198.1	28;43
Oxycodone	LGC	+	316.0	256.0	241.0	24;28
6-Acetylmorphine	LGC	+	328.0	165.0	196.0	37;26
Norfentanyl	Cerilliant	+	233.0	84.0	55.0	17;35
Hydromorphone	Cerilliant	+	286.0	185.0	157.0	30;40
Pethidine	Cerilliant	+	247.8	219.7	173.7	30;35
Pentazocine	Cerilliant	+	286.0	218.0	175.5	19;25
Norbuprenorphine	Cerilliant	+	414.5	414.5	396.2	30;33
Methadone	Cerilliant	+	310.5	265.4	105.4	10;28
Fentanyl	LGC	+	337.0	105.0	188.0	36;22
Buprenorphine	Cerilliant	+	468.6	468.6	414.4	20;35
Cannabinoids (S8)						
JWH-018 5-pentanoic acidmetabolite	Cerilliant	+	372.0	155.0	143.9	33;40
JWH-018 N-(5-hydroxy-pentyl) metabolite	Cerilliant	+	358.2	127.2	-	53
CP 47, 497-C8-hydroxy, C8-homolog	CaymanChemicals	-	347.1	159.1	185.0	72;66

11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid	Cerilliant	+	355.3	58.1	-	73
HU-210	CaymanChemicals	-	385.2	338.8	301.3	10;20
Glucocorticoids (S9)						
Triamcinolone	LGC	-	439.0	345.0	321.0	27;30
6-β-OH-budesonide	TRC	+	447.3	339.2	-	17
Prednisolone	LGC	-	405.0	329.0	295.0	35;35
Betamethasone	Dr. Ehrenstorfer	+	393.0	355.0	337.0	20;20
20(R)-Hydroxyprednisolone	TRC	+	363.0	345.0	267.3	10;25
Budesonide	Sigma	+	431.3	147.0	173.0	32;32
Triamcinoloneacetonide	LGC	+	435.0	213.0	321.0	30;13
Beta-blockers (P1)						
Atenolol	LGC	+	267.0	190.0	145.0	25;25
Nadolol	LGC	+	310.2	254.2	236.2	25;29
Acebutolol	LGC	+	337.2	116.2	98.2	31;31
Pindolol	LGC	+	249.2	116.2	144.2	25;25
Bisoprolol	Sigma	+	326.2	116.2	144.8	27;35

[a][M+H]$^+$ and [M-H]$^-$

Table ESI3. Eluent conditions investigated for the separation of the selected doping agents onto columns working in the RP and HILIC modes.

Stationary phase	**PFP, PH, C18, C8, HILIC-RP**			**HILIC**		
[a]**Mobile phase composition**	A: water B: acetonitrile			A: water B: acetonitrile		
	[b]A: water B: methanol					
	A: water (2.5 mM ammonium acetate) B: acetonitrile			A: water (2.5 mM ammonium acetate) B: acetonitrile		
	[b]A: water (2.5 mM ammonium acetate) B: methanol					
Mobile phase gradient	**Total time (min)**	**A%**	**B%**	**Total time (min)**	**A%**	**B%**
	0.5	90	10	0.5	10	90
	25	20	80	25	50	50
	26	10	90	28	60	40
	38	10	90	30	60	40
	[c]5	90	10	[c]5	10	90

[a]A and B with 0.1% formic acid in all cases
[b]Mobile phase compositions tested for all columns working in RP mode, except for the HILIC-RP column
[c]Equilibration time

Table ESI4. One-way ANOVA for differences in mean retention factors of selected compounds (mean, 95%confidence interval) on different columns and mobiles phases. Different letters mean significant differences (Games-Howell approach, p=0.05).

Factor	Mean	Group									
C18 (MeOH-water NH_4Ac)	19,7	**A**									
C18 (ACN-water)	17,0	**A**	B								
C18 (MeOH-water)	15,1	**A**	B	C							
C18 (ACN-water NH_4Ac)	13,3		B	C	D	E					
PH (MeOH-water)	12,8		B	C	D						
C8 MeOH-water NH_4Ac)	12,0			C	D	E	F	G			
PFP (ACN-water)	11,9			C	D						
PH (ACN-water)	10,8				D	E	F	G	H		
PH (MeOH-water NH_4Ac)	10,6				D	E	F	G	H	**I**	
PFP (MeOH-water NH_4Ac)	10,2				D	E	F	G	H	**I**	
C8 (MeOH-water)	9,7				D	E	F	G	H	**I**	
PH (ACN-water NH_4Ac)	9,7					E		G	H	**I**	
PFP (MeOH-water)	9,3						F	G	H	**I**	
C8 (ACN-water NH_4Ac)	9,1								H	**I**	
C8 (ACN-water)	8,9								H	**I**	
PFP (ACN-water NH_4Ac)	8,4									**I**	
HILIC-RP (ACN-water)	5,9										J
HILIC-RP (ACN-water NH_4Ac)	5,8										J
HILIC (ACN-water)	4,0										J
HILIC (ACN-water NH_4Ac)	2,3										**K**

ESI5. Number of doping agents eluting in the interval of retention factors from 1 to 10 (n=93) investigated stationary and mobile phases.

	Stationary phase					
e phase	**PFP**	**PH**	**C18**	**C8**	**HILIC-RP**	**HILIC**
ater	26	41	40	31	59	47
-water	58	35	33	48		
ater	57	52	32	49	55	47
c)						
-water	40	45	16	34		
c)						

Figure ESI1. Plots of log P versus k obtained for the 93 anti-doping agents selected in the six stationary phases investigated in a mobile phase made up of ACN-water (NH4Ac) and formic acid (0.1%)

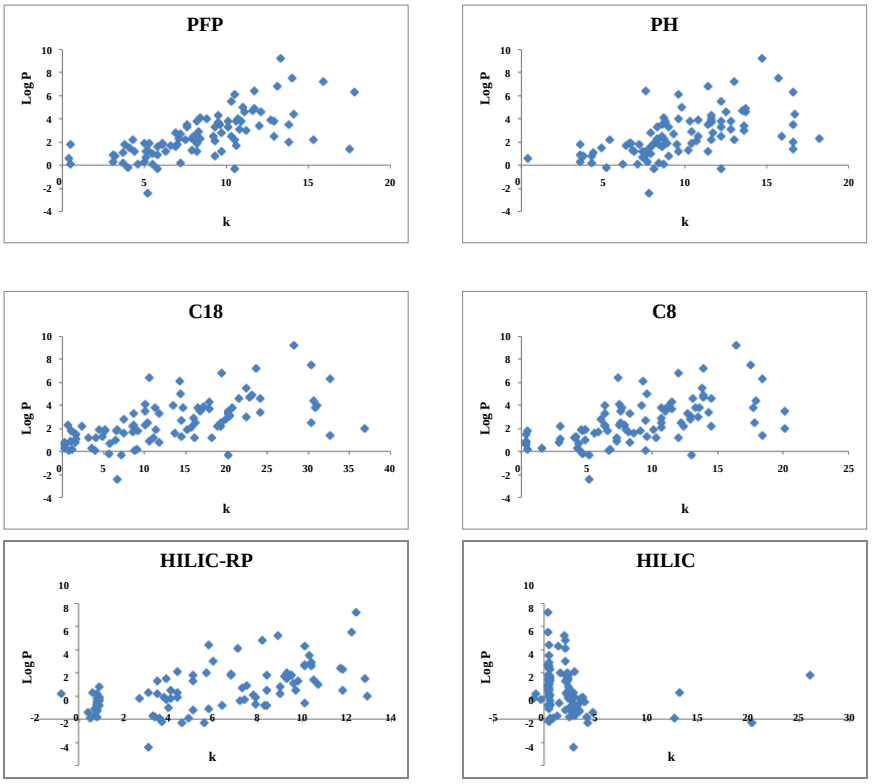


Figure ESI2. Plots of log P versus k obtained for the 93 anti-doping agents selected in four stationary phases in a mobile phase made up of methanol-water and formic acid (0.1%).

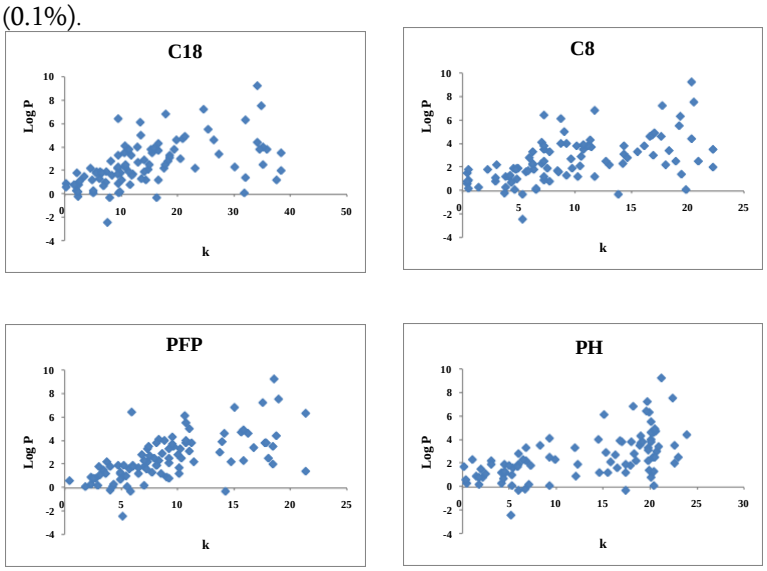


Figure ESI3. Plots of log P versus k obtained for the 93 anti-doping agents selected in four stationary phases in a mobile phase made up of methanol-water(NH4Ac) and formic acid (0.1%).

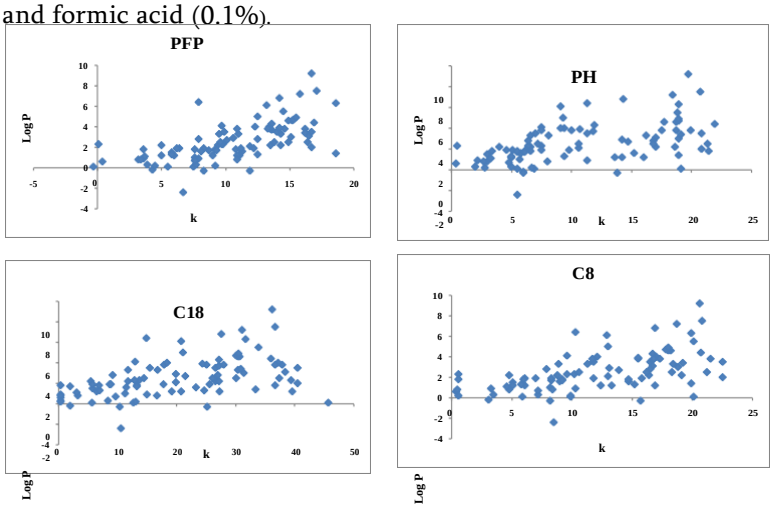


Figure ESI4. Plots of log P versus retention times obtained for 146 endogenous metabolites and banned substances in (A) PFP using ACN-water (0.1% formic acid) as eluent, and (B) HILIC, using ACN-water(NH4Ac 20 mM) as eluent. Data extracted from reference 30 in the manuscript.

A) **B)**

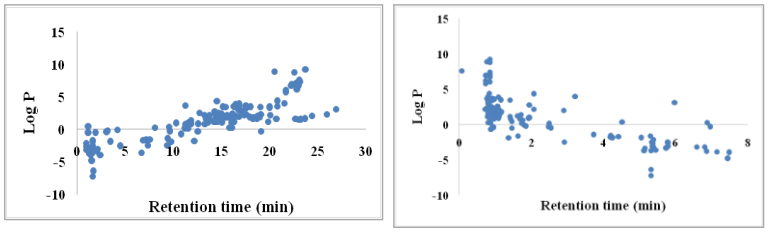
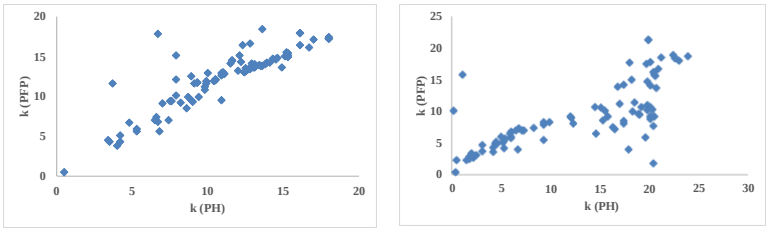
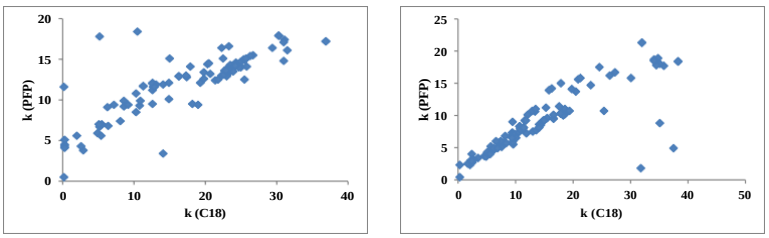


Figure ESI5. Plots showing the comparison of k values obtained in four stationary phases (PFP, PH, C18 and C8) using eluents made up of (A1-A6) ACN-water and (B1-B6) methanol-water, both in formic acid 0.1%, for 93 anti- doping agents.

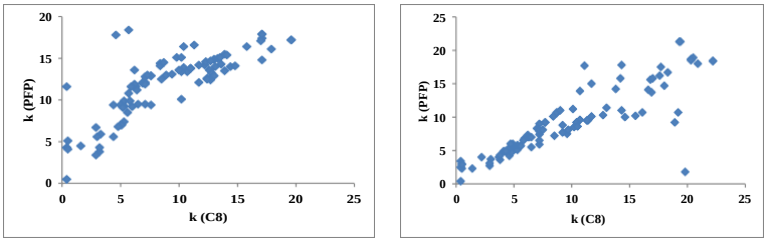
A1) **B1)**



A2) **B2)**

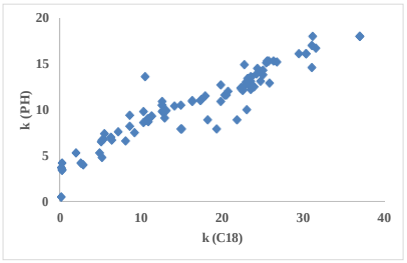


A3) **B3)**

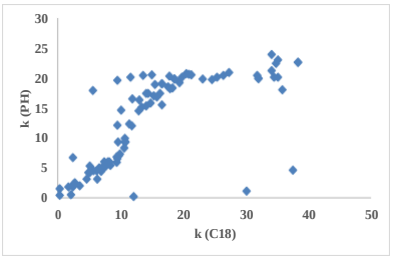


A4) **B4)**

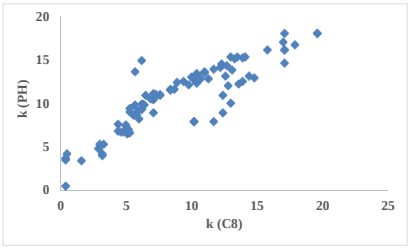
A4)



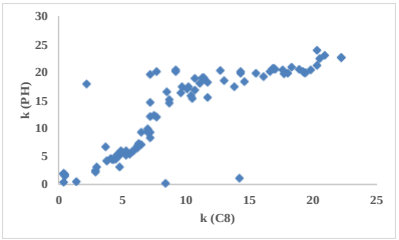
B4)



A5)



B5)



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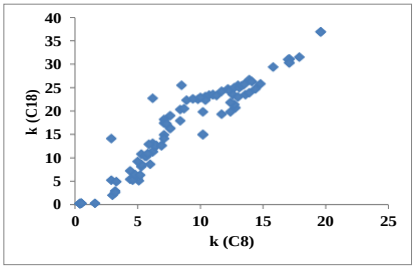


B6)

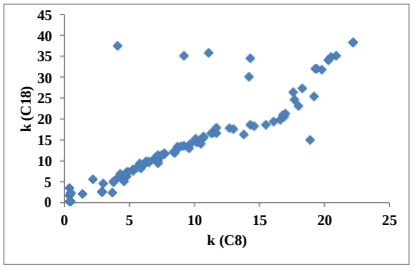


Figure ESI6. Plots showing the comparison of k values obtained for acidic anti-doping agentsin the following stationary and mobile phases: (A) PFP and PH in ACN-water; (B) PFP and PH in methanol-water; (C) C18 and C8 in ACN-water(NH4Ac); (D) C18 and C8 in methanol- water(NH4Ac); (E) PFP and HILIC-RP in ACN-water and (F) PFP and HILIC-RP in ACN- water(NH4Ac). All mobile phases contained formic acid 0.1%,

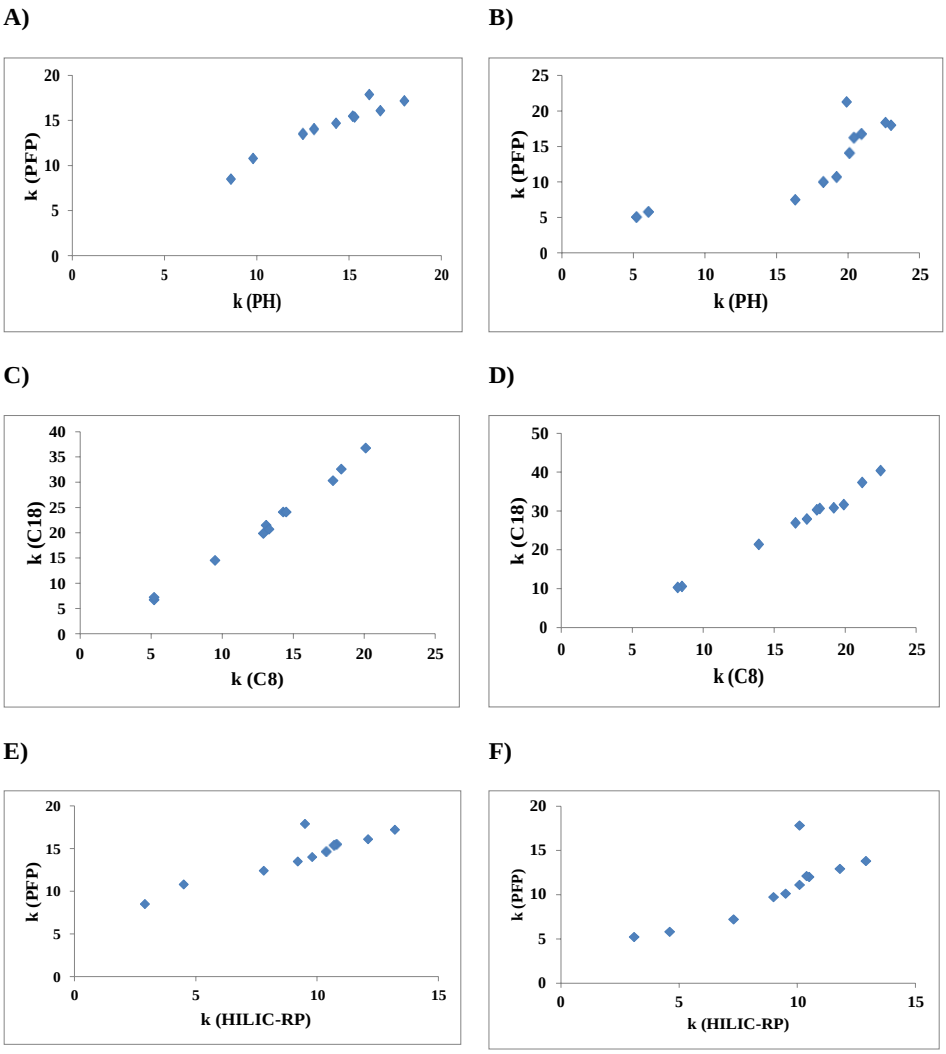


Figure ESI7. Plots showing the comparison of k values obtained in the six stationary phases investigated using eluents made up of ACN-water and ACN-water (NH4Ac), both in formic acid 0.1%, for the 93 anti-doping agents selected.

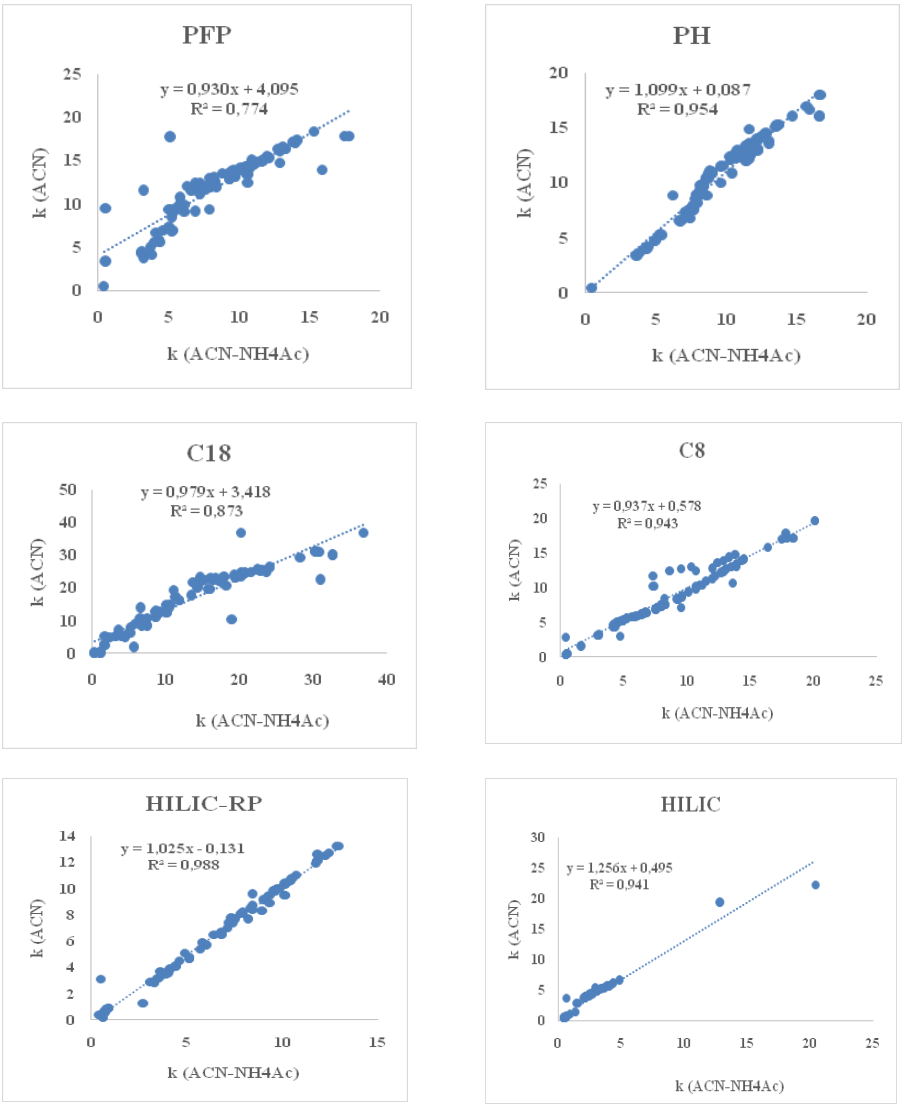
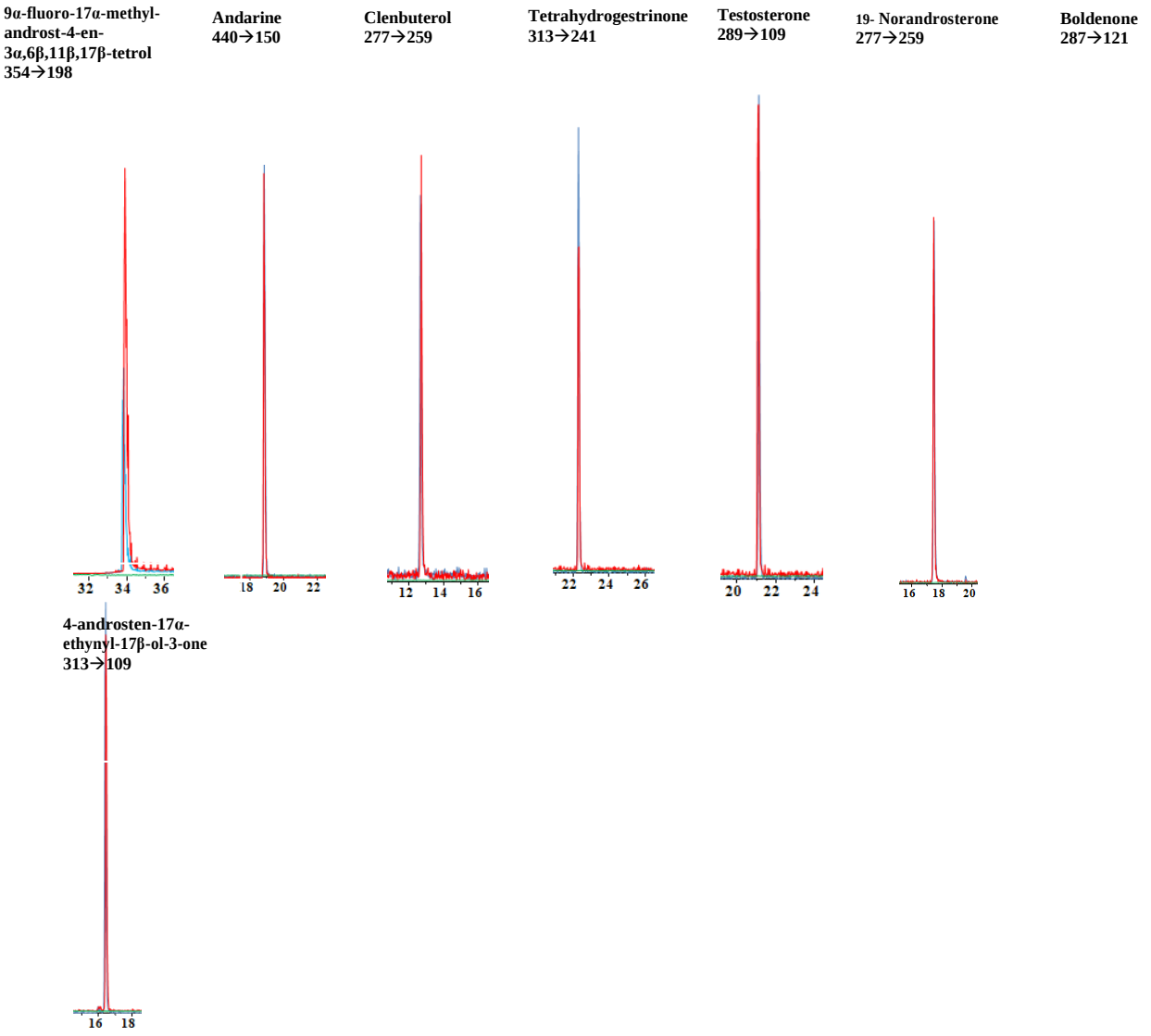
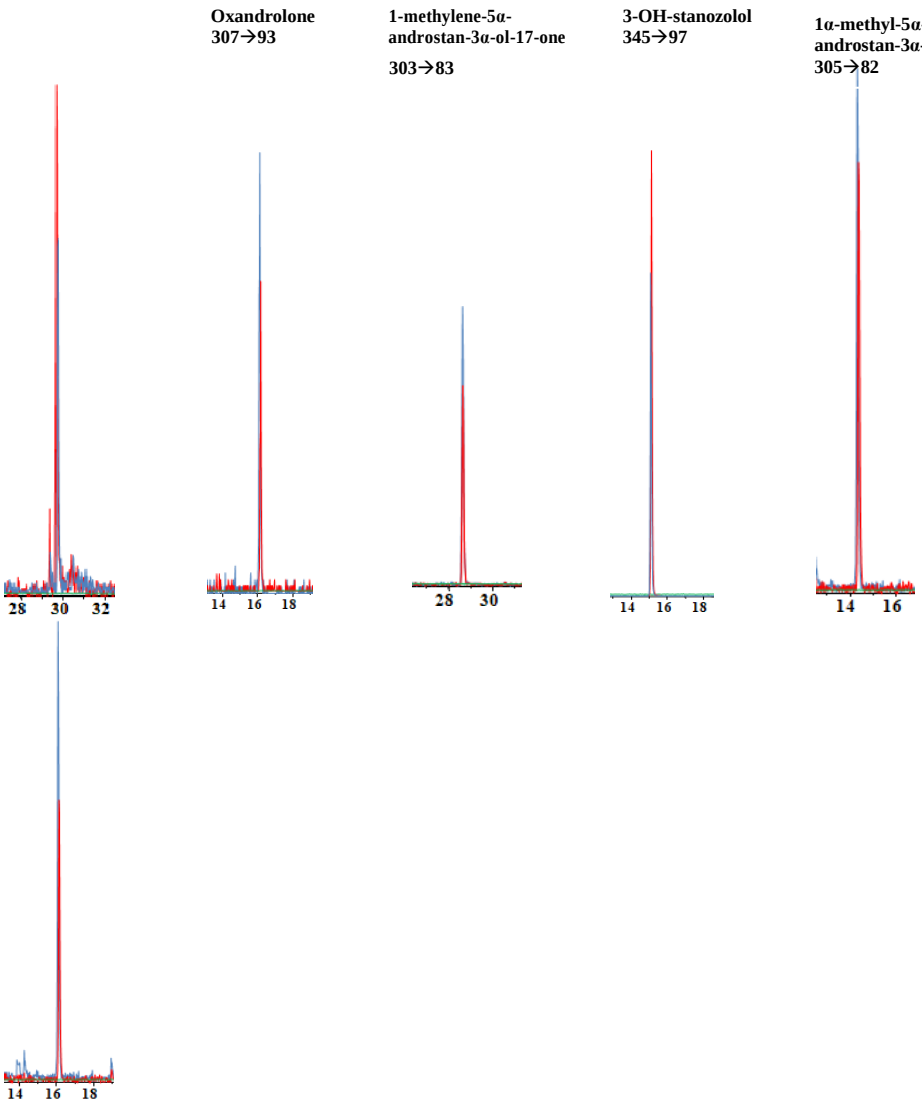


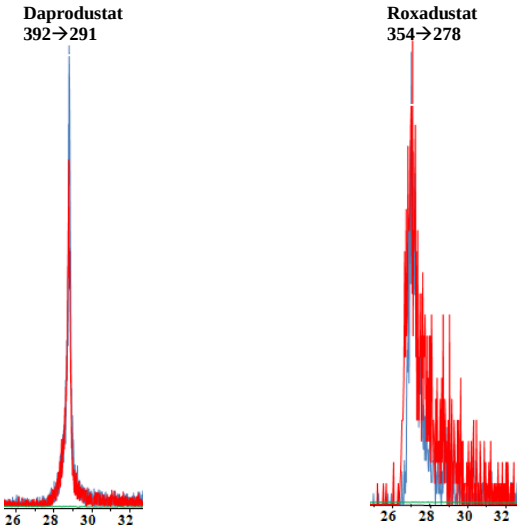
Figure ESI8. Extracted ion chromatograms obtained in the PFP column for the 93 anti-doping agents selected at a concentration of 25 ng mL^{-1} each, usingas eluent methanol-water, both in formic acid 0.1%. The chromatographic peaks correspond to standards in methanol (red), 4-fold diluted fortified urine (blue), and 4-fold diluted urine (green).

S1

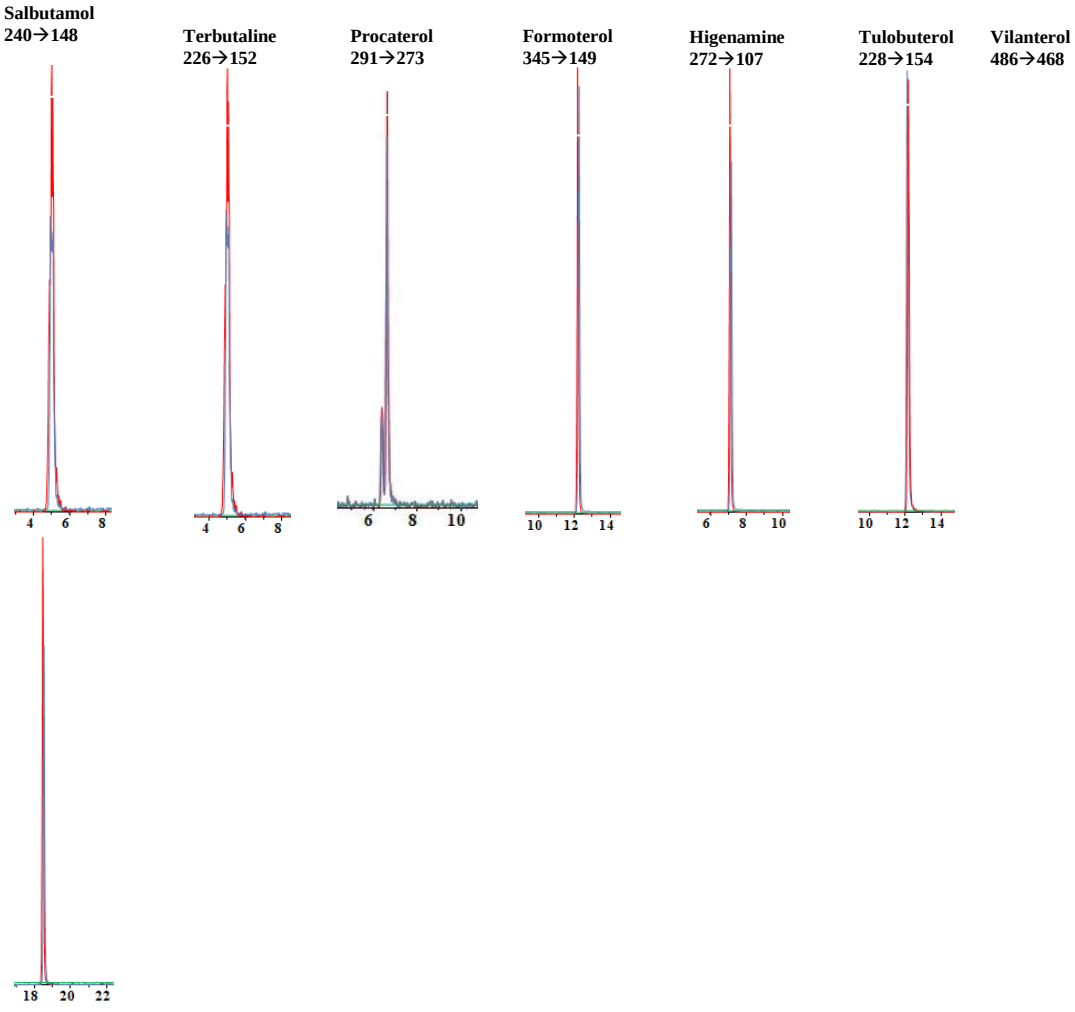


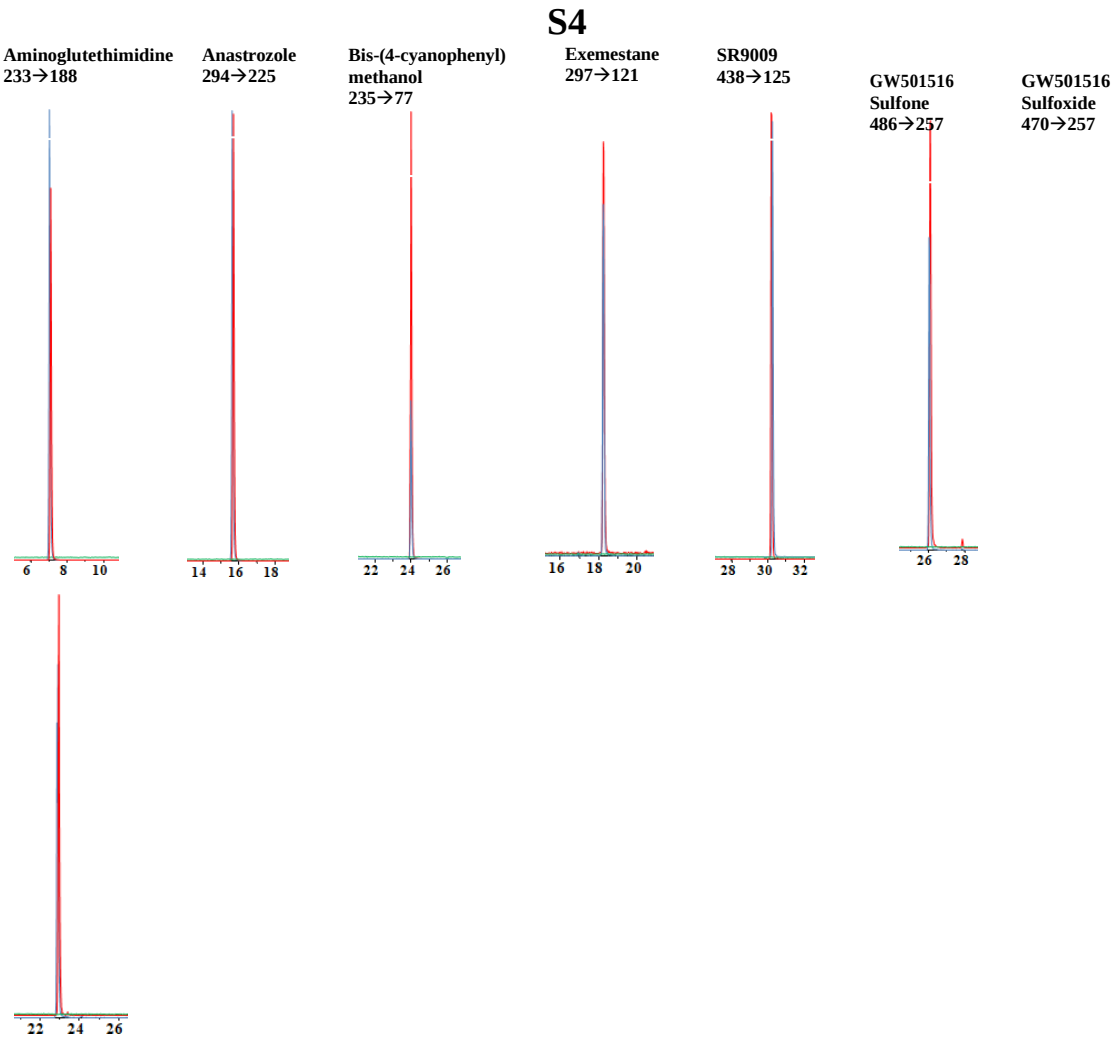

Oxandrolone
307→93
1-methylene-5α-androstan-3α-ol-17-one
303→83
3-OH-stanozolol
345→97
1α-methyl-5α-androstan-3α-ol-17-one
305→82
17α-methyl-5
androstane-3
307→289
28 30 32
14 16 18
28 30
14 16 18
14 16
14 16 18

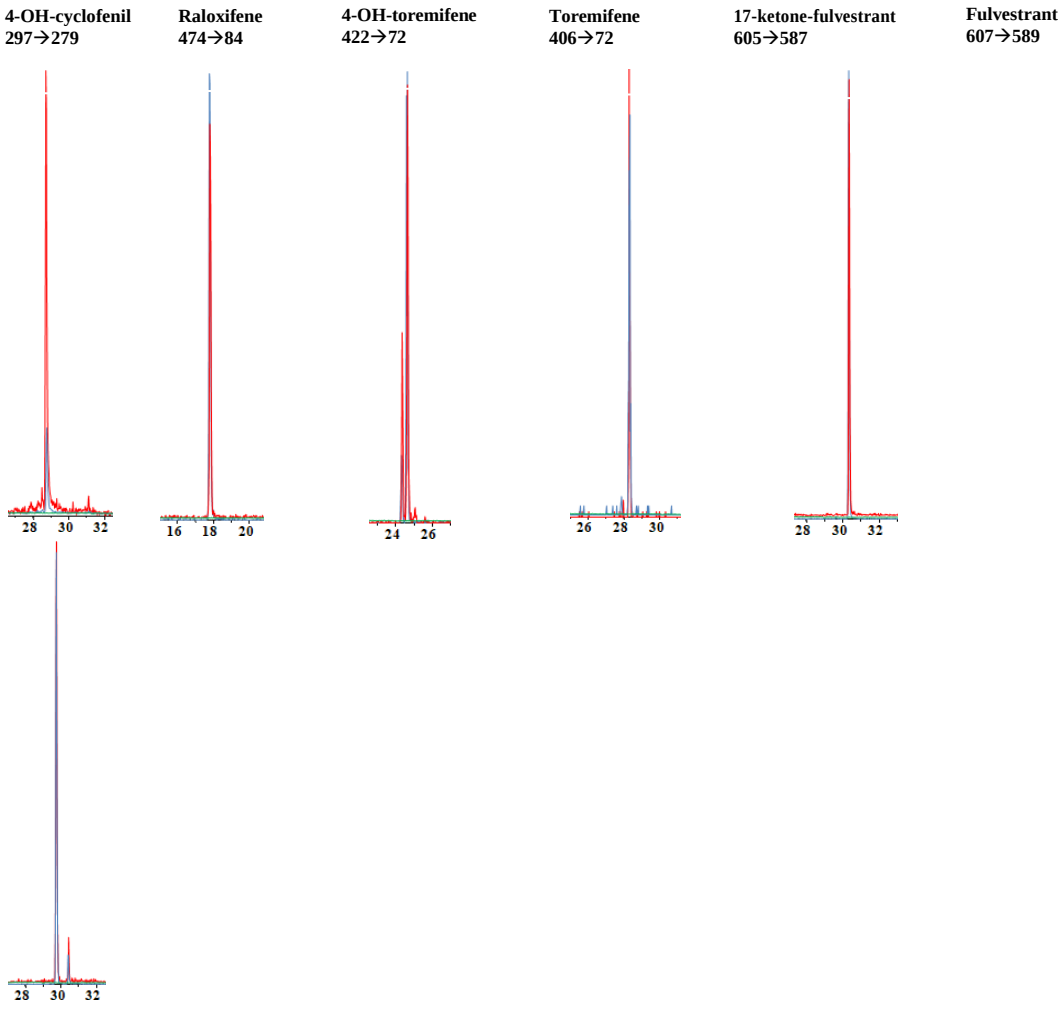
S2


Daprodustat
392→291
26 28 30 32
Roxadustat
354→278
26 28 30 32

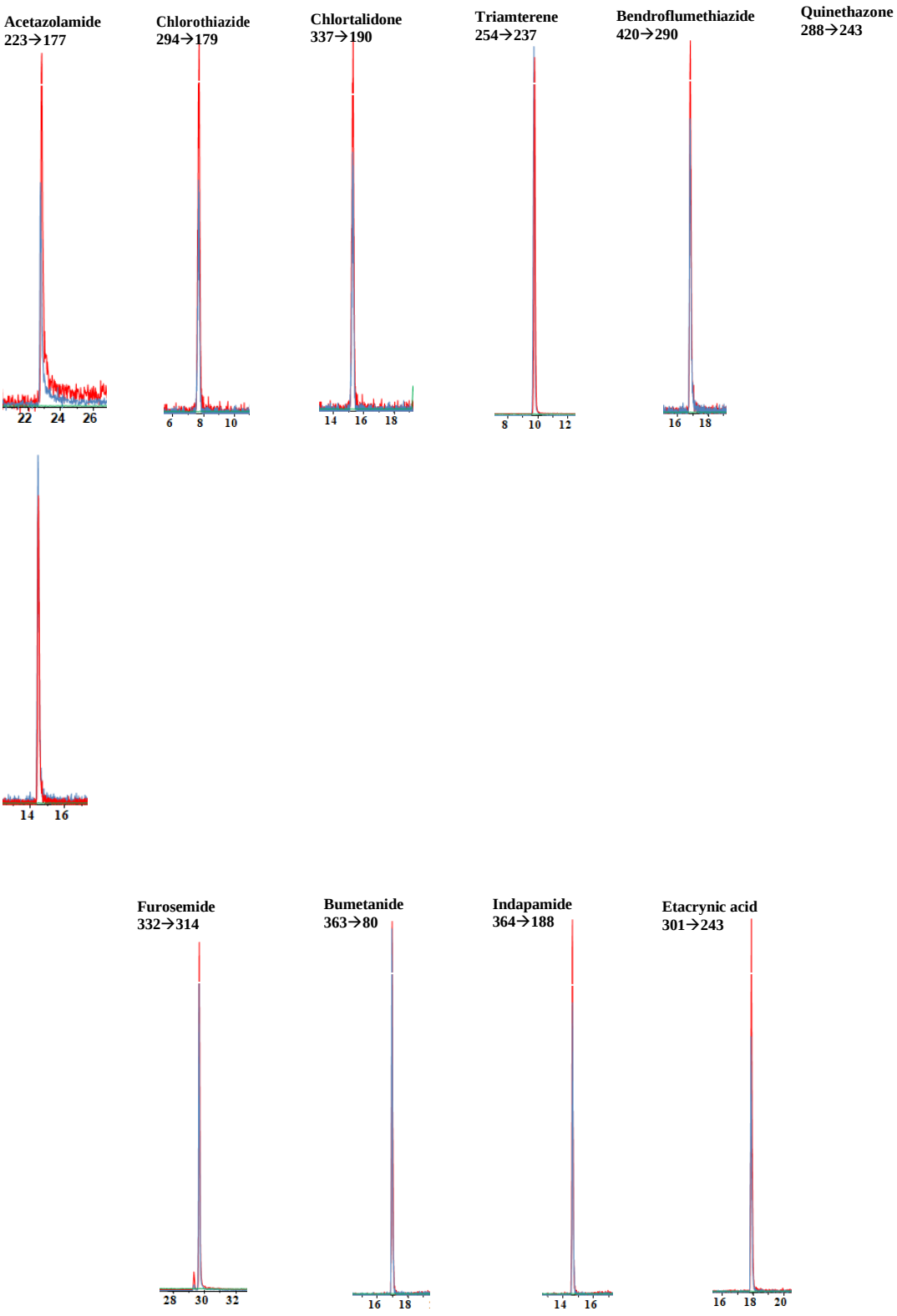
S3


Salbutamol
240→148
4 6 8
Terbutaline
226→152
4 6 8
Procaterol
291→273
6 8 10
Formoterol
345→149
10 12 14
Higenamine
272→107
6 8 10
Tulobuterol
228→154
10 12 14
Vilanterol
486→468
18 20 22

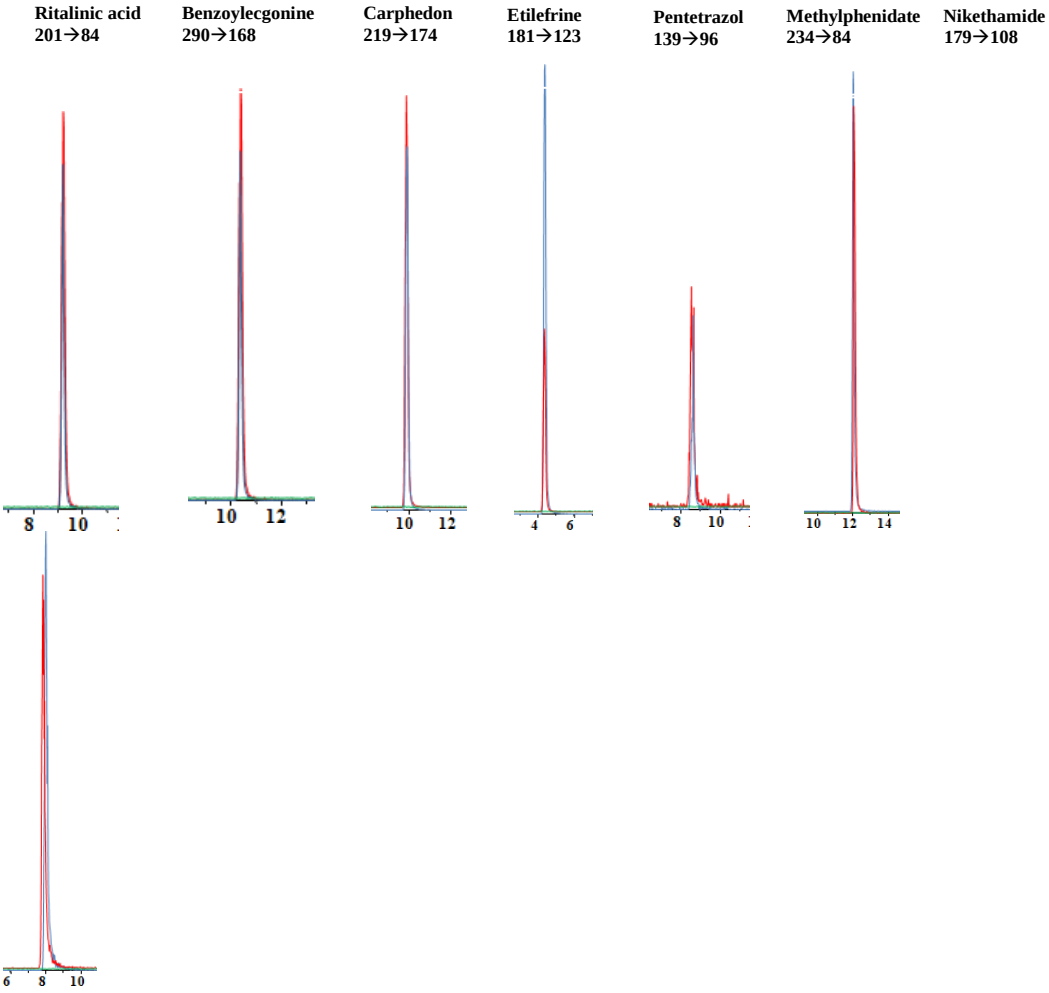

S4
Aminoglutethimidine
233→188
Anastrozole
294→225
Bis-(4-cyanophenyl)
methanol
235→77
Exemestane
297→121
SR9009
438→125
GW501516
Sulfone
486→257
GW501516
Sulfoxide
470→257
6 8 10
14 16 18
22 24 26
16 18 20
28 30 32
26 28
22 24 26

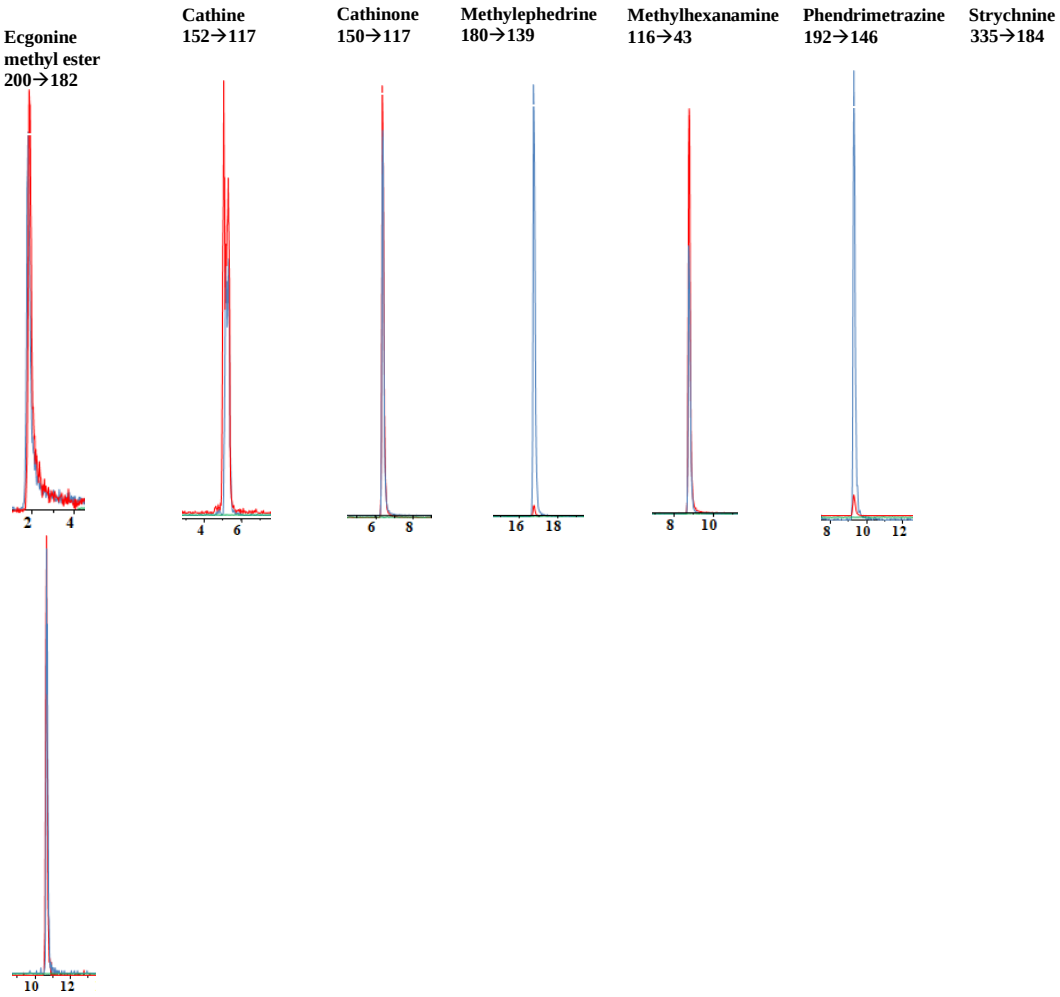

4-OH-cyclofenil
297→279
Raloxifene
474→84
4-OH-toremifene
422→72
Toremifene
406→72
17-ketone-fulvestrant
605→587
Fulvestrant
607→589
28 30 32
16 18 20
24 26
26 28 30
28 30 32
28 30 32

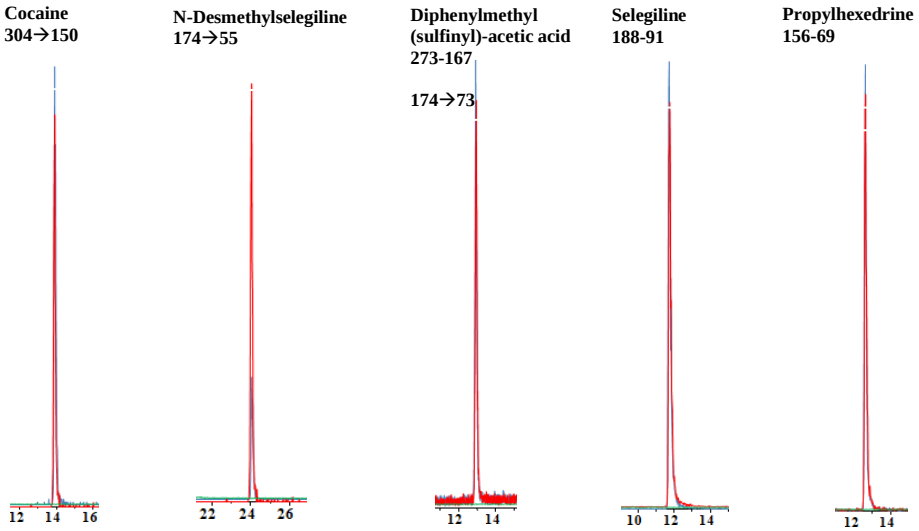
S5


Acetazolamide
223→177
Chlorothiazide
294→179
Chlortalidone
337→190
Triamterene
254→237
Bendroflumethiazide
420→290
Quinethazone
288→243
Furosemide
332→314
Bumetanide
363→80
Indapamide
364→188
Etacrynic acid
301→243

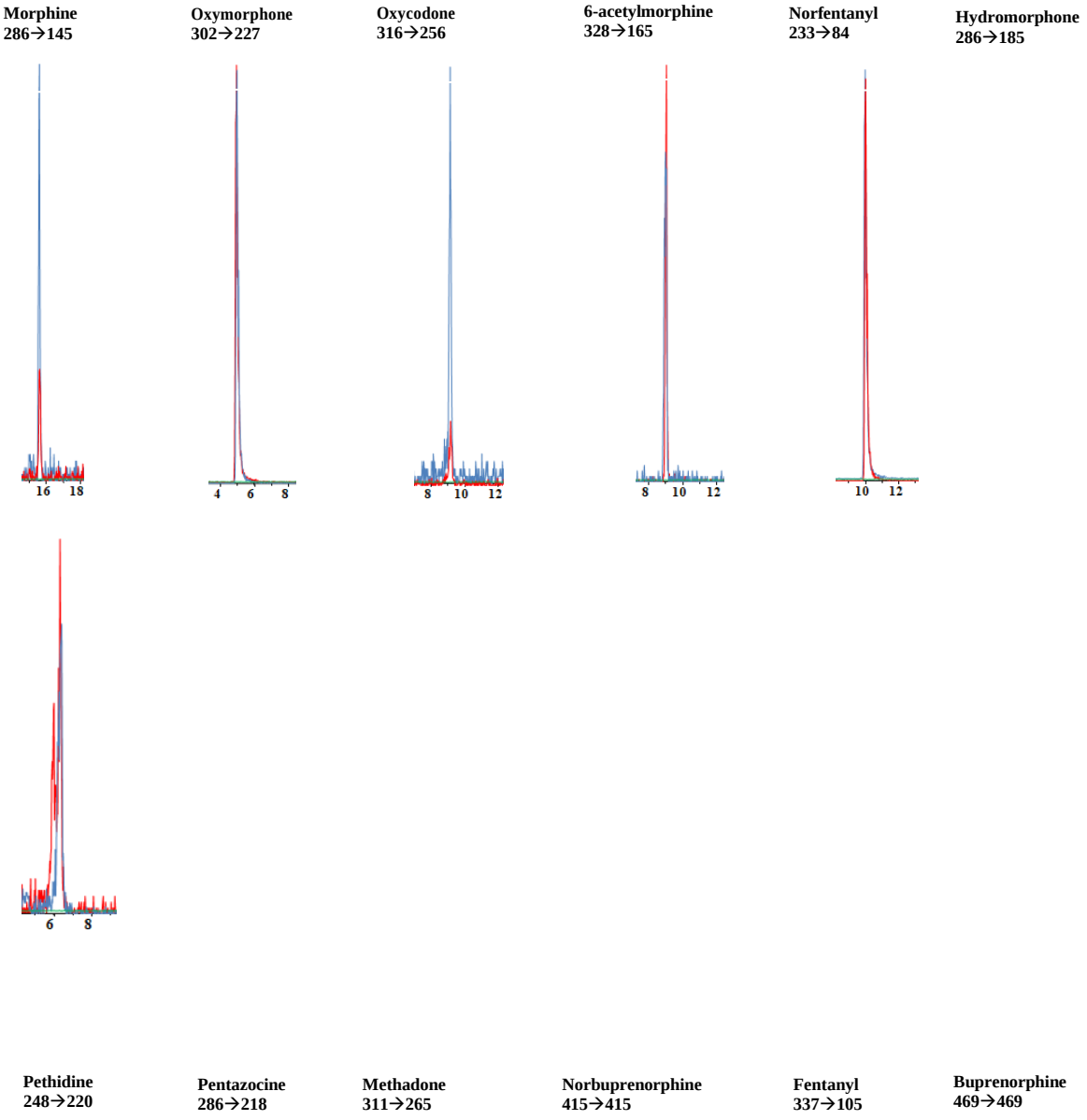
S6

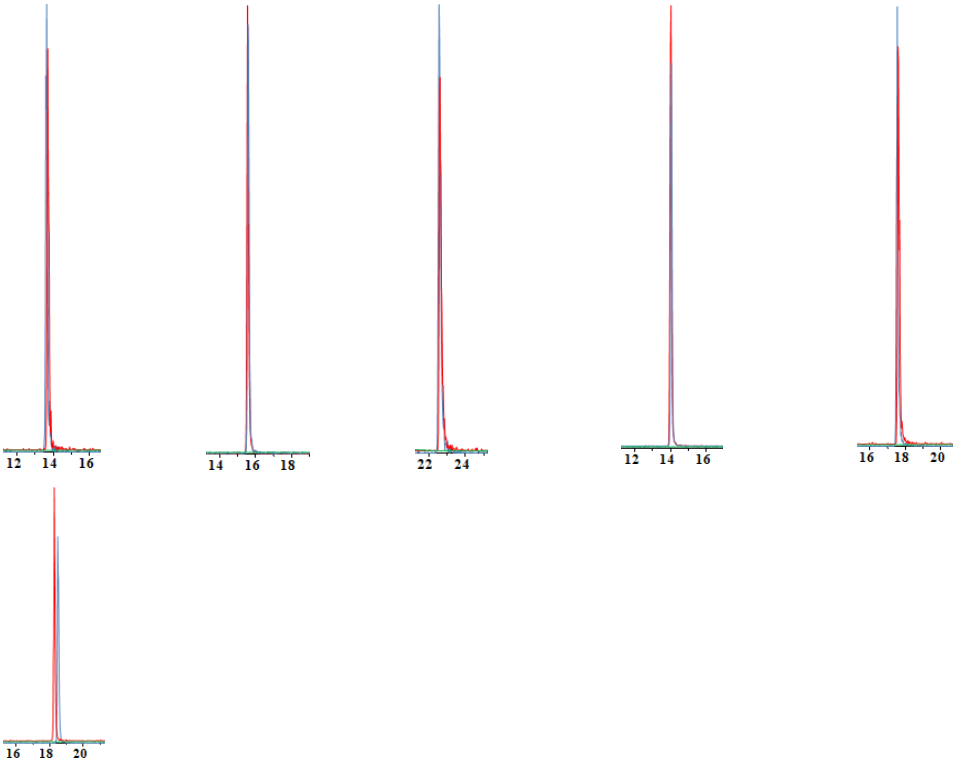

Ritalinic acid
201→84
Benzoylecgonine
290→168
Carphedon
219→174
Etilefrine
181→123
Pentetrazol
139→96
Methylphenidate
234→84
Nikethamide
179→108


Ecgonine methyl ester 200→182
Cathine 152→117
Cathinone 150→117
Methylephedrine 180→139
Methylhexanamine 116→43
Phendrimetrazine 192→146
Strychnine 335→184
2
4
4
6
6
8
16
18
8
10
8
10
12
10
12


Cocaine
304→150
12 14 16
N-Desmethylselegiline
174→55
22 24 26
Diphenylmethyl
(sulfinyl)-acetic acid
273-167
174→73
12 14
Selegiline
188-91
10 12 14
Propylhexedrine
156-69
12 14

S7

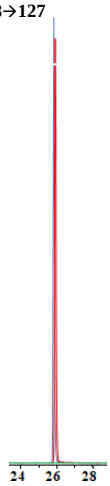

Morphine
286→145
Oxymorphone
302→227
Oxycodone
316→256
6-acetylmorphine
328→165
Norfentanyl
233→84
Hydromorphone
286→185
Pethidine
248→220
Pentazocine
286→218
Methadone
311→265
Norbuprenorphine
415→415
Fentanyl
337→105
Buprenorphine
469→469


12 14 16
14 16 18
22 24
12 14 16
16 18 20
16 18 20

S8

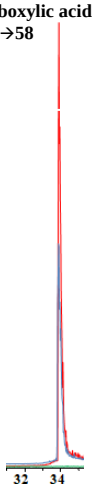
JWH-018 5-pentanoic acid metabolite
372→155

JWH-018 N-(5-hydroxy-pentyl) metabolite
358→127

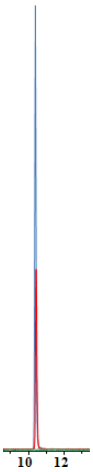


CP 47, 497-C8-hydroxy, C8-homolog
347→159

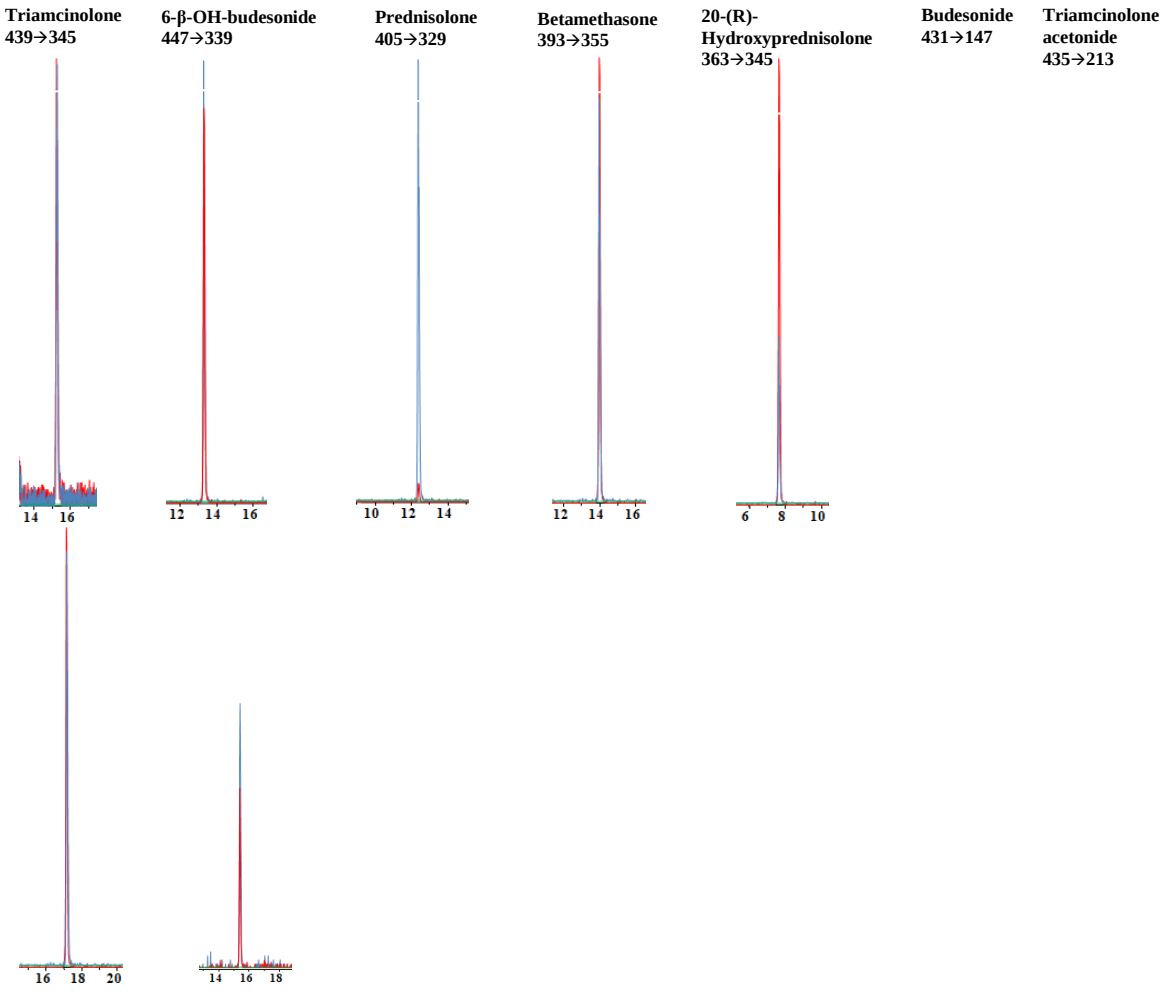
11- nor-Δ9-tetrahydrocannabinol-9-carboxylic acid
355→58



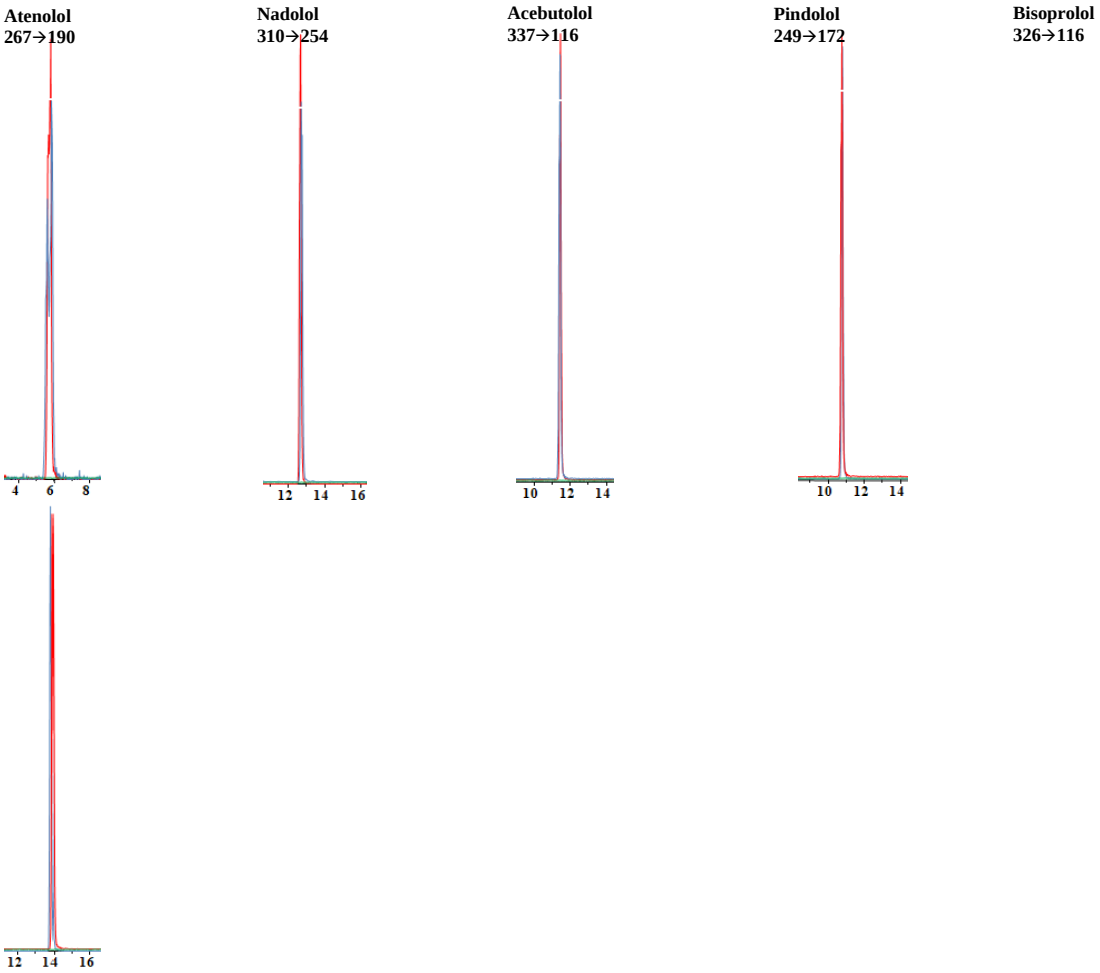
HU-210
385→339



S9


Triamcinolone
439→345
6-β-OH-budesonide
447→339
Prednisolone
405→329
Betamethasone
393→355
20-(R)-
Hydroxyprednisolone
363→345
Budesonide
431→147
Triamcinolone
acetonide
435→213
14 16
12 14 16
10 12 14
12 14 16
6 8 10
16 18 20
14 16 18

P1


Atenolol
267→190
Nadolol
310→254
Acebutolol
337→116
Pindolol
249→172
Bisoprolol
326→116
4
6
8
12
14
16
10
12
14
10
12
14
12
14
16

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Cubosomic Supramolecular Solvents: Synthesis, Characterization, and Potential for High-Throughput Multiclass Testing of Banned Substances in Urine

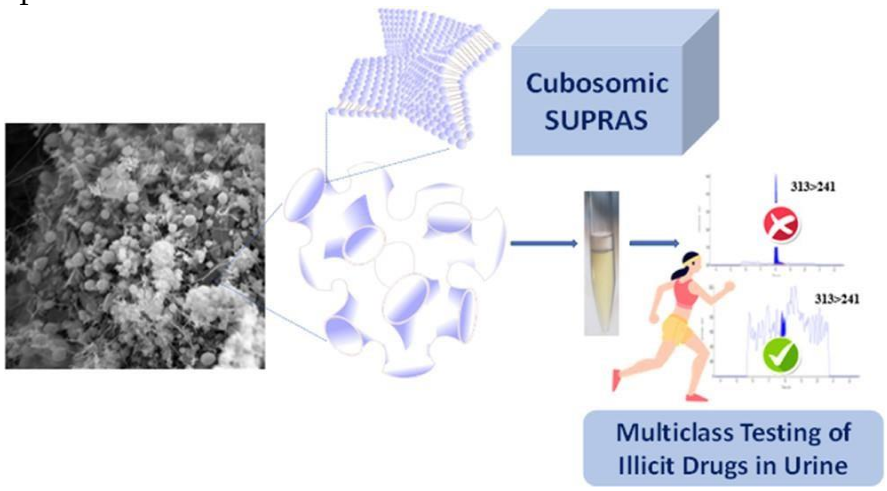
Soledad González-Rubio[1], Ana Ballesteros-Gómez[1,*], Gloria Muñoz[2], Soledad Rubio[1]

[1] Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, Marie Curie Building (Annex), Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

[2]Anti-doping control laboratory. Spanish Agency of Health Protection in Sport (AEPSAD)

*Corresponding author, email: ana.ballesteros@uco.es

Graphical Abstract



Abstract

The search for sample treatments in human sport drug testing, able to efficiently extract multiclass prohibited substances while keeping utmost selectivity and sample throughput, is a major challenge yet unsolved. In this paper, this challenge was addressed by using supramolecular solvents (SUPRASs) made up of cubosomes. These SUPRASs, here firstly reported, were synthesized by the salt-induced coacervation of 1,2-hexanediol in urine. The formation of square and rounded cubosomes with a size range of 140-240 nm was confirmed by electron microscopy. These nanostructures consisted of 1,2-hexanediol, salt and a high water content (36-61%, w/w). Their applicability in multiclass determinations was investigated by the extraction of 92 prohibited substances (log P from - 2.4 to 9.2) belonging to ten categories of the World Anti-doping Agency (WADA) list. Variables influencing both recoveries and matrix effects were optimized. Cubosomic SUPRASs showed a high extraction efficiency and interference removal capability which was attributed to their large hydrophilicity and surface area. Both features were superior to that of other eleven SUPRAS that were based on sponge droplets and inverted hexagonal aggregates and to that of conventional organic solvents. A sport drug testing method based on cubosomic SUPRASs-LC-ESI-MS/MS was proposed and validated. For the ten urine samples analyzed, around 82-95% were efficiently extracted (recoveries 70-120%) and 81-92% did not present matrix effects. Detection limits (0.001-4.2 ng mL^{-1}) were all far below

WADA`s limits. The proposed SUPRAS-based sample treatment is as simple as QuEChERS but the distinctive features of cubosomes confer them high capability in multiclass determinations.

Introduction

The list annually published by the World Anti-Doping Agency (WADA) on prohibited substances in sports encompasses a large number of compounds covering a wide range of polarity and physicochemical properties.[1] This list is notexhaustive and extends to their metabolites or biomarkers and any substance showing similar structure or effect. So, hundreds of substances are of potential interest in anti-doping testing programs.

According to WADA´s rules, drug testing in doping control laboratories involvesthe use of Initial Testing Procedures (ITPs) for the screening of all the substances covered by the Prohibited List. [2] Then, confirmatory methods are applied to the analysis of positive samples. Most of ITPs involve multiclass screening and they are mainly based on liquid chromatography coupled to both high and low resolution mass spectrometry (LC-HRMS, LC-MS/MS).[3] Increasing sample throughput and shortening turnaround and reporting times, while guaranteeing utmost specificity and sensitivity, have been long sought-after goals in drug testinglaboratories.[4,5]

A major current limitation to develop multiclass ITPs is related to sample preparation. At present, three strategies are primarily followed for this purpose; solid phase extraction (SPE), liquid-liquid extraction (LLE) and matrix dilution (dilute-and-shoot, D&S, methods), each with advantages and drawbacks.[6,7]

D&S methods, based on direct injection of urine after dilution with a buffer (viz 1:1 to 1:25 v/v), allow for little to none sample workup, increased throughput and

reduced cost. However, by diluting the sample, the sensitivity is only adequate for the screening of substances that are easily ionizable and for which permitted detection levels in urine are high (e.g. diuretics, stimulants and narcotics). Other drawbacks include retention time shifts, which reduce identification capability, and peak shape changes. [8] So, the use of a more extensive sample preparation based on LLE or SPE is yet the more reliable approach for the multiclass screening of sport drugs.

LLE is widely used because of its great simplicity. However, extractions are mainly based on analyte polarity and, given that water-immiscible solvents are used as extractants, LLE offers very poor recoveries for polar and very polar compounds. [6] Both, the use of acid and basic conditions and the reconstitution of the evaporated extracts with the original urine sample increase the multiclass screening capability of LLE. However, these strategies drastically reduce sample throughput or increasematrix effects. [9]

SPE is, in general, more appropriate than conventional LLE for the development of comprehensive ITPs because of the possibility of using mixed-mode sorbents that offer different interaction mechanisms for prohibited substances. As for LLE, reconstitution of the SPE extract with the original urine sample increases its multiclass screening capability.[10-13] However, SPE involves multiple steps(conditioning, loading, washing, elution and evaporation) and costs are high compared to LLE.

Within the analytical chemistry field, the search for new solvents that replace the conventional ones in analytical extractions has been particularly active in the last years.[14] Among them, supramolecular solvents (i.e. nanostructured liquids produced in colloidal suspensions of amphiphilic compounds by sequential self- assembly and coacervation, Figure SI1 in Supporting Information), feature outstanding properties for efficient LLE of multiclass substances. [15,16] Thus, they offer: (A) different polarity microenvironments where drugs may solubilize through mixed-mode mechanisms; (B) multiple binding sites that facilitate high

extraction yields at low SUPRAS/urine ratios; and (C) large surface area that provides fast solute mass transfer from the sample.

Despite these characteristics, application of SUPRASs to multicomponent determination has barely been exploited. Some applications involving the extraction of more than ten components include PAHs,[17] phenols,[18] surfactants,[19] bisphenols,[20] and perfluorinated compounds.[21] Among the SUPRASs investigated for this purpose, those based on alkanols[20,22] and alkanediols[21] are particularly promising given that alcohol groups do not ionize in ESI and have negligible signal in LC-ESI-MS/MS.[23] To the best of our knowledge, application of SUPRASs in multiclass determinations is testimonial (e.g. quantification of residual solvents in pharmaceutical formulations[24]).

In this paper, SUPRASs based on 1,2-hexanediol were firstly synthesized, characterized and their capability for extracting multiclass banned substances in urine was investigated. Our working hypothesis was that this amphiphile has a great potential to produce highly hydrophilic nanostructures able to solubilize efficiently very polar substances, while keeping their capability for solubilizing the nonpolar ones. This hypothesis is supported by the fact that aggregation of double-headed amphiphiles is limited by steric hindrance among them, which results in aggregates where the hydrocarbon chains remain highly folded and wetted.[25] On the other hand, 1,2-hexanediol, unlike other longer alkyl chains alkanediols,[21] is highly soluble in water and consequently its coacervation can be performed in aqueous media, which should additionally contribute to the formation of highly hydrophilic supramolecular nanostructures. Furthermore, this SUPRAS is also advantageous from a green chemistry perspective since it does not require the use of organic co-solvents for formation.

The coacervation of 1,2-hexanediol was tried in the presence of an inorganic salt. Phase diagrams, chemical composition and the resulting nanostructures were studied. Their capability for multiclass determination was investigated by extracting ninety-two prohibited substances (or their metabolites) belonging to ten categories (S1-S9, P1) of the WADA´s list (Table SI1, in Supporting

Information).[1] They covered a great number of functionalities (e.g. alcohol, carboxyl, ether, ester, ketone, primary/secondary/tertiary amines, amides, sulfonyls, etc.), and a wide range of polarity (log P from - 2.4 to 9.2). A sample treatment based on 1,2-hexanediol SUPRASs was optimized and both the recoveries and matrix effects were compared with those obtained by applying 11 SUPRASs synthesized from water-insoluble alkanols (C6-C10)[22] and alkanediols (C8-C10).[21] A method for the screening of banned substances in urine based on cubosomes-based SUPRAS-LC-MS/MS was developed and validated. Below, the most representative results are presented and discussed.

Experimental Section

Reagents and Solutions

All the solvents and reagents were of high purity grade and their suppliers are specified in the Experimental Section of the Supporting Information. The suppliersfor the 92 sport drugs and deuterated internal standards (IS) used in this study areshown in Table S2. The stock solutions for the individual drugs and the IS (1, 100,or 1000 μg mL–1) were prepared in methanol and stored at –20 °C. A multicomponent standard solution of the selected doping drugs at concentrationsof 100-fold their respective minimum required performance level (MRPL)26 (Table S3) was prepared inmethanol. An IS mixture solution at 2 μg mL–1 each was also prepared inmethanol. Intermediate and working solutions of drug mixtures were prepared monthly by the appropriate dilution of stock solutions in methanol, and they werekept at –20 °C until use.

Samples. Human urine samples were collected into 100 mL clean plastic containers (Sage Products, Crystal Lake, IL) by volunteers who were duly informed about the process, their rights, and other considerations. Details on urine collection and

treatment are specified in the Experimental Section of the Supporting Information.All solvents and reagents were of high purity grade and their suppliers are specified in the Experimental Section of Supporting Information (SI). The suppliers for the ninety-two sport drugs and deuterated internal standards (IS) used in this study are shown in Table SI2. Stock solutions for the individual drugs and the IS (1, 100 or 1000 μg mL^{-1}) were prepared in methanol and stored at -20ºC. A multicomponent standard solution of the selected doping drugs at concentrations of 100-fold their respective Minimum Required Performance Level (MRPL)[26] (Table SI3) was prepared in methanol. A IS mixture solution at 2 μg mL^{-1} each was also prepared in methanol. Intermediate and working solutions of drug mixtures were prepared by appropriate dilution of stock solutions in methanol and they were stored at -20ºC for at least one month.

Samples

Human urine samples were collected into 100 mL-clean plastic containers (Sage Products, Crystal Lake, IL) from volunteers that were duly informed about the process, their rights and other considerations. Details on urine collection and treatment is specified in the Experimental Section of the Supporting Information.

Synthesis and Characterization of Cubosomic SUPRASs from 1,2-Hexanediol

The formation of SUPRASs from 1,2-hexanediol was tried directly in the urine by addition of Na_2SO_4. For this purpose, the salt (0.1-2 M) was dissolved in the urine into 15 mL centrifuge tubes and then the 1,2-hexanediol (1-20%) was added. The total volume for the ternary mixture was fixed at 10 mL. The mixture was vortex- shaken for 5 min to facilitate the contact between their components, and then

centrifuged (3000g, 10 min) to accelerate phase separation. Experiments were performed in duplicate at 25 ºC. Region boundaries in the phase diagrams for SUPRAS formation were assigned by visual observation of two immiscible liquid phases that corresponded to the equilibrium solution (at the bottom) and the SUPRAS (at the top).

The volume of SUPRAS generated under different synthetic conditions was calculated by measuring its height in the cylindrical centrifuge tube with a digital calliper from Medid Precision, S.A. (Barcelona, Spain). Non-linear regression wasused to fit a model for the prediction of the volume of SUPRAS as a function the concentration of 1,2-hexanediol and Na_2SO_4 in the bulk solution. The statistics package Statgraphics Centurion XVI.II was used to fit a model. The density of SUPRASs synthesized under different conditions was calculated by weighting a given volume of coacervate in an analytical balance. All experiments were conducted in duplicate.

The chemical composition of the SUPRASs was determined as follows. Thecontent in water was measured using a coloumetric Karl Fischer titrator from Metrohm (Herisaus, Switzerland). The concentration of Na_2SO_4 incorporated into the SUPRAS was quantified by measuring the turbidity of barium sulfate (LP2000 Turbidity Meter from Hanna Instruments, Guipúzcoa, Spain) after dilution with water and addition of $BaCl_2$ and a stabilizing solution (method EPA 9038). Finally, once known the concentration of water and sulfate in the SUPRAS, the concentration of 1,2-hexanediol was calculated by difference.

The presence of coacervate droplets in the SUPRASs was investigated with a lightmicroscope (Leica model DME; Wetzlar, Germany) equipped with an automatic photocamera, using the bright field.

The investigation of the morphology of the aggregates in the SUPRAS droplets was undertaken by scanning electron microscopy (SEM). For this purpose, the SUPRAS (˜10 μL) was fixed with glutaraldehyde and then it was embedded with a 6% aqueous agarose solution. After that, the sample was washed three times with sodium cacodylate, and stained with OsO_4 (1%) for contrast enhancement. The

sample was subsequently dehydrated with a graded acetone series (30, 50, 70, 80, 90, 100 %) and then subjected to critical point drying. Finally, the sample was placed onto a SEM specimen stub using double-sided carbon tape and it was coated with Au/Pd. The accelerating voltage was set at 10 kV.

Optimization of the SUPRAS-based Sample Treatment in ITPs

First, the influence of the hydrophilicity of different SUPRAS nanostructures on the extraction efficiency of doping drugs and on the removal of interferences wascomparatively investigated. For this purpose, different types of SUPRASs were synthesized directly in urine from alkanols (C6—C10) to give rise to inverse hexagonal aggregates and from alkanediols (C8—C10) to form sponge-like SUPRASs. Fortified and unfortified hydrolyzed urines and distilled water blanks were subjected to SUPRAS extraction, both in the presence and absence of 1 M Na2SO4, and the results were compared to those obtained from standard solutions prepared in SUPRASs. General Procedures Were as Follows. (A) Fortified hydrolyzed urine samples. Aliquots of 1 mL were fortified with the doping drugs at the MRPLs (Table S3) in 2 mL microtubes Safe-Lock from Eppendorf Ibe´rica (Madrid, Spain). Those drugs for which decision limits (DLs) have been defined were tested at the MRPLs corresponding to their WADA group (i.e., salbutamol, formoterol, cathine, methylephedrine, morphine, and 11-nor-Δ9- tetrahydrocannabinol-9- carboxylic acid were tested at 20, 20, 50, 50, 25, and 1 ng $mL^{-1,}$ respectively). All the samples were fortified with the deuterated IS at 20 ng mL^{-1}. Then, the amphiphile (1-hexanol, 1-octanol, 1,2-octanediol, 1-decanol, or 1,2-decanediol) and tetrahydrofuran (THF) were added to the urine to give a finalconcentration of 20% (w/v for solid and v/v for liquid amphiphiles) and 10% THF (v/v), respectively. The water content in the urine promoted the self-assembly of the amphiphile and caused the spontaneous in situ formation of the SUPRAS. For

experiments carried out in the presence of 1 M Na2SO4, the salt was solubilized in the urine prior to the addition of the amphiphile and THF. In the case of 1,2-hexanediol, the amphiphile was directly solubilized in the urine, given its solubility in water, and the formation of the SUPRAS was induced by Na2SO4. Then, the urine samples containing the respective reagents were vortex shaken (2,000 rpm, 10 min in a vortex shaker from Heathrow Scientific, Illinois, USA) and centrifuged (3,000g, 15 min in a Minicen CE 182 centrifuge from Ortoalresa, Daganzo, Madrid, Spain). All the supramolecular extracts were less dense than urine, and the volume of the SUPRAS obtained from the different amphiphiles was in the range of 250—480 μL. The SUPRAS extracts were withdrawn using a microsyringe and an aliquot (250 μL) was diluted 1:1 with methanol. Finally, the diluted SUPRAS extracts were transferred into a sealed glass vial for subsequent LC-MS/MS analysis. (B) Unfortified hydrolyzed urine samples. Aliquots of 1 mL were extracted with SUPRAS as specified above. Then, the diluted SUPRAS extracts (250 μL of SUPRAS plus 250 μL of methanol) were fortified with the doping drugs at two fold the MRPL (Table S3) and the deuterated IS at 40 ng mL—1, and then they were analyzed by LC-MS/MS. (C) Procedural blanks of distilled water samples. The SUPRAS were synthesized in water by following the same procedure specified in (A). SUPRAS extracts were then diluted with methanol and they were fortified with the doping drugs at two fold the MRPL (Table S3) and the deuterated IS at 40 ng mL—1. Finally, they were analyzed by LC-MS/MS.
Recoveries and matrix effects for the selected doping drugs were calculated by the comparison of the relative peak areas ($A_{\text{doping substance}}/Ai_{\text{internal standard}}$) obtained from the experiments specified in A and B and B and C, respectively. In addition to this comparative study, the influence of different parameters on both recoveries and matrix effects for the selected doping drugs was investigated using the cubosome-based SUPRASs that were made up of 1,2-hexanediol. The variables investigated included the concentration of Na2SO4 (0.6—1.5 M) and 1,2-hexanediol (10—30% v/v) and the vortex-shaken extraction time (5—15 min). Experiments were conducted using fortified and unfortified urine and procedural blanks, as described

above First, the influence of the hydrophilicity of different SUPRAS nanostructures on the extraction efficiency of doping drugs and on the removal of interferences was comparatively investigated. For this purpose, different types of SUPRASs were synthesized directly in urine from alkanols (C6-C10) to give rise to inverse hexagonal aggregates and from alkanediols (C8-C10) to form sponge-like SUPRAS. Fortified and unfortified hydrolysed urines and distilled water blanks were subjected to SUPRAS extraction, both in the presence and absence of 1M Na_2SO_4, and the results were compared to those obtained from standard solutions prepared in SUPRASs. The general procedures followed are specified in Supporting Information. In addition to this comparative study, the influence of different parameters on both recoveries and matrix effects for the selected doping drugs was investigated using the cubosome based SUPRASs that were made up of 1,2-hexanediol. Variables included Na_2SO_4 (0.6-1.5 M) and 1,2-hexanediol (10-30% v/v) concentrations and vortex-shaken extraction time (5-15 min). Experiments were conducted using fortified and unfortified urine and procedural blanks, as described above.

Recommended Procedure for Sample Treatment in ITPs Based on Cubosomic SUPRASs

Hydrolyzed urine samples (1 mL), fortified with 20 ng mL^{-1} of each of the IS specified in Table SI2, were mixed with Na_2SO_4 (final concentration 1M) in 2-mL Eppendorf microtubes. After salt solubilization, 200 μL of 1,2-hexanediol were added. The mixture was vortex-shaken for 5 min at 2,000 rpm and then, centrifuged for 10 min at 3,000g. An aliquot of the supramolecular extract (about 250 μL) was withdrawn using a microsyringe and transferred to a sealed glass vial. Then, the aliquot was mixed with the same volume of Milli-Q water for subsequent LC-MS/MS analysis. Figure SI2 shows a schematic of the SUPRAS-based sample treatment.

LC-MS/MS Analysis of Doping Drugs

The separation and quantification of the selected doping drugs was conducted using a liquid chromatograph (Waters, Acquity H-Class, Milford, MA, USA) coupled to a hybrid triple quadrupole/linear ion trap (Applied Biosystems MSD Sciex, 5500QTRAP, Four Valley, ON, Canada) equipped with a TurboIonSpray (TIS) interface. All data were acquired and processed using the Analyst 1.6.2 Software. Chromatographic and mass spectrometric conditions are specified in theExperimental Section of the Supporting Information.

Method Validation

The method based on SUPRAS-LC-MS/MS was validated in terms of selectivity, recovery, matrix effects, detection, limits carry-over and SUPRAS extract stabilityby using 10 urine samples. Procedures used for method validation are specified inthe Experimental Section of the Supporting Information.

Results and discussion

Synthesis and Characterization of Cubosomic SUPRASs made up from 1,2- Hexanediol

SUPRAS Synthesis

The aggregation of amphiphiles to give colloidal suspensions (e.g. micelles, vesicles, etc.) is a key step for their posterior coacervation, a phenomenon through which SUPRASs are generated. The formation of micelles in aqueous solutions of1,2-hexanediol has long been reported in the literature.[27] These micelles have a critical aggregation concentration (cac) around 0.71-0.74 M, an aggregation number of 20, a micellar mass of 2,330, and a hydrodynamic radius of 13.5 Å.[27]

Their study by small-angle neutron scattering (SANS) has proved that molecules of 1,2-hexanediol exhibit a strong diol–diol interaction in solution and give spherical hydrated micelle-like aggregates with attractive interaction between them.[28] Specific ion effects on the micellization of 1,2-hexanediol has been deeplyinvestigated.[29] Thus, its cac has been determined in the presence of a variety of chloride salts of monovalent and divalent cations as well as sodium salts of monoatomic and polyatomic monovalent anions. In all cases, the cac decreased with increased salt concentrations in the range 0-3 M. The lowest cac was achieved for 3 M of divalent cations (0.14-016 M) followed by 3 M of monovalent cations and anions (0.21-0.39 M).[29]

The coacervation of 1,2-hexanediol from urine was tried by destruction of the hydration layer at its head groups. There is a broad consensus that by destructing this layer, the effective area per molecule at the interface diminishes and amphiphile monomers can be packed closer together leading to aggregate growthand liquid phase separation.[30] Two salts were investigated for destruction of the hydration layer of 1,2-hexanediol, namely sodium chloride and sulfate, at concentrations between zero and their maximum aqueous solubility. These salts are nontoxic and have low cost and high stability. The study was restricted to amphiphile concentrations below 20% (v/v) since they are the concentrations typically used in analytical extraction processes.[15]

Figure 1A shows the phase diagram obtained at 25 ºC from the 1,2-hexanediol- urine-sodium sulfate mixture. No coacervation was obtained under addition of sodium chloride to the colloidal suspension of the amphiphile. The phase diagram exhibited two different regions as the concentration of salt increased up to its limitof solubility in water (˜2 M); an isotropic solution and a region where two immiscible liquid phases were observed (SUPRAS formation).

The SUPRAS was less dense than the equilibrium phase under all the experimentalconditions. The minimum percentage of 1,2-hexanediol required to get coacervation was 3% (v/v) that corresponds to a concentration of 0.25 M of

amphiphile. As previously reported, aqueous micelles are formed at these concentrations of 1,2-hexanediol in the presence of salts.[29]

Although the microscopic origins of coacervation still remain elusive,[30] the results obtained for the coacervation of 1,2-hexanediol in the presence of the two salts investigated (sodium sulfate and sodium chloride) are strongly related to the dehydration power of their anions. Thus, sulfate is a highly hydrated anion whereas chloride is a poorly hydrated anion, as inferred from their Jones-Dole viscosity coefficients (B); 0.208 for sulfate and -0.007 for chloride.[31] So, the water with drawing power of sulfate is much higher than that of chloride, making it able to effectively destruct the hydration layer of the amphiphile. This basis strongly suggests that this mechanism is the dominant for 1,2-hexanediol coacervation.

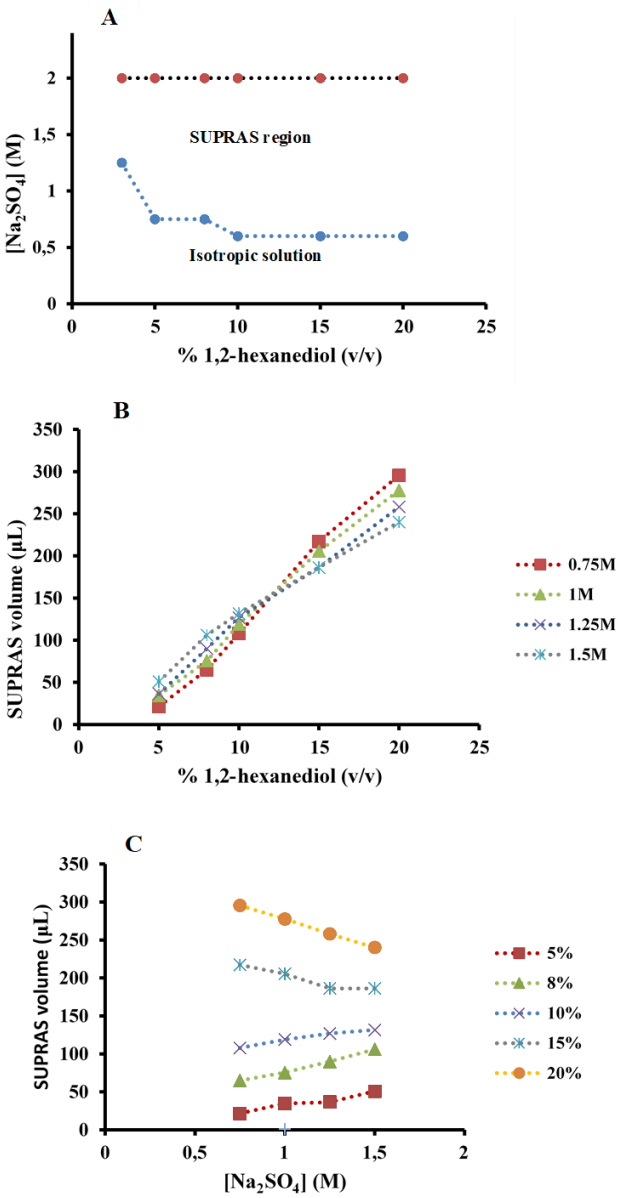


Figure 1. (A) Phase diagram for the ternary mixture 1,2-hexanediol- urine-sodium sulfate. (B) Volume of SUPRAS as a function of the initial concentration of 1,2-hexanediol (%, v/v) at different concentrations of sodium sulfate (0.75-1.5 M). (C) Volume of SUPRAS as a function of the initial concentration of sodium sulfate at different concentrations of 1,2- hexanediol (5-20%, v/v).

SUPRAS Volume

The volume of SUPRAS produced in the colloidal system (expressed as µL of SUPRAS per mL of urine) was a function of the concentration of both the amphiphile and the salt. As it can be observed in Figure 1B, this volume increased

linearly with the concentration of 1,2-hexanediol, independently of the concentration of sodium sulfate investigated. The equations and coefficients of determination for these linear dependences are shown in Table SI4. The value of the slopes of these linear graphs decreased as the concentration of salt increased (Table SI4). This decrease, along with intersection point of these plots at around 12% of amphiphile (Fig. 1B), suggested that the volume of SUPRAS produced followed different dependences at high and low concentration of amphiphile. This effect was clearly illustrated by plotting the volume of SUPRAS produced as a function of salt concentration (Figure 1C). Thus, the volume of SUPRAS decreased and increased at 1,2-hexanediol concentrations above and below 10%, respectively, as the concentration of sodium sulfate raised. Given that low sample volumes are commonly used in doping control (typically 1 mL urine) and that a minimum of around 100 μL of SUPRAS are required in chromatographic analysis using autosamplers, we decided to work at amphiphile concentrations equal or above 10% (Figure 2C). An equation was derived to calculate the volume of SUPRAS as a function of the concentrations of 1,2-hexanediol and sodium sulfate (see the section SUPRAS volume in SI) that had a good capability of prediction (Figure SI3).

SUPRAS Chemical Composition

All the SUPRASs produced in the region of analytical interest ([1,2-hexanediol] from 10% to 20%) were made of the three ingredients present in the colloidal suspension, namely water, 1,2-hexanediol and sodium sulfate. However, the ratios for these ingredients in the SUPRAS were dependent on the concentration of the coacervation-inducing agent (sodium sulfate), so these SUPRASs were environment-responsive and their composition can be tailored according to the salt concentration in the synthetic solution.

Table SI5 shows representative results. The water and amphiphile contents in the SUPRAS respectively decreased and increased when the concentration of salt in

the synthesis mixture raised. This behavior suggests that, in addition to the destruction of the hydration layer caused by the salt, salting-out effects in the bulk solution can help to coacervation.[31] The amount of sodium sulfate in the SUPRAS progressively decreased with the increase of salt in the synthesis solution (Table SI5). This behavior is in agreement with the decrease of the solubility of this salt in water-alcohol mixtures as the proportion of alcohol (i.e. 1,2-hexanediol in this case) increases.[32]

Water content in 1,2-hexanediol-based SUPRASs were above 36% (w/w) and reached concentrations as high as 61% (w/w). So, these SUPRAS have extensive hydrophilic regions in its nanostructures, which are expected to confer it a great capability for extraction of polar compounds, On the other hand, the high concentration of 1,2-hexanediol in the SUPRAS provided extensive hydrophobic environments, suitable for solubilisation of nonpolar compounds.

The amphiphile was almost fully incorporated into the SUPRAS (≥ 90%) after coacervation, except for the lowest concentration of sodium sulfate (around 40% of amphiphile incorporation for 0.6 M Na_2SO_4). This means that at the boundaries between the isotropic region and SUPRAS formation (Figure 2A), coacervation only occurred partially. All SUPRASs were less dense than water, with density values in the interval 0.90-0.95 g mL^{-1} (Table SI5).

SUPRAS Structure

A representative optical micrograph obtained for the SUPRAS is shown in Figure SI4. This micrograph illustrates that the liquid phases were produced through self-assembly and coacervation. Thus, they were not continuous phases but they were made of coacervate droplets within the interval 6-7 μm (mean value 6.5 μm).

Micrographs obtained by SEM clearly showed (Figure 2A) that the nanostructures in the SUPRAS were cubosomes with a size in the range XXX-XXX nm. These square and rounded discrete nanostructures (see magnification in Figure 2B) consist of bicontinuous domains of water and amphiphile bilayers twisted into a

three dimensional morphology (see schematic in Figure 2C). Cubosomes are usually obtained by top-down and bottom-up approaches from the cubic phases of some amphiphilic lipids (e.g. glyceryl monooleate and phytantriol) by addition of a suitable stabilizer to prevent their aggregation.[33] Thus, they possess the same microstructure as the parent cubic phase but have larger specific surface area. Their high water content along with their large surface area make cubosomes highly suitable for extracting structurally unrelated compounds covering a wide polarity range. To the best of our knowledge this is the first time that SUPRAS based on cubosomes are reported. Likewise, this is the first time that cubosomes are produced by coacervation from monomeric amphiphiles, which opens the door to their production through predictable and low-energy processes.

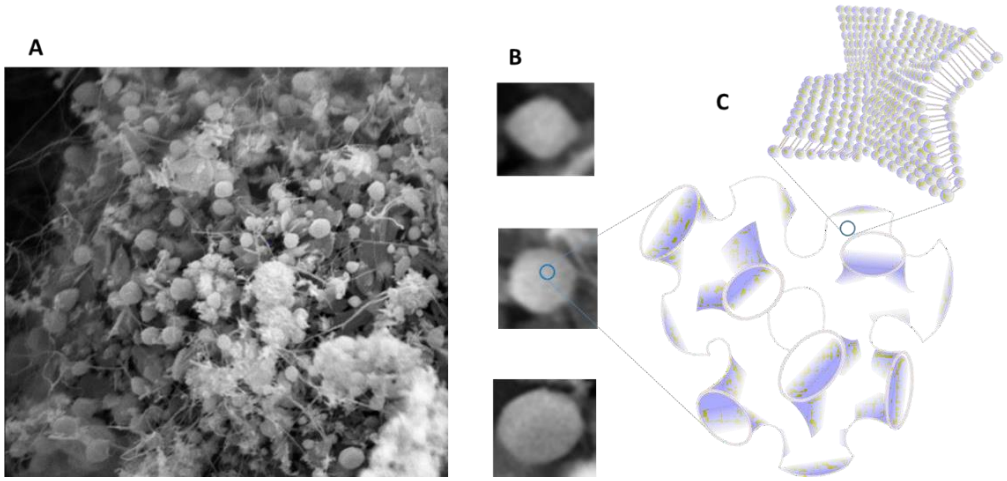


Figure 2. (A) Micrographs obtained by scanning electron microscopy for a SUPRAS produced from a mixture containing 20% (v/v) of 1,2-hexanediol and 1M Na_2SO_4. Magnification: 2 KX. (B) Amplification of some cubosomes present in (A). (C) Schematic of the bicontinuous cubic phase and the amphiphilic bilayer making up cubosomes

Optimization of the SUPRAS-Based Sample Treatment in ITPs

According to the results obtained in the previous section, the cubosomes makingup the 1,2-hexanediol-based SUPRASs are highly hydrophilic (Table SI5) and this should positively impact their capability for developing straightforward sample treatments in multiclass compound determinations. To check this

hypothesis, the influence of the hydrophilicity of SUPRAS nanostructures on the extractability of multiclass doping drugs was initially investigated. Two types of SUPRASs having different nanostructures and hydrophilicity to that of 1,2-hexanediol-based SUPRASs were selected for this purpose. The fist type was synthesized from C6- C10 alkanols in THF-urine media, in the presence and absence of salt (see the procedure at the Experimental Section in SI). It has been previously reported that these SUPRASs arrange as inverted hexagonal aggregates where the alcohol groups surround water cavities and the THF are dispersed in the hydrocarbon chains (Figure SI5, top).[22] The second type of SUPRAS was synthesized from C8-C10 1,2- alkanediols in THF-urine media, in the presence and absence of salt (see the procedure at the Experimental Section in SI). These SUPRASs have been known to have sponge nanostructures consisting in a highly convoluted and interconnected three-dimensional network of amphiphile bilayers intersected by similarly interconnected nanometer-sized aqueous channels (Figure SI5, bottom).[21] Under the synthesis conditions used, water content in the three types of SUPRASs were around 5%, 30% and 40% (w/w) for the SUPRAS made up of hexagonal, sponge and cubosome aggregates, respectively.

A total of eleven SUPRAS made up of cubosomes (1), sponges (4) and inverted hexagonal aggregates (6) were tested for the extraction of doping drugs. Table SI1 shows the name, chemical structure and molecular formula of the ninety-two prohibited substances selected for this study, ranked by WADA category (S1-S9, P1). This table also includes different parameters of interest for their extraction behavior (log P, ionization constants, number of hydrogen donors and acceptors and number of aromatic rings). The selected compounds covered a wide range of polarity (log P from - 2.4 to 9.2) and included acids, bases and neutrals.

Figure 3 shows the recoveries obtained from urine with the eleven investigated SUPRAS. Results for each SUPRAS were classified into four groups, depending on the recovery values obtained (i.e. 70-120%, 20-69%, <20% and >120%). For

comparison, the results obtained from the extraction with a conventional solvent (methyl isobutyl ketone, MIBK[6]) are also included.

As shown in Figure 3, the worse recoveries were obtained for SUPRAS made up of alkanols in the absence of salts, for which only around 45-55% of the doping drugs were within the optimal recovery interval (70-120%). Recoveries for 1- octanol and 1-decanol considerably improved under the addition of Na_2SO_4 to theurine (around 60-70% of the drugs had good recoveries), probably because of the salting-out effect. This effect was negligible for 1-hexanol, as inferred from the similar good recoveries obtained in the absence and the presence of the salt (˜50%). When considering the percentage of drugs with very low recoveries (<20 %), the best results among alkanol-based SUPRAS were obtained for 1-octanol in the presence and absence of salt, for which only around 10-15% of the drugs showed recoveries below 20%.

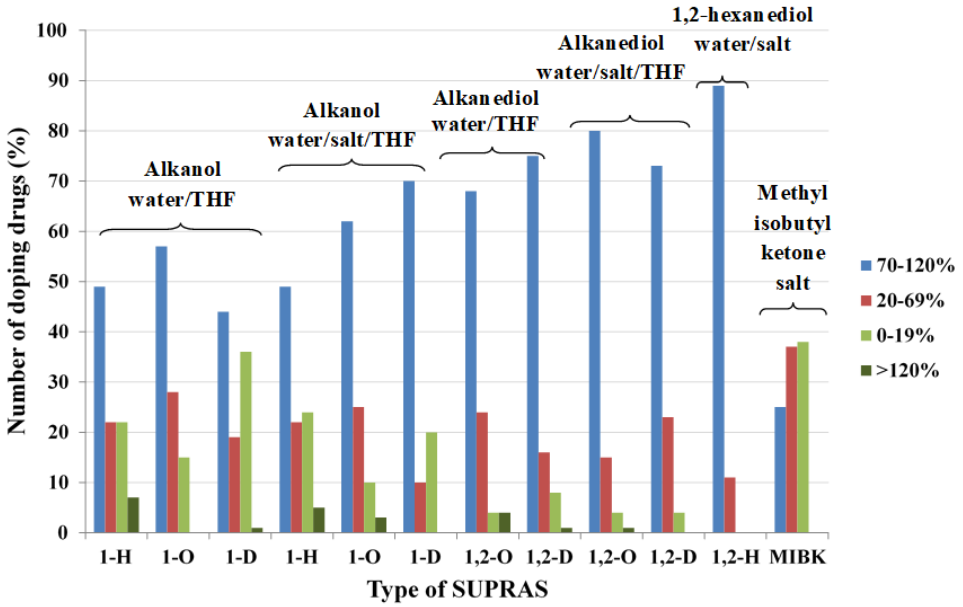


Figure 3. Percentage of prohibited substances with recoveries in the ranges 70-120% (blue), 20-69% (red), 0-19% (light green) and greater than 120% (dark green) for SUPRAS synthesized from: 1-hexanol (1-H), 1-octanol (1-O), 1-decanol (1-D), 1,2-octanediol (1,2- O), 1,2-decanediol (1,2-D), both in the absence and presence of salt, and 1-2-hexanediol (1,2-H) in the presence of salt. Results for methyl isobutyl ketone are also included.

A considerable improvement in the recovery of doping drugs was obtained with SUPRASs made up of alkanediols compared to those based on alkanols (Fig. 3). Thebest results were found for 1,2-octanediol in the presence of salt, for which the 80% of the selected drugs showed recoveries in the range 70-120%. Additionally,a valuable aspect of these SUPRAS is that they increased notably the recoveries of very polar substances. Thus, only the 4% of the selected doping drugs had recoveries below the 20% as 1,2-octanediol was used as amphiphile, both in the absence and presence of salt. The best results were obtained for 1,2-hexanediol-based SUPRAS; substances within the recommended recovery range (70-120%) approached 90% and no substances were within the very low recovery range (0-19%). As expected, recoveries with methyl isobutyl ketone were low. Only around 25% of the doping drugs were within the optimal recovery interval. It is worth noting that absolute recoveries for many of the drugs within the range 0-19% was nearly zero, despite the extraction was carried out in the presence of salt to account for the enhancement of recoveries by salting-out effects.

These results clearly show that increasing the hydrophilicity of the supramolecular nanostructures greatly increases the extractability of very polar compounds, as it can be inferred from the number of compounds falling within the interval 0-19% for each of the extractant investigated (Fig. 3). Thus, the most hydrophilic nanostructures (i.e. sponge and cubosomes) achieved the highest global recoveries for the selected compounds. On the other hand, these results also corroborate that conventional organic solvents are unable to develop efficient sample treatments for multiclass drug screening.

Given the greater extraction capability of SUPRAS based on alkanediols comparedto those based on alkanols, the suitability of the formers for the removal of matrixeffects was investigated. Results were compared with those obtained by a D&S approach (a urine sample was diluted 1:4 v/v with distilled water and readily analyzed). Matrix effects are shown in Figure 4. Matrix effects were greatly reduced after SUPRAS extraction in comparison with the D&S approach. Thus, upto 89% of the drugs could be determined in absence of matrix effects under

extraction with 1,2-hexanediol. So, in addition to excellent recoveries, the cubosome-based SUPRASs efficiently removed matrix effects from urine. Therefore, further optimization of the extraction process was performed for 1,2-hexanediol-based SUPRASs. The studied variables included the concentration of sodium sulfate used for inducing the formation of the SUPRAS (0.6-1M), the percentage of amphiphile added to the urine (10-30%) and the time for extraction (5-15 min). Selection of the optimal conditions were guided by both the recoveries and matrix effects obtained for doping drugs in urine. Representative results are shown in Figure SI6. Final optimal values were 5 min extraction, 1 M Na_2SO_4 and 200 µL of amphiphile. Under these condition around 90 % of the drugs showed recoveries in the 70-120% range and were not affected by matrix effects.

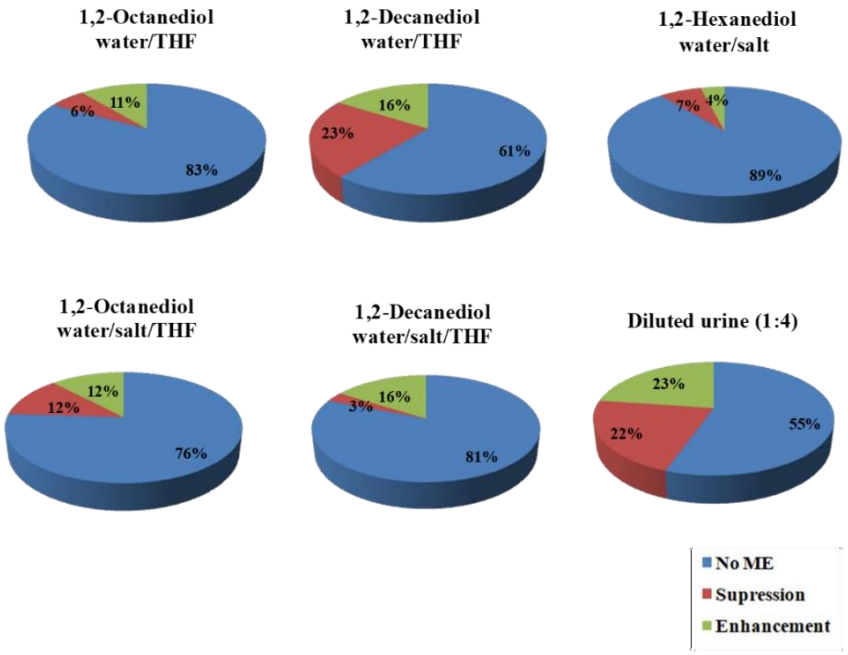


Figure 4. Percentage of prohibited substances showing no matrix effects (±20% in blue), suppression (red) and enhancement (green) for SUPRAS produced from 1,2- octanediol and 1,2-decanediol, both in the absence and presence of salt, and 1,2- hexanediol. Likewise, results obtained for diluted urine (1:4) are also shown.

Method Validation

The method developed was validated according to the procedures specified in the Experimental Section of the Supporting Information. Results for this validation are discussed in the Supporting Information (section Method Validation). Data obtained are shown in Table S3 and Figure S7 (analytical figures of the method), Figures S8 and S9 (selectivity), Figure 5A and Table S6 (recoveries), and Figure 5B and Table S7 (matrix effects). In brief, Method detection limits (MDL)s for the banned drugs were all far below the respective MRPL26 and DL34 values, the method was selective and precise, around 82—95% of drugs were efficiently extracted (recoveries 70—120%) in urine samples, and 81—92% did not present matrix effects.

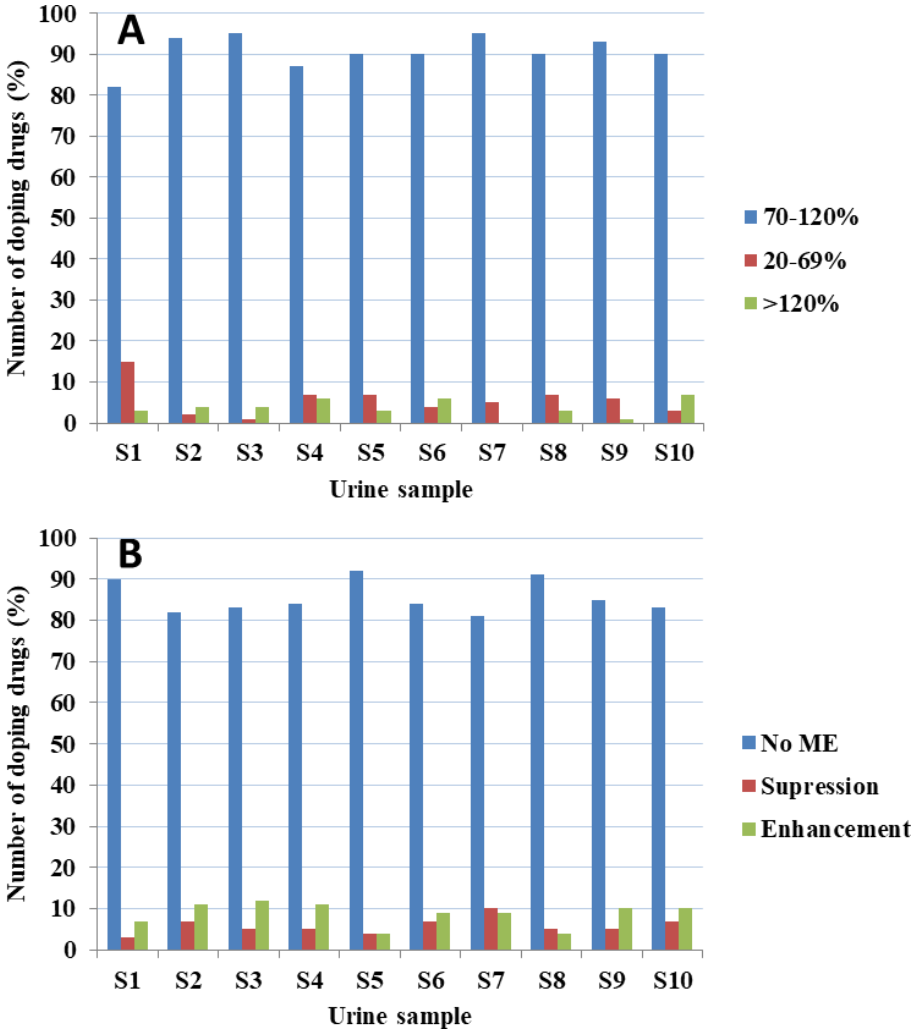


Figure 5. Percentage of prohibited substances with (A) recoveries in the ranges 70- 120% (blue), 20-69% (red), and greater than 120% (green, and (B) showing no matrix effects (± 20%, in blue), signal suppression (in red) and enhancement (in green) for the extraction of ten human urines (S1-S10) with 1,2-hexanediol-based SUPRAS.

Conclusions

The use of SUPRASs tailored with the proper nanostructures provides effective multicomponent extraction of substances covering a wide polarity range. Taking into account that SUPRASs always possess hydrophobic regions where nonpolar and medium-polar substances can be solubilized, the tailoring of their hydrophilic

regions seems essential to extend their application to the extraction of highly polar substances. In this respect, the cubosomic SUPRASs here reported have proved highly efficient for the extraction of polar substances with log P values up to 2.4 and a wide range of functionalities (e.g., alcohol, carboxyl, ether, ester, ketone, primary/secondary/tertiary amines, amides, sulfonyls, etc.). These nanostructures are characterized by high hydrophilicity, which derives from the short hydrocarbon chain and double head of the amphiphile. In our opinion, the production of highly hydrophilic SUPRASs can open the door to their effective application in multitarget liquid–liquid extractions, a field not properly covered by conventional organic solvents. Valuable practical considerations or the application of SUPRASs in multiclass extractions are simplicity and high sample throughput. Regarding the application of cubosomic SUPRASs to ITPs in human sport drug testing, the method here reported is simpler, faster, and cheaper than SPE.

Associated Content

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c00082.

Experimental methods; equation for the prediction of SUPRAS volume; schematics of SUPRAS synthesis and drug extraction; correlation between the SUPRAS volume prediction versus experimental data; optical and SEM SUPRAS micrographs; results for extraction optimization; total and extracted ion chromatograms for drugs; physicochemical characteristics, suppliers, and MS/MS parameters for the selected banned drugs; analytical figures of merit; dependences of the SUPRAS volume and chemical composition on synthesis ingredient ratios;

and single and average recoveries and matrix effects for banned substances in 10 urine samples (PDF).

Author Information

Corresponding Author

Ana Ballesteros-Gómez – Department of Analytical Chemistry, Instituto Químico para la Energía y el Medioambiente, Marie Curie Building (Annex), Campus of Rabanales, Universidad de Córdoba, 14071 Córdoba, Spain; orcid.org/0000-0003-1583-1459; Email: ana.ballesteros@uco.es

Authors

Soledad González-Rubio – Department of Analytical Chemistry, Instituto Químico para la Energía y el Medioambiente, Marie Curie Building (Annex), Campus of Rabanales, Universidad de Córdoba, 14071 Córdoba, Spain

Gloria Muñoz – Anti-Doping Control Laboratory, Spanish Agency of Health Protection in Sport (AEPSAD), 28040 Madrid, Spain

Soledad Rubio – Department of Analytical Chemistry, Instituto Químico para la Energía y el Medioambiente, Marie Curie Building (Annex), Campus of Rabanales, Universidad de Córdoba, 14071 Córdoba, Spain; orcid.org/0000-0002-9128-4787

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.2c00082

Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Cubosomic Supramolecular Solvents: Synthesis, Characterization and Potential for High Throughput Multiclass Testing of Banned Substances in Urine

Soledad González-Rubio[1], Ana Ballesteros-Gómez[*1], Gloria Muñoz[2], Soledad Rubio[1]

[1] Department of Analytical Chemistry, Instituto Químico para la Energía y el Medioambiente, Marie Curie Building (Annex), Campus of Rabanales, Universidad de Córdoba, 14071 Córdoba, Spain

[2]Anti-doping control laboratory. Spanish Agency of Health Protection in Sport (AEPSAD). C/ Pintor el Greco S/N, 28040 Madrid, Spain.

[*]Corresponding author: ana.ballesteros@uco.es

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Figure S9. Extracted ion chromatograms obtained from the qualifier transitions for the selected hormones and metabolic modulators from a urine fortified at the MRPL value and unfortified.

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EXPERIMENTAL SECTION

Reagents and Solutions

Tetrahydrofuran (THF) and methanol were provided by Fisher Scientific (Madrid, Spain). Sodium sulphate and formic acid were supplied by Sigma-Aldrich (St. Louis, MO, USA). The amphiphiles 1-Decanol, 1,2-Hexanediol, 1,2-Octanediol, and 1,2-Decanediol were provided by Sigma-Aldrich (St. Louis, MO, USA), 1-Octanol by Panreac (Darmstadt, Germany), and 1- Hexanol by Merck (Madrid, Spain). The enzyme β-glucuronidase from E. coli K12 was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Ultra-high-quality water was generated from a Milli-Q water purification system (Millipore-Sigma, Madrid, Spain). A buffer solution at pH 7 was prepared by dissolving 10.88 g of KH2PO4 and 14.24 g of Na2HPO4 .2H2O in 200 mL of Milli-Q water. The buffer was transferred into a glass bottle and was stored at 2-8ºC.

Samples

The criteria concerning data protection policy and management of biological samples established by the Ethics Committee of Andalusian´s Biomedical Research were followed. Volunteers were asked about personal information, habits and drug consumption. First, spot urine samples from 10 volunteers at the Institute of Chemistry and Nanochemistry (IUNAN, University of Córdoba) were collected and mixed at equal proportion. The resulting pooled human urine sample was used for optimization of the extraction process with SUPRASs. Second, spot urine samples from ten volunteers at the IUNAN were individually used for method validation. All samples were centrifuged (15 min at 1800 g) to remove

solids and sediments and checked for the absence of exogenous prohibited substances. When not immediately analysed, the samples were kept at - 20ºC. Before analysis, samples were subjected to enzymatic hydrolysis. For this purpose, sub-sample aliquots of 1 mL were mixed with 50 µL of the KH2PO4/Na2HPO4 buffer solution and 25 µL of the β-glucuronidase solution. Then, the urine was vortex shaken for 5 min and heated at 55ºC in a water bath for 1 hour. Finally, the urine was stored in a closed glass bottle at 4ºC until its use. S5

LC-MS/MS Analysis of Doping Drugs

The stationary phase was a perfluorophenyl (PFP) column (2.7µm, 100 mm×3.0 mm) from RESTEK (Bellefonte, Pennsylvania, USA), that was kept a 35ºC. The mobile phase consisted of solvent (A) 0.05% formic acid in water and (B) 0.025% formic acid in methanol. The flow rate was 250 µL min-1 and the injection volume was 5 µL. The elution program was as follows: isocratic conditions (95% A, 5% B) for 0.5 min, linear gradient from 5% to 100% of B in 27.5 min, and then isocratic conditions at 100% of B for 2 min. Finally, initial conditions were re-equilibrated for 5 min. Positive and negative ionization modes were used for the selected drugs. The most abundant fragment was used as quantifier ion while the second one served as qualifier. Products ions and detection parameters for each MS/MS transition are shown in Table SI2 and retention times are displayed in Table SI3. Optimal source settings were the following: source gas temperature 400ºC, capillary voltage 4500 V, nebulizer gas pressure 50 psi and curtain gas pressure 20 psi. Quantification was carried out by measuring the chromatographic peak areas of the respective quantifier transitions using the internal standard method. Five deuterated internal standards were used for this purpose. Table SI2 shows the IS used for quantification of each drug.

Method Validation

Selectivity was determined by analysing 10 negative urine samples and checking for any interfering peak in the chromatograms. Extraction recoveries for each urine were determined by analysing samples spiked before extraction with respect to those obtained in SUPRAS spiked after extraction both at the respective MRPLs (Table SI3). Values in samples that were spiked after the extraction process (two-fold the respective MRPLs, considering the concentration factor) corresponded to 100% recovery. In the case of endogenous substances, the background value was considered and it was corrected for method performance evaluation. Matrix effects were evaluated in terms of suppression and enhancement. For this purpose, the relative peak areas ($A_{analyte}/A_{IS}$) of urine samples spiked after SUPRAS extraction (2xMRPL) were compared to those obtained in SUPRAS blanks prepared in distilled water both spiked at 2xMRPL. It was checked that the ISs did not show matrix effects. To determine Method detection limits (MDLs), urine samples were spiked at 1xMRPL, 0.5xMRPL, 0.25xMRPL, and 0.1xMRPL. MDLs were defined as the lowest level at which a compound could be identified with two diagnostic ions present at a signal-tonoise ratio (S/N) greater than 3. Carry-over was evaluated by analysing a blank sample after running a sample that was spiked at 10xMRPL. SUPRAS extracts stability was evaluated during three consecutive days after extraction of four urine samples fortified at the MRPLs, by keeping the samples at 4ºC.

RESULTS AND DISCUSSION

SUPRAS Volume

Multifactorial non-linear regression was used to fit a model to predict the volume of SUPRAS produced in the sub-region of the phase diagram corresponding to 10-20% of amphiphile. For this purpose, 25 SUPRASs were synthesized under different experimental conditions and their volume were measured. The equation obtained was:

VSUPRAS= [(-15.26 [Na2SO4] + 32.52) [1,2-hexanediol] – (-210.84 [Na2SO4] + 273.12)]

where VSUPRAS (μL mL-1) is the dependent variable and the concentrations of Na2SO4 (M) and 1,2-hexanediol (%, v/v) are the independent variables. This equation is valid within the range 0.6- 1.5 M Na2SO4. Its good capability of prediction was confirmed by the coefficient of determination found (R2 = 0.9915) for the plot VSUPRAS observed versus VSUPRAS predicted (Figure SI3). This equation is of interest for application of the SUPRAS to the extraction of banned substances in urine since the preconcentration factor can be known a priori.

Method Validation

The method developed for the screening of doping drugs based on 1,2-hexanediol SUPRAS-LC-ESI-MS/MS was validated in terms of linearity, method detection limits (MDLs), intra-day and inter-day precision, selectivity, recoveries and matrix effects according to the procedures specified in the Experimental Section of Supporting Information. Linearity for each of the 92 doping substances selected was investigated by using the IS method within the interval 0.1xMRPL to 2xMRPL. Table S3 displays the values of the slopes and intercepts for the calibration curves corresponding to each of the doping substances investigated, along with their respective standard deviations. The correlation coefficients of the calibration curves, calculated by linear regression were in the interval 0.980-0.999. Retention times were highly reproducible; their standard deviations for 10 urine

samples were in the range 0.13 to 3.74x10-15 min (Table S3). Figure S7 shows a typical total ion chromatogram (TIC) obtained from the analysis of a urine sample fortified with the selected doping substances at the MRPL values. Method detection limits (LODs) for the drug selected were in the interval 0.001-4.2 ng mL-1 (Table S3). These values were all far below the respective MRPL26 and decision limits values (Table S3). The results found for intra-day and inter-day precision, expressed as relative standard deviation, are also included in Table S3. They varied in the ranges 0.1-17.6% and 1.8-19.1 %, respectively. Selectivity was determined by analyzing 10 negative urine samples and checking for any interfering peak in the chromatograms. Peaks in these chromatograms were compared to those obtained for the same urines spiked at the MRPL values. No interfering peaks were observed for any of the 92 doping substances investigated in the 10 urines analyzed. Figure S8 shows, as an example, the extracted ion chromatograms obtained for some hormones and metabolic modulators from a blank urine, both unfortified (B) and fortified at the MRPL value (A) by measuring the quantifier transitions. Figure S9 shows the extracted ion chromatograms for the same sample but measuring the qualifier transitions. S8 Regarding recoveries, the analysis of 10 urine samples spiked at the MRPL values confirmed the good extraction rates of the 1,2-hexanediol-based SUPRAS. Figure 5A depicts the obtained results and Table S6 shows the specific recovery values obtained for each of the banned substances and urine samples. The percentage of substances within the optimal recovery range (i.e. 70-120%) varied in the interval 82-95% for the ten urines (Figure5A). No substances were found to get very low recoveries (i.e. < 20%), and except for urine sample S1, the percentage of drugs showing recoveries in the range 20-69% was always below 10%. The worst extracted doping drug was ecgonine methyl ester, with recoveries ranging between 34 and 65%. The study of matrix effects (Figure 5B and Table S7) showed that a high percentage of substances were free of interferences. Thus, the relative matrix effects within the range ±20% were in the interval 81-92% for the ten urine samples, which indicates the high capability of the method for the removal of interferences arising from

matrix components. Signal enhancement was observed for around 4-11% of the substances, with enhancement percentages in the interval 22.1-63.1%. Substances showing signal suppression was around 3-10% with suppression percentages in the range 22.3-45.2%. Traces of target drugs were not detected in non-spiked blank urine samples analyzed after the determination of samples spiked with the 92 doping drugs at 10-fold the MRPLs, thus demonstrating the absence of carry-over issues. Finally, it was proved that the selected doping drugs were stable in the SUPRAS extracts at least during three consecutive days at 4ºC.

FIGURES

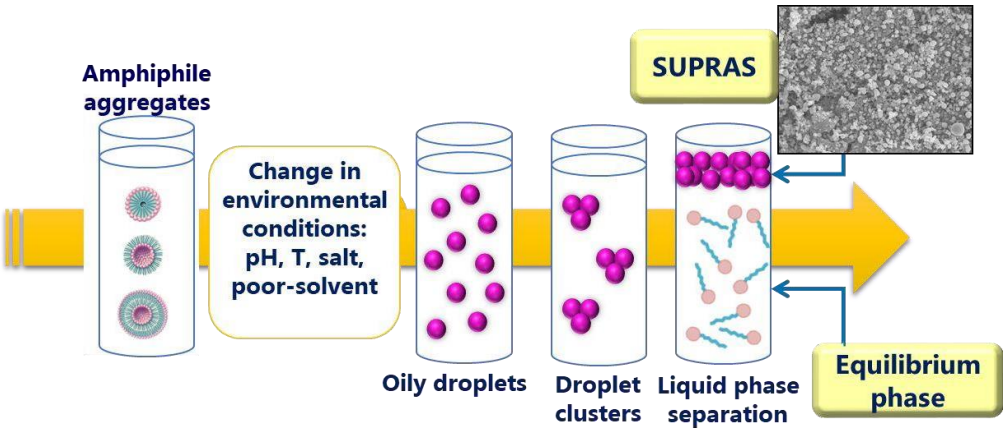


Figure S1. Schematic diagram of the general process for SUPRAS synthesis

Figure S2. Schematic of the extraction of doping drugs with SUPRAS of 1,2-hexanediol

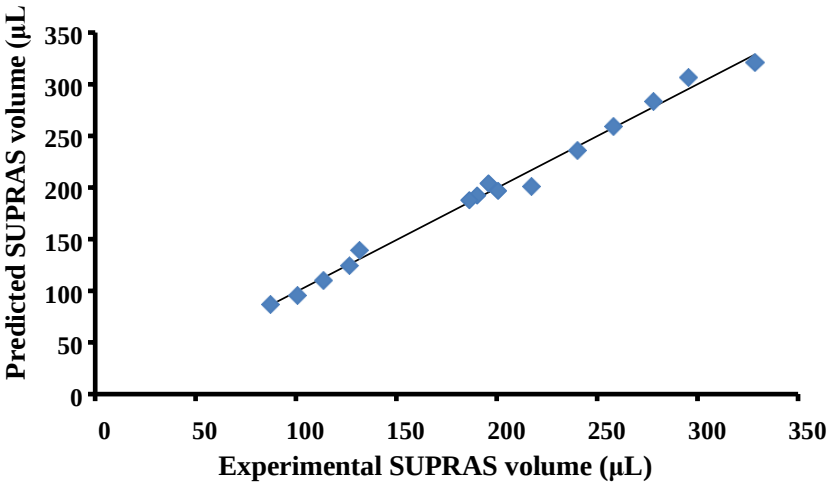


Figure S3. Agreement between the volume of SUPRAS predicted by the proposedequation andthe volume of SUPRAS obtained experimentally using 1 mL of urine.

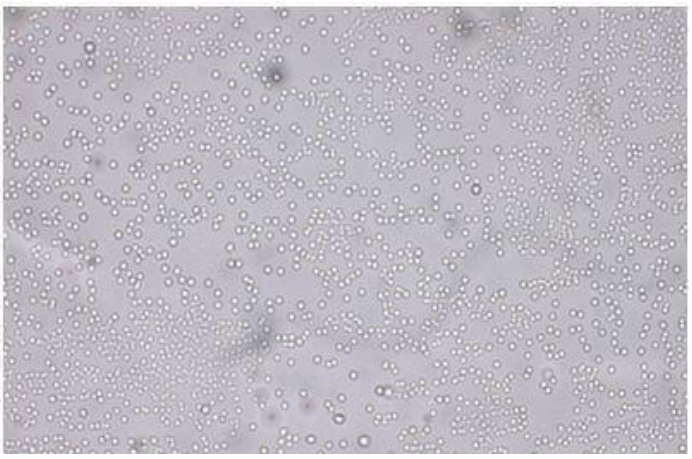


Figure S4. Micrograph obtained by optical microscopy for a SUPRAS producedfrom a mixturecontaining 20% (v/v) of 1,2-hexanediol and 1M Na2SO4.
Magnification: 20X

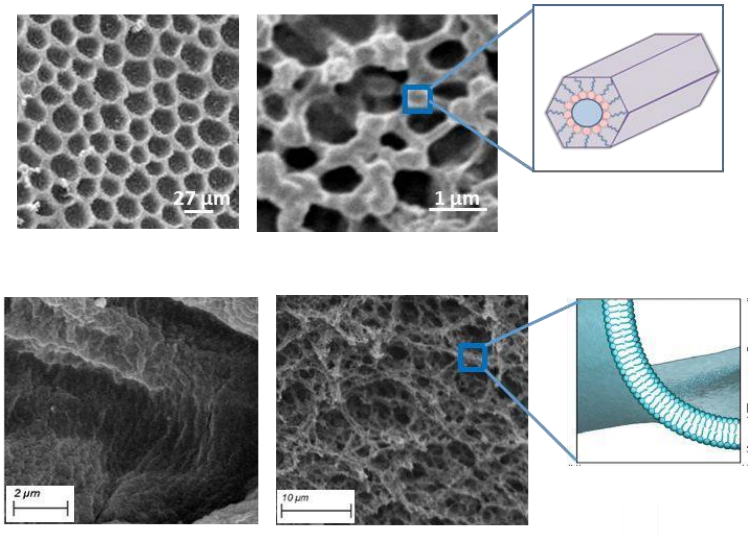


Figure S5. Scanning electron microscopy (SEM) of alkanols (top) and long hydrocarbon chains alkanediols (bottom) along with the schematic of the respective nanostructures, which were inverted hexagonal and sponge aggregates, respectively.

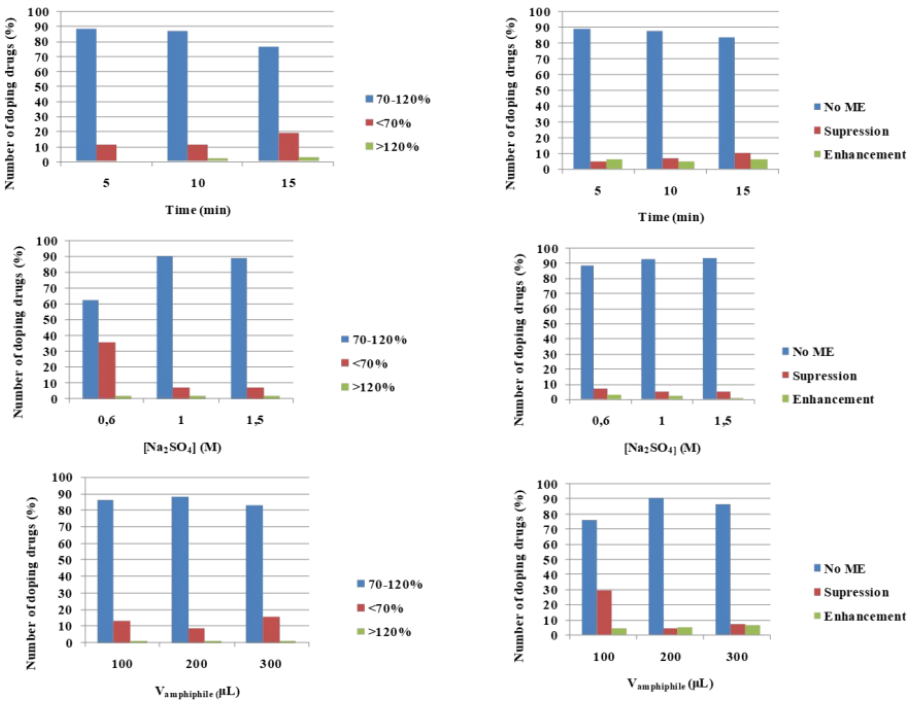


Figure S6. Recoveries (left) and matrix effects (right) obtained for the extraction of the 92prohibited substances (Table SI1) as a function of different experimental variables.

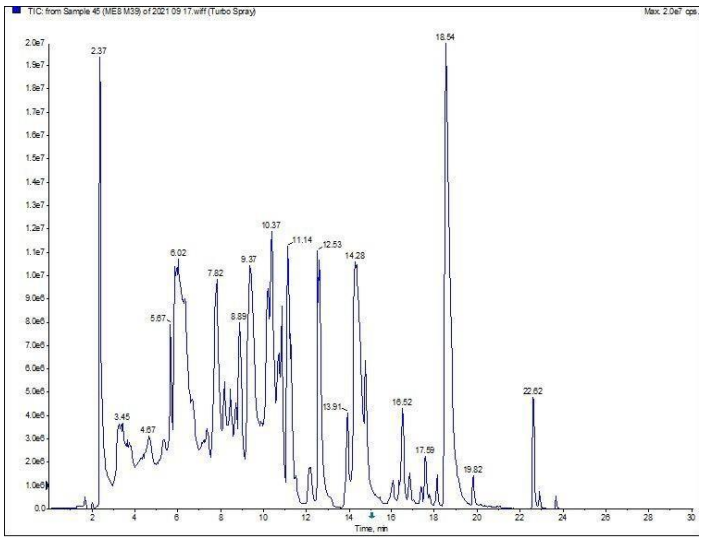


Figure S7. Total ion chromatogram (TIC) obtained for a urine sample fortified with the 92banned substances selected at the respective MRPL values

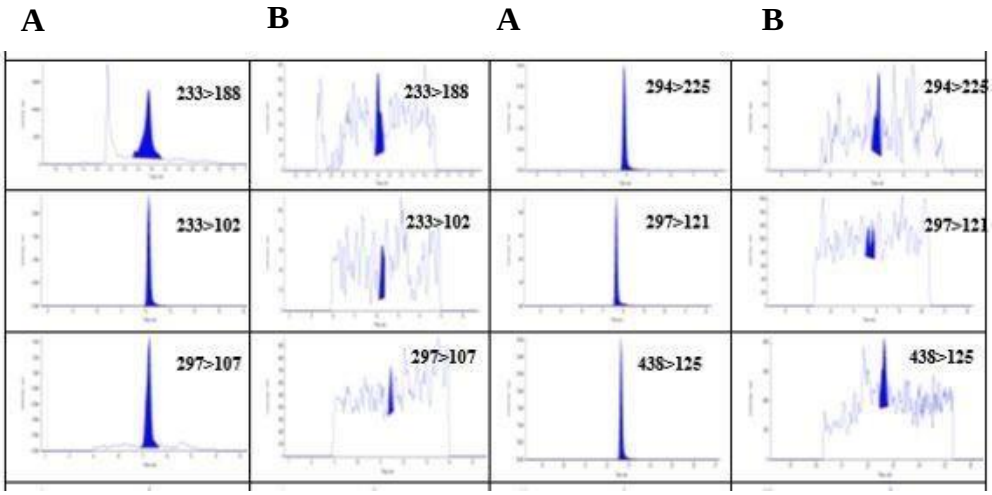


Figure S8. Extracted ion chromatograms obtained for some hormones and metabolic modulators from a urine (A) fortified at the MRPL value and (B) unfortified. Quantifier transitions for the doping drugs were: Aminoglutethimide (233→188), Anastrozole (294→225), Bis-(4- cyanophenyl) methanol (233→102), Exemestane (297→121), 4-OH-cyclofenil (297→107), SR9009 (438→125).

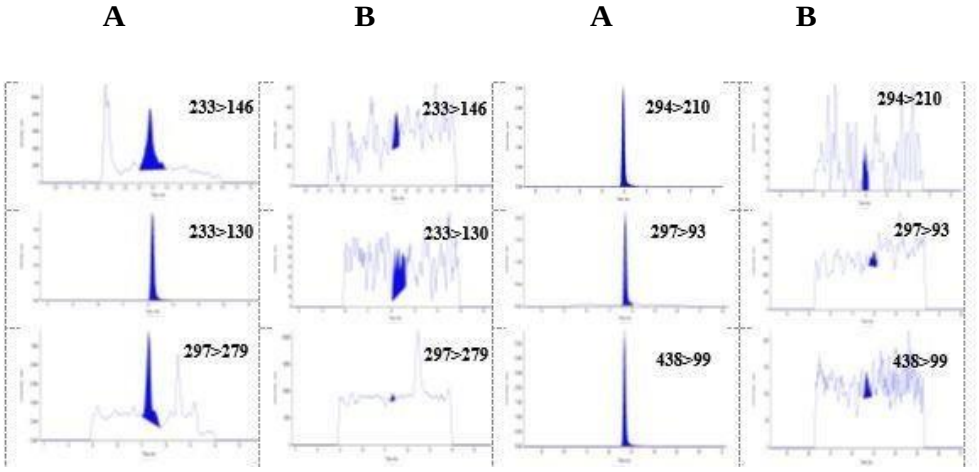
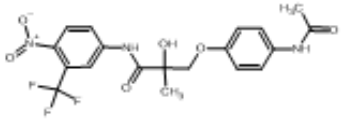
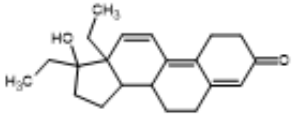
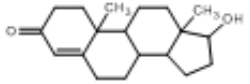
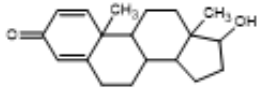
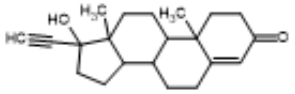
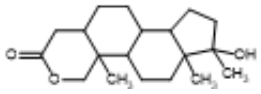
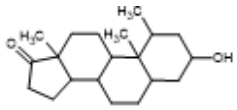
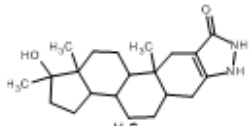
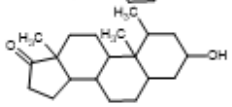
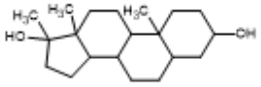
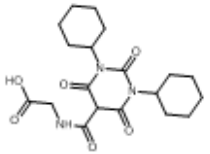
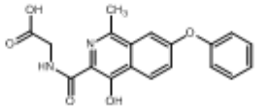
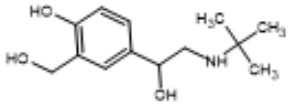
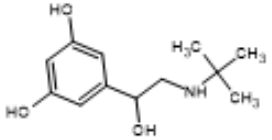
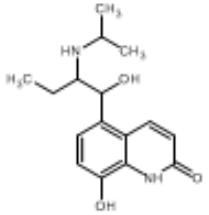
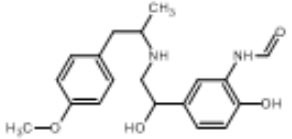
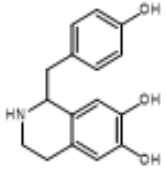
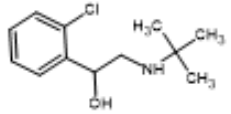
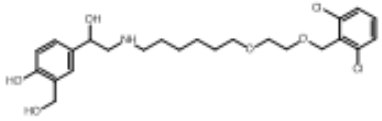


Figure S9. Extracted ion chromatograms obtained for some hormones and metabolic modulatorsfrom a urine (A) fortified at the MRPL value and (B) unfortified. Qualifier transitions for the doping drugs were: Aminoglutethimide (233→146), Anastrozole (294→210), Bis-(4- cyanophenyl) methanol (233→130), Exemestane (297→93), 4- OH-cyclofenil (297→279), SR9009 (438→99).

Table S1. Chemical structure and molecular formula for the selected WADA´s prohibited substances or metabolites, ranked by WADA categories (S1-S9, P1), as well as different parameters of interest for their extraction behaviour.

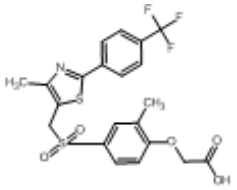
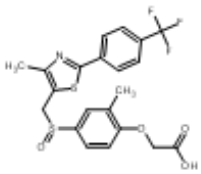
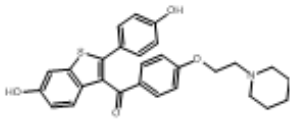
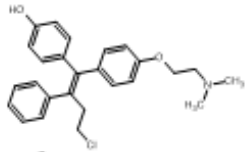
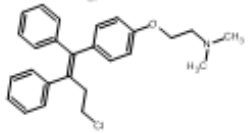
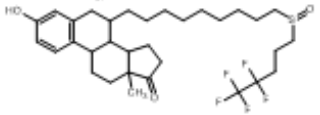
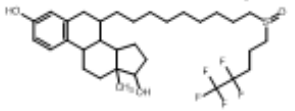
Selected WADA´s prohibited substances or their metabolites (n=92)	Chemical structure	Molecular formula	[a]Log P	[a,b]pKa (acid)	[a,b]pKa (basic)	[a]H-bond donors	[a]H-bond acceptors	[a]Number of aromatic rings
Anabolic agents (S1)								
Andarine		$C_{19}H_{18}F_3N_3O_6$	2.2	11,2	-4	3	9	2
Tetrahydrogestrinone		$C_{21}H_{28}O_2$	3.0	18,9		1	2	0
Testosterone		$C_{19}H_{28}O_2$	3.3	18,5		1	2	0
Boldenone		$C_{19}H_{26}O_2$	3.5	18,9		1	2	0
4-Androsten-17α-ethynyl-17β-ol-3-one (Metabolite of Danazol)		$C_{21}H_{28}O_2$	3.5	n/a	-	1	2	0
Oxandrolone		$C_{19}H_{30}O_3$	3.7	15,1	-	1	3	0

1-methylene-5α-androstan-3α-ol-17-one (Metabolite of Metenolone)		$C_{20}H_{30}O_2$	3.8	n/a	-	1	2	0
3-OH-stanozolol (Metabolite of Stanozolol)		$C_{21}H_{32}N_2O_2$	4.0	11,5	2,3	3	3	0
1α-methyl-5α-androstan-3α-ol-17-one (Metabolite of Mesterolone)		$C_{20}H_{32}O_2$	4.1	n/a	-	1	2	0
17α-methyl-5α-androstane-3α-17β-diol (Metabolite of Methyltestosterone)		$C_{20}H_{34}O_2$	4.3	n/a	-	2	2	0
Hypoxia-inducible factor (HIF) activating agents (S2)								
Daprodustat		$C_{19}H_{27}N_3O_6$	2.5	0,35	-9,1	2	6	0
Roxadustat		$C_{19}H_{16}N_2O_5$	3.4	2,8	3,8	3	6	3
Beta-2 Agonists (S3)								

Salbutamol		$C_{13}H_{21}NO_3$	0.3	10,3	9,4	4	4	1
Terbutaline		$C_{12}H_{19}NO_3$	0.9	8,9	9,7	4	4	1
Procaterol		$C_{16}H_{22}N_2O_3$	1.5	8,5	9,9	4	4	1
Formoterol		$C_{19}H_{24}N_2O_4$	1.8	8,6	9,8	4	5	2
Higenamine		$C_{16}H_{17}NO_3$	2.2	8,7	9,6	4	4	2
Tulobuterol		$C_{12}H_{18}ClNO$	2.3	13,9	9,6	2	2	1
Vilanterol		$C_{24}H_{33}Cl_2NO_5$	3.8	10,1	9,4	4	6	2

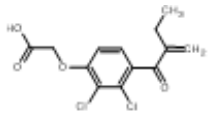
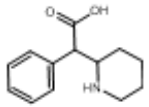
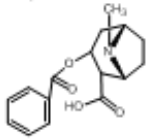
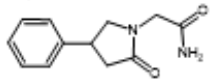
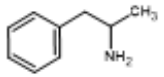
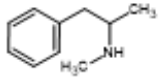
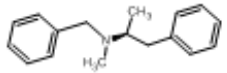
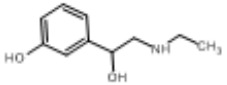
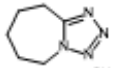
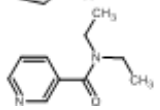
Hormone and metabolic modulators (S4)

Aminoglutethimide		$C_{13}H_{16}N_2O_2$	1.2	11,7	4,3	2	3	1
Anastrozole		$C_{17}H_{19}N_5$	2.1	-	2	0	4	2
Bis-(4-cyanophenyl) metanol (Metabolite of Letrozole)		$C_{15}H_{10}N_2O$	2.2	12,8	-	1	3	2
Exemestane		$C_{20}H_{24}O_2$	3.1	20		0	2	1
4-OH-cyclofenil (Metabolite of Cyclofenil)		$C_{19}H_{20}O_3$	3.8	n/a	-	3	3	2
SR9009		$C_{20}H_{24}ClN_3O_4S$	4.4	-	6,1	0	6	2

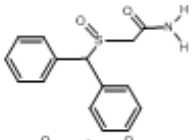
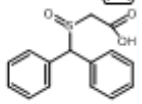
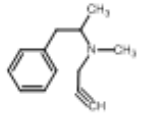
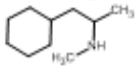
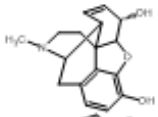
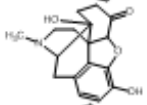
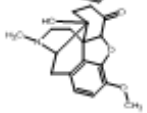
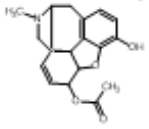
GW501516 Sulfone (Metabolite of GW501516)		$C_{21}H_{18}F_3NO_5S_2$	4.6	3	-	1	10	3
GW501516 Sulfoxide (Metabolite of GW501516)		$C_{21}H_{18}F_3NO_4S_2$	4.6	3	-	1	10	3
Raloxifene		$C_{28}H_{27}NO_4S$	6.1	8,9	7,9	2	6	3
4-OH-toremifene (Metabolite of Toremifene)		$C_{26}H_{28}ClNO_2$	6.8	n/a	n/a	1	3	3
Toremifene		$C_{26}H_{28}ClNO$	7.2	-	8,8	0	2	3
17-ketone fulvestrant (Metabolite of Fulvestrant)		$C_{32}H_{45}F_5O_3S$	7.5	n/a	-	-	-	1
Fulvestrant		$C_{32}H_{47}F_5O_3S$	9.2	10,3		2	9	1

Diuretic and masking agents (S5)

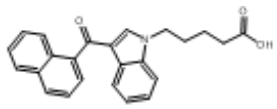
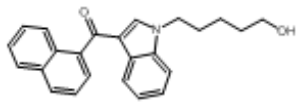
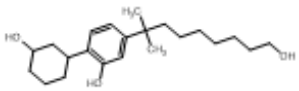
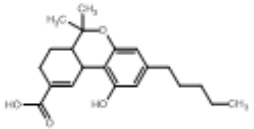
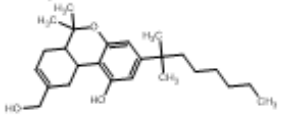
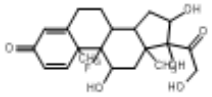
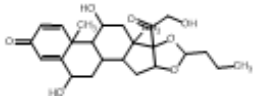
Acetazolamide		$C_4H_6N_4O_3S_2$	-0.3	-	7,2	2	7	1
Chlorothiazide		$C_7H_6ClN_3O_4S_2$	-0.2	-	6,9	2	6	1
Chlortalidone		$C_{14}H_{11}ClN_2O_4S$	0.9	-	9,4	3	5	2
Triamterene		$C_{12}H_{11}N_7$	1.0	-	6,2	3	7	2
Bendroflumethiazide		$C_{15}H_{14}F_3N_3O_4S_2$	1.2	-	8,5	3	10	1
Quinethazone		$C_{10}H_{12}ClN_3O_3S$	1.2	-	9,3	3	5	1
Furosemide		$C_{12}H_{11}ClN_2O_5S$	2.0	3,5	-1,5	3	7	2
Bumetanide		$C_{17}H_{20}N_2O_5S$	2.8	3,6	7,7	3	7	2
Indapamide		$C_{16}H_{16}ClN_3O_3S$	2.9	-	8,8	2	5	2

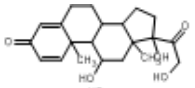
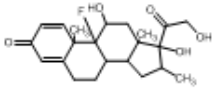
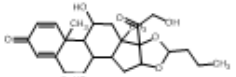
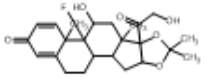
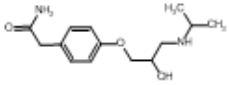
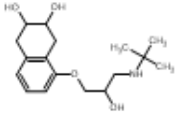
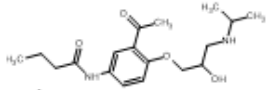
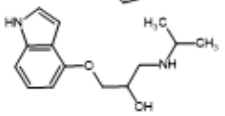
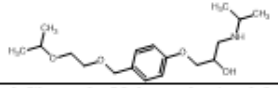
Etacrynic acid		$C_{13}H_{12}Cl_2O_4$	3.8	3,5	-5	1	4	1
Stimulants (S6)								
Ritalinic acid (Metabolite of Methylphenidate)		$C_{13}H_{17}NO_2$	-2.4	3,7	10,1	2	3	1
Benzoylecgonine (Metabolite of Cocaine)		$C_{16}H_{19}NO_4$	-0.3	3,2	9,5	1	5	1
Carphedon		$C_{12}H_{14}N_2O_2$	0.1	-	-2	1	2	1
Amphetamine		$C_9H_{13}N$	1.8	-	9,9	1	1	1
Methamphetamine		$C_{10}H_{15}N$	2.1	-	9,9	1	1	1
Benzphetamine		$C_{17}H_{21}N$	4.1	-	8,8	0	1	2
Etilefrine		$C_{10}H_{15}NO_2$	0.1	9,1	9,7	3	3	1
Pentetrazol		$C_6H_{10}N_4$	0.1	-	-0,5	0	3	1
Nikethamide		$C_{10}H_{14}N_2O$	0.3	-	3,6	0	2	1

Ecgoninemethylester (Metabolite of Cocaine)		$C_{10}H_{17}NO_3$	0.6	14,6	9	1	4	0
Cathine		$C_9H_{13}NO$	0.8	13,9	9,4	2	2	1
Cathinone		$C_9H_{11}NO$	1.1	18,6	7,5	1	2	1
Methylephedrine		$C_{11}H_{17}NO$	1.7	13,9	9,3	1	2	1
Methylhexaneamine		$C_7H_{17}N$	1.9	-	10,6	1	1	0
Phendimetrazine		$C_{12}H_{17}NO$	1.9	-	7,3	0	2	1
Strychnine		$C_{21}H_{22}N_2O_2$	1.9	-	8,3	0	3	1
Cocaine		$C_{17}H_{21}NO_4$	2.3	-	8,6	0	5	1
N-Desmethylselegiline (Metabolite de Selegiline)		$C_{12}H_{15}N$	2.3	-	n/a	1	1	1

Modafinil		$C_{15}H_{15}NO_2S$	0.6	-	-6.9	1	3	2
Diphenylmethyl (sulfinyl)-acetic acid (Metabolite of Modafinil)		$C_{15}H_{14}O_3S$	2.7	4,1	-	1	4	2
Selegiline		$C_{13}H_{17}N$	2.8	-	8,7	0	1	1
Propylhexedrine		$C_{10}H_{21}N$	3.5	-	10,6	1	1	0
Narcotics (S7)								
Morphine		$C_{17}H_{19}NO_3$	0.8	10,2	8,2	2	4	1
Oxymorphone		$C_{17}H_{19}NO_4$	0.8	10,1	8,2	2	5	1
Oxycodone		$C_{18}H_{21}NO_4$	1.2	13,6	8,7	1	5	1
6-Acetylmorphine (Metabolite of Diamorphine or heroin)		$C_{19}H_{21}NO_4$	1.3	10,2	9	1	5	0

Norfentanyl		$C_{14}H_{20}N_2O$	1.6	-	10	1	2	1
Hydromorphone		$C_{17}H_{19}NO_3$	1.8	10,1	8,6	1	4	1
Pethidine		$C_{15}H_{21}NO_2$	2.5	-	8,7	0	3	1
Pentazocine		$C_{19}H_{27}NO$	3.3	7,6	12,4	1	2	0
Norbuprenorphine (Metabolite of Buprenorphine)		$C_{24}H_{33}NO_4$	3.8	9,8	10,5	3	5	0
Methadone		$C_{21}H_{27}NO$	3.9	-	9,2	0	2	2
Fentanyl		$C_{22}H_{28}N_2O$	4.0	-	9	0	2	2
Buprenorphine		$C_{29}H_{41}NO_4$	5.0	7,5	12,5	2	5	0
Cannabinoids (S8)								

JWH-018 5-pentanoic acid metabolite (Metabolite of JWH-018)		$C_{24}H_{21}NO_3$	4.7	n/a	n/a	1	3	2
JWH-018 N-(5-hydroxy-pentyl) metabolite (Metabolite of JWH-018)		$C_{24}H_{23}NO_2$	4.9	n/a	n/a	1	2	2
CP 47, 497-C8-hydroxy, C8-homolog (Metabolite of CP 47, 497-C8-homolog)		$C_{22}H_{36}O_3$	5.5	-	-	3	3	1
11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid		$C_{21}H_{28}O_4$	6.3	4,2	-	2	4	1
HU-210		$C_{25}H_{38}O_3$	6.4	9,7	-	2	3	1
Glucocorticoids (S9)								
Triamcinolone		$C_{21}H_{27}FO_6$	1.2	13,4	-	4	7	1
6-β-OH-budesonide (Metabolite of Budesonide)		$C_{25}H_{34}O_7$	1.3	13,7	-	3	7	1

Prednisolone		$C_{21}H_{28}O_5$	1.6	12,6	-	3	5	1
Betamethasone		$C_{22}H_{29}FO_5$	1.8	13,4	-	3	6	1
Budesonide		$C_{25}H_{34}O_6$	2.5	13,7	-	2	6	1
Triamcinolone acetonide		$C_{24}H_{31}FO_6$	2.5	13,4	-	2	7	1
Beta-blockers (P1)								
Atenolol		$C_{14}H_{22}N_2O_3$	0.2	14,1	9,7	3	4	1
Nadolol		$C_{17}H_{27}NO_4$	0.7	13,6	9,8	4	5	1
Acebutolol		$C_{18}H_{28}N_2O_4$	1.7	13,9	9,6	3	5	1
Pindolol		$C_{14}H_{20}N_2O_2$	1.8	14,1	9,7	3	3	1
Bisoprolol		$C_{18}H_{31}NO_4$	1.9	14,1	9,7	2	5	1

a: Values obtained from PubChem; b: Values obtained from DrugBank; n/a: non available values

Table S2. Suppliers for the selected prohibited substances, internal standards used for calibration and MS parameters.

Selected prohibited substances, metabolites or markers	Supplier	Internal Standard used for quantification	ESI Polarity	Precursor ion	Fragment ion		[a]DP (V)	[b]CE (V)	[c]CXP (V)
					Quantifier	Qualifier			
Anabolic agents (S1)									
Andarine	TRC	Raloxifene-d4	-	440	261	150	-40;-40	-30;-40	-3;-3
Tetrahydrogestrinone	NMI	Raloxifene-d4	+	313	241	295	30;30	30;30	9;9
Testosterone	LGC	Raloxifene-d4	+	289	97	109	71;71	29;31	14;12
Boldenone	LGC	Cathine-d3	+	287	121	91	30;30	32;44	9;9
4-Androsten-17α-ethynyl-17β-ol-3-one	Steraloids	Raloxifene-d4	+	313	97	109	126;126	29;35	10;12
Oxandrolone	Dr. Ehrenstorfer	Raloxifene-d4	+	307	289	77	141;141	15;87	26;12
1-methylene-5α-androstan-3α-ol-17-one	NMI	Cathine-d3	+	303	267	91	56;56	15;63	30;10
3-OH-stanozolol	NMI	Raloxifene-d4	+	345	97	165	71;71	55;57	14;18
1α-methyl-5α-androstan-3α-ol-17-one	NMI	Raloxifene-d4	+	305	287	91	71;71	13;73	12;14
17α-methyl-5α-androstane-3α-17β-diol	NMI	Raloxifene-d4	+	307	197	57	66;66	31;61	10;8
Hypoxia-inducible factor (HIF) activating agents (S2)									
Daprodustat	TRC	Raloxifene-d4	-	392	291	122	-105;-50	-28;-40	-13;-16
Roxadustat	TRC	Raloxifene-d4	+	353	278	222	51;51	27;45	30;10

Beta-2 Agonists (S3)											
Salbutamol	LGC	Oxycodone-d3	+	240	148	222	86;86	27;15	16;24		
Terbutaline	LGC	Oxycodone-d3	+	226	152	107	30;30	21;41	12;12		
Procaterol	Sigma-Aldrich	Raloxifene-d4	+	291	273	231	30;30	19;27	12;12		
Formoterol	NMI	Raloxifene-d4	+	345	149	327	30;30	30;5	12;12		
Higenamine	Chromadex	Raloxifene-d4	+	227	161	107	30;30	27;31	12;12		
Tulobuterol	TRC	Raloxifene-d4	+	228	154	119	30;30	23;39	12;12		
Vilanterol	TRC	Raloxifene-d4	+	486	468	450	30;30	23;27	12;12		
Hormone and metabolic modulators (S4)											
Aminoglutethimide	LGC	Cathine-d3	+	233	188	146	81;81	21;29	18;20		
Anastrozole	TRC	Raloxifene-d4	+	294	225	210	30;30	33;47	12;12		
Bis-(4-cyanophenyl) methanol	NMI	Raloxifene-d4	-	233	102	130	-140;-140	-26;-20	-11;-11		
Exemestane	TRC	Raloxifene-d4	+	297	121	93	30;30	25;43	12;12		
4-OH-cyclofenil	NMI	Raloxifene-d4	+	297	107	279	46;46	25;13	10;12		
SR9009	Caymanchemicals	Raloxifene-d4	+	438	125	99	30;30	19;117	12;12		
GW501516 Sulfone	TRC	Raloxifene-d4	+	486	257	188	30;20	40;60	12;12		
GW501516 Sulfoxide	TRC	Raloxifene-d4	+	470	257	188	20;40	20;60	12;12		
Raloxifene	LGC	Raloxifene-d4	+	474	112	84	30;30	41;83	12;12		
4-OH-toremifene	TRC	Raloxifene-d4	+	422	72	301	30;30	35;40	10;10		
Toremifene	TRC	Raloxifene-d4	+	406	72	43	30;30	23;36	9;9		
17-ketone fulvestrant	TRC	Raloxifene-d4	+	605	587	491	30;30	25;30	9;9		

Fulvestrant	LGC	Raloxifene-d4	+	607	589	467	30;30	25;37	12;12
Diuretic and masking agents (S5)									
Acetazolamide	Pharmacopeia USP	Oxycodone-d3	+	223	181	164	26;26	21;29	18;16
Clorothiazide	DrEherestofer	Raloxifene-d4	-	294	179	115	-56;-56	-66;-78	-17;-11
Chlortalidone	Pharrmacopeia USP	Raloxifene-d4	-	337	190	146	-25;-30	-20;-22	-9;-35
Triamterene	LGC	Cathine-d3	+	254	237	195	30;30	35;35	9;9
Bendroflumethiazide	LGC	Raloxifene-d4	-	420	289	328	-50;-50	-40;-40	-16;-16
Quinethazone	LGC	Raloxifene-d4	-	288	245	78	-130;-130	-26;-64	-19;-9
Furosemide	LGC	Raloxifene-d4	-	329	285	205	-70;-70	-22;-30	-23;-15
Bumetanide	Pharmacopeia USP	Raloxifene-d4	-	363	80	238	-46;-46	-44;-26	-5;-23
Indapamide	LGC	Raloxifene-d4	-	364	189	132	-96;-96	-38;-36	-11;-9
Etacrynicacid	Pharmacopeia USP	Raloxifene-d4	-	301	243	207	-5;-50	-40;-40	-16;-16
Stimulants (S6)									
Ritalinicacid	LGC	Raloxifene-d4	+	220	84	56	30;30	30;60	12;12
Benzoylecgonine	LGC	Raloxifene-d4	+	290	168	105	30;30	29;55	12;12
Carphedon	NMI	Raloxifene-d4	+	219	174	129	30;30	15;25	10;10
Amphetamine	LGC	Oxycodone-d3	+	136	91	119	11;11	25;13	10;14
Methamphetamine	LGC	Raloxifene-d4	+	150	91	119	66;66	27;15	8;14
Benzphetamine	TRC	Raloxifene-d4	+	240	91	65	66;66	3779	12;10

Etilefrine	Pharmacopeia	Betamethasone-d5	+	182	164	91	61;61	17;37	16;10	
Pentetrazol	LGC	Raloxifene-d4	+	139	96	55	30;30	13;26	10;10	
Nikethamide	LGC	Raloxifene-d4	+	179	108	72	30;30	18;26	9;9	
Ecgoninemethylester	LGC	Salbutamol-d3	+	200	182	150	30;30	20;25	9;9	
Cathine	Cerilliant	Oxycodone-d3	+	152	117	115	10;10	23;23	9;9	
Cathinone	Cerilliant	Betamethasone-d5	+	150	117	89	30;30	36;61	10;12	
Methylephedrine	LGC	Betamethasone-d5	+	180	162	91	86;86	19;45	14;10	
Methylhexaneamine	Sigma	Raloxifene-d4	+	116	57	99	41;41	17;9	12;10	
Phendimetrazine	Cerilliant	Raloxifene-d4	+	192	146	91	41;41	37;45	9;9	
Strychnine	Sigma	Raloxifene-d4	+	335	184	194	30;30	63;65	10;10	
Cocaine	LGC	Raloxifene-d4	+	304	150	154	30;30	25;35	12;12	
N-Desmethylselegiline	LGC	Raloxifene-d4	+	174	91	119	31;31	33;15	10;14	
Diphenylmethyl (sulfinyl)-aceticacid	TRC	Raloxifene-d4	-	273	167	165	-105;-105	-28;-56	-17;-21	
Modafinil	TRC	Raloxifene-d4	+	274	167	165	36;36	23;59	14;20	
Selegiline	Cerilliant	Raloxifene-d4	+	188	91	119	30;30	21;15	10;10	
Propylhexedrine	LGC	Raloxifene-d4	+	156	69	83	30;30	20;30	9;9	
Narcotics (S7)										
Morphine	LGC	Salbutamol-d3	+	286	152	165	101;101	83;65	10;10	
Oxymorphone	LGC	Oxycodone-d3	+	302	227	242	30;30	28;26	12;12	
Oxycodone	LGC	Oxycodone-d3	+	316	241	256	30;30	28;24	9;9	
6-Acetylmorphine	LGC	Oxycodone-d3	+	328	165	211	30;30	37;25	9;9	
Norfentanyl	Cerilliant	Raloxifene-d4	+	233	84	55	66;66	25;51	10;8	
Hydromorphone	Cerilliant	Oxycodone-d3	+	286	185	153	30;30	30;40	9;9	

Pethidine	Cerilliant	Raloxifene-d4	+	248	220	174	81;81	29;29	22;16
Pentazocine	Cerilliant	Raloxifene-d4	+	286	69	218	30;30	26;19	9;9
Norbuprenorphine	Cerilliant	Raloxifene-d4	+	414	55	152	96;96	97;129	8;12
Methadone	Cerilliant	Raloxifene-d4	+	310	265	105	66;66	21;35	24;10
Fentanyl	LGC	Raloxifene-d4	+	337	105	188	30;30	36;22	9;9
Buprenorphine	Cerilliant	Raloxifene-d4	+	486	55	396	66;66	99;55	10;12
Cannabinoids (S8)									
JWH-018 5-pentanoic acidmetabolite	Cerilliant	Raloxifene-d4	+	372	155	127	30;30	33;77	12;12
JWH-018 N-(5-hydroxy-pentyl) metabolite	Cerilliant	Raloxifene-d4	+	358	155	127	130;130	53;33	9;9
CP 47, 497-C8-hydroxy, C8-homolog	CaymanChemicals	Raloxifene-d4	-	331	313	259	-220;-220	-38;-48	-31;-23
11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid	Cerilliant	Raloxifene-d4	+	345	327	299	136;136	23;27	6;22
HU-210	CaymanChemicals	Raloxifene-d4	-	385	363	301	-205;-205	-40;-48	-15;-27
Glucocorticoids (S9)									
Triamcinolone	LGC	Raloxifene-d4	-	393	345	363	-160;-160	-24;-14	-15;-13
6-β-OH-budesonide	TRC	Raloxifene-d4	+	447	77	121	61;61	121;59	12;14
Prednisolone	LGC	Raloxifene-d4	-	405	329	280	-30;-30	-35;-39	-19;-17
Betamethasone	Dr. Ehrenstorfer	Raloxifene-d4	+	393	355	337	30;30	20;20	9;9
Budesonide	Sigma	Raloxifene-d4	+	431	413	147	91;91	17;37	20;20
Triamcinoloneacetonide	LGC	Raloxifene-d4	+	435	213	321	30;30	30;30	9;9

Beta-blockers (P1)										
Atenolol	LGC	Oxycodone-d3	+	267	145	56	96;96	37;45	14;14	
Nadolol	LGC	Raloxifene-d4	+	310	254	201	30;30	25;33	12;12	
Acebutolol	LGC	Raloxifene-d4	+	337	116	74	30;30	31;45	12;12	
Pindolol	LGC	Raloxifene-d4	+	249	116	172	30;30	25;25	12;12	
Bisoprolol	Sigma	Betamethasone-d5	+	326	116	74	30;30	27;43	12;12	
Internal standards										
Salbutamol-d3	LGC		+	243	151	124	30;30	25;28	9;9	
Cathine-d3	LGC		+	155	137	119	46;46	15;23	14;12	
Oxycodone-d3	LGC		+	319	301	244	126;126	27;41	14;22	
Betamethanose-d5	TRC		+	398	360	342	30;30	20;20	9;9	
Raloxifene-d4	TRC		+	478	116	84	46;46	41;107	14;6	

[a] DP: declustering potential; [b] CE: Collision energy; [c] CXP: Collision cell exit potential. The entrance potential (EP) was 10 and -10 V for substances analysed in positive and negative mode, respectively.

Table S3. Retention times (n=10) for the selected prohibited substances, calibration parameters, method detection limits (MDL), WADA approved decision limits (DL) and minimum required performance levels (MRPL), and intra-day and inter-day precision.

Selected WADA´s prohibited substances or their metabolites (n=92)	Retention time (SD) (min)	Slope ± SD (ng mL^{-1})	Intercept ± SD (ng mL^{-1})	r^2	MDL (ng mL^{-1})	[a]DL or MRPL (ng mL^{-1})	Intra-day precision (RSD, %)	Inter-day precision (RSD, %)
Anabolic agents (S1)								
Andarine	16.10 (3.74x10^{-15})	0.350±0.007	-0.005±0.004	0.999	0.001	2.5	8.0	13.2
Tetrahydrogestrinone	18.10 (4.80x10^{-2})	0.09±0.01	0.004±0.003	0.993	0.06	2.5	1.7	11.0
Testosterone	16.90 (3.16x10^{-2})	0.247±0.003	0.012±0.002	0.999	0.05	2.5	3.0	14.6
Boldenone	16.10 (3.74x10^{-15})	0.512±0.007	0.005±0.004	0.999	0.07	2.5	4.3	8.4
4-Androsten-17α-ethynyl-17β-ol-3-one (Metabolite of Danazol)	17.10 (4.21x10^{-2})	0.069±0.004	0.016±0.003	0.991	0.2	2.5	2.8	10.1
Oxandrolone	15.92 (1.87x10^{-15})	0.112±0.001	-0.002±0.001	0.999	0.1	2.5	0.9	16.7
1-methylene-5α-androstan-3α-ol-17-one (Metabolite of Metenolone)	17.60 (3.16x10^{-2})	0.035±0.006	0.016±0.004	0.970	0.5	2.5	10.0	14.8
3-OH-stanozolol (Metabolite of Stanozolol)	16.70 (3.16x10^{-2})	0.186±0.007	0.008±0.004	0.997	0.03	1	5.1	12.3
1α-methyl-5α-androstan-3α-ol-17-one (Metabolite of Mesterolone)	18.50 (3.74x10^{-15})	0.052±0.002	0.009±0.001	0.998	0.2	2.5	1.5	18.8

17α-methyl-5α-androstane-3α-17β-diol (Metabolite of Methyltestosterone)	23.70 ($3.74x10^{-15}$)	0.002±0.0005	0.0101±0.0005	0.900	0.05	2.5	0.1	10.6
HIF Activating Agents (S2)								
Daprodustat	20.42 ($3.74x10^{-15}$)	0.033±0.001	-0.0006±0.0004	0.999	0.005	2	3.3	15.2
Roxadustat	19.20 ($3.74x10^{-15}$)	0.156±0.004	0.0004±0.0021	0.998	0.01	2	4.0	17.1
Beta-2 Agonists (S3)								
Salbutamol	2.37 ($2.04x10^{-2}$)	0.319±0.003	-0.002±0.001	0.998	0.4	1200[a]	0.3	9.9
Terbutaline	3.42 ($1.39x10^{-2}$)	0.182±0.004	-0.004±0.002	0.999	0.7	20	0.8	8.9
Procaterol	6.31 ($3.34x10^{-2}$)	0.26±0.02	0.02±0.01	0.987	0.5	20	2.3	13.3
Formoterol	10.20 ($5.16x10^{-2}$)	5.9±0.1	-0.03±0.06	0.999	0.01	50[a]	1.1	18.0
Higenamine	4.43 ($1.03x10^{-2}$)	0.033±0.005	0.004±0.003	0.970	3.2	10	1.0	13.4
Tulobuterol	10.70 ($5.16x10^{-2}$)	8.2±0.1	-0.09±0.06	0.999	0.007	20	3.0	9.1
Vilanterol	16.40 ($3.74x10^{-15}$)	0.689±0.009	-0.011±0.005	0.999	0.05	10	1.0	13.7
Hormone and metabolic modulators (S4)								
Aminoglutethimide	3.89 ($2.07x10^{-2}$)	0.123±0.005	0.0005±0.0026	0.997	0.5	20	2.1	12.7

Anastrozole	13.90 ($1.87x10^{-15}$)	3.8±0.1	-0.02±0.05	0.998	0.003	20	0.7	10.7
Bis-(4-cyanophenyl) metanol (Metabolite of Letrozole)	12.10 ($1.87x10^{-15}$)	0.43±0.01	0.001±0.007	0.998	0.01	20	4.4	14.3
Exemestane	17.70 ($3.16x10^{-2}$)	0.669±0.004	-0.005±0.002	0.999	0.06	20	1.0	13.6
4-OH-cyclofenil (Metabolite of Cyclofenil)	11.30 ($3.16x10^{-2}$)	0.441±0.008	-0.002±0.005	0.999	0.08	20	1.3	14.4
SR9009	22.60 ($3.16x10^{-2}$)	4.71±0.07	-0.04±0.04	0.999	0.005	20	0.9	9.3
GW501516 Sulfone(Metabolite of GW501516)	18.60 ($3.16x10^{-2}$)	3.34±0.07	-0.02±0.04	0.999	0.02	2	2.8	13.1
GW501516 Sulfoxide (Metabolite of GW501516)	18.20 ($5.16x10^{-2}$)	1.15±0.02	-0.014±0.008	0.999	0.02	2	3.8	11.8
Raloxifene	16.60 ($3.16x10^{-2}$)	1.79±0.03	-0.03±0.02	0.999	0.01	20	1.7	10.7
4-OH-toremifene (Metabolite of Toremifene)	17.60 ($3.74x10^{-15}$)	2.29±0.04	-0.02±0.023	0.999	0.002	20	3.0	10.2
Toremifene	19.90 ($3.16x10^{-2}$)	1.42±0.02	-0.02±0.02	0.999	0.005	20	0.1	16.0
17-ketone fulvestrant (Metabolite of Fulvestrant)	23.70 ($3.74x10^{-15}$)	0.405±0.005	-0.006±0.003	0.999	0.02	20	3.7	13.0
Fulvestrant	22.90 ($3.74x10^{-15}$)	0.53±0.01	-0.003±0.006	0.999	0.02	20	5.6	14.4

Diuretic and masking agents (S5)								
Acetazolamide	2.36 ($3.74x10^{-15}$)	2.5±0.2	-0.1±0.1	0.980	0.5	20	7.9	9.2
Chlorothiazide	2.38 ($0.53x10^{-2}$)	7.7±0.2	0.3±0.1	0.998	0.006	200	2.3	14.2
Chlortalidone	7.62 ($0.63x10^{-2}$)	0.98±0.03	-0.01±0.02	0.997	0.08	200	1.0	19.1
Triamterene	8.18 ($1.76x10^{-2}$)	21.8±2.1	-1.6±1.6	0.980	0.2	20	2.7	18.5
Bendroflumethiazide	11.30 ($1.87x10^{-15}$)	3.81±0.07	-0.02±0.04	0.999	0.07	200	5.9	10.5
Quinethazone	5.36 ($0.63x10^{-2}$)	0.502±0.005	-0.010±0.003	0.999	0.1	200	3.3	14.4
Furosemide	10.90 ($4.22x10^{-2}$)	8.0±0.3	0.07±0.17	0.997	0.06	20	3.0	11.4
Bumetanide	14.80 ($3.74x10^{-15}$)	4.5±0.1	0.002±0.064	0.998	0.03	20	3.3	8.8
Indapamide	10.50 ($4.22x10^{-2}$)	4.6±0.2	0.1±0.1	0.995	0.03	200	3.6	13.0
Etacrynic acid	14.30 ($3.16x10^{-2}$)	1.39±0.04	0.002±0.023	0.998	0.1	200	2.2	9.1
Stimulants (S6)								
Ritalinic acid (Metabolite of Methylphenidate)	6.35 ($3.68x10^{-2}$)	20±1	-0.5±0.6	0.994	0.08	50	15.4	16.1
Benzoylecgonine (Metabolite of Cocaine)	8.90 ($1.19x10^{-2}$)	13.1±0.3	-0.02±0.21	0.998	0.1	50	0.1	10.0
Carphedon	5.89 ($1.23x10^{-2}$)	24±1	0.5±0.8	0.995	0.04	50	2.5	9.3
Amphetamine	3.70 ($1.55x10^{-2}$)	5.5±0.3	-0.2±0.2	0.993	2	50	3.0	15.0

Methamphetamine	5.69 ($1.26x10^{-2}$)	5.5±0.2	0.02±0.01	0.998	0.08	50	7.4	18.4
Benzphetamine	14.40 ($6.66x10^{-2}$)	41±2	0.4±1.2	0.995	0.02	50	0.4	8.4
Etilefrine	2.36 ($0.63x10^{-2}$)	65±4	2.7±2.3	0.992	0.09	50	17.6	16.9
Pentetrazol	5.64 ($0.63x10^{-2}$)	1.79±0.03	-0.005±0.021	0.999	0.1	50	1.7	9.6
Nikethamide	7.78 ($0.84x10^{-2}$)	16.3±0.4	-0.022±0.249	0.998	0.03	50	2.6	8.6
Ecgonine methyl ester (Metabolite of Cocaine)	3.34 ($10.06x10^{-2}$)	35±2	1.0±1.1	0.993	0.05	50	9.4	8.3
Cathine	2.38 ($0.53x10^{-2}$)	7.3±0.3	-0.1±0.2	0.996	0.01	6000[a]	2.7	20.8
Cathinone	3.28 ($1.50x10^{-2}$)	2.98±0.03	-0.07±0.01	0.999	1.2	50	0.7	11.7
Methylephedrine	5.35 ($3.47x10^{-2}$)	16±2	3.9±0.9	0.979	0.3	11000[a]	8.7	8.0
Methylhexanamine	4.68 ($1.55x10^{-2}$)	6.6±0.2	-0.3±0.1	0.998	0.1	50	0.2	11.8
Phendimetrazine	5.91 ($10.83x10^{-2}$)	5.1±0.1	-0.01±0.07	0.998	0.1	50	5.1	8.9
Strychnine	9.92 ($2.18x10^{-2}$)	1.14±0.04	0.02±0.02	0.997	0.03	50	3.8	9.9
Cocaine	12.20 ($4.71x10^{-2}$)	1.84±0.06	0.002±0.034	0.998	0.04	10	1.5	9.9
N-Desmethylselegiline (Metabolite de Selegiline)	7.39 ($12.94x10^{-2}$)	26.9±0.8	-0.5±0.5	0.998	0.1	50	3.1	17.1
Modafinil	9.52 ($1.71x10^{-2}$)	0.55±0.03	0.01±0.02	0.995	0.1	50	14.0	8.7

Diphenylmethyl (sulfinyl)-acetic acid (Metabolite of Modafinil)	9.80 ($1.81x10^{-2}$)	26.9±0.8	-0.5±0.5	0.998	0.08	10	2.8	12.9
Selegiline	9.41 ($4.72x10^{-2}$)	32±1	0.1±0.8	0.996	0.09	50	1.9	8.0
Propylhexedrine	10.30 ($5.16x10^{-2}$)	16.8±0.2	-0.08±0.13	0.999	0.1	50	2.0	9.8
Narcotics (S7)								
Morphine	2.39 ($0.69x10^{-2}$)	1.42±0.03	0.03±0.02	0.999	0.008	1300[a]	0.3	20.0
Oxymorphone	3.87 ($1.81x10^{-2}$)	0.149±0.009	0.009±0.005	0.992	1.3	25	2.4	11.8
Oxycodone	7.21 ($2.17x10^{-2}$)	2.26±0.09	-0.04±0.05	0.996	0.4	25	3.3	18.1
6-Acetylmorphine (Metabolite of Diamorphine or heroin)	6.91 ($2.41x10^{-2}$)	0.80±0.02	-0.02±0.02	0.997	0.4	25	1.0	11.0
Norfentanyl	8.70 ($2.54x10^{-2}$)	13.19±0.09	-0.37±0.04	0.999	0.002	1	14.6	14.9
Hydromorphone	4.74 ($1.50x10^{-2}$)	0.532±0.003	0.002±0.001	1.000	1	25	9.8	14.8
Pethidine	11.20 ($4.71x10^{-2}$)	11.1±0.2	-0.1±0.1	0.999	0.005	25	2.0	8.7
Pentazocine	14.00 ($4.83x10^{-2}$)	1.77±0.03	-0.02±0.02	0.999	0.1	25	2.8	12.4
Norbuprenorphine (Metabolite of Buprenorphine)	12.20 ($3.16x10^{-2}$)	0.93±0.02	-0.02±0.01	0.999	0.3	25	3.1	10.0
Methadone	18.60 ($3.16x10^{-2}$)	44±4	2±2	0.987	0.005	25	0.7	9.5
Fentanyl	16.10 ($5.16x10^{-2}$)	0.233±0.004	-0.0005±0.0021	0.999	0.02	1	3.5	8.1

Buprenorphine	16.00 ($4.83x10^{-2}$)	0.203±0.002	0.002±0.001	0.999	0.1	2.5	5.8	12.0
Cannabinoids (S8)								
JWH-018 5-pentanoic acid (Metabolite of JWH-018)	18.60 ($3.74x10^{-15}$)	0.154±0.004	0.004±0.002	0.999	0.01	1	1.0	14.6
JWH-018 N-(5-hydroxy-pentyl) (Metabolite of JWH-018)	18.80 ($3.74x10^{-15}$)	0.067±0.002	-0.0003±0.0013	0.998	0.02	1	1.4	14.0
CP 47, 497-C8-hydroxy, C8-homolog (Metabolite of CP 47, 497-C8-homolog)	20.80 ($3.74x10^{-15}$)	0.0027±0.0001	0.0003±0.0001	0.998	0.02	1	5.8	16.6
11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid	19.00 ($3.74x10^{-15}$)	0.039±0.006	0.007±0.003	0.959	4.2	180[a]	6.0	11.1
HU-210	20.70 ($4.21x10^{-2}$)	0.0046±0.0002	0.0001±0.0001	0.995	0.03	1	2.9	15.5
Glucocorticoids (S9)								
Triamcinolone	10.20 ($1.87x10^{-15}$)	0.56±0.01	-0.005±0.006	0.999	0.03	30	7.7	13.8
6-β-OH-budesonide (Metabolite of Budesonide)	14.60 ($3.16x10^{-2}$)	0.046±0.001	0.0015±0.0004	0.999	0.7	45	5.3	15.1
Prednisolone	11.50 ($3.74x10^{-15}$)	0.705±0.009	-0.013±0.005	0.999	0.01	100	6.1	11.7
Betamethasone	13.00 ($8.75x10^{-2}$)	0.244±0.004	-0.004±0.002	0.999	0.3	60	3.1	12.8

Budesonide	17.40 ($3.74x10^{-15}$)	0.66±0.01	0.002±0.008	0.999	0.2	30	0.9	9.6
Triamcinolone acetonide	15.40 ($1.87x10^{-15}$)	0.115±0.001	-0.0011±0.0004	0.999	0.2	15	7.7	12.2
Beta-blockers (P1)								
Atenolol	5.16 ($1.23x10^{-2}$)	1.68±0.02	-0.03±0.01	0.999	0.6	50	2.3	11.7
Nadolol	8.45 ($1.94x10^{-2}$)	2.97±0.03	-0.07±0.02	0.999	0.02	50	6.0	13.0
Acebutolol	10.40 ($5.27x10^{-2}$)	16.2±0.7	0.2±0.4	0.995	0.01	50	2.6	17.3
Pindolol	7.85 ($2.32x10^{-2}$)	4.55±0.05	-0.06±0.03	0.999	0.04	50	6.7	10.5
Bisoprolol	12.60 ($4.83x10^{-2}$)	323±51	43±29	0.982	0.001	50	1.2	1.8

Table S4. Equations and coefficients of determination (R^2) calculated from the linear regression of the plots Volume of SUPRAS (y, µL· mL^{-1} urine) versus 1,2-hexanediol percentage in the colloidal system (x, v/v), at different concentrations of sodium sulphate (M).

Synthesis conditions [Na_2SO_4]	Equation	Coefficient of determination (R^2)
0.75 M	y = 189x – 782	0.994
1.00 M	y = 166x – 498	0.996
1.25 M	y = 147x – 324	0.994
1.50 M	y = 131x – 117	0.981

Table S5. Chemical composition of SUPRASs formed from representative sodium sulfate concentrations.

[a]Synthesis conditions	SUPRAS composition			SUPRAS density±SD (g mL^{-1})
Na_2SO_4 (M)	H_2O ± SD (%, w/w)	1,2-hexanediol ± SD (%, w/w)	Na_2SO_4 ± SD (%, w/w)	
0.6	61±4	35.8±0.7	5.3±0.1	0.98±0.02
0.75	45.7±0.7	50.0±0.9	4.29±0.08	0.95±0.05
1.0	39±1	58.9±0.2	3.0±0.2	0.90±0.04
1.5	36±1	62.6±0.9	1.49±0.05	0.92±0.05

[a] 1,2-hexanediol: 20% (v/v)

Table S6. Recoveries values obtained for ten urine samples (S1-S10) fortified with the selected 92 banned substances at the respective MRPL values by extraction with cubosomic SUPRAS.

Selected WADA´s prohibited substances or their metabolites	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	Mean recovery
Anabolic agents (S1)											
Andarine	104	102	107	111	105	120	95	100	110	97	105
Tetrahydrogestrinone	107	107	111	109	99	117	97	90	97	110	104
Testosterone	128	99	121	103	125	112	93	123	94	121	112
Boldenone	98	129	104	94	108	109	75	75	86	120	100
4-Androsten-17α-ethynyl-17β-ol-3-one (Metabolite of Danazol)	113	93	125	116	100	106	96	91	104	109	105
Oxandrolone	93	114	108	114	90	131	96	95	120	87	105
1-methylene-5α-androstan-3α-ol-17-one (Metabolite of Metenolone)	99	119	90	123	100	106	70	112	76	107	100
3-OH-stanozolol (Metabolite of Stanozolol)	100	100	92	114	99	115	97	95	100	101	101
1α-methyl-5α-androstan-3α-ol-17-one(Metabolite of Mesterolone)	108	97	86	105	90	120	114	87	113	98	102

17α-methyl-5α-androstane-3α-17β-diol (Metabolite of Methyltestosterone)	81	116	118	116	132	96	70	114	84	63	99
Hypoxia-inducible factor (HIF) activating agents (S2)											
Daprodustat	101	98	104	104	99	111	93	90	96	100	100
Roxadustat	101	92	98	101	93	118	93	88	99	94	98
Beta-2 Agonists (S3)											
Salbutamol	65	102	95	66	67	83	74	71	97	79	80
Terbutaline	77	92	82	47	76	54	76	58	60	132	75
Procaterol	56	81	91	59	77	86	77	72	112	80	79
Formoterol	101	93	98	89	104	119	91	100	92	112	100
Higenamine	73	100	81	81	82	95	63	94	111	115	90
Tulobuterol	79	88	96	95	80	105	87	75	87	86	88
Vilanterol	105	110	105	108	98	116	107	97	106	104	106
Hormone and metabolic modulators (S4)											
Aminoglutethimide	122	72	81	73	106	116	89	114	84	81	94
Anastrozole	105	101	99	105	102	113	102	88	96	98	101

Bis-(4-cyanophenyl) methanol (Metabolite of Letrozole)	93	114	108	118	97	104	103	91	108	103	104
Exemestane	103	102	105	110	94	118	96	93	110	102	103
4-OH-cyclofenil (Metabolite of Cyclofenil)	98	101	111	119	92	105	96	86	105	101	101
SR9009	97	105	104	104	103	119	91	91	108	105	103
GW501516 Sulfone (Metabolite of GW501516)	106	103	106	98	96	118	107	94	99	100	103
GW501516 Sulfoxide (Metabolite of GW501516)	103	94	105	115	93	123	100	91	101	94	102
Raloxifene	99	106	109	114	93	115	97	96	104	99	103
4-OH-toremifene (Metabolite of Toremifene)	108	102	109	114	100	120	102	90	97	95	104
Toremifene	108	101	106	100	96	118	99	90	105	103	103
17-ketone fulvestrant (Metabolite of Fulvestrant)	107	101	108	105	100	113	96	86	99	99	101
Fulvestrant	105	101	104	106	99	109	96	90	97	101	101
Diuretic and masking agents (S5)											
Acetazolamide	58	128	82	93	75	88	115	97	99	112	95
Chlorothiazide	45	72	86	91	39	63	70	30	66	64	63

Chlortalidone	83	102	77	118	94	119	89	77	96	94	95
Triamterene	120	88	105	106	83	77	93	100	47	125	94
Bendroflumethiazide	96	95	105	123	101	110	103	91	101	97	102
Quinethazone	91	92	100	115	88	102	87	84	98	98	96
Furosemide	91	106	105	111	92	107	96	86	101	102	100
Bumetanide	100	103	106	115	97	114	99	84	107	101	103
Indapamide	89	107	108	128	103	106	102	85	107	103	104
Etacrynic acid	98	98	108	113	96	107	93	84	95	100	99
Stimulants (S6)											
Ritalinic acid (Metabolite of Methylphenidate)	74	91	76	73	72	87	70	66	74	73	76
Benzoylecgonine (Metabolite of Cocaine)	78	79	90	83	79	97	80	75	83	86	83
Carphedon	87	94	100	125	93	109	92	86	97	96	98
Amphetamine	71	130	120	63	120	95	96	111	101	126	103
Methamphetamine	90	78	109	82	59	119	88	59	83	99	87
Benzphetamine	99	108	112	118	101	116	102	92	104	107	106
Etilefrine	76	74	72	80	28	103	75	29	79	71	69

Pentetrazol	66	72	76	76	74	81	72	64	70	72	72
Nikethamide	81	88	93	92	82	104	85	80	87	90	88
Ecgonine methyl ester (Metabolite of Cocaine)	43	44	34	47	47	54	39	61	50	34	45
Cathine	73	118	93	74	75	86	115	91	104	106	94
Cathinone	60	71	82	74	78	98	68	61	70	62	72
Methylephedrine	57	78	70	98	58	125	66	76	91	76	80
Methylhexaneamine	75	118	127	100	90	114	55	84	80	76	92
Phendimetrazine	72	78	99	80	79	101	91	72	79	89	84
Strychnine	71	74	93	83	73	88	81	75	78	80	80
Cocaine	84	87	98	95	83	105	89	82	89	91	90
N-Desmethylselegiline (Metabolite de Selegiline)	95	115	135	128	101	126	88	96	25	113	102
Modafinil	67	102	71	112	105	111	116	111	109	91	100
Diphenylmethyl (sulfinyl)-acetic acid (Metabolite of Modafinil)	91	97	107	109	93	104	95	81	99	95	97
Selegiline	91	95	107	105	99	114	101	93	102	104	101
Propylhexedrine	88	92	100	99	91	112	93	88	98	98	96

Narcotics (S7)											
Morphine	87	90	131	96	83	80	96	81	99	116	96
Oxymorphone	43	89	78	36	64	46	72	75	79	86	67
Oxycodone	64	100	91	91	114	72	81	90	59	95	86
6-Acetylmorphine (Metabolite of Diamorphine or heroin)	76	130	87	74	108	120	119	125	129	125	109
Norfentanyl	71	78	86	82	72	92	75	70	79	77	78
Hydromorphone	55	113	75	63	75	77	89	87	91	110	84
Pethidine	81	87	100	93	84	106	87	80	93	92	90
Pentazocine	96	100	98	97	97	119	94	92	93	101	99
Norbuprenorphine (Metabolite of Buprenorphine)	102	93	106	104	95	113	97	88	103	101	100
Methadone	106	107	111	114	101	119	109	103	114	115	110
Fentanyl	102	96	105	105	91	109	95	89	107	99	100
Buprenorphine	101	94	102	102	95	127	103	100	113	105	104
Cannabinoids (S8)											
JWH-018 5-pentanoic acid metabolite (Metabolite of JWH-018)	124	108	112	110	95	109	105	83	95	96	104

JWH-018 N-(5-hydroxy-pentyl) metabolite (Metabolite of JWH-018)	100	98	97	100	100	113	95	94	101	111	101
CP 47, 497-C8-hydroxy, C8-homolog (Metabolite of CP 47, 497-C8-homolog)	101	113	93	117	123	102	84	108	102	109	105
11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid	102	113	127	104	106	117	112	97	105	108	109
HU-210	119	104	102	108	112	116	88	95	106	118	107
Glucocorticoids (S9)											
Triamcinolone	92	102	101	120	91	102	96	78	97	100	98
6-β-OH-budesonide (Metabolite of Budesonide)	107	96	106	92	111	112	86	94	102	109	102
Prednisolone	96	99	109	125	94	104	104	86	107	105	103
Betamethasone	104	93	107	111	98	108	95	93	99	106	101
Budesonide	103	102	103	119	96	112	98	90	104	108	104
Triamcinolone acetonide	96	100	104	105	91	115	85	94	100	106	100
Beta-blockers (P1)											
Atenolol	43	91	70	60	60	71	71	84	85	105	74
Nadolol	67	64	78	74	63	86	64	65	71	74	71

Acebutolol											
Pindolol	129	90	96	93	113	106	90	131	92	125	107
Bisoprolol	88	86	91	97	90	98	83	92	91	98	91
	100	106	112	98	120	122	120	104	108	128	112

Table S7. Relative matrix effect values obtained for ten urine samples (S1-S10) fortified with 92 banned substances at the respective MRPL values by extraction with cubosomic SUPRAS.

Selected WADA´s prohibited substances or their metabolites (n=92)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	Mean Matrix Effects
Anabolic agents (S1)											
Andarine	16.0	40.5	41.7	45.3	20.0	58.3	43.9	19.8	46.1	56.2	38.8
Tetrahydrogestrinone	0.8	12.2	10.1	15.1	17.6	19.4	13.5	17.8	19.4	17.0	14.3
Testosterone	6.0	-2.0	-14.1	-11.0	-13.1	11.2	-18.1	11.0	17.4	-1.2	-1.4
Boldenone	-20	11.3	12.4	-19.5	19.0	19.8	-18.6	13.0	8.0	0.4	2.6
4-Androsten-17α-ethynyl-17β-ol-3-one (Metabolite of Danazol)	-0.01	5.6	-2.9	-1.8	11.3	17.3	11.6	18.4	16.0	19.0	9.4
Oxandrolone	19.1	48.7	15.1	49.6	19.3	38.6	49.6	10.6	54.6	36.6	34.2

1-methylene-5α-androstan-3α-ol-17-one (Metabolite of Metenolone)	8.9	50.0	-13.9	51.2	33.8	17.7	14.5	37.1	-6.2	9.2	20.2
3-OH-stanozolol (Metabolite of Stanozolol)	6.6	16.6	16.6	18.5	16.5	16.5	11.3	19.1	14.2	16.8	15.3
1α-methyl-5α-androstan-3α-ol-17-one (Metabolite of Mesterolone)	15.0	32.4	45.4	14.8	15.0	19.1	19.7	28.8	14.1	32.0	23.6
17α-methyl-5α-androstane-3α-17β-diol (Metabolite of Methyltestosterone)	18.9	41.9	-12.4	32.9	-14.1	-3.7	12.4	-10.9	-18.4	47.6	9.4
Hypoxia-inducible factor (HIF) activating agents (S2)											
Daprodustat	18.5	10.8	15.4	13.8	30.0	19.5	33.8	34.6	45.4	43.8	26.6
Roxadustat	5.1	17.7	16.3	18.2	18.5	10.5	19.4	13.9	11.3	10.8	14.2
Beta-2 Agonists (S3)											
Salbutamol	13.5	-10.8	16.4	14.4	-10.6	-31.4	-28.2	-28.2	-8.8	-11.2	-8.5
Terbutaline	11.5	-10.5	5.1	7.2	-34.8	-34.5	-45.2	-19.2	-33.0	-32.6	-18.6
Procaterol	-10.5	-17.9	19.3	14.6	10.5	15.8	15.1	14.9	-6.1	4.4	6.0
Formoterol	11.5	30.5	50.1	12.5	11.2	35.1	33.9	14.9	46.1	17.0	26.3
Higenamine	-13.3	-18.3	5.6	-12.5	3.5	-19.6	10.3	14.0	-12.7	-11.0	-5.4
Tulobuterol	0.5	-4.2	7.2	-3.9	15.4	11.9	15.3	19.7	19.6	17.3	9.9

Vilanterol	3.8	18.8	18.0	12.4	17.6	16.3	21.0	19.1	18.9	15.3	16.1
Hormone and metabolic modulators (S4)											
Aminoglutethimide	-13.1	-16.5	18.0	60.0	11.1	63.1	-41.6	12.7	-19.5	-40.2	3.4
Anastrozole	-0.02	10.1	19.2	13.4	16.6	16.4	17.3	12.5	19.6	12.9	13.8
Bis-(4-cyanophenyl) metanol (Metabolite of Letrozole)	-2.6	-14.6	4.9	-13.3	14.3	-1.4	-0.3	10.3	6.1	11.0	1.4
Exemestane	3.2	-3.6	10.3	18.5	18.6	19.6	19.6	14.5	16.1	19.3	13.6
4-OH-cyclofenil(Metabolite of Cyclofenil)	-5.1	-11.1	-6.2	-14.5	17.7	5.1	1.7	18.8	-0.6	11.5	1.7
SR9009	6.1	10.4	11.6	12.7	14.3	19.7	15.5	11.7	12.3	16.0	13.0
GW501516 Sulfone(Metabolite of GW501516)	3.0	15.0	15.6	17.8	17.6	15.3	14.1	12.3	13.6	19.4	14.4
GW501516 Sulfoxide(Metabolite of GW501516)	7.2	18.3	13.1	16.4	12.5	13.0	20.0	19.0	18.9	17.4	15.6
Raloxifene	1.5	9.9	16.6	14.2	19.0	14.5	14.5	10.1	18.0	19.1	13.7
4-OH-toremifene (Metabolite of Toremifene)	2.6	13.7	18.0	19.1	13.3	16.6	15.7	16.6	17.5	12.1	14.5
Toremifene	1.5	20.9	13.3	10.3	12.9	41.1	10.4	42.7	40.2	41.4	23.5

17-ketone fulvestrant (Metabolite of Fulvestrant)	15.2	1.2	9.6	10.1	6.5	17.9	18.0	19.5	19.6	12.4	13.0
Fulvestrant	-0.7	2.1	16.3	19.6	15.8	11.0	17.2	10.5	15.7	18.1	12.6
Diuretic and masking agents (S5)											
Acetazolamide	-18.1	-45.2	-39.7	-34.6	-25.0	-37.4	-44.4	-34.5	-36.2	-39.0	-35.4
Chlorothiazide	-30.6	-42.2	-39.4	-34.6	-31.6	-39.0	-38.5	-27.2	-41.1	-33.0	-35.7
Chlortalidone	-6.3	-19.8	-4.4	-14.1	15.6	-14.7	-7.3	12.6	-9.4	-14.8	-6.3
Triamterene	-26.5	-36.2	19.0	-33.5	-5.9	-17.1	-26.1	-12.6	-16.8	-32.1	-18.8
Bendroflumethiazide	8.6	-7.9	15.7	-7.9	12.2	12.4	17.0	12.2	10.9	18.8	9.2
Quinethazone	-14.4	-19.6	-16.9	-18.6	10.8	12.3	-5.3	10.8	-15.9	-4.3	-6.1
Furosemide	-9.0	-13.7	-12.4	-15.0	17.3	-8.4	-0.3	13.2	4.3	12.4	-1.2
Bumetanide	-0.02	-12.9	-1.7	-12.8	12.3	10.1	6.7	11.8	12.3	17.9	4.4
Indapamide	-4.0	-16.4	-2.4	-15.3	19.4	-4.5	3.4	17.3	6.6	12.0	1.6
Etacrynic acid	4.0	-17.3	2.2	-9.2	19.3	14.6	14.3	16.7	16.3	19.7	8.1
Stimulants (S6)											
Ritalinic acid (Metabolite of Methylphenidate)	13.3	14.8	18.4	10.8	19.1	19.9	19.6	13.4	18.4	15.9	16.4

Benzoylecgonine (Metabolite of Cocaine)	1.1	12.1	15.1	15.6	15.5	17.2	10.5	17.6	10.7	13.7	12.9
Carphedon	-2.1	-3.0	7.2	-11.8	20.7	12.2	13.9	11.5	9.7	19.8	7.8
Amphetamine	46.8	-11.5	39.3	48.0	-6.7	-10.0	-10.2	-11.2	-2.6	-5.3	7.7
Methamphetamine	12.5	12.2	10.7	10.2	9.3	14.6	17.9	10.4	15.5	-1.4	11.2
Benzphetamine	-3.7	-3.7	-13.4	-17.1	15.9	-0.03	6.1	11.3	13.4	17.7	2.6
Etilefrine	-5.6	-35.0	-37.8	-4.6	-16.9	-18.2	-33.9	-19.8	-20.0	-16.2	-20.8
Pentetrazol	-7.7	-11.3	1.5	-14.5	17.2	3.1	2.1	19.1	4.3	12.3	2.6
Nikethamide	-2.8	0.5	12.2	1.1	12.8	14.8	16.9	16.5	18.3	14.5	10.5
Ecgonine methyl ester (Metabolite of Cocaine)	11.1	46.2	43.0	34.4	17.1	20.4	-19.6	17.0	-17.8	-1.9	15.0
Cathine	32.6	-18.1	7.3	-0.6	4.7	-32.5	-28.7	-16.2	-18.6	1.0	-6.9
Cathinone	-11.3	-4.1	-19.9	-11.5	-11.3	-13.1	-18.3	9.1	-17.2	-11.2	-10.9
Methylephedrine	-2.9	-18.4	3.5	-13.7	7.6	-13.7	-1.4	1.6	-18.6	-16.1	-7.2
Methylhexaneamine	4.7	-15.7	8.6	-16.9	12.0	10.4	46.3	-0.4	44.1	19.8	11.3
Phendimetrazine	-4.0	-2.0	7.5	-1.5	14.4	3.0	7.0	23.9	2.0	6.5	5.7
Strychnine	-9.1	-12.6	-6.3	-18.8	11.4	3.5	5.4	16.8	12.3	13.0	1.6
Cocaine	0.2	6.5	5.5	3.7	10.9	10.1	18.2	13.9	14.0	19.3	10.2

N-Desmethylselegiline (Metabolite of Selegiline)	62.7	51.3	27.9	35.3	29.7	45.1	44.6	31.9	46.5	33.9	40.9
Modafinil	11.3	1.1	17.5	-4.7	20.0	19.3	15.3	11.9	13.2	11.5	11.6
Diphenylmethyl (sulfinyl)-acetic acid (Metabolite of Modafinil)	-7.3	-18.7	-12.4	-18.1	19.8	-4.3	-1.0	13.8	4.6	16.7	-0.7
Selegiline	-0.01	-3.9	4.7	-3.9	19.4	10.9	12.4	13.3	14.7	11.7	7.9
Propylhexedrine	0.4	-4.7	11.9	3.7	25.4	19.9	18.7	18.4	13.5	16.9	12.4
Narcotics (S7)											
Morphine	-13.6	-19.3	-14.3	-19.7	-13.7	-17.7	-17.0	-7.2	-11.5	-12.8	-14.7
Oxymorphone	-18.6	-12.5	-7.8	-12.5	-6.2	-4.7	-12.5	-10.1	-18.6	-14.0	-11.8
Oxycodone	-11.0	-41.6	-31.8	-29.7	-48.1	-41.5	-36.9	-41.6	-25.7	-28.9	-33.7
6-Acetylmorphine (Metabolite of Diamorphine or heroin)	32.7	0.4	34.4	42.3	2.6	-14.7	-8.5	-12.5	13.8	5.9	9.6
Norfentanyl	0.4	6.0	18.2	3.7	18.8	12.6	15.7	13.8	15.0	10.2	11.4
Hydromorphone	33.1	-11.9	40.1	34.0	1.4	-15.6	-17.6	-16.8	13.8	-8.8	5.2
Pethidine	0.2	2.5	13.1	5.7	14.7	11.7	11.0	18.1	19.9	19.0	11.6
Pentazocine	-7.7	-11.3	1.5	-14.5	17.2	3.1	2.1	19.1	4.3	12.3	2.6

Norbuprenorphine (Metabolite of Buprenorphine)	-2.0	-19.1	13.1	10.4	15.2	15.8	19.3	18.2	15.0	12.8	9.9
Methadone	-3.0	8.0	11.9	10.8	18.0	19.1	15.8	11.9	11.9	11.3	11.6
Fentanyl	15.7	11.4	16.8	19.7	12.8	17.5	16.4	15.0	19.6	16.0	16.1
Buprenorphine	12.8	17.3	14.9	16.1	18.5	17.3	10.9	14.9	14.9	14.6	15.2
Cannabinoids (S8)											
JWH-018 5-pentanoic acid metabolite (Metabolite of JWH-018)	-8.9	8.6	19.5	18.1	18.2	19.9	19.7	19.4	12.0	18.8	14.5
JWH-018 N-(5-hydroxy-pentyl) metabolite(Metabolite of JWH-018)	-2.0	12.0	12.7	10.5	17.2	14.2	18.3	13.8	13.4	13.8	12.4
CP 47. 497-C8-hydroxy. C8-homolog (Metabolite of CP 47. 497-C8-homolog)	6.6	49.0	25.1	13.0	22.6	31.5	31.6	16.1	19.3	12.0	22.7
11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid	-1.9	30.4	12.0	13.1	19.2	19.4	18.2	25.3	37.1	22.1	19.5
HU-210	-12.2	16.6	14.0	11.7	14.6	11.5	14.1	16.7	13.6	12.3	11.3
Glucocorticoids (S9)											
Triamcinolone	-14.9	-32.7	-16.7	-22.3	11.6	-11.3	-6.8	16.6	-1.8	8.6	-7.0
6-β-OH-budesonide (Metabolite of Budesonide)	9.5	50.3	38.4	17.0	12.8	34.5	39.8	15.4	14.9	23.9	25.7

Prednisolone	-15.3	-18.6	-9.5	-11.8	12.4	-10.2	-0.9	14.6	-7.0	12.7	-3.4	
Betamethasone	6.1	6.2	16.6	5.3	15.3	15.9	14.1	12.4	11.2	17.6	12.1	
Budesonide	2.2	0.4	4.5	-0.7	12.8	12.1	16.9	19.2	12.6	18.1	9.8	
Triamcinolone acetonide	2.2	10.2	19.7	7.8	15.8	12.1	20.0	12.1	16.4	16.9	13.3	
Beta-blockers (P1)												
Atenolol	32.5	-7.4	40.2	15.5	-13.2	-15.4	-18.1	-19.2	4.2	-19.4	0.0	
Nadolol	-6.0	15.4	17.4	7.7	16.2	19.7	11.0	16.2	20.8	16.2	13.5	
Acebutolol	-17.4	13.6	19.4	14.6	-11.1	11.5	10.6	-10.5	14.7	-10.2	3.5	
Pindolol	-2.5	13.0	15.2	-10.2	11.9	12.5	11.8	14.1	13.0	15.8	9.5	
Bisoprolol	19.9	-12.8	17.0	12.8	-4.4	2.6	10.5	-2.0	7.3	-1.9	4.9	

Supramolecular solvents for making comprehensive liquid-liquid microextraction in multiclass screening methods based on liquid chromatography-high resolution mass spectrometry

Soledad González-Rubio[1], Noelia Caballero-Casero[1], Ana Ballesteros-Gómez[1,*], Darío Cuervo[2], Gloria Muñoz[2], Soledad Rubio[1]

[1] Department of Analytical Chemistry, Instituto Químico para la Energía y el Medioambiente, Marie Curie Building (Annex), Campus of Rabanales, Universidad de Córdoba, 14071 Córdoba, Spain

[2]Anti-doping control laboratory. Health Institute Carlos III C/ Pintor el Greco S/N, 28040 Madrid, Spain.

[*]Corresponding author: a42caasn@uco.es

Highlights

- Multiclass screening methods lack of more comprehensive sample treatments
- Supramolecular solvents extract efficiently extract structurally unrelated compounds
- Combination of SUPRAS with LC-ESI-TOF is valuable for multiclass screening methods
- Recovery for 93% of eighty urine drugs (log P -2.4 to 9.2) in SUPRASwas 70-120%
- A multiclass screening method for doping control in urine is developed and validated

Abstract

Multiclass screening methods involving hundreds of structurally unrelated compounds are becoming essential in many control labs and research areas. Accurate mass screening of a theoretically unlimited number of chemicals canbe undertaken using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS), but the lack of comprehensive sample treatments hinders this unlimited potential. In this research, the capability of supramolecular solvents (SUPRAS) for making comprehensive liquid-liquid microextraction (LLME) in multiclass screening methods based on LC-HRMSwas firstly explored. For this purpose, a SUPRAS made up of 1,2-hexanediol,sodium sulphate and water was synthesized directly in the urine and applied tocompound extraction and interference removal in the screening of eighty prohibited substances in sports by LC-electrospray ionization-time of flight mass spectrometry (LC-ESI-TOF). Selected substances included a wide range

of polarities (log P from -2.4 to 9.2), functionalities (e.g. alcohol, amine, amide, carboxyl, ether, ester, ketone, sulfonyl, etc.) and permitted concentrations (from 1 ng mL^{-1} to 11 μg mL^{-1}). No interfering peaks were observed for any of the 80 substances investigated. Around 84-93% of drugs were efficiently extracted (recoveries 70-120%) and 83-94% of the analytes did not show matrix effects (±20 %) in the ten tested urines. Method detectionlimits for the drugs were in the interval 0.002-12.9 ng mL^{-1}. The applicability of the method was evaluated by the screening of thirty-six blinded and anonymized urine samples, previously analyzed by gas chromatography-triple quadrupole (GC-QQQ) and LC-QQQ. Seven of these samples gave positive analytical findings. This research proves that LLME based on SUPRAS constitutes an efficient and simple sample treatment in multiclass screening methods, an application that is unaffordable for conventional organic solvents.

Keywords: screening methods, supramolecular solvents, LC-ESI-TOF, doping control, liquid-liquid microextraction, urine

1. Introduction

Screening of multiclass substances has become relevant for control laboratories that are routinely confronted with hundreds of substances for which maximum permitted levels have been set and decisions on positive and negative samples have to be taken quickly (e.g. agrifood or anti-doping labs) [1,2]. Screening methods are also relevant for environmental monitoring, epidemiological studies, exposomics, metabolomics, etc. where the detection of as many toxics as possible in a single analysis is highly valuable [3,4].

High resolution mass spectrometry (HRMS), particularly combined with liquid chromatography, is currently the technique of choice for target, suspect and non-target screening of thousands of compounds. Instruments such as Orbitrap and time-of-flight (TOF) provide both high mass accuracy and resolution in full scan mode, enabling accurate mass screening of a theoretically unlimited number of chemicals [5].

By its very nature, multiclass screening methods should be comprehensive, and given the huge number of analyses usually required, they should allow high throughput sample processing and be cost-effective and green. In this respect, one of the greatest challenges of screening methods is how to reduce matrix effects while preventing the loss of chemicals with very different physicochemical properties during sample purification, and do so with good sensitivity. Thus, although sample extraction and purification should be minimal and as non-selective as possible, matrix components can strongly influence the sensitivity of screening methods, and consequently, a compromise is required [3].

The dilute and shoot (D&S) approach is theoretically ideal for screening analysis because information on sample composition remains unchanged. However, although D&S may work for chemicals present in samples at relatively high concentration, for which dilution does not compromise sensitivity, it suffers from significant and variable matrix effects, particularly in biological and environmental samples (e.g. urine, serum, surface water, etc.) [3]. So, this approach gives low sensitivity and repeatability for screening methods and consequently, most of them still require an effective sample treatment for both sample concentration and removal of matrix interferences. Solid phase extraction (SPE) is currently the technique of choice for sample preparation in screening methods owing to the variety of available sorbents. However, the obtained information will be highly dependent on the type of selected sorbent, as excellently proved in a recent publication dealing with the unknown screening of urban waters [6]. Thus, if sample processing is intended to extract compounds covering a wide range of physicochemical properties, the recommended strategy is to use a multilayer cartridge combining several

phases [5,7], or using different sorbents in parallel and then mixing the eluates[8]. However, SPE is far from simple, fast, cost-effective and green. Thus, all the required steps (conditioning, washing, eluting) require the consumption of organic solvents (e.g. a total volume of 20-30 mL per sample) and final eluateshave to be evaporated. Unfortunately, organic solvent-based extraction, particularly liquid-liquid extraction (LLE), although faster than SPE, is not a viable solution for multiclass screening since extraction will be conditioned by the similarity of the polarity of analytes and solvents [9]. On the other hand, tailored neoteric solvents such as ionic liquids [10] or deep eutectic solvents [11] have not yet been applied in multiclass screening methods.

Because of the distinctive features of supramolecular solvents (SUPRASs), their application in the development of innovative sample treatments in analytical chemistry is deserving closer attention in the last few years [12]. Thus, the different polarity microenvironments present in the amphiphilic nanostructures of SUPRASs render them excellent candidates for the extraction of analytes in a wide polarity range [13]. By their own nature, SUPRASs are non-selective extractants, as required in screening methods, however, they can be tailored to remove major matrix macrocomponents (e.g. proteins, humic acids, carbohydrates, etc.), which reduces interferences and variability among samples [14]. On the other hand, the high number of bindingsites derived from the large concentration of amphiphile in the SUPRAS, together with the mixed mechanisms offered for solute solubilisation, allow efficient extractions using low SUPRAS volume, which makes unnecessary sample extract evaporation. Moreover, SUPRAS tailoring has allowed progress in making them greener [15] and compatible with LC-ESI-MS/MS [16] and ambient mass spectrometry [17]. To the best of our knowledge, SUPRASs have not been used for screening of multiclass substances by LC- HRMS that, as mentioned above, is the current technique of choice forscreening methods.

This work was intended to explore the potential of SUPRASs for comprehensive multiclass extraction in screening methods based on LC-

HRMS. For this purpose, the application of SUPRASs to the testing of banned substances in urine was considered a proof of concept of this strategy since antidoping control is affected by the major challenges facing multiclass screening methods. Thus, substances prohibited in sports include a large number of compounds belonging to 10 categories (the World Anti-Doping Agency (WADA) list) that cover a wide range of polarity, physicochemical properties, and threshold concentrations [18-20]. This list also extends to their metabolites and any substance showing a similar structure or effect. So, hundreds of substances are of potential interest in anti-doping testing in WADA-accredited laboratories. On the other hand, urine composition shows large inter- and intra-individual variability. From an operational point of view,screening is the first step in doping control laboratories according to WADA´s guidelines [21], and only positive samples would be subjected to confirmatorymethods.

The research here developed included the selection of eighty prohibited substances and/or their metabolites from the 10 categories of the WADA list (Table S1); the selection and optimization of the SUPRAS-based LLE on the basis of SUPRAS efficiency for multiclass extraction and removal of matrix effects; and the validation of the method according to WADA guidelines. The suitability of this method for screening analysis was explored in the analysis of thirty-six blinded and anonymized urine samples received from the anti- doping control laboratory Health Institute Carlos III (ISCIII), located in Madrid. To the best of our knowledge, SUPRAS have not been applied so far in wide screening analysis based on LC-HRMS.

2. Experimental

2.1. Reagents and solutions

All solvents and reagents were of high purity grade. Methanol (MS grade) was supplied by Fisher Scientific (Madrid, Spain). Sodium sulphate, formic acid and 1,2-hexnediol were provided by Sigma-Aldrich (St. Louis, Missouri, USA). The enzyme β-glucuronidase from *E. coli* K12 (140 units per mL) was supplied by Roche Diagnostics GmbH (Mannheim, Germany). Ultra-high- quality water was generated from a Milli-Q water purification system (Millipore-Sigma, Madrid, Spain). A pH 7 buffer solution was prepared by mixing 10.88 g of KH_2PO_4 and 14.24 g of $Na_2HPO_4{\cdot}2H_2O$ in 200 mL of Milli- Q water. The buffer was transferred into a bottle of glass and was kept at 2- 8ºC. Table S2 lists the suppliers for the analytical standards of the eighty drugs and metabolites selected as well as the deuterated internal standards (IS). Stock solutions for the individual drugs and the IS (at concentrations of 1, 100 or 1000 μg mL^{-1}) were prepared in methanol and stored at -20ºC. A multi- component standard solution of the selected doping drugs at concentrations of 50-fold their respective Minimum Required Performance Level (MRPL) was prepared in methanol [19]. An IS mixture solution at 2 μg mL^{-1} each was prepared in methanol as well. Intermediate and working solutions of doping drug mixtures were prepared by appropriate dilution of stock solutions in methanol and they were stored at -20ºC for at least one month.

2.2. Urine samples

Spot urine samples were collected from 20 volunteers at the Institute of Chemistry for Energy and Environment (Córdoba) and were used for both optimization of the SUPRAS-based extraction and method validation. All volunteers were informed of the purpose of the sample collection, their rights and other concerns. The data protection policy and management of biological

samples were according to the Ethics Committee of Andalusian´s Biomedical Research. The collection of urine samples was carried out in clean plastic containers of 100 mL (Sage Products, Crystal Lake, IL). For method optimization, a pooled urine sample was obtained by mixing ten spot urines atequal proportion. The other ten spot urine samples were used individually for method validation. The samples were centrifuged for 15 min at 1,800 g and when not immediately analysed, they were stored at -20ºC. Before analysis, samples were subjected to enzymatic hydrolysis. For this purpose, 1 mL-sub- sample aliquot was mixed with 50 μL of the KH_2PO_4/Na_2HPO_4 buffer solution and 25 μL of the β-glucuronidase enzyme solution. Then, the urine was shaken in a vortex for 5 min and it was incubated in a water bath at 55ºC for 1 hour. Finally, the urine was kept in a closed glass bottle at 4ºC until its use. To provethe applicability of the method for the screening of the banned substances, 36 blind-urine samples were kindly provided by the WADA-accredited laboratory ISCIII. The samples were labelled on the basis of their pH, sex and specific gravity. All of them were non-spiked urine. No information about the identityof the athletes and the presence/absence of sport drugs was given by ISCIII. Table S3 shows the physicochemical parameters for each of these urinesamples. Before analysis, they were hydrolyzed following the same protocol specified above and properly stored at -20ºC.

2.3. Sample treatment based on SUPRAS

The hydrolyzed urine samples (1 mL), containing 20 ng mL^{-1} of the IS specified in Table S2, were mixed with Na_2SO_4 (142 mg) in Eppendorf microtubes of 2 mL. Once the salt was solubilized, 1,2-hexanediol (200 μL) was added. The mixture was vortex-shaken for 5 min at 2,000 rpm and then, centrifuged at 3,000g for 10 min. The SUPRAS extract (~250 μL) was withdrawn using a microsyringe, transferred to a sealed glass vial and mixed

with an equal volume of Milli-Q water for subsequent LC/TOF-MS analysis. Figure 1 shows a scheme of the SUPRAS-based sample treatment.

2.4. LC-HRMS Analysis of Doping Drugs

Separation and quantification of the sport drugs were conducted using an Elute UHPLC coupled to a Time-of-Flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (ESI) operating in positive and negative modes. A perfluorophenyl (PFP) column (2.7μm, 100 mm×3.0 mm) from RESTEK (Bellefonte, Pennsylvania, USA) was used as the stationary phase. The mobile phase consisted of solvent (A) 0.05% formic acid in water and (B) 0.025% formic acid in methanol. The elution program was the following: isocratic conditions (95% A, 5% B) for 0.5min, linear gradient from 5% to 100% of B in 27.5 min, and then isocratic conditions at 100% of B for 2 min. Finally, initial conditions were re- equilibrated for 5 min. The injection volume was 5 μL and the column temperature was kept constant at 40ºC. The flow rate was 0.25 mL min^{-1}. Theacquisition method was developed in full-scan MS mode. For both positive andnegative polarity modes, optimal source parameters were the following: end plate offset, 500V; capillary voltage, ±3500V; nebulizer gas pressure, 4.0 bar; dry gas, 10.0 L/min; dry temperature, 220 ºC; vaporizer temperature, 350ºC. Automatic instrument recalibration was performed at the beginning of each analysis by direct infusion of the calibration solution to ensure mass accuracy. The acquisition parameters were set for m/z range from 100 to 1500 at a scan rate of 4 scans/s. An in-house spectral library was built by direct injection of individual analytical standards of the selected sport drug under the aforementioned mentioned chromatographic and spectral conditions. The in- house library included retention time and MS spectral information for more than 90 sport drugs. The in-house library was used as a test to demonstrate the usefulness of the proposed strategy for suspect screening analyses. Identification of compounds was done by searching the acquired sample

analyses against the in-house spectral library on the basis of the following criteria: mass error below 5 ppm and retention time match (±0.1 min). Table S2 shows the theoretical and experimental masses for each compound, as well as their mass errors. Also, retention times for samples and standards in the library, and retention time errors are included. Urine samples and controls (e.g blanks) were analysed at random in batches of 45 samples under the describedconditions. All data were acquired and processed using the Compass DataAnalysis 5.3 and TASQ 2.2 software (Bruker Daltonics, Bremen, Germany).

2.5. Method Validation

The method based on SUPRAS-LC-TOF-MS was validated according to the guidelines set forth by the World Anti-Doping Agency (WADA). The following parameters were evaluated using 10 spot urine samples; linearity, selectivity, extraction recoveries, matrix effects, detection limits, carry over, and SUPRAS extract stability.

Linearity for non-threshold substances was determined using the IS method within the interval 0.1xMRPL to 2xMRPL (see MRPL values in Table S4) by fortifying SUPRAS prepared in distilled water. For threshold substances, at which the decision limits (DL) apply (Table S4), linearity was tested in the same interval of concentrations that the non-threshold substances belonging to their WADA group. *Selectivity* was evaluated by analysing 10 negative urine samples and checking for interfering peaks in the chromatograms. *Extraction recoveries* for each urine were investigated by comparing relative peak areas (Aanalyte/AIS) obtained for analytes in samples spiked before extraction (at the MRPL) with those obtained in SUPRAS spiked after extraction (at 2xMRPL to consider sample concentration). In the case of endogenous substances (e.g. testosterone), the obtained relative peak area (Aanalyte/AIS) for a non-spiked

sample was subtracted from the obtained relative peak area ($A_{analyte}/A_{IS}$) in spiked samples. *Matrix effects* were determined in terms of signal suppression and enhancement. For this purpose, the relative peak areas ($A_{analyte}/A_{IS}$) of urine samples spiked after SUPRAS extraction (2xMRPL) were compared to those obtained in SUPRAS blanks prepared in distilled water spiked at 2xMRPL. *Precision* was evaluated in terms of repeatability and within- laboratory reproducibility. For this purpose, the analysis of a spiked urine sample (at the MRPL) was repeated six times for three consecutive days (n = 18). Repeatability, expressed as relative standard deviation (RSD), was calculated as the square root of the average value of the intra-day variances, and within-laboratory reproducibility as the square root of the inter-day variance. To calculate the *method detection limits* (MDLs), urine samples were spiked at 0.5xMRPL, 0.25xMRPL, and 0.1xMRPL. MDLs were defined as the lowest level at which a compound could be identified with a signal-to-noise ratio (S/N) greater than 3. *Carry over* was evaluated by analysing a blank sample after analysis of a sample fortified at 10xMRPL. *SUPRAS extracts stability* was evaluated for three consecutive days for a urine sample fortified at the MRPLs, by storing the sample at 4ºC after analysis.

2.6. Screening analysis methods used for comparison in the analysis of urine samples

The thirty-six urine samples specified in Table S3 were subjected to the screening procedure based on SUPRAS-LC-TOF-MS (sections 2.3 and 2.4). Results were compared to those obtained by the ISCIII lab, which uses three protocols for the screening of the substances included in this study. The first protocol aims to determine anabolic agents (S1), some anti-estrogenic substances (S4), some conjugated stimulants (S6), narcotics (S7) and cannabinoids (S8) and consists of the extraction of the hydrolysed urine with tert-butyl methyl ether, evaporation of the extract to dryness, and derivatization with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), followed by

analysis with GC-QQQ-MS in MRM mode. The second protocol aims to determine unconjugated stimulants and consists of the extraction of the urine with ethyl acetate, evaporation of the extract and analysis by GC-MS in the SIM mode. The third protocol is intended to detect some anabolic agents (S1), beta-2 agonists (S3), anti-estrogens (S4), diuretics (S5), some conjugated stimulants (S6), cannabinoids (S8), glucocorticoids (S9) and beta-blockers (P1) and consists in the 5-fold dilution of the hydrolysed sample and analysis by LC-QQQ in the MRM acquisition mode.

3. Results and discussion

3.1. Selection of multiclass substances for evaluation of SUPRAS in screening methods based on LC-HRMS

As commented in Introduction, doping control has ideal characteristics to test the potential of SUPRAS in screening methods using LC-HRMS. Thus, screening is always the first step in WADA-accredited labs and only those urine samples with adverse analytical findings are subjected to confirmatory analysis. This screening, which involves hundreds of substances covering a wide range of physico-chemical properties and concentrations, should be reliable and avoid false negative and positive results while being simple, fast, cost-effective and green. The combination of different methods involving low resolution mass spectrometry coupled to LC and GC have been traditionally used for this purpose, and although the use of LC-HRMS is progressively having more prominence in this field, its potential is not yet fully exploited, partly because of the lack of comprehensive sample treatments.

Table 1 shows representative methods reported in the last decade for the testing of banned substances in urine by LC-HRMS [9, 22-35]. None of these methods was applied to the 10 drug categories (S1-S9, P1) of the WADA list,and many of them did not report studies about matrix effects. The typical three strategies used in general screening methods for sample treatment (e.g. SPE, LLE and dilute and shoot) have been also used in doping control. Although no information is generally reported on the interval of the polarity of the substances selected for screening, LLE methods using diethyl ether, methyl tert-butyl ether or ethyl acetate are not effective for extraction of polar substances [26,32,34], and addition of original urine to the solvent extract is sometimes used for extending the range of polarity of the detected analytes [34]. As expected, direct injection of urine [31] or dilute and shoot methods [27-29] are affected by significant interferences, unless high dilution factors are applied. SPE, commonly using mixed sorbents [9,23-25], has been the moststraightforward approach for sample treatment in doping screening analysis, although, as above mentioned, the SPE procedure is far from being simple, fastand green.

In this research, we selected eighty substances or their metabolites belonging to the 10 drug categories of the WADA list (Table S1). The target compoundswere selected with the aim of covering a wide range of polarities (log P from -

2.4 to 9.2), functionalities (e.g. alcohol, amine, amide, carboxyl, ether, ester, ketone, sulfonyl, etc.) and acid/base characteristics (pK_a acid: 2.8 to 18.6; pKa basic: -6.9 to 12.5). Likewise, the minimum required performance level (MRPLs) for the selected non-threshold substances (1-200 ng mL^{-1}) anddecision limits (DLs) for the threshold substances (50-11.000 ng mL^{-1}) varied in a wide concentration range (Table S4). So, this selection was considered representative for investigating the potential of SUPRAS for LLE extraction in screening methods based on LC-HRMS.

3.2. SUPRAS selection and optimization

Among SUPRAS properties, the following three characteristics were highlighted for SUPRAS application in screening analysis. (1) *High hydrophilicity*, which should favour the extraction of very polar substances while keeping the ability to solubilize nonpolar compounds in the hydrophobic chains of the amphiphilic nanostructures. (2) *Negligible signal in LC-ESI-TOF(MS),* which encourages the use of amphiphiles containing polar groups that do not ionize in ESI. (3) *No need for organic cosolvents in sample treatment,* which would make the process advantageous from a green chemistry perspective.

With these characteristics in mind, we selected SUPRASs produced directly inthe urine samples by the addition of 1,2-hexanediol as the amphiphile and sodium sulphate as the coacervation-inducing agent. It has been previously reported that these SUPRASs consist of cubosomic nanostructures made up of1,2-hexanediol, sodium sulphate and water, whose relative concentrations depend on the concentration of the coacervation-inducing agent added to the urine [16]. Thus, SUPRASs containing variable and high content of water (36-61%, w/v) can be easily synthesised at urinary concentrations of sodium sulphate in the interval 0.6-1.5 M. On the other hand, it is known that long chain alcohols do not significantly ionize in ESI [36], so it is expected they give a negligible signal in the analysis of SUPRAS extracts by LC-ESI- TOF(MS). Finally, conventional organic solvents are not necessary for SUPRAS formation or sample treatment.

The influence of the *chemical composition of the SUPRAS* on its capability for extracting the eighty drug/metabolites selected (Table S1) was investigated by adding to the pooled fortified urine sample (section 2.2) a fixed volume of 1,2- hexanediol (200 μL), and variable amounts of sodium sulphate (final concentration 0.6-1.5 M). **Figure 2A** shows the recoveries obtained for SUPRAS synthesised at three sulphate concentrations, which gave SUPRASswith the following composition in 1,2-hexanediol, water and sodium sulphate (%, w/v): 35.8±0.7, 61±4, 5.3±0.1 for 0.6 M, 58.9±0.2, 39±1, 3.0±0.2 for 1M, and 62.6±0.9, 36±1, 1.49±0.05 for 1.5M. The results show that SUPRASs

containing a high concentration of amphiphile (~60%, w/v) were able to efficiently extract 90% of the selected drugs (recoveries in the range 70-120%).The high extent of the hydrophilic area provided by the double-headed amphiphile and the surrounding water molecules (near 40%, w/v) were considered responsible for these results, from which it is worth noting that anyof the selected drugs were extracted with recoveries below 30%.

The influence of the *volume of SUPRAS* that is produced in the urine on the extraction efficiency of the drugs was investigated by adding different volumes of the amphiphile to the pooled fortified urine sample (section 2.2) while keeping constant the concentration of sodium sulphate (1 M). Under these conditions, the chemical composition of the SUPRAS remained unchanged butits volume varied linearly according to the following equation [16]:

$$V_{SUPRAS} = (-15.26\ [Na_2SO_4] + 32.52)\ [1,2\text{-hexanediol}] - (-210.84\ [Na_2SO_4] + 273.12)$$

where V_{SUPRAS} is the volume of SUPRAS (μL per mL of sample), and $[Na_2SO_4]$ and [1,2-hexanediol] represent their concentration in the urine, expressed as M and % ,v/v, respectively. Figure 2B shows representative results for SUPRAS prepared from different volumes of 1,2-hexanediol. Although no great differences in recoveries were obtained in the tested interval, a higher percentage of drugs were extracted with recoveries within the optimal interval (70-120%) using 200 μL of 1,2-hexanediol and this volume was selected for further experiments.

On the other hand, it was found (Figure 2C) that, as usual, extraction equilibrium conditions were quickly reached (e.g. 5 min of vortex-shaking) since SUPRAS-based extraction is intrinsically dispersive liquid extraction because of the coacervate droplets making up these solvents.

The superiority of SUPRASs compared to conventional organic solvents for extraction of multiclass substances in doping control can be inferred from the results previously reported in the literature [26,32,34], and those obtained from

the extraction of the drugs selected in this study with methyl isobutyl ketone (MIBK) (Figure 2D). Only 22% of the drugs had good recoveries, while the 40% of them had recoveries below 20% (many of them approaching zero).

Matrix effects were investigated under the same experimental conditions as those used for determining the extraction efficiency of SUPRASs. For simplicity, Figure 2E shows only the results at the value of the variable selectedas optimal. No matrix effects were detected for around 90-92% of the drugs, which is highly valuable if these results are compared with the dilution and shoot approach for the same drugs (Figure 2F). No great differences were obtained in matrix effects as varying the chemical composition or volume of the SUPRAS or the vortex-shaking time. Thus, the percentage of substances not showing matrix effects varied in the intervals 89-92%, 77-91% and 83- 90% as the concentration of sodium sulphate, volume of 1,2-hexanediol and vortex-shaking time varied in the ranges 0.6-1.5 M, 100-300 μL and 5-15 min,respectively.

3.3. Method validation

Method validation was carried out according to the procedure specified in section 2.5. Table S4 gives the values of the slopes and intercepts, along with their respective standard deviations, for the calibration curves obtained from each of the doping substances. Calibrations were run in SUPRAS prepared from distilled water. Correlation coefficients for these calibrations were calculated by linear regression and gave values in the interval 0.982-0.999. Method detection and quantification limits (MDLs and MQLs) for the drug selected were in the intervals 0.002-12.9 ng mL^{-1} and 0.007-43.2 ng mL^{-1}, respectively. Since these values were all below the respective MRPLs and decision limits (Table S4), the SUPRAS-LC-QTOF method meets the requirements to be used for the screening of banned substances in doping control. The intra-day and inter-day precision were expressed as relative

standard deviation and they varied in the range 0.5-16.4% and 0.9-19.7 %, respectively.

The selectivity of the SUPRAS-LC-QTOF method was checked for each of the banned substances by analysing ten fortified (at the respective MRPL value) and unfortified urine samples. Figure S1 shows, as an example, the extracted ion chromatograms for urine samples fortified with banned substances belonging to different groups (S1-S9, P1) of the WADA list (A) and those from their respective blanks (B). The ordinate axes in blank chromatograms are 1 or 2 orders of magnitude below the respective chromatograms corresponding to fortified samples. No interfering peaks were observed for any of the 80 investigated doping substances in the urine samples.

With regard to recovery studies, Figure 3A shows the whole picture of the obtained results and Table S5 depicts the specific recoveries for the tested substances in ten urine samples fortified at their respective MRPLs. Around 84-93% of drugs were efficiently extracted (recoveries 70-120%) in the urine samples. The only substance with recoveries below 30% for some urine samples was ecgonine methyl ester. It is worth noting that ritalinic acid (log P -2.4) was extracted with recoveries within the range 72-93% in the ten urines. The study of matrix effects (Figure 3B and Table S6) proved the high ability of the selected SUPRAS for eliminating matrix components that potentially can provoke undesired effects on the detection of the analytes. Thus, as depicted in Fig. 2B, around 83-94% of the analytes did not show matrix effects (±20 %) in the ten urines. Only three compounds, acetazolamide, chlorothiazole and aminoglutethimide underwent ion suppression/enhancement effects out of this range for most of the urine samples. When we focused on the individual sample results per analyte, the matrix effect affected 35 analytes out of 80 (43.8 %) in at least one sample. However, this percentage decreased down to 17.5 % when only compounds with matrix effect for more than two samples were taken into account, revealing the high potential of the SUPRAS as a general sample treatment prior to screening analysis.

The selected drugs were stable in the SUPRAS extracts, kept at 4º C, for at least three days. On the other hand, these drugs were undetected in blank urine samples that were analysed following the screening of urines fortified with the80 doping substances, at a concentration of 10-fold the respective MRPLs. Therefore, there were not carry-over effects.

3.4 Analysis of anonymized urine samples

The SUPRAS-LC-TOF(MS) screening method was applied to the analysis of 36 urine samples taken from athletes during antidoping controls and donated by the ISCIII laboratory (Table S3). As specified in section 2.4, the identification of compounds was done by searching the acquired sampleanalyses against the in-house spectral library taking into account the followingcriteria: mass error below 5 ppm and retention time match (±0.1 min). Table 2 shows the prohibited substances detected in the different urine samples by both the SUPRAS-LC-TOF-MS protocol and the different protocols used by the ISCIII lab (section 2.6). Besides the detection, the ISCIII lab had also confirmed the presence of these substances in the urine samples. Thus, the SUPRAS-LC-TOF-MS method was able to detect all the positive analytical findings using a single screening analysis per sample, which proves its capability for setting reliable doping ITPs. All substances identified were non- threshold, for which minimum required performance levels (MRPLs) have been set by WADA. Table 2 includes the MDLs obtained for the detected substances using the SUPRAS-LC-TOF-MS screening method as well as their respective MRPL values and the level of concentrations found (below or abovethe MRPL value).

Seven out of thirty-six urine samples gave positive analytical findings (Table 2). The SUPRAS-LC-TOF-MS method was able to detect doping drugs belonging to seven different groups of the WADA prohibited list. Figure 4 and Figure 2S show the extracted ion chromatograms obtained by TOF full scan for the urines with positive results.

4. Conclusions

The availability of multiclass screening methods, which are fast, simple and reliable, is highly valuable for many control labs and research areas. One of the greatest challenges to be faced in multiclass screening methods is how to be comprehensive without compromising sensitivity, selectivity and simplicity. LLME is a very simple technique, with capacity for analyte concentration and removal of interferences but conventional organic solvents can only extract substances in a limited range of polarity and they fail in extracting polar and highly polar substances, as it has been here proved for methyl isobutyl ketone. This research shows that SUPRAS-based sample treatments are a valuable strategy for screening methods involving multiclass substances covering a wide range of polarities and physicochemical properties. The SUPRAS screening method here developed features high recoveries and low matrix effects and it has been proved that it works for an area (i.e. dopingcontrol) where multiclass analysis is mandatory. An important feature of SUPRAS-based sample treatment is the immediate applicability of this procedure in routine laboratories without significant effort, since the analytical approach does not involve any important change compared to the current practices, skills and equipment commonly used in doping control.

CRediT authorship contribution statement

Soledad González-Rubio: XXXXX. **Noelia Caballero-Casero:** Conceptualization, Methodology, Supervision, Writting-review & editing, **Ana Ballesteros-Gómez:** Conceptualization, Writting-review & editing, Supervision. **Darío Cuervo:** XXXXX. **Gloria Muñoz:** XXXXX. **Soledad Rubio:** Conceptualization, Writting-review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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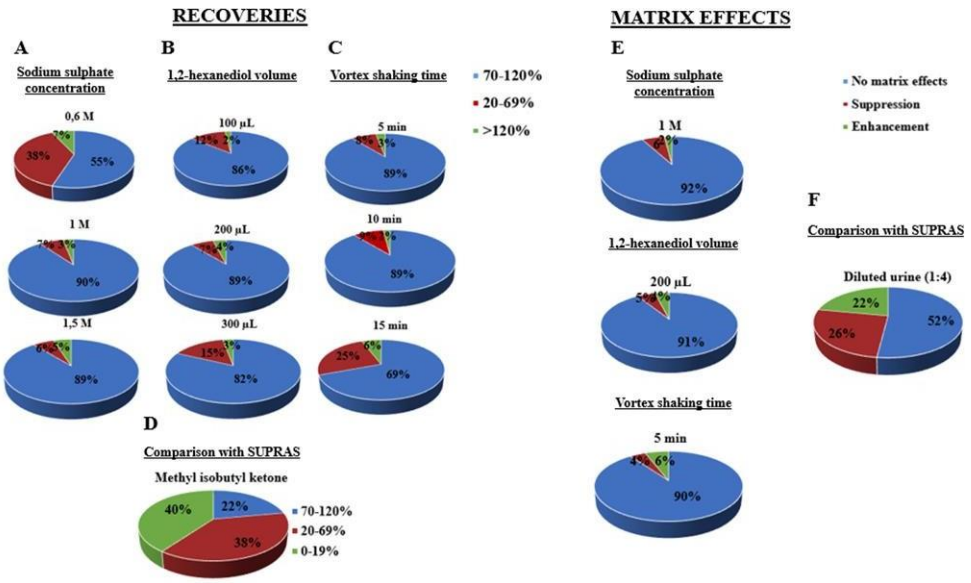
Figure Captions

Figure 1. Schematic of the SUPRAS-based sample treatment for screening analysis of multiclass substances in urine by LC-ESI-TOF.

Figure 2. (A-D) Percentage of banned substances with recoveries in the ranges of 70-120% (blue), 20-69% (red) and >120% (green) as a function of the experimental conditions used for SUPRAS formation: (A) sodium sulphate concentration, (B) volume of 1,2-hexanediol, and (C) vortex-shaking time, and (D) for methyl isobutyl ketone extraction. (E-F) Percentage of banned substances showing no matrix effects (±20%, in blue), signal suppression (red) and enhancement (green) for (E) SUPRAS formed in different experimental conditions and (F) analysing diluted urine (1:4).

Figure 3. Percentage of prohibited substances with (A) recoveries in the ranges of 70-120% (blue), 20-69% (red), and >120% (green), and (B) showing no matrix effects (± 20%, in blue), signal suppression (red), and enhancement (green) for the extraction of 10 human urine samples (S1-S10) with SUPRAS. **Figure 4.** Extracted ion chromatograms obtained for (A) 3-OH stanozolol, (B) furosemide, (C) ritalinic acid, (D) terbutaline, (E) bis-(4-cyanophenyl) methanol and (F) atenolol in the screening of the urine samples numbered as 7, 10, 15, 18, 22 and 25, respectively, using the SUPRAS-LC-ESI-TOFmethod.

Figure 1. Schematic of the SUPRAS-based sample treatment for screening analysis of multiclasssubstances in urine by LC-ESI-TOF.



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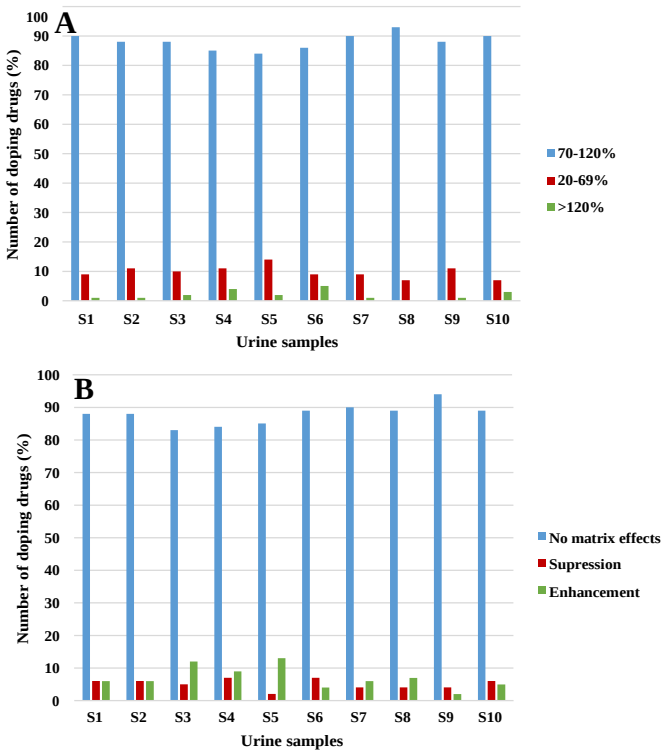

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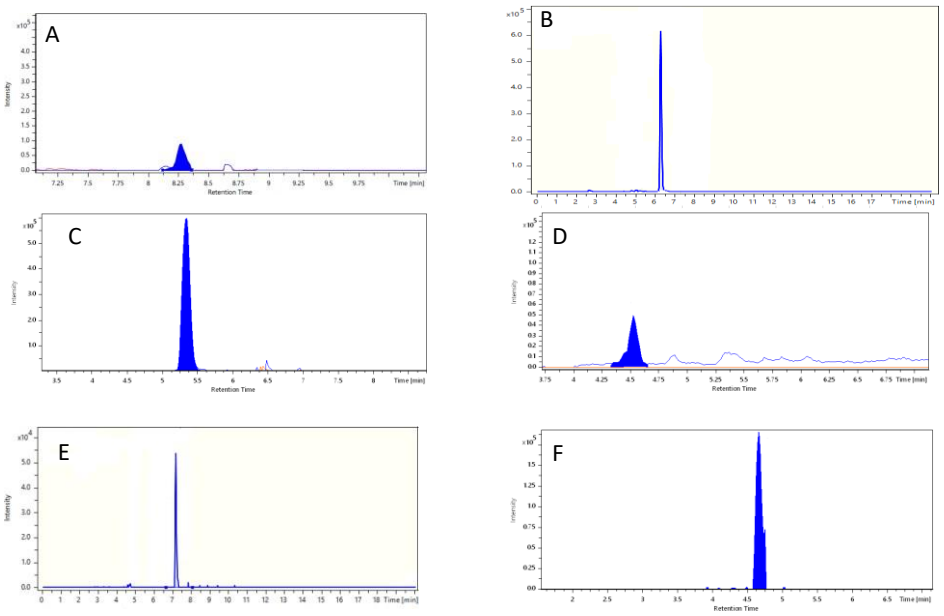


Figure 4. Extracted ion chromatograms obtained for (A) 3-OH stanozolol, (B) furosemide, (C) ritalinic acid, (D) terbutaline, (E) bis-(4-cyanophenyl) methanol and (F) atenolol in the screening of the urine samples numbered as 7, 10, 15, 18, 22 and 25, respectively, using the SUPRAS-LC-ESI-TOF method.

Table 1. Sample preparation strategies and analytical parameters obtained for representative methods reported in the last decade for screening ofbanned substances in urine using LC-HRMS.

WADA class (number of drugs)	Sample treatment	Chemicals involved in sample treatment	Limits of detection (ng mL^{-1})	LC-HRMS technique	Recoveries (%)	Matrix effects (%)	Ref.
S1, S5, S6, S7, S8, P1 (124)	• Hydrolysis • SPE (Cation exchange/C8) • Solvent evaporation	MeOH: 3 mL	27 out of 124 drugs no detected at their MRPL value.	LC-TOF	33-98 (median 58%). Five compounds 0-9%	Ion suppression of 50% for compounds at retention times below 2 min. An unspecified decrease in intensity for the rest of the compounds.	[23]
S1, S3, S4 (29)	• Hydrolysis LLE • Solvent evaporation	Diethyl ether: 5 mL	>0.1	LC-Orbitrap	23-97 (mean: 80)	No reported	[26]
S4, S5, S6, S7, P1 (103)	• Dilute and shoot (D&L)	Dilution factors from 2 to 10	1-500	LC-QTOF		73% of compounds presented ion suppression	[27]

S1, S3, S4, S5, S6, S7, S8, S9, P1 (197)	• Hydrolysis • Mixed-mode SPE (HCX and HCA cartridges) • Solvent evaporation	MeOH: 4 mL MeOH/water 50/50: 1mL	6 out of 20 drugs deeper studied for validation could not be detected at their MRPL values.	LC-TOF	83-115%	No reported	[24]
S1, S3, S4, S5, S6, S7, S9, P1 (241)	• Hydrolysis • LLE • Solvent evaporation	Diethyl ether: 5 mL	<1-100	LC-TOF	1-103%	Ion suppression depended on the specific urine matrix that was analyzed, resulting in great variability between the six different matrices tested.	[33]
S1, S3, S5, S6, S7 (56)	• Hydrolysis • Solvent extraction • Clean up based on SPE (Oasis MCX cartridge)	Diethyl ether; MeOH: 3 mL Acetone/water 1/1: 3 mL Ammonia-ethyl acetate: 3 mL	CC_{α}[b]=2.5-192	LC-TOF	68-143	No reported	[32]
S5, S6 (122)	• D&S	Dilution factors of 2,4, 10, 100	For diuretics: 25-250. For stimulants: 5-500	LC-Orbitrap		From -56 to +68.4	[28]

S1, S2, S3, S5, S6, S7, S9 (182)	• Hydrolysis (1h, 50ºC) • Solvent extraction • SPE (mixed-mode cation exchange) • Solvent evaporation	MeOH (0.5 mL) 3% ammonium hydroxide in MeOH:acetonitrile (50:50, v/v, 3mL) MeOH in water (20%, v/v, 1 mL)	0.5-200	LC-Q/Orbitrap	No reported	No reported	[25]
S1, S3, S4, S5, S6, S7, S9 (189)	different sample procedures: • SPE (PLEXA polymeric cartridge) • SPE (Oasis HLB cartridge) • SPE (Discovery DSC-18 cartridge) • SPE (Oasis MCX mixed mode cartridge) • SPE (Oasis MAX mixed mode cartridge) • LLE • Sorbent-su liquid (S	**SPE PLEXA** MeOH:MeCN (50 :50, 8 mL) ; 5% MeOH in ultra pure water (4 mL) ; MeOH/water (10:90(v/v), 0.5 mL) **SPE Oasis HLB and SPE C18** MeOH (8 mL); 5% MeOH in Mi mL); M (10	No reported	LC-TOF	Compoun with		

S4, S5, S6, (27)	• D&S	Dilution factor of 4	<5-100	LC-Q/Orbitrap		ion suppression ≥50% for 75% of the compounds	[29]
S1, S3, S4, S5, S6, S7, S8, S9, P1 (200)	• SPE (Bond Elut PLEXA cartridge) • Solvent evaporation	MeOH:MeCN (1:1.8 mL); Ultra pure water (4 mL); 5% MeOH in water (4 mL); MeOH:water (1:90, v/v, 0.5 mL)	0.005-7.6	LC-TOF	70-120	20% of the compounds presented matrix effects.	[22]

S1, S2, S3, S4, S5, S6, S7 (200)	• Urine direct injection		0.1-25	LC-Q/Orbitrap		150 out of 200 compounds presented matrix effects (75%).	[31]
S1, S3, S4, S5, S6, S7, S8, S9, P1 (81)	• D&S	Water/MeCN (95/5) at two levels (1:20 and 1:50)	LOQ: 5	NanoLC- Q-Orbitrap		Negligible matrix effects (0-10%) at a factor dilution of 1:50.	[30]
S1, S2, S3, S4, S6, S7, S8, S9, P1 (304)	• Hydrolysis • LLE • Sample freezing (-80ºC) • Mixture of reconstituted extract and original urine	Ethyl acetate: 5 mL	0.025-12.5	LC-Q/Orbitrap/MS	2.1-101	Only 62 substances were measured for matrix effects and all of them presented significant effects.	[34]
S1, S6, S7, S8, S9, P1 (300)	• Hydrolysis • SPE • Extract evaporation • Reconstitution	Methanol: 5.5 mL	0.5-100	LC-Q/Orbitrap/MS	0.6-185.7	0.04-5.28 (RSD,%)	[35]

Table 2. Prohibited substances detected by the SUPRAS-LC-ESI-TOF method in the screening analysis of 36 blinded urine samples.

Urine internal sample number	Prohibited substances detected by SUPRAS-LC-ESI-TOF	Group of WADA list	MDL ng mL^{-1}	MRPL ng mL^{-1}	Drug concentration in the blinded urine	[a]Prohibited substances detected and confirmed by GC-MS or LC-QQQ
7	3-OH-stanozolol	S1	0.2	1	> MRPL	3-OH-stanozolol
10	Furosemide	S5	0.3	20	> MRPL	Furosemide
15	Ritalinic acid	S6	0.7	50	> MRPL	Ritalinic acid
18	Terbutaline	S3	0.1	20	> MRPL	Terbutaline
22	Bis (4-cyanophenyl) methanol	S4	1.4	20	> MRPL	Bis (4-cyanophenyl) methanol
25	Atenolol	P1	0.5	50	< MRPL	Atenolol
28	Prednisolone	S9	1.5	100	< MRPL	Prednisolone

[a] Methods specified in section 2.6

SUPPORTING INFORMATION

Supramolecular solvents for making comprehensive liquid-liquid microextraction in multiclass screening methods based on liquid chromatography-high resolution mass spectrometry

Soledad González-Rubio[1], Noelia Caballero-Casero[1], Ana Ballesteros-Gómez[1], Darío Cuervo[2], Gloria Muñoz[2], Soledad Rubio[1].

[1] Department of Analytical Chemistry, Instituto Químico para la Energía y el Medioambiente, Marie Curie Building (Annex), Campus of Rabanales, Universidad de Córdoba, 14071 Córdoba, Spain[2]Anti-doping control laboratory. Spanish Agency of Health Protection in Sport (AEPSAD). C/ Pintor el Greco S/N, 28040 Madrid, Spain.

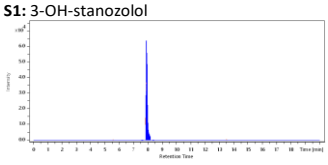
*Corresponding author: a42caasn@uco.es

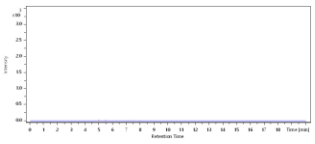
CONTENT

Urine samples fortified with the specified drugs or metabolites at the respective MRP value

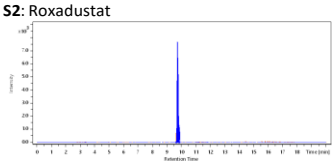
Unfortified urine

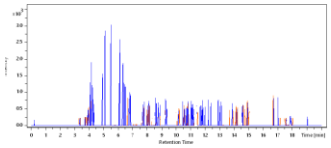
S1: 3-OH-stanozolol



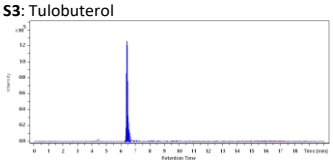


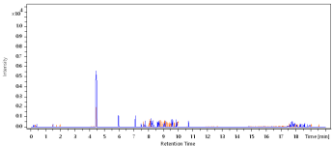
S2: Roxadustat



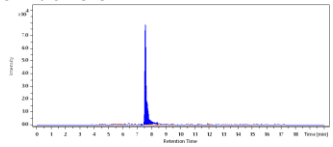


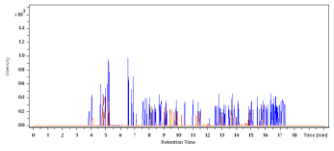
S3: Tulobuterol



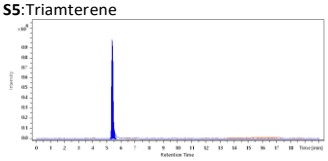


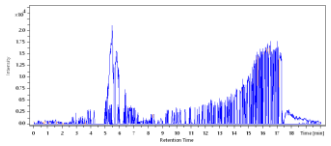
S4: Raloxifene



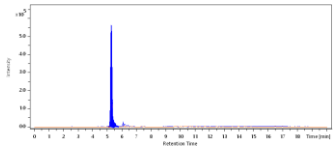


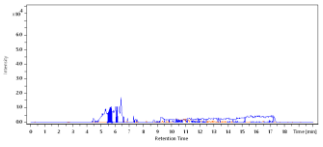
S5:Triamterene





S6: Ritalinic acid





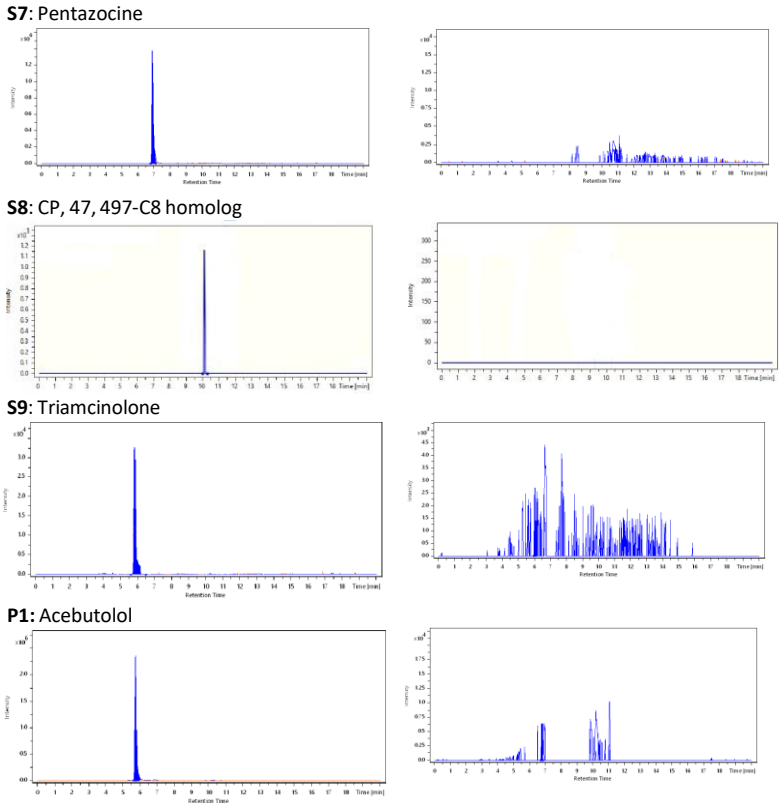


Figure S1. Extracted ion chromatograms obtained by SUPRAS-LC-ESI-TOFfor some of the drugs investigated from a urine both fortified at the MRPL and unfortified.

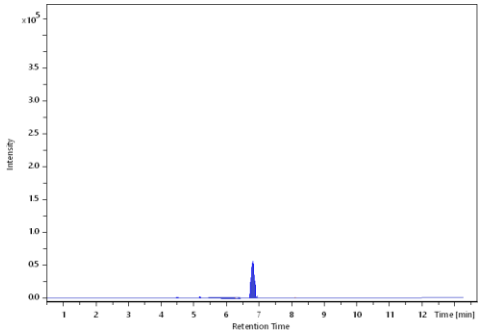
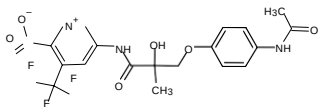
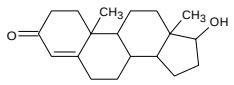
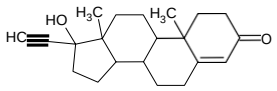
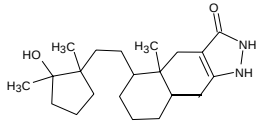
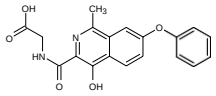
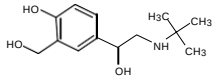
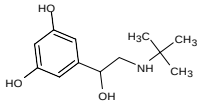
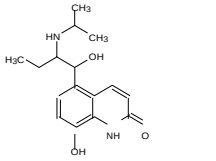
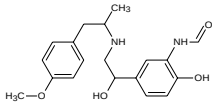


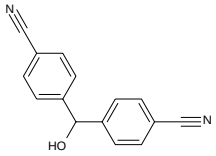
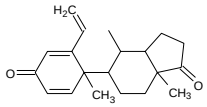
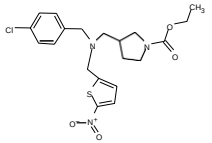
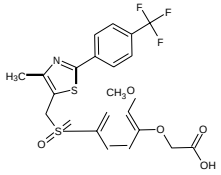
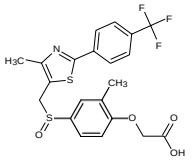
Figure S2. Extracted ion chromatogram obtained for prednisolone in the screening of the urine sample number 28 using the SUPRAS-LC-ESI-TOF method.

Table S1. Chemical structure for the selected WADA´s prohibited substances or metabolites, ranked by WADA categories (S1-S9,P1) as well as different parameters of interest for their extraction behavior

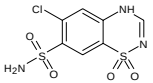
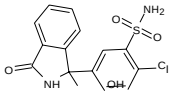
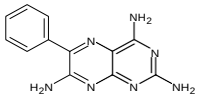
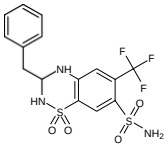
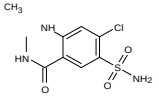
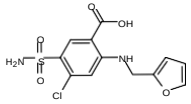
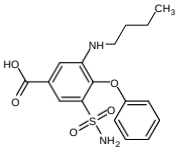
Selected WADA´s prohibited substances or their metabolites (n=80)	**Chemical structure**	**[a]Log P**	**[a,b]pKa (acid)**	**[a,b]pKa (basic)**	**[a]H-bond donors**	**[a]H-bond acceptors**	**[a]Number of aromatic rings**
Anabolic agents (S1)							
Andarine		2.2	11.2	-4	3	9	2
Testosterone		3.3	18.5	-	1	2	0
4-Androsten-17α-ethynyl-17β-ol-3-one (Metabolite of Danazol)		3.5	n/a	-	1	2	0
3-OH-stanozolol (Metabolite of Stanozolol)		4.0	11.5	2.3	3	3	0
Hypoxia-inducible factor (HIF) activating agents (S2)							

Roxadustat		3.4	2.8	3.8	3	6	3
Beta-2 Agonists (S3)							
Salbutamol		0.3	10.3	9.4	4	4	1
Terbutaline		0.9	8.9	9.7	4	4	1
Procaterol		1.5	8.5	9.9	4	4	1
Formoterol		1.8	8.6	9.8	4	5	2

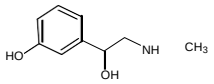
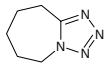
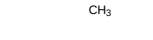
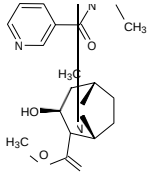
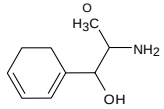
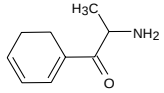
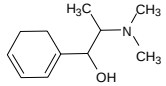
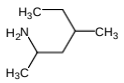
Higenamine		2.2	8.7	9.6	4	4	2
Tulobuterol		2.3	13.9	9.6	2	2	1
Vilanterol		3.8	10.1	9.4	4	6	2
Hormone and metabolic modulators (S4)							
Aminoglutethimide		1.2	11.7	4.3	2	3	1
Anastrozole		2.1	-	2	0	4	2

Bis-(4-cyanophenyl) metanol (Metabolite of Letrozole)		2.2	12.8	-	1	3	2
Exemestane		3.1	20	-	0	2	1
SR9009		4.4	-	6.1	0	6	2
GW501516 Sulfone (Metabolite of GW501516)		4.6	3	-	1	10	3
GW501516 Sulfoxide (Metabolite of GW501516)		4.6	3	-	1	10	3

Raloxifene		6.1	8,9	7,9	2	6	3
4- OH-toremifene (Metabolite of Toremifene)		6.8	n/a	n/a	1	3	3
Toremifene		7.2	-	8.8	0	2	3
17-ketone fulvestrant (Metabolite of Fulvestrant)		7.5	n/a	-	-	-	1
Fulvestrant		9.2	10.3	-	2	9	1
Diuretic and masking agents (S5)							
Acetazolamide		-0.3	-	7.2	2	7	1

Chlorothiazide		-0.2	-	6.9	2	6	1
Chlortalidone		0.9	-	9.4	3	5	2
Triamterene		1.0	-	6.2	3	7	2
Bendroflumethiazide		1.2	-	8.5	3	10	1
Quinethazone		1.2	-	9.3	3	5	1
Furosemide		2.0	3.5	-1.5	3	7	2
Bumetanide		2.8	3.6	7.7	3	7	2

Indapamide		2.9	-	8.8	2	5	2
Etacrynic acid		3.8	3.5	-5	1	4	1
Stimulants (S6)							
Ritalinic acid (Metabolite of Methylphenidate)		-2.4	3.7	10.1	2	3	1
Benzoylecgonine (Metabolite of Cocaine)		-0.3	3.2	9.5	1	5	1
Carphedon		0.1	-	-2	1	2	1
Amphetamine		1.8	-	9.9	1	1	1
Methamphetamine		2.1	-	9.9	1	1	1

Etilefrine		0.1	9.1	9.7	3	3	1
Pentetrazol		0.1	-	-0.5	0	3	1
Nikethamide		0.3	-	3.6	0	2	1
Ecgoninemethylester (Metabolite of Cocaine)		0.6	14.6	9	1	4	0
Cathine		0.8	13.9	9.4	2	2	1
Cathinone		1.1	18.6	7.5	1	2	1
Methylephedrine		1.7	13.9	9.3	1	2	1
Methylhexaneamine		1.9	-	10.6	1	1	0

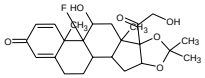
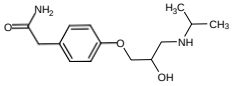
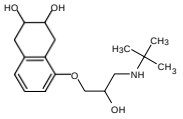
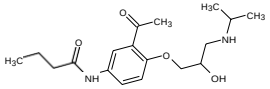
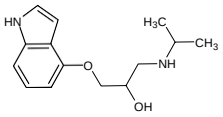
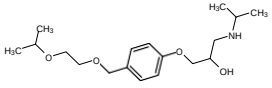
Phendimetrazine		1.9	-	7.3	0	2	1
Strychnine		1.9	-	8.3	0	3	1
Cocaine		2.3	-	8.6	0	5	1
N-Desmethylselegiline (Selegiline)		2.3	-	n/a	1	1	1
Selegiline		2.8	-	8.7	0	1	1
Modafinil		0.6	-	-6.9	1	3	2
Propylhexedrine		3.5	-	10.6	1	1	0

Narcotics (S7)

Morphine		0.8	10.2	8.2	2	4	1
Oxymorphone		0.8	10.1	8.2	2	5	1
Oxycodone		1.2	13.6	8.7	1	5	1
6-Acetylmorphine (Metabolite of Diamorphine or heroin)		1.3	10.2	9	1	5	0
Norfentanyl		1.6	-	10	1	2	1
Hydromorphone		1.8	10.1	8.6	1	4	1
Pethidine		2.5	-	8.7	0	3	1

Pentazocine		3.3	7.6	12.4	1	2	0
Norbuprenorphine (Metabolite of Buprenorphine)		3.8	9.8	10.5	3	5	0
Methadone		3.9	-	9.2	0	2	2
Fentanyl		4.0	-	9	0	2	2
Buprenorphine		5.0	7.5	12.5	2	5	0
Cannabinoids (S8)							
JWH-018 5-pentanoic acid metabolite (Metabolite of JWH-018)		4.7	n/a	n/a	1	3	2

CP 47, 497-C8-hydroxy, C8-homolog (Metabolite of CP 47, 497-C8-homolog)		5.5	-	-	3	3	1
HU-210		6.4	9.7	-	2	3	1
Glucocorticoids (S9)							
Triamcinolone		1.2	13.4	-	4	7	1
6-β-OH-budesonide (Metabolite of Budesonide)		1.3	13.7	-	3	7	1
Prednisolone		1.6	12.6	-	3	5	1
Betamethasone		1.8	13.4	-	3	6	1
Budesonide		2.5	13.7	-	2	6	1

Triamcinolone acetonide		2.5	13.4	-	2	7	1
Beta-blockers (P1)							
Atenolol		0.2	14.1	9.7	3	4	1
Nadolol		0.7	13.6	9.8	4	5	1
Acebutolol		1.7	13.9	9.6	3	5	1
Pindolol		1.8	14.1	9.7	3	3	1
Bisoprolol		1.9	14.1	9.7	2	5	1

[a]Values obtained for PubChem; [b] Values obtained for DrugBank; n/a: non available

Table S2. Suppliers for the 80 selected prohibited substances, internal standards, HRMS parameters and retention times

Analytes (n=80)	Supplier	IS used for quantification	Molecular formula	Theoretical m/z	Experimental m/z	Mass error (ppm)	ESI polarity	Rt standard (min)	Rt sample (min)	Rt error (min)
Anabolic agents (S1)										
Andarine	TRC	Raloxifene-d4	$C_{19}H_{18}F_3N_3O_6$	440.1064	440.1071	1.6	-	7.97	7.95	-0.02
Testosterone	LGC	Raloxifene-d4	$C_{19}H_{28}O_2$	289.2162	289.2162	0.0	+	8.40	8.37	-0.03
4-Androsten-17α-ethynyl-17β-ol-3-one	Steraloids	Raloxifene-d4	$C_{21}H_{28}O_2$	313.2162	313.2164	0.6	+	8.93	8.85	-0.08
3-OH-stanozolol	NMI	Raloxifene-d4	$C_{21}H_{32}N_2O_2$	345.2536	345.2534	0.6	+	8.27	8.27	0.00
Hypoxia-inducible factor (HIF) activating agents (S2)										
Roxadustat	TRC	Raloxifene-d4	$C_{19}H_{16}N_2O_5$	353.1132	353.1134	0.6	+	9.77	9.76	-0.01
Beta-2 Agonists (S3)										
Salbutamol	LGC	Oxycodone-d3	$C_{13}H_{21}NO_3$	239.1521	239.1519	0.8	+	4.43	4.43	0.00
Terbutaline	LGC	Raloxifene-d4	$C_{12}H_{19}NO_3$	226.1438	226.1446	3.5	+	4.53	4.53	0.00
Procaterol	Sigma-Aldrich	Raloxifene-d4	$C_{16}H_{22}N_2O_3$	291.1703	291.1703	0.0	+	4.56	4.56	0.00
Formoterol	NMI	Raloxifene-d4	$C_{19}H_{24}N_2O_4$	345.1809	345.1809	0.0	+	5.78	5.80	+0.02
Higenamine	Chromadex	Raloxifene-d4	$C_{16}H_{17}NO_3$	272.1281	272.1282	0.4	+	4.47	4.55	+0.08

Tulobuterol	TRC	Raloxifene-d4	$C_{12}H_{18}ClNO$	228.1150	228.1151	0.4	+	6.46	6.50	+0.04
Vilanterol	TRC	Raloxifene-d4	$C_{24}H_{33}Cl_2NO_5$	486.1809	486.1798	2.3	+	7.97	7.95	-0.02
Hormone and metabolic modulators (S4)										
Aminoglutethimide	LGC	Cathine-d3	$C_{13}H_{16}N_2O_2$	233.1285	233.1286	0.4	+	4.90	4.87	-0.03
Anastrozole	TRC	Raloxifene-d4	$C_{17}H_{19}N_5$	294.1713	294.1715	0.7	+	6.70	6.70	0.00
Bis-(4-cyanophenyl) methanol	NMI	Raloxifene-d4	$C_{15}H_{10}N_2O$	233.0709	233.0717	3.4	-	7.18	7.18	0.00
Exemestane	TRC	Raloxifene-d4	$C_{20}H_{24}O_2$	297.1849	297.1850	0.4	+	8.68	8.66	-0.02
SR9009	Cayman Chemicals	Raloxifene-d4	$C_{20}H_{24}ClN_3O_4S$	438.1249	438.1245	0.9	+	11.37	11.35	-0.02
GW501516 Sulfone	TRC	Raloxifene-d4	$C_{21}H_{18}F_3NO_5S_2$	486.0651	486.0640	2.3	+	8.94	8.92	-0.02
GW501516 Sulfoxide	TRC	Raloxifene-d4	$C_{21}H_{18}F_3NO_4S_2$	470.0702	470.0693	1.9	+	8.77	8.74	-0.03
Raloxifene	LGC	Raloxifene-d4	$C_{28}H_{27}NO_4S$	474.1734	474.1722	2.5	+	7.58	7.56	-0.02
4-OH-toremifene	TRC	Raloxifene-d4	$C_{26}H_{28}ClNO_2$	422.1881	422.1875	1.4	+	8.36	8.35	-0.01
Toremifene	TRC	Raloxifene-d4	$C_{26}H_{28}ClNO$	406.1932	406.1929	0.7	+	10.04	10.03	-0.01
17-ketone fulvestrant	TRC	Raloxifene-d4	$C_{32}H_{45}F_5O_3S$	605.3082	605.3071	1.8	+	11.62	11.60	-0.02
Fulvestrant	LGC	Raloxifene-d4	$C_{32}H_{47}F_5O_3S$	607.3239	607.3231	1.3	+	11.08	11.06	-0.02
Diuretic and masking agents (S5)										

Acetazolamide	Pharmacopeia USP	Oxycodone-d3	$C_4H_6N_4O_3S_2$	222.9924	222.9914	4.5	+	4.90	4.95	-0.05
Clorothiazide	Dr Eherestofer	Raloxifene-d4	$C_7H_6ClN_3O_4S_2$	293.9404	293.9416	4.1	-	4.38	4.39	+0.01
Chlortalidone	Pharrmacopeia USP	Raloxifene-d4	$C_{14}H_{11}ClN_2O_4S$	337.0044	337.0055	3.3	-	5.36	5.44	+0.08
Triamterene	LGC	Raloxifene-d4	$C_{12}H_{11}N_7$	254.1149	254.1148	0.4	+	5.39	5.41	+0.02
Bendroflumethiazide	LGC	Raloxifene-d4	$C_{15}H_{14}F_3N_3O_4S_2$	422.0451	422.0445	1.4	+	6.01	5.99	-0.02
Quinethazone	LGC	Raloxifene-d4	$C_{10}H_{12}ClN_3O_3S$	288.0204	288.0216	4.2	-	4.92	4.93	+0.01
Furosemide	LGC	Raloxifene-d4	$C_{12}H_{11}ClN_2O_5S$	328.9993	329.0005	3.6	-	6.34	6.31	-0.03
Bumetanide	Pharmacopeia USP	Raloxifene-d4	$C_{17}H_{20}N_2O_5S$	363.1009	363.1021	3.3	-	6.95	6.93	-0.02
Indapamide	LGC	Raloxifene-d4	$C_{16}H_{16}ClN_3O_3S$	364.0517	364.0525	2.2	-	5.95	5.94	-0.01
Etacrynic acid	Pharmacopeia USP	Raloxifene-d4	$C_{13}H_{12}Cl_2O_4$	301.0029	301.0044	5.0	-	7.34	7.30	-0.04
Stimulants (S6)										
Ritalinic acid	LGC	Raloxifene-d4	$C_{13}H_{17}NO_2$	220.1332	220.1333	0.4	+	5.31	5.30	-0.01
Benzoylecgonine	LGC	Raloxifene-d4	$C_{16}H_{19}NO_4$	290.1387	290.1388	0.4	+	5.85	5.84	-0.01
Carphedon	NMI	Raloxifene-d4	$C_{12}H_{14}N_2O_2$	219.1128	219.1131	1.4	+	5.55	5.52	-0.03

Amphetamine	LGC	Raloxifene-d4	$C_9H_{13}N$	136.1121	136.1122	0.4	+	5.08	5.17	+0.09
Methamphetamine	LGC	Raloxifene-d4	$C_{10}H_{15}N$	150.1277	150.1278	0.4	+	5.30	5.31	+0.01
Etilefrine	Pharmacopeia	Betamethasone-d5	$C_{10}H_{15}NO_2$	182.1176	182.1176	0.0	+	6.46	6.48	+0.02
Pentetrazol	LGC	Raloxifene-d4	$C_6H_{10}N_4$	139.0978	139.0975	2.2	+	5.07	5.07	0.00
Nikethamide	LGC	Raloxifene-d4	$C_{10}H_{14}N_2O$	179.1179	179.1178	0.4	+	5.55	5.54	-0.01
Ecgonine methyl ester	LGC	Salbutamol-d3	$C_{10}H_{17}NO_3$	200.1281	200.1281	0.0	+	3.62	3.70	+0.08
Cathine	Cerilliant	Oxycodone-d3	$C_9H_{13}NO$	152.1070	152.1071	0.4	+	4.48	4.46	-0.02
Cathinone	Cerilliant	Betamethasone-d5	$C_9H_{11}NO$	150.0913	150.0914	0.4	+	4.54	4.63	+0.09
Methylephedrine	LGC	Betamethasone-d5	$C_{11}H_{17}NO$	180.1383	180.1383	0.0	+	5.05	5.02	-0.03
Methylhexaneamine	Sigma	Raloxifene-d4	$C_7H_{17}N$	116.1434	116.1433	0.4	+	5.40	5.42	+0.02
Phendimetrazine	Cerilliant	Raloxifene-d4	$C_{12}H_{17}NO$	192.1383	192.1384	0.4	+	5.29	5.31	+0.02
Strychnine	Sigma	Raloxifene-d4	$C_{21}H_{22}N_2O_2$	335.1754	335.1752	0.6	+	5.70	5.72	+0.02
Cocaine	LGC	Raloxifene-d4	$C_{17}H_{21}NO_4$	304.1543	304.1542	0.4	+	6.46	6.51	+0.05
N-Desmethylselegiline	LGC	Raloxifene-d4	$C_{12}H_{17}N$	174.1277	174.1278	0.4	+	5.60	5.61	+0.01
Modafinil	TRC	Raloxifene-d4	$C_{15}H_{15}NO_2S$	274.0896	274.0906	3.6	+	5.90	5.87	-0.03
Selegiline	Cerilliant	Raloxifene-d4	$C_{13}H_{17}N$	188.1434	188.1436	0.6	+	5.74	5.75	+0.01

Propylhexedrine	LGC	Raloxifene-d4	$C_{10}H_{21}N$	156.1747	156.1747	0.0	+	6.52	6.59	+0.07
Narcotics (S7)										
Morphine	LGC	Salbutamol-d3	$C_{17}H_{19}NO_3$	286.1438	286.1439	0.4	+	4.42	4.46	+0.04
Oxymorphone	LGC	Oxycodone-d3	$C_{17}H_{19}NO_4$	302.1387	302.1389	0.7	+	4.36	4.36	0.00
Oxycodone	LGC	Raloxifene-d4	$C_{18}H_{21}NO_4$	316.1543	316.1545	0.6	+	4.91	4.95	+0.04
6-Acetylmorphine	LGC	Raloxifene-d4	$C_{19}H_{21}NO_4$	328.1543	328.1543	0.0	+	4.70	4.80	+0.10
Norfentanyl	Cerilliant	Raloxifene-d4	$C_{14}H_{20}N_2O$	233.1648	233.1648	0.0	+	5.64	5.65	+0.01
Hydromorphone	Cerilliant	Oxycodone-d3	$C_{17}H_{19}NO_3$	286.1438	286.1439	0.4	+	4.32	4.29	-0.03
Pethidine	Cerilliant	Raloxifene-d4	$C_{15}H_{21}NO_2$	248.1645	248.1644	0.4	+	6.24	6.34	+0.10
Pentazocine	Cerilliant	Raloxifene-d4	$C_{19}H_{27}NO$	286.2165	286.2164	0.4	+	6.96	6.93	-0.03
Norbuprenorphine	Cerilliant	Raloxifene-d4	$C_{25}H_{35}NO_4$	414.2639	414.2634	1.2	+	6.51	6.53	+0.02
Methadone	Cerilliant	Raloxifene-d4	$C_{21}H_{27}NO$	310.2165	310.2165	0.0	+	9.66	9.63	-0.03
Fentanyl	LGC	Raloxifene-d4	$C_{22}H_{28}N_2O$	337.2274	337.2280	1.8	+	8.11	8.11	0.00
Buprenorphine	Cerilliant	Raloxifene-d4	$C_{29}H_{41}NO_4$	463.3108	463.3124	3.5	+	7.82	7.78	-0.04
Cannabinoids (S8)										
JWH-018 5-pentanoic acid metabolite	Cerilliant	Raloxifene-d4	$C_{24}H_{21}NO_3$	372.1594	372.1585	2.4	+	5.71	5.71	0.00

CP 47, 497-C8-hydroxy, C8-homolog	Cayman Chemicals	Raloxifene-d4	$C_{22}H_{36}O$	331.2632	331.2634	0.3	-	10.53	10.44	-0.09
HU-210	Cayman Chemicals	Raloxifene-d4	$C_{25}H_{38}O_3$	387.2894	387.2882	3.1	-	10.05	10.05	0.00
Glucocorticoids (S9)										
Triamcinolone	LGC	Raloxifene-d4	$C_{21}H_{27}FO_6$	395.1864	395.1867	0.8	-	5.85	5.82	-0.03
6-β-OH-budesonide	TRC	Raloxifene-d4	$C_{25}H_{34}O_7$	447.2377	447.2379	0.4	+	6.95	6.98	+0.03
Prednisolone	LGC	Raloxifene-d4	$C_{21}H_{28}O_5$	361.2010	361.2009	0.3	-	6.80	6.90	+0.10
Betamethasone	Dr. Ehrenstorfer	Raloxifene-d4	$C_{22}H_{29}FO_5$	393.2072	393.2075	0.8	+	7.29	7.33	+0.04
Budesonide	Sigma	Raloxifene-d4	$C_{25}H_{34}O_6$	431.2428	431.2419	2.1	+	8.60	8.58	-0.02
Triamcinolone acetonide	LGC	Raloxifene-d4	$C_{24}H_{31}FO_6$	435.2177	435.2165	2.8	+	7.59	7.56	-0.03
Beta-blockers (P1)										
Atenolol	LGC	Oxycodone-d3	$C_{14}H_{22}N_2O_3$	267.1703	267.1711	2.9	+	4.64	4.66	+0.02
Nadolol	LGC	Raloxifene-d4	$C_{17}H_{27}NO_4$	310.2013	310.2013	0.0	+	5.33	5.34	+0.01
Acebutolol	LGC	Raloxifene-d4	$C_{18}H_{28}N_2O_4$	337.2122	337.2123	0.3	+	5.76	5.76	0.00
Pindolol	LGC	Raloxifene-d4	$C_{14}H_{20}N_2O_2$	249.1598	249.1596	0.8	+	5.39	5.40	+0.01

Bisoprolol	Sigma	Betamethasone-d5	$C_{18}H_{31}NO_4$	326.2326	326.2324	0.6	+	6.83	6.81	-0.02
Internal standards										
Salbutamol-d3	LGC		$C_{13}H_{18}D_3NO_3$	243.1782	243.1785	1.2	+	4.43	4.40	-0.03
Cathine-d3	LGC		$C_9H_{10}D_3NO$	155.1258	155.1255	1.9	+	4.48	4.46	-0.02
Oxycodone-d3	LGC		$C_{18}H_{18}D_3NO_4$	319.1732	319.1728	1.2	+	4.90	4.96	+0.06
Betamethanose-d5	TRC		$C_{22}H_{24}D_5FO_5$	398.2386	398..2374	3.0	+	6.73	6.76	+0.03
Raloxifene-d4	TRC		$C_{28}H_{23}D_4NO_4S$	478.1912	478.1910	0.4	+	7.57	7.56	-0.01

Table S3. Physico-chemical parameters for the anonymized urine samplesprovided by the anti-doping control ISCIII laboratory.

Sample	pH	Specific gravity	Sex
01	6.40	1.017	Male
02	5.65	1.013	Female
03	6.67	1.024	Male
04	5.35	1.029	Male
05	5.66	1.029	Male
06	6.01	1.018	Male
07	5.10	1.015	Male
08	5.26	1.027	Female
09	6.17	1.030	Male
10	5.50	1.015	Male
11	6.45	1.024	Male
12	5.33	1.010	Male
13	6.66	1.012	Female
14	6.52	1.019	Male
15	6.01	1.020	Male
16	6.46	1.016	Male
17	6.70	1.012	Male
18	6.68	1.012	Male
19	6.37	1.019	Male
20	6.85	1.011	Male
21	7.09	1.007	Female
22	6.10	1.016	Male
23	6.02	1.016	Male
24	6.17	1.020	Male
25	5.80	1.012	Male
26	6.06	1.013	Female
27	5.64	1.004	Female
28	6.40	1.013	Male
29	6.38	1.021	Female
30	5.67	1.012	Male
31	6.85	1.018	Female
32	6.46	1.009	Female
33	6.95	1.022	Female
34	5.39	1.032	Male
35	5.30	1.031	Male
36	5.87	1.013	Female

[a] DL: Decision limits for threshold substances, [b] MRPL: minimum required performance limits for non-threshold substances

Table S4. Analytical parameters for the selected prohibited substances, method detection limits (MDL), WADA approved decision limits (DL) and minimum required performance levels (MRPL), and intra-day and inter-day precision.

Analytes **(n=80)**	**Slope ± SD (mL ng^{-1})**	**Intercept ± SD (ng mL^{-1})**	**r^2**	**MDL (ng mL^{-1})**	**MQL (ng mL^{-1})**	**[a]DL or [b]MRPL (ng mL^{-1})**	**Intra-day precision (RSD, %)**	**Inter-day precisión (RSD, %)**
Anabolic agents (S1)								
Andarine	0.0189±0.0005	-0.08±0.03	0.996	0.05	0.2	2.5	1.9	6.7
Testosterone	0.0049±0.0002	0.03±0.01	0.992	0.07	0.2	2.5	3.8	3.3
4-Androsten-17α-ethynyl-17β-ol-3-one (Metabolite of Danazol)	0.0066±0.0003	-0.09±0.02	0.989	0.3	1.1	2.5	3.5	5.9
3-OH-stanozolol (Metabolite of Stanozolol)	0.019±0.001	-0.29±0.07	0.980	0.2	0.5	1	14.9	16.7
Hypoxia-inducible factor (HIF) activating agents (S2)								
Roxadustat	0.0018±0.0001	-0.002±0.004	0.991	0.08	0.2	2	11.1	11.3
Beta-2 Agonists (S3)								
Salbutamol	0.0159±0.0008	-0.02±0.04	0.991	0.1	0.4	1200[a]	2.8	4.1
Terbutaline	0.0163±0.0006	-0.01±0.03	0.995	0.1	0.4	20	3.1	3.9
Procaterol	0.0143±0.0005	-0.09±0.03	0.993	0.5	1.8	20	4.6	7.3

Formoterol	0.27±0.01	-0.2±0.6	0.992	0.3	1.1	50[a]	2.2	5.2
Higenamine	0.0177±0.0008	-0.13±0.04	0.993	1.3	4.4	10	10.7	19.7
Tulobuterol	0.033±0.001	-0.04±0.06	0.995	0.009	0.03	20	1.9	2.3
Vilanterol	0.0239±0.0009	-0.05±0.05	0.993	0.03	0.1	10	4.8	19.0
Hormone and metabolic modulators (S4)								
Aminoglutethimide	0.273±0.009	-1.9±0.5	0.994	0.2	0.8	20	2.2	4.8
Anastrozole	0.085±0.003	-0.3±0.1	0.995	0.9	3.0	20	3.3	3.5
Bis-(4-cyanophenyl) metanol (Metabolite of Letrozole)	0.0110±0.0004	-0.06±0.02	0.993	1.4	4.6	20	2.5	3.6
Exemestane	0.0189±0.0007	0.07±0.03	0.995	0.3	1.0	20	4.3	4.6
SR9009	0.0533±0.0007	-0.19±0.03	0.999	0.04	0.1	20	1.5	1.8
GW501516 Sulfone (Metabolite of GW501516)	0.0252±0.0006	-0.04±0.04	0.997	0.02	0.07	2	2.9	7.1
GW501516 Sulfoxide (Metabolite of GW501516)	0.0133±0.0001	-0.031±0.009	0.999	0.02	0.06	2	3.6	4.1
Raloxifene	0.026±0.001	-0.4±0.1	0.982	0.2	0.5	20	0.5	4.2
4-OH-toremifene (Metabolite of Toremifene)	0.104±0.004	-0.2±0.2	0.992	0.1	0.4	20	2.7	4.1

Toremifene	0.176±0.003		0.998			20	0.9	2.0
17-ketone fulvestrant	0.0077±0.0003	0.005±0.017	0.993	1.3	4.4	20	0.9	1.0
(Metabolite of Fulvestrant)								
Fulvestrant	0.0054±0.0002	-0.007±0.012	0.993	0.7	2.3	20	3.5	4.6
Diuretic and masking agents (S5)								
Acetazolamide	0.067±0.003	-0.3±0.2	0.990	1.0	3.4	20	2.9	10.9
Chlorothiazide	0.35±0.01	-0.9±0.9	0.990	2.5	8.3	200	1.3	2.0
Chlortalidone	0.197±0.003	-0.6±0.2	0.999	12.7	42.5	200	1.7	2.2
Triamterene	2.16±0.09	5.3±5.3	0.991	0.1	0.5	20	4.0	4.5
Bendroflumethiazide	0.0186±0.0005	0.003±0.029	0.996	5.0	16.8	200	1.2	2.6
Quinethazone	0.0233±0.0006	0.05±0.03	0.997	0.2	0.5	200	5.5	8.2
Furosemide	0.214±0.004	-0.9±0.2	0.999	0.3	0.9	20	2.8	3.4
Bumetanide	0.371±0.005	-1.6±0.3	0.999	0.08	0.3	20	1.5	2.6
Indapamide	0.47±0.02	-0.7±1.2	0.991	3.0	10.0	200	1.7	3.9
Etacrynic acid	0.0357±0.0007	-0.14±0.04	0.998	12.9	43.2	200	2.2	2.2
Stimulants (S6)								

Ritalinic acid (Metabolite of Methylphenidate)	0.166±0.006	-0.3±0.3	0.993	0.7	2.3	50	2.6	3.7
Benzoylecgonine (Metabolite of Cocaine)	0.32±0.01	-0.8±0.7	0.993	1.4	4.6	50	3.7	4.9
Carphedon	0.0118±0.0003	-0.04±0.02	0.997	2.6	8.8	50	3.9	4.1
Amphetamine	0.0325±0.0008	-0.09±0.04	0.998	4.7	15.7	50	1.1	6.4
Methamphetamine	0.0224±0.0008	-0.03±0.05	0.994	0.6	2.2	50	3.9	4.5
Etilefrine	0.170±0.009	-0.6±0.5	0.990	2.0	6.8	50	4.7	7.0
Pentetrazol	0.0179±0.0005	-0.15±0.02	0.997	2.2	7.4	50	3.1	8.1
Nikethamide	0.277±0.008	-0.7±0.5	0.996	0.6	2.2	50	1.2	3.3
Ecgonine methyl ester (Metabolite of Cocaine)	0.224±0.008	-0.5±0.5	0.994	4.1	13.8	50	1.7	4.9
Cathine	0.027±0.001	-0.05±0.05	0.994	3.6	12.1	6000[a]	8.8	13.1
Cathinone	0.144±0.005	-0.4±0.3	0.995	2.6	7.2	50	3.3	5.8
Methylephedrine	0.45±0.02	5.5±0.7	0.995	4.4	14.6	11000[a]	3.2	4.3
Methylhexanamine	0.075±0.002	-0.2±0.1	0.996	0.02	0.07	50	2.0	2.7
Phendimetrazine	0.212±0.005	-0.4±0.3	0.997	0.6	2.0	50	2.6	2.7
Strychnine	0.38±0.01	-0.1±0.6	0.995	0.9	2.9	50	1.1	2.4

Cocaine	0.72±0.03	-1.4±1.6	0.993	0.09	0.3	10	2.8	5.7
N-Desmethylselegiline	0.0120±0.0004	-0.04±0.02	0.996	2.9	9.7	50	2.9	3.3
(Metabolite de Selegiline)								
Modafinil	0.145±0.005	-0.4±0.3	0.995	4.7	15.6	50	7.6	8.5
Selegiline	0.053±0.002	-0.09±0.12	0.993	0.01	0.05	50	1.5	3.6
Propylhexedrine	0.161±0.004	10.6±0.1	0.998	3.3	10.9	50	1.8	2.8
Narcotics (S7)								
Morphine	0.219±0.009	-0.6±0.5	0.991	0.7	2.4	1300[a]	1.8	2.5
Oxymorphone	0.129±0.005	-0.4±0.2	0.995	0.6	1.9	25	14.0	19.2
Oxycodone	0.088±0.003	-0.4±0.2	0.995	1.6	5.5	25	2.6	3.2
6-Acetylmorphine (Metabolite of Diamorphine or heroin)	0.141±0.006	-1.0±0.3	0.994	0.9	2.9	25	3.7	4.5
Norfentanyl	0.160±0.005	-0.6±0.3	0.994	0.02	0.06	1	4.0	4.5
Hydromorphone	0.161±0.008	-0.8±0.4	0.990	0.6	1.9	25	1.8	2.5
Pethidine	0.37±0.01	-0.9±0.8	0.993	0.002	0.007	25	0.9	2.2
Pentazocine	0.43±0.01	-1.3±0.8	0.995	0.02	0.08	25	2.3	3.3
Norbuprenorphine (Metabolite of Buprenorphine)	0.213±0.007	-0.3±0.4	0.996	0.3	1.0	25	0.7	2.4

Methadone	0.369±0.009	-0.7±0.5	0.997	0.2	0.7	25	0.6	2.1
Fentanyl	0.0091±0.0003	0.03±0.02	0.996	0.04	0.1	1	14.0	19.0
Buprenorphine	0.0117±0.0003	-0.03±0.02	0.996	0.03	0.1	2.5	2.4	2.9
Cannabinoids (S8)								
JWH-018 5-pentanoic acid (Metabolite of JWH-018)	0.0033±0.0001	-0.039±0.008	0.990	0.01	0.04	1	1.7	4.9
CP 47, 497-C8-hydroxy, C8-homolog (Metabolite of CP 47, 497-C8-homolog)	0.000266±0.000008	-0.0013±0.0004	0.996	0.06	0.2	1	6.6	8.4
HU-210	0.00074±0.00005	-0.003±0.002	0.989	0.2	0.8	1	7.0	11.9
Glucocorticoids (S9)								
Triamcinolone	0.0059±0.0001	-0.010±0.008	0.997	1.2	4.0	30	11.1	13.1
6-β-OH-budesonide (Metabolite of Budesonide)	0.00264±0.00008	-0.015±0.005	0.996	3.3	11.0	45	7.9	19.0
Prednisolone	0.0157±0.0007	0.09±0.04	0.996	1.5	4.9	100	1.7	2.6
Betamethasone	0.0041±0.0002	-0.045±0.009	0.992	4.0	13.4	60	2.1	12.7
Budesonide	0.0084±0.0002	0.06±0.01	0.997	2.9	9.6	30	16.4	18.2
Triamcinolone acetonide	0.0065±0.0002	-0.02±0.02	0.992	0.1	0.4	15	2.1	8.2

Beta-blockers (P1)								
Atenolol	0.58±0.03	-7.4±1.6	0.991	0.5	1.8	50	3.7	19.5
Nadolol	0.36±0.01	-1.3±0.6	0.996	2.9	9.7	50	1.4	1.9
Acebutolol	0.57±0.02	-1.2±1.3	0.992	0.1	0.4	50	1.9	4.4
Pindolol	0.37±0.01	0.2±0.8	0.992	3.4	11.2	50	0.9	2.2
Bisoprolol	3.9±0.2	-12.3±10.2	0.990	1.7	5.6	50	0.9	0.9

Table S5. Recoveries values obtained for ten urine samples (S1-S10) fortified with the selected 80 banned substances at the respective MRPL values.

Selected WADA´s prohibited substances or their metabolites (n=80)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	Mean recovery
Anabolic agents (S1)											
Andarine	87	107	105	92	88	106	84	108	86	98	**96.1**
Testosterone	128	116	118	123	104	104	99	108	102	130	**113.2**
4-Androsten-17α-ethynyl-17β-ol-3-one (Metabolite of Danazol)	114	126	103	109	104	108	100	99	91	128	**108.2**
3-OH-stanozolol (Metabolite of Stanozolol)	103	85	86	101	82	88	101	119	86	118	**96.9**
Hypoxia-inducible factor (HIF) activating agents (S2)											

Roxadustat	102	108	113	80	94	108	98	116	90	87	**99.6**
Beta-2 Agonists (S3)											
Salbutamol	49	39	52	54	34	76	57	57	59	46	**52.3**
Terbutaline	103	112	119	87	87	126	100	114	80	113	**104.1**
Procaterol	97	83	85	94	72	66	85	94	96	96	**86.8**
Formoterol	78	89	106	87	113	93	83	110	89	110	**95.8**
Higenamine	112	71	98	92	118	126	105	96	124	112	**105.4**
Tulobuterol	81	88	116	79	94	107	89	85	82	84	**90.5**
Vilanterol	106	102	117	86	92	119	92	88	88	90	**98.0**
Hormone and metabolic modulators (S4)											
Aminoglutethimide	90	102	97	114	95	105	106	104	83	100	**99.6**
Anastrozole	102	101	117	101	108	107	104	98	93	96	**102.7**
Bis-(4-cyanophenyl) methanol (Metabolite of Letrozole)	79	108	98	73	85	91	87	102	89	98	**91.0**
Exemestane	111	105	115	97	94	111	93	98	88	95	**100.7**
SR9009	99	95	117	84	94	119	94	88	89	89	**96.8**
GW501516 Sulfone (Metabolite of GW501516)	109	105	113	90	102	104	98	89	88	91	**98.9**

GW501516 Sulfoxide (Metabolite of GW501516)	110	107	119	92	10	109	102	93	93	95	**93.0**
Raloxifene	95	98	104	107	88	114	89	85	85	85	**95.0**
4-OH-toremifene (Metabolite of Toremifene)	95	92	94	84	90	116	92	90	90	92	**93.5**
Toremifene	96	92	92	85	88	115	91	90	89	91	**92.9**
17-ketone fulvestrant (Metabolite of Fulvestrant)	98	100	107	88	99	115	97	85	91	91	**97.1**
Fulvestrant	100	94	119	81	96	97	96	92	95	93	**96.3**
Diuretic and masking agents (S5)											
Acetazolamide	92	72	94	78	118	71	103	120	77	76	**90.0**
Chlorothiazide	92	71	71	92	99	87	72	109	64	97	**85.3**
Chlortalidone	74	97	111	77	124	112	79	113	90	118	**99.5**
Triamterene	86	101	74	79	82	126	100	89	79	84	**90.1**
Bendroflumethiazide	91	102	105	101	96	118	85	89	76	95	**95.8**
Quinethazone	95	90	80	123	104	111	107	75	64	119	**96.8**
Furosemide	92	95	86	83	108	120	97	83	81	77	**92.1**
Bumetanide	93	97	83	85	92	101	100	117	89	113	**97.1**

Indapamide	71	105	79	112	123	99	85	119	62	116	**97.0**
Etacrynic acid	111	96	101	74	114	118	103	105	115	110	**104.6**
Stimulants (S6)											
Ritalinic acid (Metabolite of Methylphenidate)	79	73	93	90	73	90	72	93	78	76	**81.6**
Benzoylecgonine (Metabolite of Cocaine)	91	82	95	101	109	91	92	73	74	101	**90.9**
Carphedon	85	96	110	86	72	116	85	103	80	106	**93.8**
Amphetamine	102	74	91	90	75	86	82	76	73	81	**83.0**
Methamphetamine	77	76	91	95	120	95	83	93	74	82	**88.5**
Etilefrine	83	93	97	74	71	80	71	75	89	73	**80.5**
Pentetrazol	84	55	74	59	65	98	72	75	76	75	**73.1**
Nikethamide	87	86	106	88	87	109	83	71	72	73	**86.0**
Ecgonine methyl ester (Metabolite of Cocaine)	19	28	50	32	22	31	20	23	23	31	**27.8**
Cathine	68	58	57	56	50	83	57	47	71	64	**61.2**
Cathinone	83	67	119	124	55	103	73	95	113	109	**93.4**
Methylephedrine	77	106	94	80	80	106	80	80	93	84	**87.9**
Methylhexaneamine	91	86	86	88	89	110	85	114	83	98	**92.9**

Phendimetrazine	78	95	100	97	119	84	101	92	99	88	**95.2**
Strychnine	79	82	99	78	95	83	72	97	107	95	**88.7**
Cocaine	78	87	93	74	79	71	79	74	77	73	**78.4**
N-Desmethylselegiline (Metabolite de Selegiline)	95	79	101	82	102	112	94	95	93	84	**93.6**
Modafinil	93	102	112	98	80	119	88	102	84	77	**95.4**
Selegiline	92	91	117	80	98	118	93	89	92	97	**96.7**
Propylhexedrine	90	78	110	71	93	113	90	84	95	91	**91.5**
Narcotics (S7)											
Morphine	57	49	45	38	48	55	55	64	59	60	**53.0**
Oxymorphone	47	39	54	37	31	51	40	47	31	41	**41.7**
Oxycodone	79	87	64	68	99	85	79	97	76	80	**81.2**
6-Acetylmorphine (Metabolite of Diamorphine or heroin)	73	62	74	59	60	49	73	105	71	72	**69.8**
Norfentanyl	106	83	76	72	112	94	72	84	119	102	**92.0**
Hydromorphone	57	49	60	71	45	65	75	64	71	60	**61.6**
Pethidine	81	91	89	81	83	104	81	77	81	78	**84.4**

Pentazocine	88	94	114	87	87	110	90	77	84	80	**91.1**
Norbuprenorphine (Metabolite of Buprenorphine)	98	100	78	76	80	108	92	108	99	97	**93.5**
Methadone	100	98	120	87	96	123	95	87	91	91	**98.9**
Fentanyl	108	82	119	115	72	95	85	93	115	72	**95.4**
Buprenorphine	106	106	94	101	101	103	93	98	93	97	**99.3**
Cannabinoids (S8)											
JWH-018 5-pentanoic acid metabolite (Metabolite of JWH-018)	90	98	95	85	80	92	97	87	88	100	**91.2**
CP 47, 497-C8-hydroxy, C8-homolog (Metabolite of CP 47, 497-C8-homolog)	77	103	84	100	105	113	94	98	95	93	**96.0**
HU-210	103	103	101	85	91	117	93	87	92	83	**95.4**
Glucocorticoids (S9)											
Triamcinolone	94	102	109	110	87	115	89	89	115	72	**98.1**
6-β-OH-budesonide (Metabolite of Budesonide)	117	104	128	76	112	124	123	109	79	108	**108.0**
Prednisolone	96	119	101	95	105	120	83	119	106	116	**106.0**
Betamethasone	95	95	100	74	105	115	84	100	115	112	**99.2**

Budesonide	90	108	105	91	100	115	112	112	102	108	**104.1**
Triamcinolone acetonide	94	94	107	101	76	113	75	83	92	79	**91.2**
Beta-blockers (P1)											
Atenolol	79	70	48	68	65	54	49	75	44	71	**62.4**
Nadolol	50	75	82	91	65	76	64	83	59	81	**72.6**
Acebutolol	75	86	91	87	76	108	81	107	96	94	**90.0**
Pindolol	76	85	71	73	98	93	77	92	85	73	**82.1**
Bisoprolol	92	96	117	114	92	112	91	79	87	80	**95.8**

Table S6. Relative matrix effect values obtained for ten urine samples (S1-S10) fortified with the selected 80 banned substances at the respective MRPL values.

Selected WADA´s prohibited substances or their metabolites (n=80)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	Mean
Anabolic agents (S1)											
Andarine	-10	-6	12	-28	15	-9	-5	11	-15	-18	**-5.4**
Testosterone	12	-13	12	10	39	-14	8	15	-19	-16	**3.4**
4-Androsten-17α-ethynyl-17β-ol-3-one (Metabolite of Danazol)	17	32	7	-6	-8	17	-19	-17	-2	1	**2.2**
3-OH-stanozolol (Metabolite of Stanozolol)	-20	-20	0	-13	-10	-13	-15	-16	0	-14	**-12.1**
Hypoxia-inducible factor (HIF) activating agents (S2)											
Roxadustat	11	1	-1	-2	-15	-6	-20	24	11	31	**3.4**
Beta-2 Agonists (S3)											
Salbutamol	14	1	1	15	20	0	-11	12	20	11	**8.3**
Terbutaline	-36	-20	19	20	19	-34	19	-7	-16	11	**-2.4**
Procaterol	15	-33	10	19	30	-14	19	-8	-16	11	**3.4**
Formoterol	-20	-25	10	19	3	9	-3	11	-7	-17	**-2.0**
Higenamine	-9	-2	-16	6	5	-11	4	16	-9	18	**0.08**
Tulobuterol	20	-9	-13	13	18	-10	-17	-20	-13	-17	**-7.2**

Vilanterol	18	-3	19	20	32	-7	15	13	-16	-3	**8.9**
Hormone and metabolic modulators (S4)											
Aminoglutethimide	-13	-38	-39	-32	-31	-14	2	-37	-24	-20	**-24.4**
Anastrozole	3	-7	-1	-10	-18	-6	7	9	-6	-2	**-3.0**
Bis-(4-cyanophenyl) methanol (Metabolite of Letrozole)	-18	-19	9	-15	-17	-30	10	9	-19	-8	**-9.9**
Exemestane	-11	5	10	10	10	-11	7	12	14	4	**5.0**
SR9009	14	-5	5	11	4	-17	1	4	-15	-14	**-2.3**
GW501516 Sulfone (Metabolite of GW501516)	25	9	26	28	12	-5	15	12	-4	14	**13.2**
GW501516 Sulfoxide (Metabolite of GW501516)	15	9	15	13	9	-2	18	20	1	15	**11.3**
Raloxifene	10	-1	-12	11	5	4	8	6	5	-2	**3.4**
4-OH-toremifene (Metabolite of Toremifene)	23	-11	26	59	24	-6	12	8	-5	2	**13.4**
Toremifene	16	3	-12	-8	12	-3	17	8	8	14	**5.5**
17-ketone fulvestrant (Metabolite of Fulvestrant)	14	7	19	15	14	0	18	17	7	17	**12.7**
Fulvestrant	12	12	17	18	16	7	-2	1	14	-8	**8.8**

Diuretic and masking agents (S5)											
Acetazolamide	-31	-43	31	-40	-31	-40	-37	-40	-33	-47	**-30.9**
Chlorothiazide	54	59	49	-45	53	37	-38	34	29	19	**25.1**
Chlortalidone	-32	29	-31	-17	20	-29	29	20	-45	32	**-2.4**
Triamterene	-9	-14	-11	-18	-19	-15	-16	-18	-14	-15	**-14.9**
Bendroflumethiazide	-8	-9	-18	-17	-16	-9	19	15	-11	-4	**-5.8**
Quinethazone	-15	17	14	13	16	19	25	16	11	-38	**7.9**
Furosemide	-15	25	-17	-2	9	3	11	14	-3	-15	**1.1**
Bumetanide	-15	-16	-4	9	15	14	12	20	12	-20	**2.7**
Indapamide	1	-17	-11	-2	-19	-12	-11	-9	-5	-17	**-10.1**
Etacrynic acid	-10	2	-14	-16	3	-13	-13	14	-15	-12	**-7.3**
Stimulants (S6)											
Ritalinic acid (Metabolite of Methylphenidate)	-6	14	17	16	17	0	-2	15	7	-6	**7.3**
Benzoylecgonine (Metabolite of Cocaine)	-11	-6	-4	19	-4	-1	-3	8	4	-7	**-0.5**
Carphedon	2	-15	-12	-6	33	-17	-1	-10	-5	-12	**-4.1**
Amphetamine	-3	8	20	-3	16	9	-4	-20	8	12	**4.2**

Methamphetamine	-7	13	14	-8	-13	-7	2	15	-4	-9	**-0.2**
Etilefrine	14	-14	4	-6	-3	-17	-17	-20	7	-6	**-5.8**
Pentetrazol	16	-16	14	17	0	-13	-13	-12	-15	-12	**-3.6**
Nikethamide	-14	-8	10	-10	16	-14	-2	10	-2	-8	**-2.2**
Ecgonine methyl ester (Metabolite of Cocaine)	4	12	35	46	16	17	16	8	11	2	**16.4**
Cathine	-4	8	39	35	41	-44	12	6	5	1	**9.9**
Cathinone	18	-16	0	17	16	-5	3	-12	-13	-10	**-0.3**
Methylephedrine	-12	6	-16	2	-10	-4	10	3	9	2	**-1.2**
Methylhexaneamine	7	-12	9	-7	12	10	37	0	35	20	**11.1**
Phendimetrazine	4	2	11	15	17	9	2	8	17	19	**10.5**
Strychnine	-18	-11	-17	-18	-11	-20	-16	-18	-11	-10	**-14.9**
Cocaine	14	4	-1	-7	-19	-16	15	-7	-10	9	**-2.4**
N-Desmethylselegiline (Metabolite de Selegiline)	-23	37	31	17	19	-10	38	41	-2	41	**18.7**
Modafinil	-4	-13	-14	-4	-11	-14	-16	-19	-11	-7	**-11.1**
Selegiline	-6	13	20	-3	-7	-3	15	19	13	16	**7.7**
Propylhexedrine	12	12	-11	-11	20	1	12	30	7	26	**9.8**

Narcotics (S7)											
Morphine	6	14	-7	16	19	14	19	-30	-6	13	**5.8**
Oxymorphone	25	-10	37	10	47	-9	18	15	-12	-5	**11.6**
Oxycodone	-3	7	3	15	-8	3	13	12	11	11	**6.2**
6-Acetylmorphine (Metabolite of Diamorphine or heroin)	2	-14	-17	-34	19	43	18	32	-13	17	**5.2**
Norfentanyl	-15	-9	-11	-15	-11	-8	-9	-7	2	-10	**-9.2**
Hydromorphone	-20	-15	10	19	3	9	-3	11	-7	-17	**-1.0**
Pethidine	15	-1	-18	19	4	-4	16	8	4	9	**5.2**
Pentazocine	8	2	14	27	8	-14	11	9	4	12	**8.0**
Norbuprenorphine (Metabolite of Buprenorphine)	-18	-15	-3	-9	-15	-17	-11	12	-9	-14	**-9.8**
Methadone	13	1	-12	-13	8	-9	10	4	-3	6	**0.5**
Fentanyl	-5	1	1	7	14	-5	14	-6	7	12	**4.0**
Buprenorphine	16	17	16	10	15	14	3	6	7	8	**11.0**
Cannabinoids (S8)											
JWH-018 5-pentanoic acid metabolite (Metabolite of JWH-018)	9	-18	12	9	15	-12	-18	-13	-17	13	**-2.4**

CP 47, 497-C8-hydroxy, C8-homolog (Metabolite of CP 47, 497-C8-homolog)	15	-12	12	14	-10	1	17	-6	-15	-8	**1.2**
HU-210	-8	1	-14	-17	11	1	16	18	10	-15	**0.3**
Glucocorticoids (S9)											
Triamcinolone	-28	17	-2	-10	16	-6	-12	9	8	-15	**-2.1**
6-β-OH-budesonide (Metabolite of Budesonide)	7	9	-1	19	18	-5	15	12	-4	14	**8.4**
Prednisolone	-10	18	43	-16	29	40	-14	40	-15	-34	**8.1**
Betamethasone	18	-9	6	25	35	-14	10	17	-3	4	**9.0**
Budesonide	26	-4	18	20	18	-19	-5	4	-15	-14	**3.0**
Triamcinolone acetonide	17	-2	18	30	21	-13	-15	-8	-15	-25	**0.7**
Beta-blockers (P1)											
Atenolol	19	-18	25	-38	-15	-37	-28	14	-18	-17	**-11.3**
Nadolol	-11	-18	-27	2	-18	-7	-19	-18	4	-16	**-12.5**
Acebutolol	-8	-14	-1	7	-16	-8	-13	-9	-4	-18	**-8.5**
Pindolol	-12	-29	-34	-20	-15	-11	-19	-12	-9	-25	**-18.5**
Bisoprolol	19	-2	16	-8	-10	14	36	19	8	7	**9.8**

CONCLUSIONES

Las investigaciones realizadas durante el desarrollo de esta Tesis Doctoral han tratado de dar respuesta a los retos inicialmente planteados en los objetivos programados, en los cuales se ponía el foco en el desarrollo de metodologías innovadoras para el cribado y/o cuantificación de sustancias reguladas de interés para laboratorios de control agroalimentario y dopaje, así como de contaminantes emergentes en matrices ambientales. Para ello, en el ámbito del control de sustancias reguladas se han desarrollado métodos para la determinación de coccidiostatos en todas las matrices alimentarias legisladas y el cribado/cuantificación de sustancias prohibidas en orina, incluyendo sustancias de todos los grupos estructurales contemplados en la lista prohibida por la Agencia Mundial Antidopaje. En el área de medioambiente, se han desarrollado métodos para la biomonitorización de residuos de contaminantes emergentes, concretamente bisfenoles y benzofenonas, en aves rapaces, y la cuantificación de compuestos perfluorados en aguas superficiales.

Las innovaciones metodológicas más destacadas en las investigaciones realizadas han estado relacionadas con el desarrollo de disolventes supramoleculares (SUPRASs) a partir de moléculas anfifílicas de doble grupo polar. De acuerdo a la hipótesis de trabajo planteada en los objetivos de esta tesis, estos SUPRASs contienen una región hidrófila extensa, lo que ha permitido ampliar el intervalo de polaridad de los compuestos que pueden extraerse eficientemente. Así, se han desarrollado SUPRASs con estructura esponjosa a partir de 1,2-decanediol que ha permitido la extracción de compuestos perfluorados con valores de log P en el intervalo 0.4-11.6, así como SUPRASs cubosómicos a partir de 1,2-hexanediol, que ha permitido la extracción de sustancias prohibidas en deporte con valores de log P en el intervalo -2.4 a 9.2. Otra innovación metodológica desarrollada durante lasinvestigaciones de esta tesis ha sido la combinación de SUPRASs con extracción en

fase sólida dispersiva, lo que ha permitido la determinación selectiva de coccidiostatos ionóferos en todas las matrices alimentarias legisladas.

Las conclusiones generales más relevantes que pueden extraerse a partir de las investigaciones realizadas se exponen brevemente a continuación.

- Los SUPRASs cubosómicos y con estructura esponja permiten la extracción eficiente de compuestos que cubren un amplio intervalo de polaridad y por lo tanto constituyen una excelente herramienta para el desarrollo de plataformas de tratamiento de muestra para análisis de multicomponentes, lo que es relevante en control antidopaje y análisis ambiental.
- Los SUPRASs con propiedades de acceso restringido (SUPRAS-RAM), combinados con extracción en fase sólida dispersiva, constituyen una estrategia eficaz para la eliminación de efectos matriz en LC-(ESI)-MS/MS, lo que es relevante para la determinación de sustancias reguladas en matrices alimentarias.
- Los SUPRASs formados a partir de alcoholes y dioles no se ionizan en ESI y por tanto son ideales para su combinación con detección/cuantificación mediante LC-ESI-MS/MS o LC-ESI-TOF.
- Los tratamiento de muestras desarrollados sólo requieren un bajo volumen de SUPRAS (inferior a 0.5 mL), lo cual es relevante para incrementar la sostenibilidad de los procesos analíticos.
- La eficacia de extracción de los SUPRAS en extracciones líquido-liquido es muy superior a la de los disolventes orgánicos convencionales y permite su aplicación en áreas no abordables por éstos (ej. análisis de multicomponentes).
- La biomonitorización de contaminantes emergentes en aves rapaces, aunque esencial para la evaluación del impacto de los mismos, es un área que requiere nuevos desarrollos analíticos y más investigaciones para establecer las tendencias espaciales y temporales.

A continuación se exponen las conclusiones derivadas de cada una de las investigaciones desarrolladas.

Bloque A. Biomonitorización de contaminantes emergentes en aves rapaces europeas

Capítulo I. Revisión sobre contaminantes emergentes en aves rapaces Europeas (2002-2020)

- En Europa, la mayoría de las investigaciones publicadas sobre contaminantes emergentes en aves rapaces se han realizado por investigadores procedentes de Noruega y España.
- Los compuestos perfluorados (PFASs) y los nuevos retardantes de llama (NFRs) fueron los contaminantes de nuevo interés (CECs) más estudiados en huevos, plumas y plasma.
- Otros contaminantes emergentes encontrados en aves rapaces incluyen neonicotinoides, filtros ultravioleta, parafinas cloradas y bisfenoles. Sin embargo, dada la baja cantidad de datos disponibles en la literatura, se urge a la necesidad de recolectar más información sobre estos compuestos para evaluar su impacto ambiental.
- La mayoría de los estudios temporales realizados para PFASs se han llevado a cabo en huevos de aves rapaces Europeas. Estos estudios han reportado concentraciones crecientes de perfluorocarboxilatos (PFCAs) a lo largo del tiempo y resultados no concluyentes para el perfluorooctano sulfonato (PFOS).
- En cuanto a la recurrencia de los NFR), la mayoría de los estudios en los que las muestras de las aves rapaces (huevos, plumas y plasma) fueron recolectadas después del 2010, reportaron mayores concentraciones que de PBDEs, estando esto en concordancia con la retirada y restricciones impuestos a este último grupo de contaminantes.

- El águila de cola blanca, el halcón peregrino, el azor del Norte y el búho leonado han sido las especies de aves rapaces más estudiadas en Europa. Sin embargo, apenas existen estudios en el ratonero común, el cual es considerado una especie prioritaria para la biomonitorización de contaminantes prioritarios.
- No existe una matriz óptima para el monitoreo de contaminantes en aves rapaces y la selección de la matriz depende de los objetivos de cada estudio, el estado de conservación de la especie y el compuesto/s de interés. Para el análisis de PFASs se recomienda el uso de huevos, sangre y plasma. Las plumas son una matriz no destructiva muy atractiva, pero su uso no está recomendado para algunos contaminantes y además debe prestarse una atención especial a la contaminación externa que se puede dar sobre la superficie de la pluma.
- Una matriz que puede ser relevante es la glándula sebácea, considerada como el mayor órgano excretor de contaminantes emergentes, y que podría constituir una matriz de referencia para la biomonitorización de contaminantes.
- Las metodologías analíticas más usadas combinan la extracción con disolventes y cromatografía de gases o de líquidos acoplada a espectrometría de masas. Otros tratamientos de muestra alternativos (como QuECheRS o extracción con líquidos presurizados) se han investigado escasamente en este campo.

A partir de la revisión bibliográfica realizada, se destaca la necesidad de ampliar los estudios a otras regiones Europeas (principalmente Europa del Este), con el objetivo de mejorar nuestro conocimiento sobre la evolución temporal y espacial de los contaminantes emergentes. También se recomienda estudiar otras matrices y especies de aves rapaces y desarrollar nuevas estrategias analíticas más sensibles y selectivas. Finalmente, los efectos toxicológicos y ecológicos no han recibido mucha atención hasta el momento, y este aspecto se considera esencial para determinar qué contaminantes emergentes podrían ejercer mayor impacto.

Capítulo II. Potencial de bioacumulación de bisfenoles y benzofenonas de filtro ultravioleta: un enfoque multirresiduo en tejidos de aves rapaces.

Este estudio tuvo como objetivo investigar el perfil de la incidencia de bisfenolesy benzofenonas en diferentes tejidos de aves rapaces para determinar el potencial de bioacumulación de estos contaminantes. Para ello se determinaron 8 bisfenoles y 5 benzofenonas utilizadas como filtro UV en 6 tipos de tejidos, incluyendo músculo, riñón, hígado, cerebro, adiposo y glándula sebácea. Se analizaron 44 muestras de tejido en 20 aves rapaces pertenecientes a 3 especies diferentes colectadas en el periodo 1997-2011. Las conclusiones extraídas de este estudio son:

- Se demostró que el hígado, riñón y glándula sebácea eran matrices adecuadas para el biomonitoreo de estos contaminantes.
- La incidencia de los resultados obtenidos en los tejidos de tres especies de aves rapaces sugirieron un patrón de incremento de tasas de detección y medianas de concentraciones a medida que aumenta el tamaño de la especie de ave rapaz y su posición en la cadena trófica, aunque esto debe ser confirmado en un futuro a partir del análisis de un tamaño de muestra mayor.
- Se encontró que la glándula sebácea de las aves rapaces es el mayor órgano excretor de bisfenoles y benzofenonas
- Los contaminantes encontrados a mayor concentración en las aves rapaces analizadas fueron bisfenol A (BPA), bisphenol F (BPF), benzofenona-8 (BzP-8) y 4-hidroxibenzofenona (4-OH BzP).
- Los resultados obtenidos en este estudio confirman que los bisfenoles y las benzofenonas tienen potencial de bioacumulación en determinados tejidos de aves rapaces.

Bloque B. Desarrollo de plataformas genéricas de tratamiento de muestra para el control de contaminantes en alimentos

Capítulo III. Una nueva estrategia de tratamiento de muestra, basada en la combinación de un disolvente supramolecular con extracción en fase sólida dispersiva, para la determinación de coccidiostatos ionóforos en todos los alimentos legislados.

Se ha desarrollado un método para la extracción de residuos de coccidiostatos ionóforos en alimentos basado en el uso de la combinación de disolventes supramoleculares con propiedades de materiales de acceso restringido con extracción en fase sólida dispersiva. La formación del disolvente se produce espontáneamente a temperatura ambiente mediante la adición de hexanol, THF y agua a la muestra. La extracción en fase sólida dispersiva se realiza utilizando amina primaria secundaria (PSA) y sulfato de magnesio. El método se optimizó y validó para la determinación de los coccidiostatos ionóforos en todos los tipos de matrices legisladas (leche, huevos, tejido graso, hígado, riñón, y músculo de pollo y buey). A continuación, se exponen las conclusiones más relevantes derivadas de este estudio.

- La combinación de un SUPRAS-RAM y dSPE permite eliminar eficazmente los componentes de la matriz de la muestra (proteínas, lípidos y carbohidratos) lo que permite el desarrollo de metodologías que son independientes de la composición de las muestras y por tanto aplicables a una gran variedad de alimentos.
- El tratamiento de muestra sólo requiere la adición de pequeñas cantidades/volúmenes de reactivos (184 µL de hexanol, 84 µL de THF, 25 mg de PSA y 150 mg de $MgSO_4$), la agitación de la muestra durante 2 min y su centrifugación durante 10 min. El extracto de SUPRAS obtenido se analiza directamente mediante LC-ESI-MS/MS. El método por tanto es simple, rápido y económico.
- Esta estrategia constituye una buena alternativa a los métodos convencionales desarrollados para la determinación de coccidiostatos ionóforos, y se prevé que

se pueda extender a la extracción de diferentes contaminantes en los respectivos alimentos legislados lo que daría lugar al desarrollo de tratamientos de muestra genéricos.

Bloque C. Desarrollo de plataformas de tratamiento de muestra para la monitorización ambiental de contaminantes en un amplio intervalo de polaridad

Capítulo IV. Gotas de esponja basadas en anfifilos de doble cabeza polar: síntesis, caracterización y potencial en la extracción de compuestos con un amplio intervalo de polaridad.

Este capítulo recoge los resultados obtenidos en el estudio de la coacervación de 1,2-decanediol en medio hidro-orgánico, en la presencia y ausencia de sal. Se determinaron las condiciones de formación del SUPRAS, se desarrollaron las ecuaciones que predicen el volumen de SUPRAS formado en función de lasconcentraciones relativas de los ingredientes que lo constituyen, se determinó su composición química, se elucidaron las nanoestructuras que lo conforman y se investigó su aplicabilidad a la extracción de compuestos con un amplio intervalo de polaridad. Las conclusiones más relevantes de este estudio se exponen a continuación.

- Se demuestra que los SUPRASs formado a partir de 1,2-decanediol contiene mayor cantidad de agua en su estructura que los sintetizados a partir de decanol, y que por lo tanto, su región hidrofílica es más amplia, lo cual debería incrementar la eficiencia de extracción para compuestos muy polares.
- Acorde con su composición, las nanoestructuras de los SUPRAS de 1,2-decanediol son esponjas mientras las de los SUPRAS de 1-decanol son estructuras hexagonales inversas.

- Se demuestra que los SUPRAS de 1,2-decanediol extraen más eficientemente compuestos perfluorados con log P en el intervalo 0.4-11.6 que los SUPRAS formados a partir de 1-decanol.
- El método desarrollado para la extracción de PFCs en aguas superficiales presenta características analíticas y operacionales superiores a los métodos reportados previamente. Así, los PFCs se determinaron con una sensibilidad similar a otros métodos desarrollados en los que se requieren altos volúmenes de agua (en torno a 1L), utilizando menor tiempo de extracción (15 min en vez de 5.6-8.5 h) y evitando la etapa de evaporación.

Con esta investigación, se demostró el potencial que tienen los SUPRASs obtenidos a partir de anfifilos de doble cabeza polar en el análisis de multicomponentes.

Bloque D. Desarrollo de plataformas de tratamiento de muestra para el análisis de multicomponentes en control antidopaje

Se ha investigado la influencia de diferentes fases estacionarias y móviles tanto en los parámetros cromatográficos como efectos matriz en la determinación de drogas de abuso incluidas en la Lista Prohibida de la Word Antidoping Agency (WADA) mediante LC-(ESI)-MS/MS (Capitulo (V). Asimismo, se han desarrollado dos métodos analíticos para la detección y/o confirmación de estas drogas de abuso en muestras de orina de deportistas de alto rendimiento utilizando LC-(ESI)-MS/MS (Capítulo (V) y LC-ESI-TOF (Capítulo VII). En estas investigaciones, se ha puesto especial interés en el diseño de procedimientos de tratamiento de muestra de multicomponentes simples y rápidos, un aspecto fundamental para los laboratorios de control del dopaje, en los que se requiere el análisis de un gran número de muestras en un corto intervalo de tiempo. Para ello, se han diseñado disolventes supramoleculares basados en disoluciones salinas coloidales de anfifilos de doble cabeza polar (1,2-hexanediol). Un aspecto a destacar en los métodos que se presentan en los capítulos VI y VII es que el

volumen de SUPRAS necesario para la extracción eficiente de las drogas de abuso es de 250 µL de SUPRAS por muestrade orina (1 mL). A continuación, se detallan las conclusiones específicas de cada uno de los estudios.

Capítulo V. Influencia de diferentes mecanismos de retención en el comportamiento de drogas de abuso en cromatografía de líquidos acoplada a espectrometría de masas.

En este estudio, se investigó el efecto de fases estacionarias en fase reversa (C18, C8, pentafluorofenil PFP y fenilhexil PH), modo mixto hidrofílico/RP (HILIC-RP) e hidrofílico (HILIC). Las fases móviles utilizadas fueron agua con metanol o acetonitrilo como modificadores orgánicos, en presencia y ausencia de acetato amónico. En todos los casos se añadió ácido fórmico al 0.1%. Entre las drogas de abuso, se seleccionaron 93 sustancias prohibidas (o sus metabolitos) pertenecientesa 10 categorías de la lista de la WADA (S1-S9 además de la P1) con diferentes polaridades (log P desde -2.4 hasta 9.2) y funcionalidades. Las principales conclusiones se muestran a continuación.

- Aunque ninguna fase estacionaria/móvil fue óptima para todos los parámetros cromatográficos y efectos matriz estudiados, el uso de columnas como PFP, PH y HILIC-RP, que ofrecen mecanismos de retención adicionales a la dispersión, fue más eficaz para la separación de un elevado número de drogas de abuso, estableciendo así alternativas a la fase estándar utilizada (C18).
- Las interacciones π-π, dipolo-dipolo y de puente de hidrógeno proporcionadas por la fase reversa pentafluorofenilpropil (PFP) son particularmente interesantes en el cribado de drogas de abuso dado al gran número de las mismas que presentan anillos aromáticos y enlaces de hidrógeno.
- El uso de metanol en vez de acetonitrilo incrementa la retención de las drogas de abuso en PFP, mejorando así la separación.

- La columna HILIC proporcionó una separación eficiente para los compuestos muy polares y no aromáticos y por tanto, proporciona separación ortogonal en relación a PFP.

Capítulo VI. Disolventes supramoleculares cubosómicos: síntesis, caracterización y potencial en el análisis multicomponentes de sustancias prohibidas en orina.

Este capítulo recoge los resultados obtenidos en el estudio de la coacervación de 1,2-hexanediol en medio acuoso en la presencia de sal. Se determinaron las condiciones de formación del SUPRAS, se desarrollaron las ecuaciones que predicen el volumen de SUPRAS formado en función de las concentraciones relativas de los ingredientes que lo constituyen, se determinó su composición química, se elucidaron las nanoestructuras que lo conforman y se investigó su aplicabilidad a la extracción de 92 drogas de abuso en orina de deportistas de alto rendimiento, pertenecientes a 10 grupos diferentes de la lista prohibida de la Word Antidoping Agency (WADA). Las conclusiones más relevantes de este estudio se exponen a continuación.

- Se describen por primera vez SUPRASs formados por cubosomas cuyo contenido en agua puede alcanzar hasta el 61% w/w. Estos disolventes, inmiscibles en líquidos que contienen una elevada proporción de agua (ej. orina), presentan un elevado potencial para la extracción de multicomponentes en extracción líquido-líquido, un área en la que los disolventes orgánicos no son aplicables.
- Entre un 82-95% de las 92 drogas seleccionadas se extrajeron de forma eficiente (recuperaciones entre 70-120%) en muestras de orina y entre el 81-92% de estas drogas no presentaron efectos matriz.
- El método de tratamiento de muestra desarrollado es más simple, rápido y económico que la extracción en fase sólida (SPE).

- La baja toxicidad de los ingredientes del SUPRAS (1,2-hexanodiol, agua y sulfato de sodio) así como su simplicidad en la síntesis hacen que los SUPRAS cubosómicos cumplan con los principios de la química verde.

Capítulo VII. Disolventes supramoleculares para la microextracción líquido-líquido de multicomponentes para métodos de cribado basados en cromatografía de líquidos acoplada a espectrometría de masas de alta resolución.

El objetivo de las investigaciones recogidas en este capítulo ha sido demostrar el potencial de los SUPRASs cubosómicos para el cribado de multicomponentes mediante LC-(ESI)TOF. Para ello, se ha desarrollado y validado un método para el cribado de sustancias de abuso en el control del dopaje. A continuación se muestran las conclusiones más relevantes.

- El tratamiento de muestra requiere la adición de mínimos volúmenes/cantidades de reactivos (200 µL de 1,2-hexanediol y 142 mg de Na_2SO_4) a 1 mL de orina, la agitación durante 5 min y la centrifugación durante 10 min.
- El método de cribado basado en SUPRAS-LC-ESI-TOF ofrece altas recuperaciones y bajos efectos matriz.
- Se demuestra en el análisis de 36 muestras de orina anonimizadas de atletas que se obtienen resultados comparables con el uso combinado de tres métodos basados en GC-MS y LC-MS/MS. Por lo tanto, el uso de SUPRAS-LC-ESI-TOF simplifica de forma importante el cribado de muestras de orina en control antidopaje.

CONCLUSIONS

The research developed in this Doctoral Thesis has addressed the challenges described in the programmed objectives. These were focused on the developmentof innovative methodologies for the screening and/or quantification of regulated substances of interest in agrifood and doping control laboratories, as well as of emerging contaminants in environmental matrices. For this purpose and concerning the regulated substances, methods were developed for the determination of coccidiostats in all the legislated foods and for the screening/quantification of prohibited substances in urine, including compounds of all the structural groups covered in the Prohibited List of the World Antidoping Agency (WADA). Regarding emerging environmental contaminants, methods for biomonitoring of bisphenols and benzophenones in raptors and for quantification of perfluorinated compounds in surface waters were developed.

The most important methodological innovations in the research carried out in this Thesis were related to the development of supramolecular solvents (SUPRASs) formed from double-headed amphiphile molecules. According to the raised hypothesis, these SUPRASs have a wide hydrophilic region, that has allowed the increase of the polarity range of the compounds that can be efficiently extracted. Thus, SUPRASs with a spongy structure formed from 1,2-decanediol molecules were developed. These SUPRASs were applied to the extraction of perfluorinated compounds with log P values in the range 0.4-11.6. The development of cubosomic SUPRASs from 1,2-hexanediol amphiphiles allowed the extraction of prohibited substances in urine with log P values from -2.4 to 9.2. Another methodological innovation was the combination of SUPRASs with dispersive solid phase extraction, which enabled the selective determination of ionophore coccidiostats in all the regulated food matrices.

The most important general conclusions from this Thesis are described as follows:

- SUPRASs with a cubosomic or spongy structure allow the efficient extraction of compounds in a wide polarity range, making them an excellent tool for the

development of sample treatment platforms for the analysis of multicomponents in antidoping control and environmental analysis.

- SUPRASs with restricted access properties (SUPRAS-RAM), combined with dispersive solid phase extraction, are an effective strategy for the removal of matrix effects in LC-(ESI)-MS/MS. These SUPRASs are suitable for the determination of regulated substances in food matrices.
- SUPRASs formed from alcohols and diols don´t ionize in electrospray (ESI), thus making them ideal for their combination with detection/quantification techniques, such as LC-ESI-MS/MS or LC-ESI-TOF.
- The developed sample treatments only require a low volume of SUPRAS (less than 0.5 mL), making them relevant for the increase of the sustainability of the analytical processes.
- The extraction efficacy of the SUPRASs in liquid-liquid extraction is higher than the efficacy of other conventional organic solvents, thus allowing their application in those areas that are not approachable by the latter (e.g. multicomponent analysis).
- The biomonitoring of emerging contaminants in raptors is essential for the assessment of their impact and it is a field that requires new analytical developments and more research to establish temporal and spatial trends.

Below the conclusions derived from each research activity are shown.

Block A. Biomonitoring of emerging contaminants in European raptors.

Chapter I. A review on contaminants of emerging concern in European raptors (2002-2020).

- In Europe, most of the studies on contaminants of emerging concern (CECs) published in raptors were carried out in Norway and Spain.

- Perfluorinated compounds (PFASs) and new flame retardants (NFRs) were the most studied CECs in eggs, feathers and plasma.
- The detection of other miscellaneous CECs (neonicotinoids, UV-filters, chlorinated paraffins and bisphenols) and the limited data available in the literature suggest an urgent need to collect more information on these compounds in order to evaluate the environmental threat that they may pose.
- Most temporal trend studies con PFASs in European raptor eggs reported increasing concentrations of perfluorocarboxylates (PFCAs) over time and inconclusive results for perfluorooctane sulfonate (PFOS).
- Regarding the occurrence of NFRs, most of the studies in which raptor matrices (eggs, feathers and plasma) were collected after 2010, reported higher concentrations than that of polybrominated diphenyl ethers (PBDEs), being in accordance with the phase out and restrictions imposed on these persistent organic pollutants.
- White-tailed eagle, Peregrine falcon, Northern sparrowhawk and tawny owl were the most studied European species. However, studies on the common buzzard, which is considered a priority specie for the biomonitoring of CECs, barely exist.
- There is not an optimal all-purpose matrix for monitoring contaminants in raptors and the selection of the matrix depends on the objectives of each study, the conservation status of the species and the compound/s of interest. For the analysis of PFASs the use of eggs, blood and plasma is recommended. Feathers are a popular non-destructive sampling matrix for raptors, but their use may not be suitable for some CECs and specific attention is needed to avoid potential external contamination on the feather surface.
- A recent study highlighted the role of the preen gland as a major excretory organ for CECs, which can be further investigated as a potential matrix for the analysis of contaminants in raptors.
- The most used analytical methodologies usually combine solvent extraction with gas or liquid chromatography in tandem with mass spectrometry. Other

sample treatments (such as QuEChERS or pressurized liquid extraction) have been only scarcely investigated in this field.

The results of this study highlight the need for further research on CECs in other European regions (mainly Eastern Europe), with the aim to improve the assessment of temporal and spatial trends. It is also advisable to further examine suitable matrices and other species and to develop new and sensitive analytical strategies for their analysis (especially for CECs) in raptors. Finally, the toxicological effects and ecological impact of exposure to CECs on birds of prey and owls in Europe have not received much attention thus far and this would be essential to guide which CECs should be investigated in the future.

Chapter II. Bioaccumulation potential of bisphenols and benzophenone UV filters: A multiresidue approach in raptor tissues

The aim of this study was to investigate the profile of bisphenols (BPs) and benzophenones (BzPs) in different raptor tissues in order to determine the bioaccumulation profile of these contaminants. For this purpose, 8 BPs and 5 UV-filter BzPs were determined in 6 types of tissues, including muscle, kidney, liver, brain, fat and preen gland. Forty-four raptor tissues were analyzed in twenty raptors belonging to three different species and collected in the period 1997-2011. The conclusions derived from the study are the following:

- It was shown that the liver, kidney and preen gland tissues are suitable for the monitoring of CECs in raptors.
- The occurrence data in the tissues of the three raptor species suggested a pattern of increasing detection rates (DRs) and median concentrations with the increase of the raptor species size and the expected trophic position, although this needs to be further confirmed with larger sample sizes.
- The potential role of the preen gland as a major excretory organ for BPs and BzPs was suggested.

- Bisphenol A, bisphenol F, benzophenone-8 and 4-hydroxybenzophenone were the dominant contaminant analogues in the tissues.
- The results indicate that BPs and BzPs have bioaccumulation potential in specific raptor tissues.

Block B. Development of generic sample treatment platforms for the control of contaminants in foods.

Chapter III. A new sample treatment strategy based on simultaneous supramolecular solvent and dispersive solid-phase extraction for the determination of ionophore coccidiostats in all legislated foodstuffs.

A method for the extraction of ionophore coccidiostats in foods based on the combination of supramolecular solvents with restricted access materials (SUPRAS- RAM) and dispersive solid phase extraction (dSPE) has been developed. The solvent is spontaneously produced at room temperature through the addition of hexanol, THF and water to the sample. dSPE uses a primary secondary amine (PSA) and magnesium sulfate. The method was optimized and validated for the determination of ionophore coccidiostats in all the legislated matrices (milk, eggs, fat, liver, kidney and chicken/beef muscle). The most important conclusions derived from this study are shown below.

- The combination of a SUPRAS-RAM with dSPE allows to efficiently remove matrix effects from the sample (proteins, lipids, and carbohydrates) which allows the development of methodologies that are independent of the sample composition and applicable to a wide variety of foods.
- The sample treatment only requires the addition of low amounts/volumes of reagents (184 μL of hexanol, 84 μL of THF, 25 mg of PSA and 150 mg of $MgSO_4$), the shaking of the sample during 2 min and its centrifugation during

10 min. The SUPRAS extract is directly injected into the LC-ESI-MS/MS system. The method is simple, rapid and economic.

- This strategy constitutes a good alternative to conventional methods for the determination of ionophore coccidiostats and it is expected that it could be applied to the extraction of different contaminants in the legislated foods thus resulting in the development of generic sample treatments.

Block C. Development of sample treatment platforms for the environmental monitoring of contaminants in a wide polarity range.

Chapter IV. Double-headed amphiphile-based sponge droplets: synthesis, characterization and potential for the extraction of compounds over a wide polarity range

This chapter collects the results obtained from the study of the 1,2-decanediol coacervation in hydro-organic media and in the presence and absence of salt. TheSUPRAS formation conditions were developed as well as the equations that predict the SUPRAS volume that is generated as a function of the relative concentration of the reagents employed for its synthesis. SUPRASs were also characterized in terms of chemical composition and nanostructures. Finally they were applied to the extraction of compounds in a wide polarity range. The most relevant conclusions of this study are shown below.

- It is proved that SUPRASs formed from 1,2-decanediol molecules have a higher water content in their structure than those synthesized from decanol and, therefore, their hydrophilic region is wider and the extraction efficacy for very polar compounds is higher.
- According to their composition, the nanostructures of the 1,2-decanediol SUPRASs are spongy while those formed from 1,2-decanol present inverted hexagonal structures.

- It is demonstrated that SUPRASs of 1,2-decanediol extract more efficiently perfluorinated compounds with log P values in the range 0.4-11.6 than those made up of decanol.
- The developed method for the extraction of PFCs in surface waters shows greater analytical and operational characteristics than those previously reported. PFCs were determined with a similar sensitivity to other developed methods which required a high volumen of water (around 1L) and slow extraction times (5.6-8.5 h). Also, it was possible to avoid an evaporation step.

To conclude, the potential of SUPRASs formed from double-headed amphiphiles for the analysis of multicomponents was demonstrated.

Block D. Development of sample treatment platforms for the analysis of multicomponents in antidoping control.

The influence of different stationary and mobile phases on the chromatographic parameters and matrix effects for the determination of drugs of abuse was investigated. These compounds belong to the Prohibited List of the World Antidoping Agency (WADA) and they were analyzed with LC-(ESI)-MS/MS (Chapter V). Likewise, two analytical methods for the detection and/or confirmation of these drugs of abuse in urine samples of athletes using LC-(ESI)-MS/MS (Chapter VI) and LC-ESI-TOF (Chapter VII) were later developed. There is a special interest in designing simple and fast sample treatment procedures for multicomponents. This is critical for antidoping control laboratories, in which the analysis of a high number of samples in a short period of time is required. For this purpose, SUPRASs based on salty colloidal solutions of double-headed amphiphiles (1,2-hexanediol) were designed. An important aspect to mention regarding the methods presented in Chapters VI and VII is that the SUPRAS volume necessary for the efficient extraction of drugs of abuse is around 250 μL per urine sample (1 mL). The specific conclusions of each study are detailed below.

Chapter V. A comprehensive study on the performance of different retention mechanisms in sport drug testing by liquid chromatography tandem mass spectrometry

In this work, the effect of stationary reverse phases (C18, C8, pentafluorophenyl PFP and phenyl hexyl PH), mixed-mode hydrophilic/reverse phases (HILIC-RP) and hydrophilic phases (HILIC) was studied. The mobile phases consisted in water and methanol or acetonitrile in the presence and absence of ammonium acetate as organic modifier. In all cases, formic acid was added at 0.1%. Along the drugs of abuse, 93 prohibited substances (or their metabolites) belonging to ten categories of the WADA List (S1-S9 and P1) with different polarities (log P values from -2.4 to 9.2) and functionalities were selected. The most important conclusions derived from the study are shown below.

- Although there is not an optimal stationary/mobile phase that suits all the evaluated chromatographic parameters and matrix effects, the use of some columns, such as the PFP, PH and HILIC-RP, which offer additional dispersion retention mechanisms, offered more efficacy for the separation of a high number of drugs of abuse, thus establishing alternatives to the standard phase used (C18).
- π-π, dipole-dipole and hydrogen bond interactions provided by the reverse phase PFP are particularly interesting in the screening of drugs of abuse due to the great number of them that have aromatic rings and hydrogen bonds in their structure.
- The use of methanol instead of acetonitrile increases the retention of the drugs of abuse with the PFP column, thus enhancing the separation.
- The HILIC column provided an efficient separation for very polar and non aromatic compounds. This allows an orthogonal separation compared to the PFP column.

Chapter VI. Cubosomic supramolecular solvents: synthesis, characterization, and potential for high-throughput multiclass testing of banned substances in urine.

This chapter collects the results from the study of the 1,2-hexanediol coacervation in water and salt mixtures. The SUPRAS formation conditions as well as the equations that predict the SUPRAS volume that is generated as a function of the relative concentrations of the reagents in the synthesis solution were determined. Their chemical composition and the characterization of their nanostructures were also investigated. These SUPRASs were applied to the extraction of 92 drugs of abuse in urine samples of athletes. These compounds belong to ten different groups of the Prohibited List of the World Antidoping Agency (WADA). The most relevant conclusions of this study are shown below.

- Cubosomic SUPRASs are described for the first time and they have a high water content of up to 61% w/w. These solvents are immiscible in liquids that have a high proportion of water (e.g. urine) and have the ability to extract multicomponents in liquid-liquid extraction, a field in which organic solvents are not applicable.
- Around 82-95% of the 92 drugs were efficiently extracted (recoveries between 70-120%) from urine samples and 81-92% of these compounds did not show matrix effects.
- The sample treatment method is more simple, rapid and economic than the solid phase extraction (SPE).
- The low toxicity of the components of the SUPRAS (1,2-hexanediol, water and sodium sulphate) and its simple synthesis make it to meet the principles of Green Chemistry.

Chapter VII. Supramolecular solvents for comprehensive liquid-liquid microextraction in multiclass screening methods based on liquid chromatography-high resolution mass spectrometry.

The objective of the research carried out in this chapter was to demonstrate the potential of the cubosomic SUPRASs for the screening of multicomponents by LC-(ESI)TOF. For this purpose, a method for the screening of substances of abuse in antidoping control was developed. Next, the most relevant conclusions are shown.

- The sample treatment requires the addition of low volumes/quantities of reagents (200 µL of 1,2-hexanediol and 142 mg of Na_2SO_4) to 1 mL of urine. The extraction by shaking takes 5 min and the centrifugation 10 min. The screening method based on SUPRAS-LC-ESI-TOF provides high recoveries and low matrix effects.
- It is proved that the results from the analysis of 36 anonymized urine samples from athletes are comparable to the results from the combined use of three methods based on GC-MS and LC-MS/MS. Thus, the SUPRAS-LC-ESI-TOF use simplifies the screening of urine samples in antidoping control.

ANEXOS

Anexo A. Otras publicaciones científicas

Además de los 7 artículos científicos incluidos en esta Memoria, la doctoranda es coautora de otros dos artículos científicos como segunda autora.

1. Salatti-Dorado, J.A., González-Rubio, S., García-Gómez, D., Lucena, R., Cárdenas, S., Rubio, S., 2019. A high thermally stable oligomer-based supramolecular solvent for universal headspace Gas Chromatography: Proof-of-principle determination of residual solvents in drugs. Anal. Chim. Acta. 1046, 132-139.

2. Almofti, N., González-Rubio, S., Ballesteros-Gómez, A., Girela, E., Rubio, S., 2022. Green nanostructured liquids for the analysis of urine in drug facilitated sexual assault cases. Anal. Bioanal. Chem. DOI: https://doi.org/10.1007/s00216-022-04358-z

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A high thermally stable oligomer-based supramolecular solvent for universal headspace Gas Chromatography: Proof-of-principle determination of residual solvents in drugs

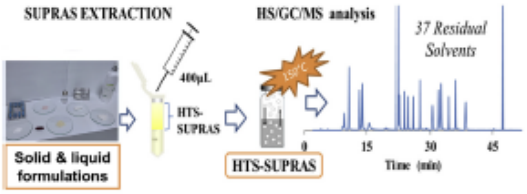
José Ángel Salatti-Dorado, Soledad González-Rubio, Diego García-Gómez*, [1], Rafael Lucena, Soledad Cárdenas, Soledad Rubio

Departamento de Química Analítica, Instituto Universitario de Investigación en Química Fina y Nanoquímica IUNQFN, Universidad de Córdoba, Campus de Rabanales, Edificio Marie Curie (anexo), E-14071, Córdoba, Spain

HIGHLIGHTS

- A supramolecular solvent with high thermal stability is described for the first time.
- This new solvent is based on poly-undecylenic acid, an oligomeric surfactant.
- It opens a new avenue for sample treatment prior to Headspace - Gas Chromatography.
- A validated determination of 37 residual solvents in 10 different drugs was achieved.

GRAPHICAL ABSTRACT



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ABSTRACT

Supramolecular solvent (SUPRAS) extraction is gaining attraction as a sample treatment technique because of its great performance in terms of effectiveness, versatility, sample clean-up, quickness, cost and sustainability. However, the nature of SUPRASs, being formed by amphiphile molecules, results in poor compatibility with Gas Chromatography (GC). Here, we show the hitherto unexplored development of a SUPRAS with high thermal stability (HTS) suitable for subsequent direct analysis by Headspace (HS)-GC. This novel HTS-SUPRAS, based on poly-undecylenic acid -an oligomeric surfactant-, tetraglyme -a polar aprotic solvent with excellent chemical and thermal stability- and water, was fully characterized in terms of composition and physical properties such as thermal stability. Subsequently, the HTS-SUPRAS developed was further successfully applied, as a proof-of-principle, to the extraction and determination of residual solvents in pharmaceutical drugs, including several solvents (class 2C) whose analysis by HS-GC has been shown to be highly complex. Analytical performance was demonstrated as mandated by the International Council for Harmonisation of technical requirements for pharmaceuticals for human use (ICH). Furthermore, excellent recoveries (70–120%) and high precisions (<20%, expressed as relative standard deviations) were obtained for the analysis of several different drug formulations spiked with the analyzed 37 residual solvents at their respective maximum residue levels.

Analytical and Bioanalytical Chemistry
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PAPER IN FOREFRONT

Green nanostructured liquids for the analysis of urine in drug-facilitated sexual assault cases

Nouman Almofti[1,2] · Soledad González-Rubio[1] · Ana Ballesteros-Gómez[1] · Eloy Girela[2] · Soledad Rubio[1]

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Abstract
In this work, we optimize and validate a simple, time-saving, and environmentally friendly sample preparation method based on supramolecular solvents (SUPRAS), green nanostructured liquids, for the extraction of selected drug-facilitated sexual assault (DFSA) substances from human urine. The methodology was fast and simple (stirring, centrifugation, and dilution). Cubosomic SUPRAS were formed by the addition of 1,2-hexanediol (200 µL) to 1.0 mL of human urine containing 1 M Na_2SO_4. SUPRAS extracts were analyzed by LC-MS/MS. The method was fully validated for 23 DFSA compounds including 10 benzodiazepines, 1 z-hypnotic drug, 5 amphetamine derivatives, 3 cocaine metabolites, and 4 miscellaneous compounds. Extraction efficiency varied between 79 and 119%, and matrix effects were acceptable (–14.3/+21.5) for 87% of the compounds. Method detection and quantification limits ranged from 0.003 to 0.75 ng/mL and from 0.01 to 2.50 ng/mL, respectively. These values were low enough for the established minimum required performance limits (MRPL) of these substances. This simple and green method has a great potential to be implemented for the monitoring of illegal drugs involved in DFSA cases by forensic laboratories.

Keywords Supramolecular solvents · Drug-facilitated sexual assault · Urine · Benzodiazepines · Cocaine · LC-MS/MS

Introduction

In the last two decades, social concern regarding drug-facilitated sexual assault (DFSA) has rapidly increased, a phenomenon in which a sexual attack is conducted under the influence of a certain pharmaceutical or other illegal substances [1, 2]. These substances incapacitate the victim to resist and bring them to a state of inability and delirium which facilitates any assault or violation [3]. DFSA cases are complicated to solve and require collaboration between the victim, police, medical staff, and scientific experts [4].

The analysis of substances involved in DFSA cases is challenging for forensic laboratories, and it has been an intensive field of research since the 1990s. DFSA compounds, like other illegal drugs, are determined in a variety of biological matrices that include blood, urine, and hair [5–8]. However, in sexual assault incidences, the time lapse between the administration of the drug and the reporting of the sexual attack is usually more than 12 h [9], which limits the use of blood as a suitable sample, since most of the DFSA compounds have short half-lives and a fast metabolism [10]. Therefore, urine is a priority sample that provides longer windows of detection for both parent compounds and their metabolites that can reach up to 96 h after the alleged sexual attack [11].

Sample preparation methods for the determination of DFSA substances in urine mostly include solid-phase extraction (SPE) [12] or liquid-liquid extraction (LLE) [6, 13], which involve multiple time-consuming and costly steps with the use of a considerable volume of organic solvents.

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In honor of Prof. Miguel Valcárcel.

✉ Ana Ballesteros-Gómez
ana.ballesteros@uco.es

[1] Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, Universidad de Córdoba, Anexo Marie Curie, Campus de Rabanales, 14071 Córdoba, Spain

[2] Section of Forensic and Legal Medicine, Department of Morphological and Sociosanitary Sciences, Faculty of Medicine and Nursing, University of Córdoba, 14071 Córdoba, Spain

Anexo B. Comunicaciones presentadas en congresos

Se han realizado un total de 12 contribuciones a congresos científicos (5 nacionales y 7 internacionales), donde se han mostrado los resultados de las investigaciones realizadas en esta Tesis. En concreto, se han presentado 3 pósteres y 9 comunicaciones orales, una de los cuales obtuvo el premio a la mejor comunicación oral del congreso.

1. Disolventes supramoleculares para la determinación rápida de coccidiostatos en leche. Autores: S. González-Rubio, D. García-Gómez, A. Ballesteros-Gómez, S. Rubio. Presentada en formato póster en la XXI Reunión de la Sociedad Española de Química Analítica (SEQA 2017). Celebrada en Valencia durante los días 5-7 de septiembre de 2017.

2. Aplicación de un disolvente supramolecular basado en hexanol para la extracción de coccidiostatos en muestras lácteas. Autores: S. González-Rubio, D. García-Gómez, A. Ballesteros-Gómez, S. Rubio. Presentada como comunicación oral en la IV Reunión de Jóvenes Investigadores en Coloides e Interfases (JICI-IV). Celebrada en Córdoba durante los días 7 y 9 de febrero de 2018.

3. Supramolecular solvent extraction + dispersive SPE as a new sample preparation technique. Application to the LC-MS/MS determination of coccidiostats in animal tissues and products of animal origin. Autores: S. González-Rubio, D. García-Gómez, A. Ballesteros-Gómez, S. Rubio. Presentada como comunicación oral en la 40th International Conference on Environmental & Food Monitoring (ISEAC-40). Celebrada en Santiago de Compostela durante los días 19-22 de junio de 2018.

4. Síntesis y caracterización de un nuevo disolvente supramolecular basado en un surfactante de doble cabeza polar. Aplicación para la extracción de compuestos perfluorados en aguas ambientales. Autores: S. González-Rubio, A.M. Ballesteros-Gómez, D. García-Gómez, S. Rubio. Presentada en formato póster en el VII Encuentro sobre Nanociencia y Nanotecnología de Investigadores Andaluces (NANOUCO VII). Celebrado en Córdoba durante los días 21 y 22 de enero de 2019.

5. Extraction of perfluorinated compounds in environmental waters with a novel nanostructured liquid. Autores: S. González-Rubio, A.M. Ballesteros-Gómez, D. García-Gómez, S. Rubio. Presentada como comunicación oral en la 17th International Conference on Chemistry and the Environment (ICCE 2019). Celebrada en Tesalónica durante los días 16-20 de junio 2019.

6. Síntesis y aplicación de un nuevo disolvente supramolecular basado en 1,2-decanodiol para la extracción de compuestos perfluorados en aguas naturales. Autores: S. González-Rubio, A.M. Ballesteros-Gómez, D. García-Gómez, S. Rubio. Presentada como comunicación oral en la XXII Reunión de la Sociedad Española de Química Analítica (SEQA 2019). Celebrada en Valladolid durante los días 17-19 de julio de 2019. Premio a la mejor comunicación oral del congreso.

7. Extraction of perfluorinated compounds in environmental waters with a novel nanostructured liquid prior to determination by LC-MS/MS. Autores: S. González-Rubio, A.M. Ballesteros-Gómez, D. García-Gómez, S. Rubio. Presentada como comunicación póster en la 4th International Mass Spectrometry School. Celebrado en Sitges (Barcelona), del 15 al 20 de septiembre de 2019.

8. A comprehensive study on the performance of different retention mechanisms in sport drug testing by liquid chromatography tandem mass spectrometry. Autores: Soledad González-Rubio, A. Ballesteros-Gómez, D.

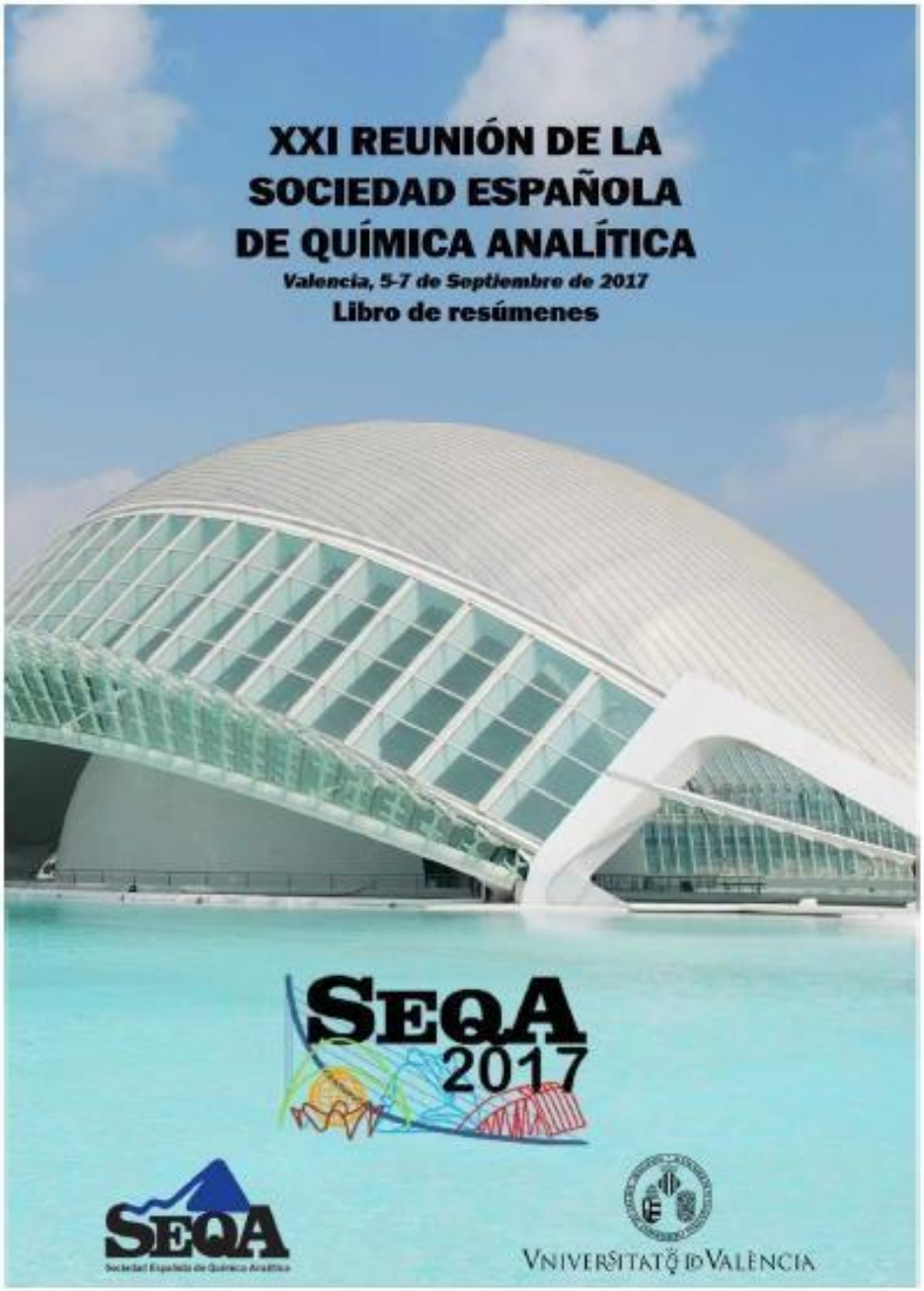
Carreras, G. Muñoz, S. Rubio Presentado como comunicación póster en la 39th Manfred Donike Workshop. Celebrado en Colonia (Alemania) del 22 al 26 de marzo de 2021.

9. Hyphenating supramolecular solvents and LC-MS/MS for high-throughput universal testing of banned substances in urine. Autores: Soledad González-Rubio, A. Ballesteros-Gómez, G. Muñoz, S. Rubio . Presentado como póster virtual en la Virtual Poster Session de la Partnership for Clean Competition (PCC). Celebrado virtualmente el 12 de julio de 2021.

10. Cubosomic supramolecular solvents: synthesis, characterization and potential for high throughput multiclass testing of banned substances in urine. Autores: S. González-Rubio, A. Ballesteros-Gómez, G. Muñoz, S. Rubio. Presentado como póster en la 40th Manfred Donike Workshop. Celebrado en Colonia (Alemania) del 28 de marzo al 1 de abril de 2022.

11. Synthesis, characterization, and potential of cubosomic supramolecular solvents for multiclass extraction of prohibited substances in urine. Autores: S. González-Rubio, N. Caballero-Casero, A.M. Ballesteros-Gómez, G. Muñoz, S. Rubio. Presentada como póster en la X Reunión de la Sociedad Española de Espectrometría de Masas (X-RSEEM). Celebrado en Córdoba durante los días 1-3 de junio de 2022.

12 A general screening method for testing of banned substances in urine based on cubosomic supramolecular solvents and liquid chromatography/time-of-flight mass spectrometry. Autores: Soledad González-Rubio, N. Caballero-Casero, A. Ballesteros-Gómez, D. Cuervo, G. Muñoz, S. Rubio Presentado como comunicación póster en el 41th Manfred Donike Workshop. Se celebrará en Colonia (Alemania) en marzo de 2023.


XXI REUNIÓN DE LA
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DISOLVENTES SUPRAMOLECULARES PARA LA DETERMINACIÓN RÁPIDA DE COCCIDIOSTATOS EN LECHE

S. González-Rubio, D. García-Gómez, A. Ballesteros-Gómez, S. Rubio

Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba, Edificio Anexo Marie Curie, Campus de Rabanales, 14071, Córdoba (España), ga1rubrs@uco.es

Los coccidiostatos ionóforos (Lasalocid, Narasin, Salinomycin, Monensin, Semduramicin y Maduramicin) son una familia de agentes antiprotozoarios obtenidos a partir de bacterias [1]. Químicamente, se caracterizan por poseer una cadena poli-éter que, de forma similar a los éteres corona, conduce a la formación de complejos con cationes monovalentes, lo que explica sus propiedades antibióticas. Actualmente, se utilizan de forma extensiva para prevenir la coccidiosis en la industria alimentaria (bovina, aviaria y láctea), por lo que sus niveles residuales máximos han sido regulados por la Comisión Europea (EC124/2009, EC37/2010 y EC86/2012). Entre las diferentes matrices legisladas, la leche presenta los límites más restrictivos, con tolerancias que varían entre 1 ng g^{-1} y 2 ng g^{-1}, dependiendo del compuesto. Es por lo tanto necesario desarrollar métodos analíticos que permitan su determinación de forma rápida, económica y fiable.

Los disolventes supramoleculares (SUPRAS) están mostrando un gran potencial como alternativa verde a los métodos tradicionales de extracción [2]. Los SUPRAS son líquidos nanoestructurados bioinspirados formados por agregados de compuestos anfifílicos organizados mediante autoensamblaje. Presentan regiones de diferente polaridad para la extracción de una amplia variedad de analitos. También se comportan como materiales de acceso restringido, lo cual permite realizar la extracción y exclusión simultánea de interferentes comunes en la muestra como, por ejemplo, macromoléculas (proteínas, polisacáridos, etc.).

En este trabajo se presenta el desarrollo, validación y aplicación de un método analítico basado en SUPRAS de hexanol como método rápido de extracción y limpieza para la determinación de coccidiostatos ionóforos en leche. Este disolvente, que presenta una nanoestructura hexagonal inversa, se forma de manera espontánea en la matriz acuosa de leche (1275 □L), en presencia del anfifilo hexanol (184 □L) y con la adición de un bajo volumen de tetrahidrofurano (84 □L), tras una simple etapa de extracción en vórtex (2 min, 3000 rpm) y centrifugación (15 min, 20000g) para acelerar la separación de fases. EL SUPRAS es directamente compatible con el sistema de detección por LC-MS/MS sin posteriores etapas de re-extracción, evaporación o limpieza. La recuperación obtenida fue cuantitativa para todos los coccidiostatos estudiados, obteniéndose un factor de preconcentración de 11. Los límites de detección variaron entre 3 pg g^{-1} para Salinomycin y 28 pg g^{-1} para Maduramicin, valores muy por debajo de los límites legislados.

La metodología desarrollada se aplicó al análisis de muestras de leche de diversa naturaleza y procedencia, encontrándose coccidiostatos en dos de las muestras a concentraciones que variaron entre 23 pg g^{-1} para Salinomycin y 189 pg g^{-1} para Monensin. En todo caso, cabe destacar que dichas concentraciones se encontraron por debajo de los límites establecidos por la legislación.

En conclusión, los SUPRAS son una interesante alternativa para la determinación de coccidiostatos en leche permitiendo desarrollar un método LC-MS/MS barato, rápido y de acuerdo a los parámetros establecidos por la legislación europea.

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IV reunión de Jóvenes Investigadores en Coloides e Interfases

Córdoba, 7-9 Febrero 2018

Universidad de Córdoba

JICI-IV

Libro de resúmenes

APLICACIÓN DE UN DISOLVENTE SUPRAMOLECULAR BASADO EN HEXANOL PARA LA EXTRACCIÓN DE COCCIDIOSTATOS EN MUESTRAS LÁCTEAS. Aplicaciones.

S. González-Rubio D. García-Gómez, A. Ballesteros-Gómez, S. Rubio

Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba, Edificio Anexo María Curie, Campus de Rabanales, 14071, Córdoba (España).
q02goruz@uco.es

Los coccidiostatos ionóforos (Lasalocid, Narasin, Salinomycin, Monensin, Semduramicin y Maduramicin) son una familia de agentes antiprotozoarios obtenidos a partir de bacterias. Químicamente, se caracterizan por poseer una cadena poli-éter, que de forma similar a los éteres corona, conduce a la formación de complejos con cationes monovalentes, lo que explica sus propiedades antibióticas. Actualmente, se utilizan de forma intensiva para prevenir la coccidiosis en la industria alimentaria (bovina, aviaria y láctea), por lo que sus niveles residuales máximos han sido regulados por la Comisión Europea (EC124/2009, EC37/2010 y EC86/2012). Entre las diferentes matrices legisladas, la leche presenta los límites más restrictivos, con tolerancias que varían entre 1 ng g^{-1} y 2 ng g^{-1}, dependiendo del compuesto. Es lo por lo tanto necesario desarrollar métodos analíticos que permitan su determinación de forma rápida, económica y fiable.

Los disolventes supramoleculares (SUPRAS) están mostrando un gran potencial como alternativa verde a los métodos tradicionales de extracción. Los SUPRAS son líquidos nanoestructurados bioinspirados formados por agregados de compuestos anfifílicos organizados mediante autoensamblaje. Presentan regiones de diferente polaridad para la extracción de una amplia variedad de analitos. También se comportan como materiales de acceso restringido, lo cual permite realizar la extracción y exclusión simultánea de interferentes comunes en la muestra como, por ejemplo, macromoléculas (proteínas, polisacáridos, etc).

En este trabajo se presenta el desarrollo, validación y aplicación de un método analítico basado en SUPRAS de hexanol como método rápido de extracción y limpieza para la determinación de coccidiostatos ionóforos en leche. Este disolvente, que presenta una nanoestructura hexagonal inversa, se forma de manera espontánea en la matriz acuosa de leche (1275 µL), en presencia del anfifilo hexanol (184 µL) y con la adición de un bajo volumen de tetrahidrofurano (84 µL), tras una simple etapa de extracción en vórtex (2 min, 3000rpm) y centrifugación (15min, 20000g) para acelerar la separación de fases. EL SUPRAS es directamente compatible con el sistema de detección por LC-MS/MS sin posteriores etapas de re-extracción, evaporación o limpieza. La recuperación obtenida fue cuantitativa para todos los coccidiostatos estudiados, obteniéndose un factor de preconcentración de 11. Los límites de detección variaron entre 3 pg g^{-1} para Salinomycin y 28 pg g^{-1} para Maduramicin, valores muy por debajo de los límites legislados.

La metodología desarrollada se aplicó al análisis de muestras de leche de diversa naturaleza y procedencia, encontrándose coccidiostatos en dos de las muestras a concentraciones que variaron entre 23 pg g^{-1} para Salinomycin y 189 pg g^{-1} para Monensin. En todo caso, cabe destacar que dichas concentraciones se encontraron por debajo de los límites establecidos por la legislación.

En conclusión, los SUPRAS son una interesante alternativa para la determinación de coccidiostatos en leche permitiendo desarrollar un método LC-MS/MS barato, rápido y de acuerdo a los parámetros establecidos por la legislación europea.

Agradecimientos: Los autores agradecen el apoyo financiero del MINECO (Proyecto CTQ2014-53539-R) y FEDER, D.G.G y A.B.G agradecen al MINECO su beca posdoctoral (FJCI-2014-20052) y Ramón y Cajal (RYC 2015-18482), respectivamente.

ISEAC-40

INTERNATIONAL CONFERENCE ON ENVIRONMENTAL & FOOD MONITORING

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The oral presentation:

"SUPRAMOLECULAR SOLVENT EXTRACTION + DISPERSIVE SPE AS A NEW SAMPLE PREPARATION TECHNIQUE. APPLICATION TO THE LC-MS/MS DETERMINATION OF COCCIDIOSTATS IN ANIMAL TISSUES AND PRODUCTS OF ANIMAL ORIGIN"

Authored by: González-Rubio S., García-Gómez D., Ballesteros-Gómez A., Rubio S.

has been presented in the 40th INTERNATIONAL CONFERENCE ON ENVIRONMENTAL & FOOD MONITORING (ISEAC-40) held in Santiago de Compostela (Spain) from 19th to 22nd of June 2018.

On behalf of the organizing committee,

Santiago de Compostela (Spain), 22 June 2018

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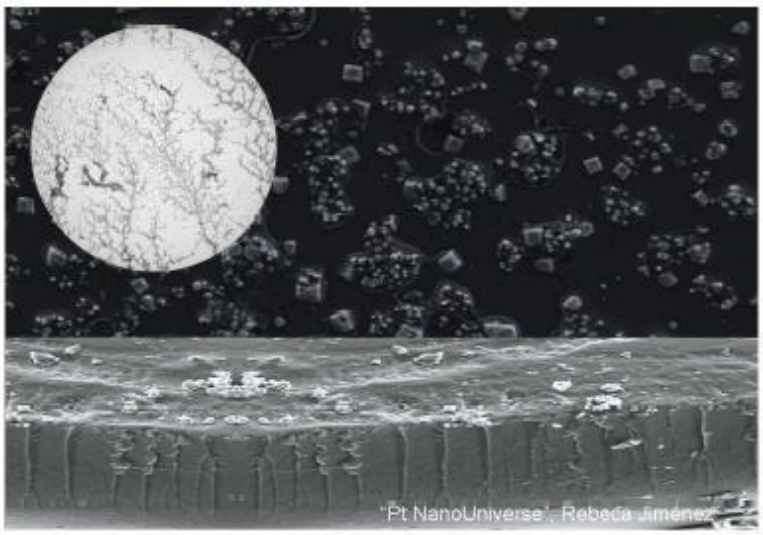

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NANOUCO VII

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P44-CA

SÍNTESIS Y CARACTERIZACIÓN DE UN NUEVO DISOLVENTE SUPRAMOLECULAR BASADO EN UN SURFACTANTE DE DOBLE CABEZA POLAR. APLICACIÓN PARA LA EXTRACCIÓN DE COMPUESTOS PEFLUORADOS EN AGUAS AMBIENTALES

Soledad González-Rubio[a], Ana María Ballesteros-Gómez[a], Diego García-Gómez[b], Soledad Rubio[a].

[a]Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba, Edificio Anexo Marie Curie, Campus de Rabanales, 14071, Córdoba (España), ana.ballesteros@uco.es

[b] Departamento de Química Analítica, Nutrición y Bromatología, Facultad de ciencias químicas, Universidad de Salamanca, Plaza de los Caídos s/n, 37008, Salamanca (España).

En los últimos años, la formación y el diseño de disolventes funcionales para su uso en procesos analíticos e industriales ha sido un campo de creciente interés por su impacto en la obtención de mayores rendimientos de producción y la disminución de costes. Una de las estrategias más utilizadas para la obtención de este tipo de disolventes consiste en procesos de síntesis basados en autoensamblaje mediante enlaces no covalentes y reversibles, a través de los cuales las moléculas se ordenan espontáneamente para formar macroestructuras

En este trabajo se ha sintetizado y caracterizado un nuevo disolvente supramolecular (SUPRAS) basado en un surfactante de doble cabeza polar, evaluándose posteriormente sus capacidades extractivas. Los SUPRAS son líquidos nanoestructurados formados por agregados anfifílicos a través de procesos de autoensamblaje y coacervación. Presentan regiones de diferente polaridad lo que permite la extracción de una amplia variedad de analitos. El uso de un surfactante con una cabeza polar extra (1,2-decanodiol) ha dado lugar a la formación de un SUPRAS con diferente estructura y composición respecto a los formados a partir de alcoholes de una sola cabeza polar. El mayor contenido en agua de estos SUPRAS, así como un mayor número de grupos funcionales polares, ha supuesto una mayor eficiencia en la extracción de contaminantes en un amplio intervalo de polaridad. En base a estas propiedades, se ha desarrollado y validado un método analítico para la determinación de sustancias perfluoroalquiladas (PFAS) en aguas ambientales. Se han determinado sulfonatos perfluoroalquilados (C_4-C_8) y ácidos perfluoroalquilados (C_5-C_{14}), destacando el bajo consumo de disolvente (250 µL de SUPRAS por 36 mL de muestra) y la rapidez (30 minutos de agitación, 5 minutos de centrifugación) de la metodología desarrollada. El nuevo SUPRAS se formó de manera espontánea en la muestra de agua (36 mL, 1 M NaCl) tras la adición de 4 mL de THF y 150 mg de 1,2-decanodiol. Posteriormente, se inyectó directamente en el sistema LC-MS/MS sin etapas posteriores de limpieza o evaporación/reconstitución. Los límites de detección alcanzados fueron de entre 0.005 y 0.010 ng L^{-1}. En todas las aguas ambientales analizadas (procedentes del sur de España) se encontraron PFAS en el intervalo 0.03-2.33 ng L^{-1}.

En conclusión, el uso y estudio de anfifilos con múltiples cabezas polares abre una nueva línea de investigación enfocada en el desarrollo y la síntesis de SUPRAS con propiedades de extracción mejoradas.

Agradecimientos: Los autores agradecen el apoyo financiero del Ministerio Español de Ciencia, Innovación y Universidades (Proyecto CTQ2017-83823R). A.B.G agradece su beca Ramón y Cajal (RYC-2015-18482) y S.G.R su contrato de inicio a la investigación.

Referencias:

[1] Bhattacharya, S.; Samanta, S.K. *J. Phys. Chem. Lett.*, **2011**, 2, 914-920.

[2] Caballo, C.; Sicilia, M.D., Rubio, S. En Supramolecular Solvents for Green Chemistry; Pena-Pereira, F., Tobiszewski, M., Eds., Elsevier: Amsterdam **2017** pp. 111-137.

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CONFERENCE PROCEEDINGS

Extraction of perfluorinated compounds in environmental waters with a novel nanostructured liquid

S. González-Rubio[1*], A. Ballesteros-Gómez[1], D. García-Gómez [1,2], S. Rubio[1]

[1]Departamento de Química Analítica, Instituto Universitario de Química Fina y Nanoquímica IUNAN, Universidad de Córdoba, Campus de Rabanales, Edificio Marie Curie (anexo), E-14071 Córdoba, España
[2]Department of Analytical Chemistry, Nutrition and Food Science, University of Salamanca, Spain.
*corresponding author: q02gorus@uco.es

Abstract

Tailored solvents with advances functional features are gaining a great interest in recent years. In this work, a double-headed surfactant based supramolecular solvent (SUPRAS) has been synthetized and its potential for analytical extractions assessed. SUPRAS are tailored nanostructured liquids made up of supramolecular aggregates. These solvents are generated through a coacervation phenomenon in which a liquid-liquid phase separation is produced in an aqueous or organic colloidal solution of amphiphiles by self-assembly processes. (Caballo et al, 2017; Ballesteros-Gómez et al., 2018). First, the preparation of a colloidal solution of amphiphiles at a concentration above the critical aggregation concentration lead to the formation of tridimensional aggregates (generally aqueous or reverse micelles, vesicles, bilayer, etc.). Secondly, a coacervation agent is added to induce an environmental change (temperature, pH, salt concentration, hydration or dehydration of the amphiphiles, etc.) that promotes the assembly of the aggregates by diminishing the repulsions among themand separate into a new highly packed phase (SUPRAS). SUPRAS have unique properties for the development of innovative extraction processes. Because of their high amphiphile content they establish multiple binding interactions with the analytes. Furthermore, the aggregates offer different polarity regions with mixed-mechanisms for analyte solubilization (dispersive, ionic, dipole-dipole, π-cation, etc.) which are determined by the amphiphile nature, the dispersion media composition and their spatial distribution among the aggregates. As a result, solutes can be extracted efficiently in a wide polarity range by using low volumes of SUPRAS (with concentration factors typically ranging from 100 to 500). They also feature restricted access properties, being able to extract low molecular weight solutes while excluding macromolecules, which are common interferents in many matrices.

In this work we design a new SUPRAS based on an alkanol with two polar heads (1,2-decanediol) dispersed in tetrahydrofuran and water. The SUPRAS is characterized in terms of formation, composition and structure. As it has been reported before, when a surfactant molecule contains a greater number of head groups, the steric hindrance results in fewer amphiphiles that can be accommodated within the aggregate. In such a situation, the aggregates are smaller and the hydrocarbon chains of the double-headed amphiphiles remain folder, leading to more open structures that allow to establish a higher number of interactions with water molecules. At the same time, the introduction of an extra alcohol group would increase hydrogen bonds and dipole-dipole interactions, being the former one of the strongest type of binding interaction. To assess the extraction potential, a wide polarity range of perfluorinated compounds (PFASs) (with sulfates and carboxylate head groups and an alkyl chain length in the range C_4-C_{18}) were selected and determined in environmental waters by LC-MS/MS.

Keywords: nanomaterials; liquid chromatography; mass spectrometry; perfluorinated compounds; environmental analysis

SEQA 2019

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MAB-O01

SÍNTESIS Y APLICACIÓN DE UN NUEVO DISOLVENTE SUPRAMOLECULAR BASADO EN 1,2-DECANODIOL PARA LA EXTRACCIÓN DE COMPUESTOS PERFLUORADOS EN AGUAS NATURALES

Soledad González-Rubio[a], Diego García-Gómez[b], Ana María Ballesteros-Gómez[a]

[a]Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba, Edificio Anexo Marie Curie, Campus de Rabanales, 14071, Córdoba (España), ga1rubrs@uco.es
[b]Departamento de Química Analítica, Nutrición y Bromatología, Facultad de ciencias químicas, Universidad de Salamanca, Plaza de los Caídos s/n, 37008, Salamanca (España).

El diseño y uso de disolventes funcionales ha sido un campo de creciente interés en los últimos años. Los disolventes supramoleculares (SUPRAS) son disolventes nanoestructurados cuya síntesis se basa en procesos de autoensamblaje y coacervación. Concretamente, se generan a partir de disoluciones coloidales acuosas u orgánicas de anfifilos, los cuales comienzan a formar agregados tridimensionales (micelas o vesículas, principalmente) a partir de una concentración de agregación crítica. Para la formación del SUPRAS como una nueva fase se añade un agente coacervante que induce un cambio ambiental (pH, temperatura, concentración de sal…), que disminuye la repulsión entre los agregados favoreciendo su ensamblaje.

En este trabajo, se sintetizó y caracterizó por primera vez un SUPRAS basado en un anfifilo de doble cabeza polar (1,2-decanodiol) en una disolución de tetrahidrofurano y agua. Con el fin de evaluar sus capacidades extractivas se aplicó, posteriormente, para la determinación de sustancias perfluoroalquiladas (PFASs), -concretamente, sulfonatos y ácidos con cadenas alquilo de longitudes comprendidas entre C_4-C_{18}-, en aguas ambientales de la provincia de Córdoba. Estos nuevos SUPRAS, en comparación con aquellos sintetizados a partir de surfactantes homólogos de una sola cabeza polar (decanol), presentan diferente estructura y composición, así como mayores capacidades extractivas para compuestos en un amplio intervalo de polaridad debido al incremento de las interacciones dipolo-dipolo y puente de hidrógeno. Estos SUPRAS son también más polares y presentan un mayor contenido en agua ya que se acomodan menos anfifilos de doble cabeza polar en un mismo agregado -debido a impedimentos estéricos- y se forman estructuras más abiertas. En esta nueva metodología desarrollada destacan el bajo consumo de disolvente (250 µL de SUPRAS por 36 mL de muestra) y la rapidez (30 min de agitación, 5 de centrifugación).

Tras la adición del agente coacervante (36 mL de muestra acuosa, 1 M NaCl) a una disolución de 4 mL de THF y 150 mg de 1,2-decanodiol, el nuevo SUPRAS se formó espontáneamente. Posteriormente, se inyectó directamente en el sistema LC-MS/MS sin etapas posteriores de limpieza o evaporación/reconstitución. Los límites de detección alcanzados fueron de entre 0.005 y 0.010 ng L^{-1}. En todas las aguas ambientales analizadas se encontraron PFAS en el intervalo 0.03-2.33 ng L^{-1}.

En conclusión, el uso y estudio de anfifilos con múltiples cabezas polares abre una nueva línea de investigación enfocada en el desarrollo y la síntesis de SUPRAS con propiedades de extracción mejoradas.

Agradecimientos: Los autores agradecen el apoyo financiero del Ministerio Español de Ciencia, Innovación y Universidades (Proyecto CTQ2017-83823R). A.B.G agradece su beca Ramón y Cajal (RYC-2015-18482) y S.G.R su contrato de inicio a la investigación.

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XXII Reunión de la Sociedad Española de Química Analítica

Valladolid 17-19 de julio de 2019

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SÍNTESIS Y APLICACIÓN DE UN NUEVO DISOLVENTE SUPRAMOLECULAR BASADO EN 1,2-DECANODIOL PARA LA EXTRACCIÓN DE COMPUESTOS PERFLUORADOS EN AGUAS NATURALES

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Soledad González-Rubio, Diego García-Gómez, Ana María Ballesteros-Gómez

ha sido premiada como mejor Comunicación en forma Oral

Y para que conste expide el presente certificado en Valladolid, 19 de julio de 2019

Dr. José Luis Pérez Pavón
Presidente de la Sociedad Española de Química Analítica

4th INTERNATIONAL
MASS SPECTROMETRY SCHOOL
Sitges, 15-20 September 2019

The author/s: **Soledad González-Rubio**

And the co-author/s: **D. García-Gómez, A. Ballesteros-Gómez**

Have presented the **Poster** entitled ***EXTRACTION OF PERFLUORINATED COMPOUNDS IN ENVIRONMENTAL WATERS WITH A NOVEL NANOSTRUCTURED LIQUID PRIOR TO DETERMINATION BY LC-MS/MS*** at the 4th International Mass Spectrometry School, held in Sitges (Spain) on 15-20 September, 2019.

Encarnación Moyano, **Chair**
University of Barcelona
SEEM Barcelona

Montserrat Carrascal, **Co-Chair**
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MDI MANFRED DONIKE WORKSHOP 2021

Poster

González-Rubio S[1], Ballesteros-Gómez AM[1], Carreras D[2], Muñoz G[2], Rubio S[1]

A comprehensive study on the performance of different retention mechanisms in sport drug testing by liquid chromatography tandem mass spectrometry.

Analytical Chemistry, University of Córdoba, Córdoba, Spain[1]
Madrid Anti-Doping Laboratory, Madrid, Spain[2]

Abstract

The WADA Prohibited List includes hundreds of compounds of very different physico-chemical properties. Laboratories needs to screen all these substances in the Initial Testing Procedures (ITPs). ITPs are mostly based on reversed-phase (RP) liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using C18 columns, which feature poor retention and peak tailing for polar and basic compounds, respectively.

In this research, a comprehensive study on the performance of six stationary phases and four eluents on different separation parameters (retention factors, asymmetry factors, co-elutions, total run times) and matrix effects (signal enhancement or suppression) in LC-MS/MS-based ITPs was performed. For this purpose, a representative group of 93 anti-doping agents (log P from -2.4 to 9.2) included in ten different classes of prohibited substances was selected. Columns working in both RP [C18, C8, phenyl hexyl (PH), pentafluorophenyl (PFP) and mixed-mode hydrophilic/RP (HILIC-RP)) and hydrophilic (HILIC)] modes were investigated. Eluents contained methanol or acetonitrile as organic modifiers, with or without the addition of ammonium acetate. The best column-mobile phase binomial for ITPs was PFP using water-methanol (0.1% formic acid) as eluent, while HILIC was the best option for highly polar non-aromatic anti-doping agents, which were poorly addressed by PFP.

This research provides valuable information to anti-doping control labs for improving LC-MS/MS-based ITPs


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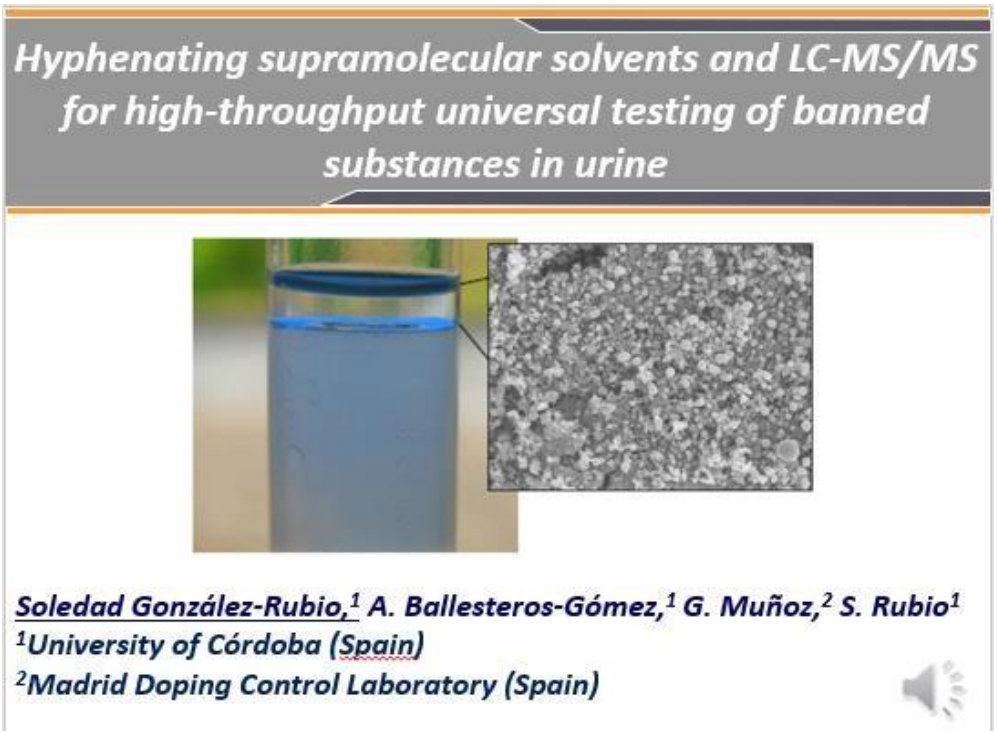
Hyphenating supramolecular solvents and LC-MS/MS for high-throughput universal testing of banned substances in urine
Soledad González-Rubio,[1] A. Ballesteros-Gómez,[1] G. Muñoz,[2] S. Rubio[1]
[1]University of Córdoba (Spain)
[2]Madrid Doping Control Laboratory (Spain)

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Poster

González-Rubio S[1], Ballesteros-Gómez A[1], Muñoz G[2], Rubio S[1]

Cubosomic supramolecular solvents: synthesis, characterization and potential for high throughput multiclass testing of banned substances in urine

Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, University of Córdoba, Córdoba, Spain[1];
Madrid Anti Doping Laboratory, Madrid, Spain[2]

Abstract

The search for sample treatments in human sport drug testing, able to efficiently extract multiclass prohibited substances while keeping utmost selectivity and sample throughput, is a major challenge yet unsolved. In this paper, this challenge was addressed by using supramolecular solvents (SUPRASs) made up of cubosomes. These SUPRASs, here firstly reported, were synthesized by the salt-induced coacervation of 1,2-hexanediol in urine. The formation of square and rounded cubosomes with a size range of 140-240 nm was confirmed by electron microscopy. These nanostructures consisted of 1,2-hexanediol, salt and a high water content (36-61%, w/w). Their applicability in multiclass determinations was investigated by the extraction of 92 prohibited substances (log P from - 2.4 to 9.2) belonging to ten categories of the World Anti-doping Agency (WADA) list. Variables influencing both recoveries and matrix effects were optimized. Cubosomic SUPRASs showed a high extraction efficiency and interference removal capability which was attributed to their large hydrophilicity and surface area. Both features were superior to that of other eleven SUPRAS that were based on sponge droplets and inverted hexagonal aggregates and to that of conventional organic solvents. A sport drug testing method based on cubosomic SUPRASs-LC-ESI-MS/MS was proposed and validated. For the ten urine samples analyzed, around 82-95% were efficiently extracted (recoveries 70-120%) and 81-92% did not present matrix effects. Method detection limits (0.001-4.2 ng/mL) were all far below WADA's limits. The proposed SUPRAS-based sample treatment is as simple as QuEChERS but the distinctive features of cubosomes confer them high capability in multiclass determinations.


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P-28. Synthesis, Characterization, and Potential of Cubosomic Supramolecular Solvents for Multiclass Extraction of Prohibited Substances in Urine

Soledad González Rubio [1], Noelia Caballero Casero[1], Ana María Ballesteros Gómez [1], Gloria Muñoz[1], Soledad Rubio [1]
1 Universidad de Córdoba, Córdoba

Área: DOPAJE DEPORTE
Tipo Comunicación: XRSEEM POSTER
Palabras Clave: Sport drugs, urine, supramolecular solvents, liquid chromatography, mass spectrometry

RESUMEN:
This work was intended to efficiently extract multiclass prohibited substances in human sport drug testing by using supramolecular solvents (SUPRASs) made up of cubosomes. These SUPRASs are synthesized by the salt-induced coacervation of 1,2-hexanediol in urine. The formation of square and rounded cubosomes with a size range of 140-240 nm was confirmed by electron microscopy. These nanostructures consisted of 1,2-hexanediol, salt, and a high water content (36-61%, w/w). Their applicability in multiclass determinations was investigated by the extraction of 92 prohibited substances (log P from 2.4 to 9.2) belonging to the 10 categories of the World Anti-Doping Agency´s (WADA) list. Variables influencing both recoveries and matrix effects were optimized. Cubosomic SUPRASs showed high extraction efficiency and interference removal capability, which was attributed to their large hydrophilicity and surface area. Both features were superior to those of other investigated SUPRASs (n=11) that were based on sponge droplets and inverted hexagonal aggregates and to those of conventional organic solvents. A sport drug-testing method based on cubosomic SUPRAS-LC-ESI-MS/MS was proposed and validated. Around 82-95% of drugs were efficiently extracted (recoveries 70-120%) in urine samples, and 81-92% did not present matrix effects. The method detection limits (0.001-4.2 ng mL-1) were all far below WADA´s limits. The proposed SUPRAS-based sample treatment is as simple as QuEChERS, but the distinctive features of cubosomes confer them high capability in multiclass determinations.

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MANFRED DONIKE WORKSHOP 2023 (PREVIEW)
Poster

González-Rubio S[1], Caballero-Casero N[1], Ballesteros-Gómez A[1], Cuervo D[2], Muñoz G[2], Rubio S[1]

A general screening method for testing of banned substances in urine based on cubosomic supramolecular solvents and liquid chromatography/time-of-flight mass spectrometry

Department of Analytical Chemistry, Institute of Chemistry for Energy and the Environment, University of Córdoba, Córdoba, Spain[1]
Madrid Anti Doping Laboratory, Health Institute Carlos III, Madrid, Spain[2]

Abstract

Multiclass screening methods in doping control should be comprehensive, allow high throughput sample processing and be cost-effective and green. In this respect, one of the greatest challenges of screening methods is how to extract efficiently the target compounds and reduce matrix effects while preventing the loss of chemicals with very different physicochemical properties during sample extraction and purification. Here, cubosomic supramolecular solvents (SUPRASs), able to extract efficiently multiclass substances while removing matrix interferences were firstly investigated for the multiclass extraction of banned substances in urine prior to their screening analysis by LC-QTOF/MS. For this purpose, eighty prohibited substances and/or their metabolites, selected from the 10 categories (S1-S9, P1) of the WADA list were tested. These substances included a wide range of polarity (log P from -2.4 to 9.2) and chemical structures (e.g. compounds with alcohol, amine, carboxyl, ether, ester, ketone, sulfonyl, etc. functional groups). The SUPRAS selected was synthesized directly in the urine by the spontaneous self-assembly and coacervation of 1,2-hexanediol, induced by sodium sulfate. Method validation was carried out according to WADA guidelines. No interfering peaks were observed for any of the 80 substances investigated. Around 84-93% of drugs were efficiently extracted (recoveries 70-120%) and 83-94% of the analytes did not show matrix effects (±20 %) in the ten urines tested. The only substance with recoveries below 30% for some urine samples was ecgonine methyl ester. It is worth noting that ritalinic acid (log P -2.4) was extracted with recoveries within the range 72.6-93.3%. Method detection and quantification limits for the drugs selected were in the intervals 0.002-12.9 ng mL^{-1} and 0.007-43.2 ng mL^{-1}, respectively. The applicability of the method was evaluated by the screening of thirty-six blinded and anonymized urine samples

Anexo C. Actividades de divulgación científica

Se han realizado diferentes actividades de divulgación científica, que incluyen la participación en Paseo por la Ciencia y en la Noche Europea de los Investigadores, organizados por la Universidad de Córdoba, entrevistas en diferentes medios de comunicación, incluyendo prensa y televisión, la divulgación en colaboración con la Unidad de Cultura Científica e Innovación y la Oficina de Proyectos Internacionales de la Universidad de Córdoba y la publicación de un artículo en la revista de la Sociedad Española de Química Analítica (SEQA)

1. Participación en el Paseo por la Ciencia, organizada por la Universidad de Córdoba el día 14 de abril de 2018.

D. Casimiro Barbado López, con DNI 8784222Q, secretario de la Asociación Profesorado de Córdoba por la Cultura Científica (APCCC), con sede social en Córdoba, C/ Doña Berenguela s/n e inscrita en el registro de asociaciones de Andalucía con el número 14/1/06230

CERTIFICO

Que Soledad González Rubio con DNI 31008591Z, doctoranda contratada del Departamento de Química Analítica de la Facultad de Ciencias (Universidad de Córdoba), ha participado en el **Paseo por la Ciencia**, una actividad de 10 horas de duración, organizada por esta asociación el día 14 de abril de 2018.

En Córdoba, a 2 de julio de 2018.

Fdo: Casimiro Barbado López

V°B°: Concepción Lara Feria
Presidenta de la APCCC

V°B°: Mª Paz Aguilar Caballos
Decana de la Facultad de Ciencias

2. Entrevista televisiva emitida el 11 de noviembre de 2020 en el programa Pequeños Científicos de Telelebrija sobre la importancia

de la determinación y control de los coccidiostatos en productos de origen animal.

http://www.lebrija.tv/category/pequenos-cientificos/page/10/

3. Participación en la Noche Europea de los Investigadores, organizada por la Universidad de Córdoba, el día 30 de septiembre de 2022.

4. Divulgación del Proyecto "Hyphenating SUPRAS and LC-MS-MS for high throughput universal testing of banned substances in urine" por la Oficina de Proyecto Internacional de la Universidad de Córdoba el día 8 de Febrero de 2021.

SUPRAS

THE SUPRAS PROJECT DESIGNS NEW, MORE EFFECTIVE SOLVENTS TO DETECT DOPING IN HIGH-PERFORMANCE SPORTS

PROJECT CODE: PCC-195500 R119-SUPRAS
CALL: PPC GRANT
PERIOD: 36 MONTHS
BUDGET: 195,000 $
PRINCIPAL INVESTIGATOR: SOLEDAD RUBIO BRAVO

Supramolecular solvents, often referred to by their acronym SUPRAS, have a great capacity to improve the selectivity and performance of extractions, thereby reducing the time and costs involved in sample analysis. These are liquids with very low toxicities and that, over the last few years, have been used in the analysis of pollutants in environmental and agri-food samples, and in the treatment of wastewater. Henceforth, in addition to these applications, these solvents will have a new one: detecting doping in high-performance sports.

The new solvents make possible the simultaneous extraction of a large number of illegal substances in urine

The SUPRAS research project, spearheaded by the University of Cordoba, will incorporate the use of these solvents into the detection of drugs in athletes, an initiative that will enhance the effectiveness of the initial screening of urine samples for the detection of positives, at the same time simplifying the process.

The number of substances on the World Anti-Doping Agency (WADA) Prohibited List is over 300, to which must be added all the compounds with similar chemical structures or biological effects, as well as any drug not approved for therapeutic use. When conventional solvents are used for the extraction of doping substances in urine samples, they only efficiently extract nonpolar compounds - those with low solubility in urine - which constitute just a small fraction of the substances prohibited by the WADA. Unlike these, the new solvents proposed in the project are also capable of extracting polar molecules; that is, those that are highly soluble in urine.

The principal investigator on the project, Soledad Rubio, emphasises that the anti-doping protocols establish a first screening of the samples, generally urine, followed by a confirmatory analysis of the positive cases, "such that the substances not detected in the first phase are not subjected to a confirmatory analysis." The preparation of the samples, she notes, "is an especially critical stage, since the reliable detection of drugs at the levels required calls for a methodology that efficiently extracts all the prohibited substances from the urine while eliminating the main components of it."

Thus, the SUPRAS project proposes the use of new solvents for the development of an analytical platform that makes possible the efficient extraction of prohibited substances and the simultaneous elimination of the urine's components through a "low cost and fast" procedure. This an important factor, considering that official anti-doping laboratories analyse about 300,000 samples annually, on which they must report reliable results within 24 hours, as a general rule.

The project, which will test the new solvents by extracting 100 representative substances from the 11 categories established by the World Anti-Doping Agency, is funded by the Partnership for Clean Competition, a non-profit organization created in 2008 by different US organizations related to sport (the Olympic Committee, the National Football League, Major League Baseball and the Anti-Doping Agency).

The project team is comprised of researchers Soledad Rubio, Ana María Ballesteros and Soledad González, from the FQM-186 group in the Department of Analytical Chemistry at the UCO; Professor Eloy Girela, from the Department of Morphological and Social Health Sciences, and Dr. Gloria Muñoz, director of the Spanish Agency for Health Protection in Sport's Doping Lab.

This report forms part of the communications strategy of the International Projects Office, to publicize notable University of Cordoba international projects.

5. Divulgación de la actividad del grupo FQM-186 en las jornadas solidarias de recaudación de fondos para la asociación Autismo Córdoba en el colegioAlmedina el día 19 de Diciembre de 2022.

La UCO participa en unas jornadas solidarias de recaudación de fondos para la asociación Autismo Córdoba

El grupo de investigación supras, junto a la presidenta, tesorera y vicepresidente de la asociación Autismo Córdoba, durante la celebración de las jornadas

El encuentro, organizado por las familias del colegio Almedina, ha contado con la participación del grupo de investigación SUPRAS, quien ha realizado varios experimentos científicos en directo para divulgar su trabajo diario

La Universidad de Córdoba (UCO) participó el pasado sábado en unas jornadas solidarias para ayudar económicamente a la asociación Autismo Córdoba. El encuentro, organizado por las Familias del colegio Almedina y celebrado en las propias instalaciones del centro escolar, ha tenido como objetivo recaudar fondos para la construcción y equipamiento de nuevas instalaciones para la entidad, que trabaja para mejorar la calidad de vida de personas con Trastorno del Espectro Autista (TEA) y sus familias.

El encuentro, en el que participaron otras organizaciones y empresas ofertando actividades para todos los públicos, contó con la participación del grupo de investigación SUPRAS del Departamento de Química Analítica de la Universidad de Córdoba, que realizó distintos experimentos en directo para divulgar sus líneas de investigación, en una jornada en la que estuvieron presentes la presidenta de Autismo Córdoba, Paqui Suárez, así como tu tesorera y vicepresidente, Raquel Rojas y Francisco Gámez.

Las investigadoras Lourdes Aigar, Celia Sánchez, Soledad González, Cristina de Dios, María Jesús Dueñas, y el investigador Luis Muñiz guiaron diversas experiencias prácticas, en las que el público asistente pudo experimentar de primera mano con líquidos newtonianos, realizar lámparas de lava y observar los efectos de la luz ultravioleta en diversos materiales.

El grupo de investigación SUPRAS, liderado por Soledad Rubio, trabaja en el desarrollo de disolventes verdes a través de procedimientos eco-eficientes con el objetivo de proporcionar alternativas sostenibles al uso de disolventes orgánicos derivados del petróleo en procesos químicos.

Estos disolventes, denominados supra moleculares, permiten el desarrollo de procedimientos innovadores para la detección y cuantificación de contaminantes en muestras de alimentos, medioambientales y biológicas y la detección de drogas en control antidopaje. A nivel industrial están siendo muy demandados, dada su inocuidad, para la obtención de compuestos bioactivos a partir de residuos agroalimentarios, microalgas y plantas, de gran interés para la industria farmacéutica, cosmética y alimentaria.

6. Publicación de un artículo titulado "Synthesis and characterization of a new double-headed amphiphile-based supramolecular solvent. Application to the extraction of perfluorinated compounds in environmental waters" en el número 67 de la revista Actualidad Analítica de la Sociedad Española de Química Analítica (SEQA) en 2019.

XXII SEQA: PREMIO ... MEJOR COMUNICACIÓN....

SYNTHESIS AND CHARACTERIZATION OF A NEW DOUBLE-HEADED AMPHIPHILE-BASED SUPRAMOLECULAR SOLVENT. APPLICATION TO THE EXTRACTION OF PERFLUORINATED COMPOUNDS IN ENVIRONMENTAL WATERS

S. GONZÁLEZ-RUBIO[1], A. BALLESTEROS-GÓMEZ [1*], D. GARCÍA-GÓMEZ[1,2]

[1] Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry , University of Córdoba, Campus of Rabanales, Marie Curie Building (Annex), E-14071 Córdoba, Spain.

[2] Current address: Department of Analytical Chemistry, Nutrition and Food Science, University of Salamanca, Spain.

*Corresponding author, email: ana.ballesteros@uco.es

The formation and design of tailored solvents with certain features for a specific application has been a field of special interest in recent years. Their use in analytical and industrial processes has meant a revolution in these areas since they have greatly improved yields, selectivity, sustainability and costs. Thus, the development of new tailor-made solvents for their further application is in constant growth.

One of the most powerful and widespread strategies for the obtainment of designed structures consists in their formation through self-assembly processes, in which different isolated components with molecular sizes arrange spontaneously into macro-ordered structures. The use of this technique has allowed the synthesis of supramolecular materials (such as supramolecular liquid crystals, polymers or solid assemblies) and, more recently, of supramolecular solvents (SUPRASs).

SUPRASs are tailored nanostructured liquids made up of supramolecular aggregates. These solvents are generated through a coacervation phenomenon in which a liquid-liquid phase separation is produced in an aqueous or organic colloidal solution of amphiphiles by self-assembly processes. First, the preparation of a colloidal solution of amphiphiles at a concentration above the critical aggregation concentration lead to the formation of tridimensional aggregates (generally aqueous or reverse micelles, vesicles, bilayer, etc.). Secondly, a coacervation agent is added to induce an environmental change (temperature, pH, salt concentration, hydration or dehydration of the amphiphiles...) that promotes the assembly of the aggregates by diminishing the repulsions among them and separate into a new highly packed phase (SUPRAS).

SUPRASs have unique properties for the development of innovative extraction processes. Because of their high amphiphile content they establish multiple binding interactions with the analytes. Furthermore, the aggregates offer different polarity regions with mixed-mechanisms for anayte solubilization (dispersive, ionic, dipole-dipole, π-cation, etc.) which are determined by the amphiphile nature, the dispersion media composition and their spatial distribution among the aggregates. As a result, solutes can be extracted efficiently in a wide polarity range by using low volumes of SUPRAS (with concentration factors typically ranging from 100 to 500). They also feature restricted access properties, being able to extract low molecular weight solutes while excluding macromolecules, which are common interferents in many matrices.

From all the possible ways to induce coacervation, the addition of a poor solvent for the amphiphile (generally water) to a water soluble organic colloidal solution has been one of the most developed strategies, mainly for the synthesis of SUPRAS from carboxylic acids and alkanols. This is due to the fact that both water and the colloidal solvent (being tetrahydrofuran the most used) are further incorporated into the SUPRAS continues network and, therefore, by changing their initial ratio, a variety of binding interactions and solubility properties can be programmed for the SUPRAS. Furthermore, the synthesis of water-induced SUPRAS is very simple, not requiring a high temperature neither extreme ionic strength or pH values that could affect the subsequent analysis.

Although the formation of alkanol-based SUPRAS in THF:water has been extensively studied , to the best of our knowledge no information has been provided regarding the coacervation of alkanediols SUPRAS.

In this work, we synthetized, characterized and applied for the first time a SUPRAS based on an alkanol with two polar heads (1,2-decanediol) dispersed in tetrahydrofuran and water. As it has been reported before, when a surfactant molecule contains a greater number of head groups, the steric hindrance results in fewer amphiphiles that can be accommodated within the aggregate. In such a situation, the aggregates are smaller and the hydrocarbon chains of the double-headed amphiphiles remain folder, leading to more open structures that allow to establish a higher number of interactions with water molecules. At the same time, the introduction of an extra alcohol group increases hydrogen bonds and dipole-dipole interactions, being the former one of the strongest type of binding interaction. Therefore, different structures and extraction capabilities are expected from SUPRAS formed from alkanediol molecules that might extent their applicability.

To assess the extraction potential of these SUPRASs, a wide polarity range of perfluorinated compounds (PFASs) (with sulfates and carboxylate head groups and an alkyl chain length in the range C4-C18) were selected and determined in environmental waters by LC-MS/MS.

Below, results on the characterization of alkanediol-based SUPRAS and its potential extraction capabilities are shown.

RESULTS AND DISCUSSION

- *Phase diagrams, composition and volumes*

As mentioned before, water-induced SUPRAS are environmental responsive and, therefore, their main properties (composition and structure) depend on the synthetic conditions. Phase diagrams were constructed to show the composition of the synthetic solution that produce SUPRAS phases (1,2-decanediol:water:organic solvent w/w, %). Both protic and dipolar aprotic solvents (isopropanol and tetrahydrofuran, respectively) were chosen as good solvents, considering their miscibility in water and their ability to dissolve alkanediols. Ternary plots exhibited three different domains that were set by visual inspection: (a) SUPRAS (b) isotropic solution at high percentages of organic solvent and (c) precipitation region at low percentages of organic solvent. Synthetic solutions containing water with 1 M NaCl and THF were also tested, in order to study the influence of the ionic strength in SUPRAS formation and in further extraction steps, since the salting-out phenomena has been widely employed to enhance extraction efficiencies in analytical methods. In general, narrower SUPRAS formation region were obtained in comparison with 1-decanol-based SUPRAS in THF:water, at which THF percentages typically varied in the range ~5-60% w/w. However, the addition of salt broadened the SUPRAS formation region due to probably the mixed effect of salting-out and the dehydration of the polar groups of the surfactant promoting the assembl. As it was expected and described before for alkyl carboxylic acids, SUPRAS in THF:water mixtures showed broader formation regions than those prepared in isopropanol. SUPRAS phases were further characterized by their chemical composition. 1,2-decanediol was almost fully incorporated into the SUPRAS phase in a wide range of compositions (above 80-90%), being the incorporation more favorable in the salty aqueous solutions, for the same reason explained above. SUPRAS composition was tested at a constant percentage of 1,2-decanediol (3-5 %w/w) and under decreasing percentages of the coacervating agent (water). The amphiphile percentage value was based on previous studies whereby the range of analytical interest was considered to be between 0.5 and 5 (%, w/w). In all cases, water contents were around 30% (w/w) and maintained invariable while changing the water percentage in the synthetic solution. These values only dropped to around 20-21 % w/w water for salty synthetic mixtures and at high percentages of organic solvent (≥ 35.4 % w/w), where the salting-out effect led to the non-miscibility of THF and water and was outside of the SUPRAS only region. These water contents were much higher than their homologues 1-decanol-based SUPRAS in THF:water (i.e. ~4-21 % w/w), the latter varying with the coacervating agent percentage in the synthetic mixture. the content of THF and isopropanol in SUPRAS increased (14-38% w/w) and decreased (20-9% w/w), respectively, when decreasing the coacervating agent in the synthetic solution. When the salt was present, values for THF varied in a wider range (6-69% w/w). The concentration of the amphiphile also changed accordingly. This means that SUPRAS composition was environment dependent and that could be tuned for obtaining SUPRAS with different solubility properties to maximize recoveries on each analytical application.

The volumes of 1,2-decanediol-based SUPRAS increased linearly with the percentage of the amphiphile in the synthetic solution. It also increased with the percentage of organic solvent, fitting well polynomial or linear regressions with and without the presence of the salt, respectively.

- *SUPRAS structure*

The external structure of SUPRAS (coacervate droplets made up of assembled aggregates) was characterized by light microscopy. The corresponding micrographs of SUPRAS showed SUPRAS made up of coacervate droplets with a not uniform size distribution. This structure was in accordance with that is generally found in other coacervation processes.

- *Potential of alkanediol-based SUPRAS for analytical method development: application to PFAS extraction from environmental waters*

Extractions of the target compounds were carried out with SUPRAS formed either from 1-decanol or from 1,2-decanediol in mixtures of THF:water. For 1,2-decanediol-based SUPRAS, recoveries were quantitative (above 60%) for PFSAs and PFCAs with carbon atoms of 6 and 8 and in the range 8-18, respectively. However, under the same conditions, the applicability of 1-decanol-based SUPRAS was much limited for both most polar and most apolar PFAS, i.e. quantitative recoveries were found only for PFCAs with carbon atoms in the range 12-16 and were always more than 10% lower than those obtained for 1,2-decanediol-based SUPRAS.

The extraction of PFAS was carried out using the three different scenarios employed in the characterization study: isopropanol:water, THF:water and THF:water (1 M NaCl). Recoveries were higher for THF:water than for isopropanol:water (specially for polar compounds: PFCAs C5-C8 and PFBS). This could be due to the lower incorporation rate of amphiphile to SUPRAS phase in the case of isopropanol at the percentage of organic solvent tested (around 70-90% for isopropanol against 90-99% for THF). This would lead to a higher concentration of amphiphile in the equilibrium aqueous solution that would compete with the SUPRAS phase for solubilization of the target compounds, affecting more strongly to the solubility

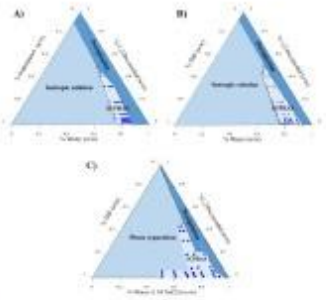
of the more polar ones. Regarding the presence of salt, recoveries were enhanced in around 10-20% for all PFASs due to the salting-out effect that it is known to decrease the solubility of the compounds in the aqueous solution. Under these conditions, recoveries were quantitative for all the compounds (from 72±8 to 117±17) except for the most polar PFPeA (40±1) and PFBS (50±5).

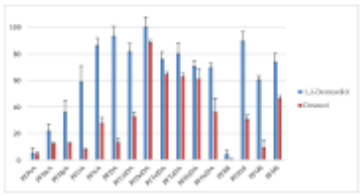
The optimal method involved a low consumption of reagents (36 mL sample, 1 M NaCl, 4 mL THF, 150 mg de 1,2-decanediol) and simple steps of stirring (30 min) and centrifugation (5 min, 3,000 rpm) for SUPRAS formation (250 µL SUPRAS) and simultaneous PFASs extraction.

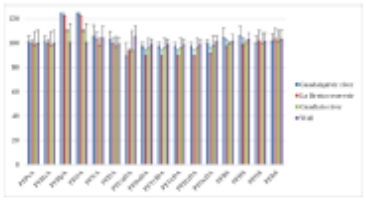
Recoveries for all PFASs were quantitative in water samples despite their wide polarity range (in the range 79-123%; median 100%; mean 100.9%, internal calibration). The precision (at 25 ng L-1 of PFASs) expressed as relative standard deviation (RSD), was between 1 and 23% (median 6, mean 7). Detection limits were in the range 0.005-0.01 ng L-1 Four natural water samples (collected in South Spain, namely La Breña water reservoir, a well located in Trassierra and Guadalquivir and Guadiato rivers) were analysed. In all cases excepting for la Breña reservoir, PFCAs concentrations were lower than PFSAs ones. Higher concentrations of PFASs were found in water collected from Guadalquivir, which flows by Córdoba city and it is more exposed to industrial contamination as well as in La Breña reservoir, in which human activities such as fishing are commonly carried out. Levels were consistent with the values generally found in the literature (0.01-5 ng L-1).

CONCLUSIONS

The use of an amphiphile with an extra polar group allowed to obtain a SUPRAS with different composition, structure and extraction properties than those made up from 1-alkanol molecules. As it was expected from the increased number of polar groups, water percentages of these types of SUPRAS were higher and maintained invariable when increasing the percentage of organic solvent in the synthetic solution. The applicability of this new SUPRAS has been established by developing an analytical methodology for the determination of PFASs in environmental waters. In this sense, the alkanediol-based SUPRAS showed better extraction capabilities in a wide range of polarity than alkanol-based SUPRAS and exhibited great analytical performance in terms of accuracy, precision and sensibility and also in terms of quickness, low cost and ease of use. In short, the use and study of multi-headed surfactants opens the door to the development and synthesis of a new line of SUPRAS with enhanced extraction properties for generic sample treatment







7. Divulgación del Proyecto “Hyphenating SUPRAS and LC-MS-MS for high throughput universal testing of banned substances in urine” por la Unidad de Cultura Científica e Innovación de la Universidad de Córdoba el día 24 de febrero de 2021.

El proyecto SUPRAS diseña nuevos disolventes más eficaces para detectar el dopaje en el deporte de alto rendimiento

Imagen de alguno de los integrantes del grupo de investigación de la UCO responsable del proyecto SUPRAS.

Los nuevos disolventes, en los que trabaja la Universidad de Córdoba en colaboración con la Agencia Española de Protección de la Salud en el Deporte, permiten la extracción simultánea de un gran número de sustancias ilegales en orina

Los disolventes supramoleculares, conocidos como SUPRAS por sus siglas en inglés, poseen una gran capacidad para mejorar la selectividad y el rendimiento de las extracciones y disminuir, así, el tiempo y los costes del análisis de muestras. Se trata de líquidos con muy baja toxicidad y que a lo largo de los últimos años se han utilizado en el análisis de contaminantes en muestras ambientales y agroalimentarias y en el tratamiento de aguas residuales. A partir de ahora, además de estas utilidades, estos disolventes contarán con una nueva aplicación: detectar el dopaje en el deporte de alto rendimiento.

El proyecto de investigación SUPRAS, liderado por la Universidad de Córdoba, incorporará el uso de estos disolventes a la detección de drogas en deportistas, una iniciativa que permitirá mejorar la eficacia del cribado inicial de muestras de orina para la detección de muestras positivas, a la vez que permitirá la simplificación del proceso.

La cifra de sustancias que figuran en la Lista de Prohibiciones de la Agencia Mundial Antidopaje (WADA) es superior a 300, a los que deben añadirse todos los compuestos con estructura química o efectos biológicos similares, así como cualquier fármaco no aprobado

para uso terapéutico. Cuando se usan disolventes convencionales para la extracción de sustancias dopantes en muestras de orina, estos sólo extraen de forma eficaz los compuestos apolares -aquellos con baja solubilidad en orina-, los cuales constituyen una mínima fracción de las sustancias prohibidas por la WADA. A diferencia de estos, los nuevos disolventes propuestos en el proyecto también son capaces de extraer las moléculas polares, es decir, aquellas que son muy solubles en la orina.

Tal y como subraya la investigadora principal del proyecto, Soledad Rubio, los protocolos antidopaje establecen un primer cribado de las muestras, generalmente de orina, seguido por un análisis confirmatorio de los casos positivos, "por lo que las sustancias no detectadas en la primera fase no se someten a un análisis confirmatorio". La preparación de las muestras, señala, "es una etapa especialmente crítica, ya que la detección de las drogas de forma fiable a los niveles requeridos exige que la metodología usada extraiga eficientemente todas las sustancias prohibidas a partir de la orina a la vez que elimina los principales componentes de la misma".

En este sentido, el proyecto SUPRAS propone el uso de los nuevos disolventes para el desarrollo de una plataforma analítica que permita la extracción eficiente de sustancias prohibidas y la eliminación simultánea de los componentes de la orina mediante un procedimiento "de bajo coste y rápido", un factor importante teniendo en cuenta que los laboratorios oficiales antidopaje analizan anualmente cerca de 300.000 muestras, para las cuales deben proporcionar resultados fiables en un periodo de tiempo que por lo general no supera las 24 horas.

El proyecto, que probará los nuevos disolventes mediante la extracción de 100 sustancias representativas de las 11 categorías establecidas por la agencia Mundial Antidopaje, está financiado por la ~~Sadosoohio for Clean Competition~~, una organización sin fines de lucro creada en 2008 por diferentes organismos estadounidenses relacionados con el deporte (el Comité Olímpico, la liga Nacional de Fútbol, la liga Mayor de Béisbol y la Agencia Antidopaje).
El equipo del proyecto está integrado por las investigadoras Soledad Rubio, Ana María Ballesteros y Soledad González, del grupo FQM-186del Departamento de Química Analítica de la UCO, el profesor Eloy ~~Girala~~, del Departamento de Ciencias Morfológicas y Sociosanitarias de la institución universitaria y la doctora Gloria Muñoz, directora del Laboratorio de Control del Dopaje de la Agencia Española de Protección de la Salud en el Deporte.
Este reportaje forma parte de la estrategia de comunicación de la Oficina de Proyectos Internacionales para dar a conocer los proyectos internacionales concedidos a la Universidad de Córdoba

8. Divulgación sobre la determinación de coccidiostatos en alimentos de origen animal por la Unidad de Cultura Científica e Innovación de la Unidad de Córdoba el día 28 de julio de 2020.

Martes, 28 Julio 2020 09:09

Desarrollan un nuevo método para extraer residuos de antibióticos en alimentos de origen animal

Escrito por UCCi

tamaño de la fuente | Imprimir | Email

La investigadora responsable del estudio, Soledad González, en el laboratorio.

El procedimiento simplifica y abarata el proceso para extraer coccidiostatos, medicamentos empleados para tratar una enfermedad intestinal en animales pero que pueden provocar riesgos en la salud de las personas a dosis elevadas

La coccidiosis es un tipo de enfermedad intestinal que afecta a distintos grupos de animales. Inhibe la absorción de nutrientes y el crecimiento por lo que, en ocasiones, se produce la muerte del animal, generándose así pérdidas económicas en la industria ganadera. Para combatir esta dolencia, se administran antibióticos como los 'coccidiostatos', fármacos eficaces para tratar la enfermedad pero que, sin embargo, pueden provocar enfermedades cardiovasculares en los seres humanos cuando su concentración es elevada en productos de consumo de origen animal.

El grupo de Investigación de Química Analítica Supramolecular de la Universidad de Córdoba ha desarrollado un nuevo método que permite la extracción y determinación de estas sustancias en alimentos de origen animal. Según señala la investigadora responsable del estudio, Soledad González, el procedimiento es capaz de extraer de forma simultánea todo el grupo de antibióticos ionóforos (es decir, de origen natural) en todos los productos de origen animal legislados por la Unión Europea, algo "realizado por primera vez en esta investigación".

Concretamente, el método emplea disolventes supramoleculares, disolventes no tóxicos, conocidos como SUPRAS por sus siglas en inglés y que tienen una gran capacidad para mejorar la selectividad y el rendimiento de las extracciones y disminuir, así, los costes de producción. Tal y como señala la investigadora Soledad González, este tipo de disolventes permite realizar de forma simultánea y en un solo paso la extracción de los residuos de antibióticos y la limpieza de la muestra. Además, "se trata de un método de bajo coste y respetuoso con el medioambiente, ya que se utiliza un menor volumen de disolvente orgánico en comparación con otras técnicas ampliamente implementadas en laboratorios de rutina", añade.

Tras aplicar la nueva metodología en distintos productos como huevos, leche y carne (hígado, riñón, músculo y grasa), se comprobó que los límites de detección alcanzados estaban muy por debajo de los límites establecidos por la ley, lo que demuestra que el procedimiento podría ser aplicable en laboratorios de control de calidad de alimentos. Además, el nuevo método, en el que también han participado las investigadoras Ana María Ballesteros, Soledad Rubio y Diego García-Gómez, éste último de la Universidad de Salamanca, ha sido validado siguiendo la normativa europea 2002/657/CE, que regula la presencia de residuos en productos de origen animal, por lo que podría incorporarse a las rutinas de control de los laboratorios.

Referencias:

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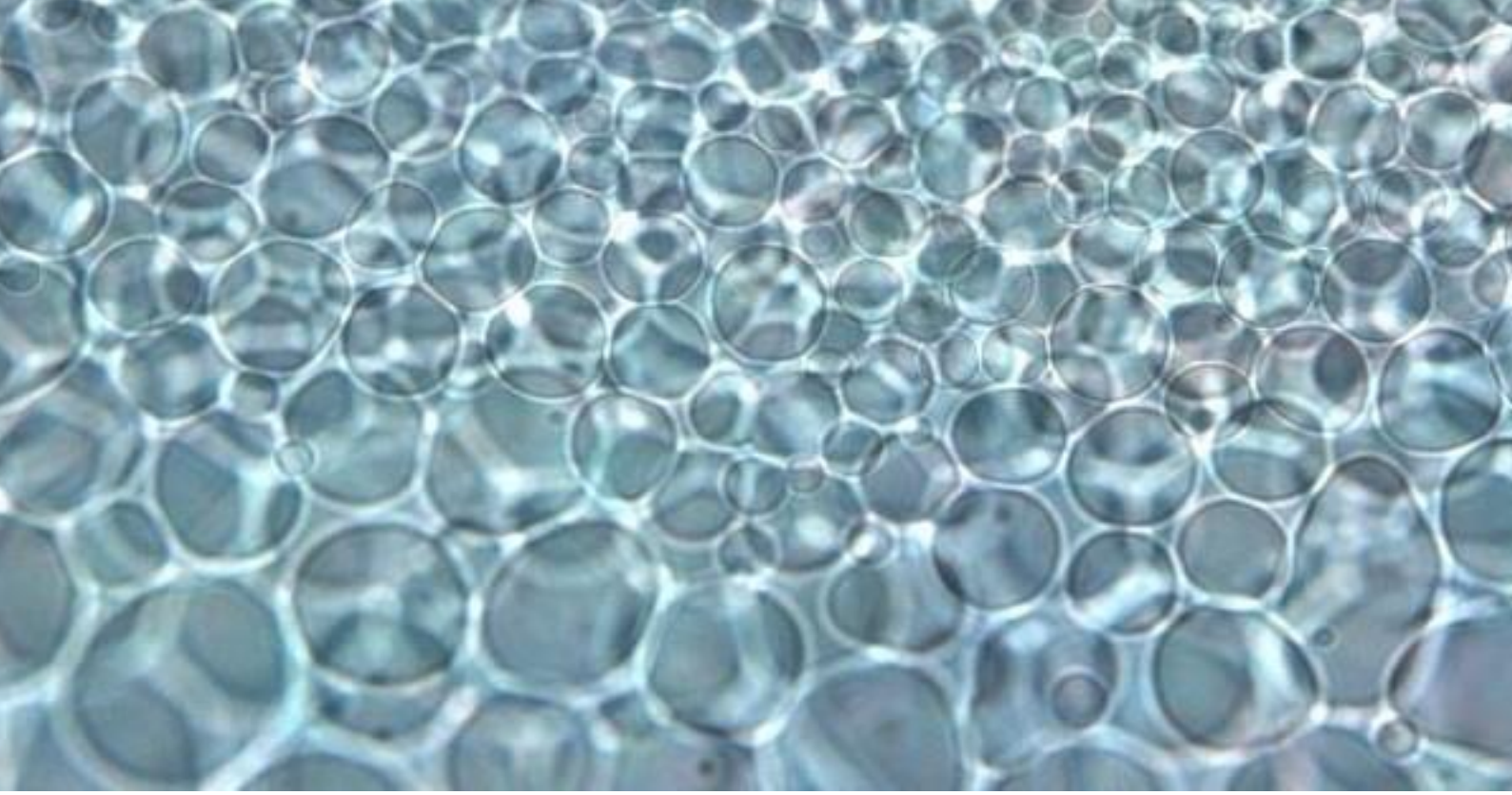

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