Presencia, destino y eliminación de filtros solares orgánicos en el ecosistema acuático

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Estudio de la presencia, destino y degradación de filtros solares orgánicos en el ecosistema acuático

Memoria presentada para optar al grado de Doctor
por la Universitat de Barcelona
Por

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ÍNDICE

PRÓLOGO – ¿POR QUÉ FILTROS UV?

Hoy en día, los compuestos orgánicos que actúan de pantalla frente a la radiación solar ultravioleta (filtros solares, UV-F) se utilizan cada vez con más frecuencia y en mayor cantidad dada la concienciación actual que existe con respecto a los efectos perjudiciales de la radiación solar. La progresiva destrucción de la capa de ozono (la cual absorbe gran parte de la radiación UVB, muy energética), unida a los largos periodos de exposición solar, principalmente en actividades de ocio, ha contribuido a una mayor incidencia de las enfermedades relacionadas con esta exposición, principalmente el cáncer de piel. El factor de protección solar (Sun Protection Factor, SPF) que aportan los productos que los contienen depende de la naturaleza del agente y de su proporción en la formulación. Prácticamente todas las formulaciones contienen una mezcla de UV-F para poder aportar un SPF elevado que cubra el espectro de radiaciones UVA y UVB. Durante años, la única preocupación con respecto a estos compuestos hacía referencia a su eficacia para atenuar los efectos nocivos de la radiación UV sin causar riesgo a la salud humana. No ha sido hasta hace muy pocos años que se dispone de los primeros datos, aunque escasos y dispersos, sobre su presencia, destino y efectos en el medio ambiente [1, 2].

Actualmente hay 28 UV-F regulados en la Unión Europea para su uso en la industria cosmética. Sin embargo, existen más UV-F que se usan en otras actividades industriales, ya que los UV-F son ampliamente utilizados como conservantes, potenciadores del sabor y el olor, fotoionizadores o inhibidores de polimerización (conservación de pinturas, plásticos, textiles, etc).

Los UV-F se integran dentro del grupo de los “contaminantes emergentes”, que engloba medicamentos, productos de uso y cuidado personal (PCPs), nanomateriales, etc. Este tipo de compuestos, a diferencia de los contaminantes los prioritarios, no están regulados en la normativa ambiental existente. Por lo general, se desconocen sus posibles efectos ecotoxicológicos y sus mecanismos de toxicidad.

Los UV-F acceden al medio acuático por vía directa, a través de actividades acuáticas (baño, natación) y por vía indirecta a través de las estaciones de tratamiento de aguas residuales (EDARs), después de haber sido transferidos del cuerpo a la vestimenta, por la excreción después de su absorción dérmica, etc y a través de las aguas residuales industriales. Dada la elevada proporción de estos aditivos que se utiliza en las

formulaciones y su frecuente uso, la cantidad de UV-F que va a parar al medio ambiente es importante. El uso de estos compuestos es especialmente alto en la costa mediterránea, donde se llevan a cabo gran cantidad de actividades acuáticas recreativas, sobre todo en temporada estival.

Hasta el momento, los estudios sobre la presencia de UV-F en el medio ambiente son muy limitados, especialmente si los comparamos con los referidos a otros contaminantes emergentes, como por ejemplo los fármacos. Algunos UV-F, entre los que se incluyen el octil metoxicinamato (EHMC), la benzofenona-3 (BP3) o el 4-metilbenciliden canfor (4MBC) (substancia prohibida para su uso en productos cosméticos en diversos países como los Estados Unidos, Dinamarca o Japón) y el octocrileno (OC) se han determinado en aguas de entrada y salida de EDARs [3-5] y, en menor concentración, en aguas superficiales [3, 6]. De todas maneras, se han detectado residuos de UV-F en invertebrados, peces y hasta en aves [7]. Los niveles ambientales encontrados varían significativamente en función del área geográfica y la estación del año, principalmente. En temporada estival, donde el uso de estos compuestos se incrementa a la vez que aumenta la actividad turística, puede haber grandes cargas puntuales en las EDARs de algunos núcleos urbanos que no están preparadas parar tratar volúmenes de agua tan grandes. Los escasos estudios que existen sobre la degradación de estos compuestos en EDARs indican que la eliminación es incompleta para los pocos UV-F investigados (tratamiento convencional de lodos activados). Esto pone en evidencia la necesidad de desarrollar nuevos procesos de tratamiento de aguas para su completa eliminación.

Actualmente, uno de los mayores retos en el estudio ambiental de los UV-F es disponer de métodos de análisis lo suficientemente sensibles para poder evaluar la presencia de tales compuestos, sus metabolitos y otros productos de transformación en el sistema agua/sedimento/biota y, también, en aguas residuales y profundizar así en su degradación. Hasta el momento, solo ha sido estudiado un número muy reducido de UV-F y existen muy pocos estudios en matrices sólidas, como sedimento, fangos de EDAR y biota. Entre ellos algunos estudios con peces demuestran el potencial de bioacumulación de estas sustancias, que en su mayoría son lipofílicas con valores de Log $K_{ow} > 5$.

Hoy en día, la clasificación toxicológica de los UV-F es heterogénea. Ensayos *in vitro* indican que una gran mayoría de estos compuestos exhiben actividad disruptora endocrina, y muchos de ellos actividad múltiple [8]. Algunos ensayos *in vivo* han demostrado la actividad disruptora endocrina de 3-benziliden canfor (3BC), benzofenona-2 (BP2), benzofenona-1 (BP1) y del ácido octil dimetil PABA (OD-PABA) en ratas y peces [9-11]. Incluso se relacionan algunas enfermedades humanas como la endometriosis con la exposición a estos compuestos [12]. Sin embargo, los ensayos se han realizado con muy pocos compuestos; así mismo no se han efectuado estudios para analizar la posible sinergia en mezclas de UV-F (situación habitual) o de otros contaminantes. Es por esto que su presencia ambiental es motivo de preocupación y es necesario obtener nuevos datos ecotoxicológicos para poder realizar una correcta evaluación de su riesgo ambiental.

OBJETIVOS GENERALES

Con esta Tesis Doctoral se pretende aportar herramientas (métodos) y conocimiento (datos) para ampliar la información disponible sobre el impacto ambiental de los filtros solares orgánicos (presencia, destino y eliminación).

Uno de los principales objetivos fue el desarrollo de nuevas metodologías analíticas basadas en la cromatografía líquida de alta resolución acoplada a espectrometría de masas en tándem (LC-MS/MS) para el análisis de UV-F y algunos de sus productos de transformación, compuestos hasta el momento muy poco investigados. Estas metodologías fueron aplicadas en el estudio de la presencia y distribución de UV-F en muestras reales de aguas naturales, aguas residuales, sedimentos y lodos de EDAR. Otro de los objetivos fue aportar nuevos datos sobre la bioacumulación de UV-F, por lo que se analizarán diferentes especies de peces e incluso mamíferos marinos (delfines).

Por otra parte, teniendo en cuenta que los procesos biológicos convencionales que se aplican en las EDARs no son, en general, muy efectivos para la degradación de estos compuestos, se pretende estudiar nuevos tratamientos, eficientes y respetuosos con el medio ambiente, para su degradación en fase líquida y sólida. Concretamente se ha investigado en la biodegradación mediante el hongo ligninolítico *Trametes versicolor* (*T. versicolor*) y mediante procesos de oxidación avanzada como la ozonización y su combinación con peróxido de hidrógeno. En estos estudios se hará especial hincapié en la identificación de los productos de transformación que se forman durante estos procesos.

ESTRUCTURA

La memoria se estructura en cuatro capítulos. El primer capítulo es una introducción, que incluye información general sobre qué son y por qué se utilizan los UV-F, cómo se clasifican, la normativa existente al respecto de la utilización de estos compuestos, su presencia en el ecosistema acuático y una revisión del estado del arte del análisis de UV-F en el medio ambiente, incluyendo la etapa de preparación de muestra, el análisis instrumental, así como los parámetros de calidad de los métodos existentes en la

literatura. También se presentan los compuestos que han sido objeto de estudio en esta Tesis Doctoral, incluyendo sus estructuras y principales propiedades fisico-químicas.

Los siguientes capítulos recogen el trabajo experimental y los resultados obtenidos a lo largo de la Tesis Doctoral. Todos ellos presentan una pequeña introducción sobre el tema que tratan, así como una descripción de sus objetivos específicos y su estructura. Los estudios se presentan en forma de publicaciones científicas y posteriormente se discute y/o añade información relevante. Al final de cada capítulo se exponen las conclusiones que se han obtenido de los estudios incluidos.

El Capítulo II describe el desarrollo de metodologías analíticas para la detección de UV-F en diferentes matrices ambientales (agua, sedimento y biota) y en aguas de entrada/salida y lodos de EDAR. En este capítulo también se incluyen datos de su presencia ambiental en las matrices mencionadas.

En el Capítulo III se presentan los estudios de biodegradación de los UV-F en lodos de EDAR y en fase acuosa con una nueva tecnología basada en la utilización del hongo *T. versicolor*. Este proceso es respetuoso con el medio ambiente, de bajo coste económico y bajo consumo energético. La identificación mediante el análisis con espectrometría de masas de alta resolución de los productos de transformación que se forman en estos procesos constituye una parte importante de estos estudios.

En el Capítulo IV se incluye el trabajo realizado en una estancia científica predoctoral en el Departamento de Química Orgánica y Tecnología de la Universidad de Gante (Bélgica). En este estudió se evaluó la aplicación de la ozonización y su combinación con el peróxido de hidrógeno para la degradación de BP3 y su principal producto de transformación BP1. En este caso, también se hace hincapié en la identificación de los productos de transformación y su comportamiento durante el proceso, especialmente importante en los procesos de oxidación avanzada.

Finalmente, se recogen las conclusiones generales de esta Tesis Doctoral. Posteriormente se incluye la bibliografía así como diversos anexos, entre los que están incluidos el índice de abreviaturas y acrónimos, el índice de Tablas y Figuras, la lista de publicaciones que ha dado lugar esta Tesis Doctoral y las contribuciones en congresos científicos.

A continuación se describe la distribución de las publicaciones incluidas en esta memoria:

- Capítulo I

- Publicación científica #1: Gago-Ferrero P., Díaz-Cruz M.S., Barceló D. (2013) Liquid chromatography-tandem mass spectrometry for the multi-residue analysis of organic UV-Filters and their transformation products in the aquatic environment. Analytical Methods 5:355-366.

- Publicación científica #2: Gago-Ferrero P., Díaz-Cruz M.S., Barceló D. (2012) An overview of UV-absorbing compounds (organic UV-Filters) in aquatic biota. Analytical and Bioanalytical Chemistry 404:2597-2610.

- Capítulo II

- Publicación científica #3: Gago-Ferrero P., Mastroianni N., Díaz-Cruz M.S., Barceló D. (2013) Fully automated determination of nine UV-Filters and transformation products in natural waters and wastewaters by on-line solid phase extraction-liquid chromatography-tandem mass spectrometry. Journal of Chromatography A. En prensa.DOI:10.1016/j.chroma.2013.04.037.

- Publicación científica #4: Gago-Ferrero P., Díaz-Cruz M.S., Barceló D. (2011) Occurrence of multiclass UV-Filters in treated sewage sludge from wastewater treatment plants. Chemosphere 84: 1158-1165.

- Publicación científica #5: Gago-Ferrero P., Díaz-Cruz M.S., Barceló D. (2011) Fast pressurized liquid extraction with in-cell purification and analysis by liquid chromatography-tandem mass spectrometry for the determination of UV-Filters and their degradation products in sediments. Analytical and Bioanalytical Chemistry 400: 2195-2204.

- Publicación científica #6: Gago-Ferrero P., Díaz-Cruz M.S., Barceló D. (2013) Multi-residue method for trace level determination of UV filters in fish based on pressurized liquid extraction and liquid chromatography-quadrupole-linear ion trap-mass spectrometry. Journal of Chromatography A 1286:93-101.

- Publicación científica #7: Gago-Ferrero P., Alonso M.B., Bertozzi C.P., Marigo J., Barbosa L., Cremer M., Secchi E.R., Azevedo A., Lailson-Brito J., Torres J.PM., Malm O., Eljarrat E. Díaz-Cruz M.S., Barceló D. (2013) First determination of UV-Filtres in marine mammals. Octocrylene levels in Franciscana dolphins. Environmental Science & Technology. Aceptado.

- Capítulo III

- Publicación científica #8: Gago-Ferrero P., Badia-Fabregat M., Olivares A., Piña B., Blànquez P., Vicent T., Caminal G., Díaz-Cruz M.S., Barceló D. (2012) Evaluation of fungal- and photo-degradation as potential treatments for the removal of sunscreens BP3 and BP1. Science of the Total Environment 427-428: 355-363.

- Capítulo IV

- Publicación científica #9: Gago-Ferrero P., Demeestere K., Díaz-Cruz M.S., Barceló D. (2013) Ozonation and peroxone oxidation process of BP3 in water: kinetics, parameter study and identification of intermediate products. Science of the Total Environment 443: 209-217.

CAPÍTULO 1

INTRODUCCIÓN

1.1. NECESIDAD DEL USO DE PROTECTORES SOLARES Y DEFINICIÓN DE LOS FILTROS UV

La radiación visible (VIS) (400-760 nm) constituye el 44.3 % de la radiación solar que llega a la superficie terrestre, mientras que el 49.5 % está constituido por radiación infrarroja (IR) (>760 nm), y tan solo un 6.2 % corresponde a radiación ultravioleta (UV) (100-400 nm), ya que la capa de ozono estratosférico hace posible su atenuación. El 98 % de la radiación UV se debe a la denominada ultravioleta A (UVA) (320-400 nm), mientras el 2 % restante se debe a la ultravioleta B (UVB) (290-320 nm); la UVC (100-290 nm), que es la más energética, no llega a la superficie terrestre [13].

Aunque la exposición solar es saludable y terapéutica cuando se practica moderadamente, ya que, por ejemplo, aumenta la producción de vitamina D, aumenta la absorción del calcio y previene el raquitismo; su exceso puede acarrear serios problemas para la salud humana. La radiación solar provoca daño cutáneo debido a que las radiaciones UV son absorbidas por el ADN, ARN, proteínas, lípidos de membranas y orgánulos celulares presentes en la epidermis y dermis, incluyendo el sistema vascular. Los efectos negativos son acumulativos y están en relación con la duración, frecuencia e intensidad de la radiación [14]. En los últimos años se ha observado una mayor incidencia del cáncer de piel en las sociedades desarrolladas, asociada principalmente a una excesiva exposición a la radiación solar [15]. Estos efectos se ven agravados por el continuo deterioro de la capa de ozono, ya que permite que la radiación UV llegue a la piel con mayor intensidad. La exposición solar también incluye otros efectos negativos como inflamaciones, quemaduras solares, hiperqueratosis, fotoenvejecimiento, fotoalergia y estados de inmunosupresión [13].

La piel dispone de mecanismos de adaptación y defensa para mitigar las agresiones sufridas por la exposición solar. Entre ellas se encuentran el espesamiento de la capa córnea, la producción de melanina, la activación de las moléculas antioxidantes, los sistemas de reparación del ADN y la secreción de citoquinas. Estos mecanismos de fotoprotección natural son insuficientes, debiéndose recurrir a fotoprotecciones artificiales, como lo son los protectores solares químicos.

Para prevenir los efectos nocivos de la radiación solar se utilizan protectores solares, productos cosméticos de aplicación tópica que contienen UV-F. Los UV-F son compuestos que protegen generalmente contra la radiación UVB y en algunos casos

contra la radiación UVA, la cual penetra en profundidad en la piel y después de una larga exposición puede llegar a dañarla considerablemente [16]. Como se ha comentado, el SPF de un producto depende de los UV-F que contiene, y de su proporción en la formulación. Hay una tendencia a aumentar el SPF aumentando la concentración de UV-F en los protectores solares. Por otra parte se intenta conseguir una protección para un amplio espectro de longitudes de onda, para lo que se utilizan mezclas de UV-F, porque sólo con un compuesto no es posible abarcar todo el espectro de radiación UV.

No obstante, algunos autores como Krause et al. [17] ponen en duda que los UV-F hayan conseguido el principal objetivo para el que son tan ampliamente utilizados, la protección frente a la aparición del melanoma maligno (MM). Aseguran que mientras no hay duda de que estos productos protegen contra las quemaduras solares, la queratosis solar y otras enfermedades de la piel [18-20], el único estudio aleatorio que evaluó el riesgo de formación de MM después del uso regular de protectores solares, no encontró evidencias estadísticas significativas para una reducción en la incidencia de melanoma primario nuevo [21]. Además, a pesar del uso de estos compuestos durante décadas, la incidencia de los MMs sigue creciendo rápidamente [22]. Considerando los estudios que advierten de los efectos disruptores endocrinos asociados a los UV-F sumado al hecho de la gran absorción de estos compuestos a través de la piel (BP3 se encontró en el 96% de muestras de orina analizadas [23] en USA y otros UV-F en el 85% de las muestras de leche materna en Suiza [24]), estos estudios llevan a plantearse la pregunta de si los potenciales efectos adversos que tienen el uso de estos compuestos son, en general, compensados por los efectos beneficiosos que aportan.

De lo que no hay duda es de su amplia utilización. Esto hace que sea necesario llevar a cabo estudios exhaustivos que profundicen en el conocimiento sobre la presencia, el destino, la eliminación y los efectos de estos compuestos en el medio ambiente.

1.2. CLASIFICACIÓN DE LOS FILTROS UV

Los UV-F se pueden dividir en dos grandes grupos teniendo en cuenta su naturaleza química:

- *Filtros inorgánicos o físicos*, son aquellos que poseen una función fundamentalmente impermeabilizante, reflejando gran parte de la radiación UV que les llega. Debido a la uniformidad de las partículas que los componen y su tamaño son fotoestables y seguros. Se han venido utilizando como tales algunos óxidos metálicos. Los más representativos son el óxido de zinc y el dióxido de titanio.

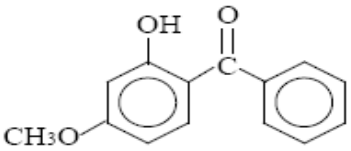
- *Filtros orgánicos o químicos*, son aquellos que absorben la radiación UV. Se trata de moléculas orgánicas constituidas por uno o varios anillos bencénicos, conjugados con dobles enlaces C-C y/o grupos carbonilo, proporcionando un sistema de alta deslocalización electrónica, que les confiere un elevado coeficiente de absortividad molar en la zona UVA (400-320 nm) y/o UVB (320-290 nm).

En el ámbito de la cosmética, los filtros físicos proporcionan una mayor protección y, al no penetrar en la piel, no constituyen ningún trastorno para el sistema inmunológico, pero forman una película impermeable que tapa los poros de la piel. Por otra parte, el coste de las materias primas es más elevado que en el caso de los filtros químicos. Los filtros químicos proporcionan menor protección. Penetran en la piel, por lo que pueden provocar en algunos casos reacciones alérgicas. Su coste es menor y permiten la transpiración de la piel. Por otra parte, estos productos son más aceptados comercialmente, debido a que suelen ser más fluidos y tienen una aplicación más confortable para el usuario, puesto que una vez absorbido no deja una capa de producto sobre la piel como ocurre en el caso de los filtros físicos. Además, para las empresas de productos cosméticos, resulta más sencillo formular productos con filtros químicos que con filtros físicos, ya que éstos últimos presentan mayor dificultad para formar emulsiones estables. Estudios recientes han demostrado su presencia en fluidos biológicos como orina [12, 25-27], plasma [28], semen [29] y leche materna [24]. Como se observa en la Tabla 1.2, tan solo existe un filtro físico (el dióxido de titanio) del total de los 28 filtros autorizados en la UE.

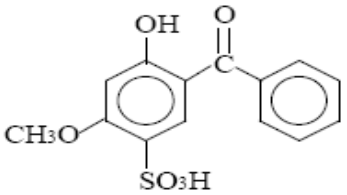
Los 27 filtros químicos permitidos por la legislación europea se clasifican en

nueve grupos atendiendo a su composición química:

1- Derivados de la benzofenona

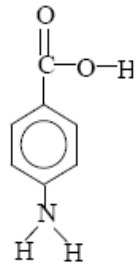


Benzofenona-3

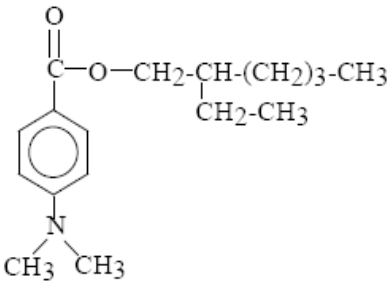


Benzofenona-4

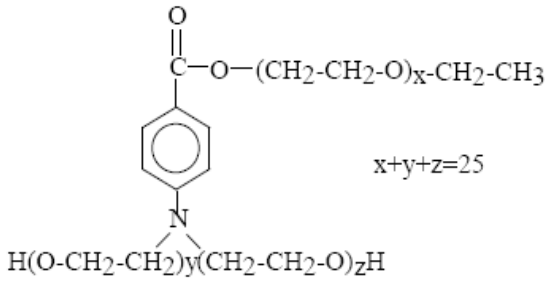
2- Ácido p-aminobenzoico (PABA) y sus derivados:



PABA

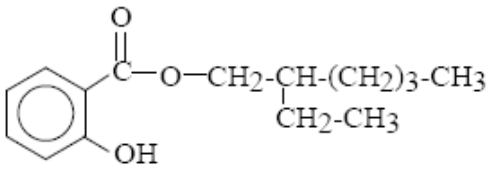


Octil dimetil PABA

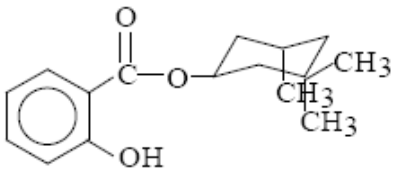


PEG-25 PABA

3- Salicilatos:

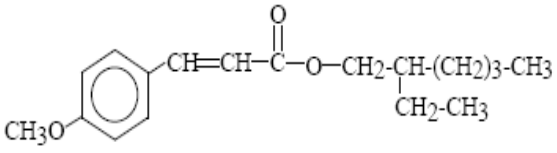


Octil salicilato

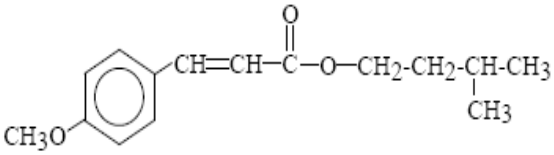


Homosalato

4- Metoxicinamatos:

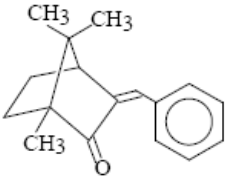


Octil metoxicinamato

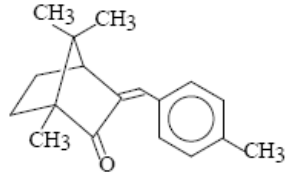


Isoamil metoxicinamato

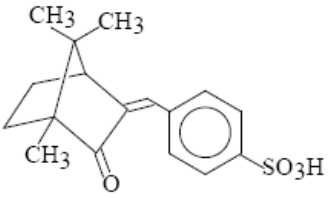
5- Derivados del canfor:



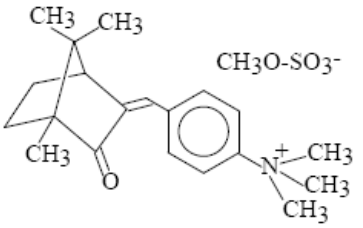
3-benciliden canfor



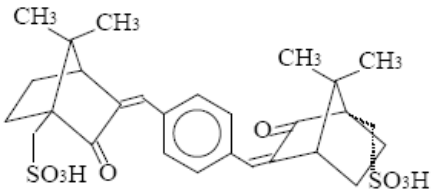
4-metilbenciliden canfor



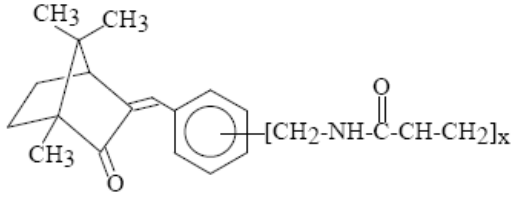
Ácido benciliden canfor sulfónico



Canfor benzalconio metosulfato

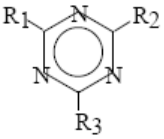


Ácido tereftaliden dicanfor sulfónico



Poliacrilamidometil benciliden canfor

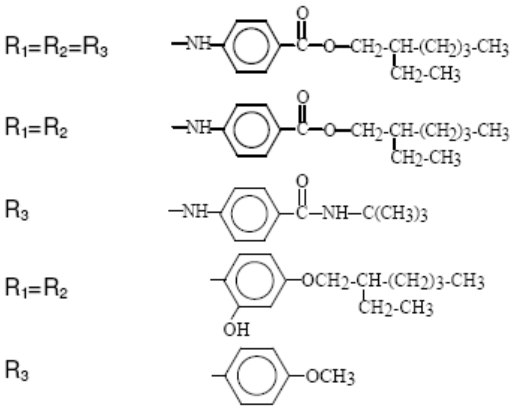
6- Derivados de la triazina:



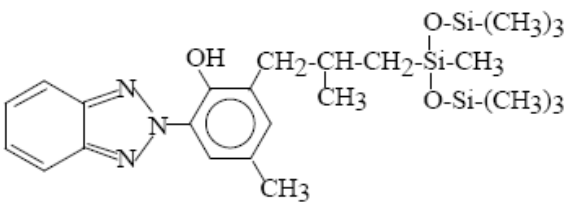
Octil triazona

Dioctil butamido triazona

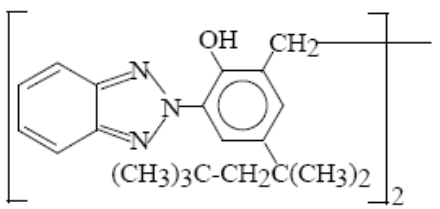
Bis-etilhexiloxifenol metoxifenil triazina



7- Derivados del benzotriazol:

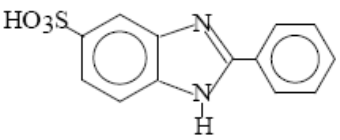


Drometrizol trisiloxano

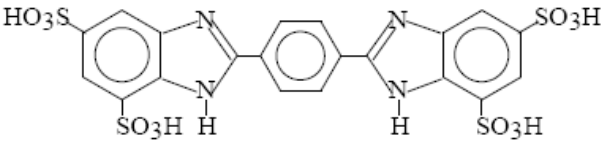


Metilen bisbenzotriazolil

8- Derivados del bencilmidazol:

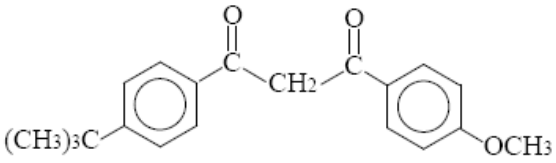


Ácido fenilbencimidazol sulfónico

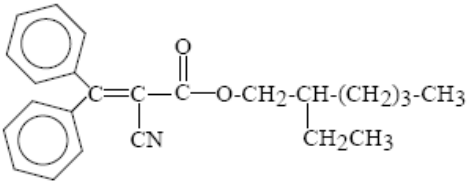


Ácido fenildibencimidazol tetrasulfónico

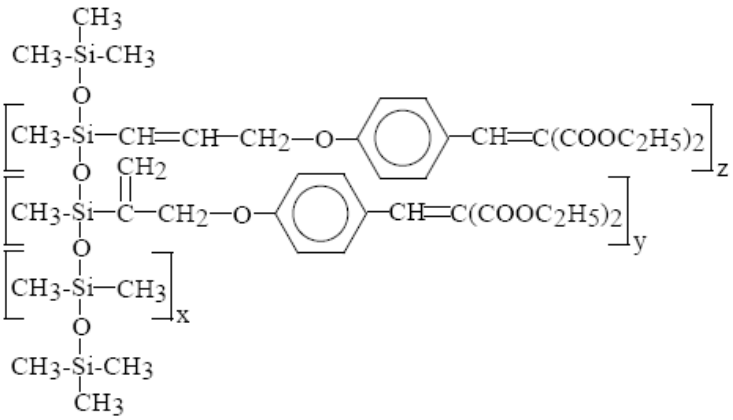
9- Otros:



Butil metoxidibenzoilmetano



Octocrileno



Polisilicona-15

1.3. NORMATIVA

La lista de compuestos permitidos en productos cosméticos por la legislación europea [European Comisión (EC), Council Directive 76/768/EEC of 27 July 1976 and its successive amendments] regula 28 UV-F de los cuales 27 son orgánicos y 1 inorgánico. La legislación estipula el porcentaje de cada filtro permitido en las formulaciones cosméticas entre el 2 y el 15% en peso de producto acabado.

Tras sucesivas modificaciones y adaptaciones, según el progreso técnico de la directiva original (76/768/CEE) en materia de productos cosméticos, y dada la importancia de los compuestos empleados como UV-F, la *Directiva Europea 83/574/CEE* del 26 de octubre de 1983 incorporó por primera vez un listado de compuestos que podían ser empleados como UV-F en los productos cosméticos, a modo de anexo (*Anexo VII de la Directiva Europea en materia de productos cosméticos*). En este anexo se incluían seis compuestos que podían formularse en los preparados cosméticos (*Primera Parte del anexo*) y 31 que podían emplearse provisionalmente (*Segunda Parte del anexo*). En la Tabla 1.1 se muestra la nomenclatura, cantidad máxima permitida y las directivas en las que se modificaron las condiciones de uso de cada uno de estos compuestos.

Tabla 1.1. Listado inicial de compuestos que podían ser empleados como UV-F en los productos cosméticos para la protección solar según la directiva 83/574/CEE.

UE[a]	Nombre químico	Nomenclatura INCI[b]	Conc máx. autorizada	Modificaciones[c]
Primera Parte. Lista de los filtros UV admitidos que podrán contener los productos cosméticos				
1.1	Ácido 4-aminobenzoico	PABA	5 %	
1.2	Metil sulfato de N,N,N-trimetil-4-(2-oxoborn-3-ilidinemetil) anilinio	Canfor benzalconio metosulfato	6 %	
1.3	Salicilato de 3,3,5-trimetil ciclohexilo *(homosalato)*	Homosalato	10 %	
1.4	2-hidroxi-4-metoxibenzofenona *(oxibenzona)*	Benzofenona-3	10 %	
1.5	Ácido 3-(1H-imidazol-4-il)-2-propenoico y su éster etílico	Ácido urocánico	2 %[d]	93/47/CEE$^{II \rightarrow I}$ 96/41/CE†
1.6	Ácido 2-fenilbencimidazol-5-sulfónico y sus sales de K^+, Na^+, TEA	Ácido fenilbencimidazol sulfónico	8 %	
Segunda Parte. Lista de los filtros UV que podrán contener provisionalmente los productos cosméticos				
2.1	4-[bis(2-hidroxipropil)amino]benzoato de etilo (mezcla de isómeros)	Etil dihidroxipropil PABA	5 %[d]	92/86/CEE†
2.2	4-aminobenzoato de etilo etoxilado	PEG-25 PABA	10 %	98/62/CE$^{II \rightarrow I}$
2.3	4-dimetilaminobenzoato de pentilo (mezcla de isómeros)	Pentil dimetil PABA	5 %	89/174/CEE†

2.4	4-aminobenzoato de glicerilo	Gliceril PABA	5 %	92/86/CEE†
2.5	4-dimetilaminobenzoato de 2-etilhexilo	Octil dimetil PABA	8 %	2000/6/CE$^{II \rightarrow I}$
2.6	Salicilato de 2-etilhexilo	Octil salicilato	5 %	98/62/CE$^{II \rightarrow I}$
2.7	2-acetamidobenzoato de 3,3,5-trimetilciclohexilo	Homomentil acetamidobenzoato	2 %	89/174/CEE†
2.8	Ácido cinámico (sal de potasio)	Ácido cinámico	2 %	89/174/CEE†
2.9	Ácido 4-metoxicinámico (sales de K^+, Na^+ y DEA)	Ácido metoxicinámico	8 %[d]	89/174/CEE†
2.10	4-metoxicinamato de isopropilo	Isopropil metoxicinamato	3 %	89/174/CEE†
2.11	Ácido salicílico (sales de K^+, Na^+ y TEA)	Ácido salicílico	2 %[d]	89/174/CEE†
2.12	4-metoxicinamato de isoamilo (mezcla de isómeros)	Isoamil metoxicinamato	10 %	98/62/CE$^{II \rightarrow I}$
2.13	4-metoxicinamato de 2-etilhexilo	Octil metoxicinamato	10 %	97/45/CE$^{II \rightarrow I}$
2.14	4-metoxicinamato de 2-etoxietilo *(cinoxate)*	Cinoxato	5 %	89/174/CEE†
2.15	3,4-dihidroxi-5-((3,4,5-trihidroxi-benzoil)-oxi)benzoato de trioleilo	Digalloil trioleato	4 %	89/174/CEE†
2.16	2-hidroxi-4-metoxi-4'-metilbenzofenona *(mexenona)*	Benzofenona-10	4 %	83/574/CEE^{II} 92/86/CEE†
2.17	Ácido 2-hidroxi-4-metoxibenzofenona-5-sulfónico *(sulisobenzona)*	Benzofenona-4	5 %[d]	83/574/CEE^{II} 2000/6/CE$^{II \rightarrow I}$
2.18	2-(4-fenilbenzoil) benzoato de 2-etilhexilo	Octil fenilbenzoilbenzoato	10 %	83/574/CEE^{II} 89/174/CEE†
2.19	5-metil-2-fenilbenzoxazol	Metil fenilbenzoxazol	4 %	83/574/CEE^{II} 89/174/CEE†
2.20	3,4-dimetoxifenilglioxilato de sodio	Dimetoxi fenilglioxilato de sodio	5 %	83/574/CEE^{II} 89/174/CEE†
2.21	4,4'-dimetoxi dibenzoilmetano	Dimetoxi dibenzoilmetano	6 %	83/574/CEE^{II} 89/174/CEE†
2.22	5-(3,3-dimetil-2-norborniliden)-3-penten-2-ona	Bornelona	3 %	83/574/CEE^{II} 89/174/CEE†
2.23	Ácido α-(2-oxoborn-3-yliden)-xilen-4- sulfónico	Ácido metilbenciliden canfor sulfónico	6 %	83/574/CEE^{II} 89/174/CEE†
2.24	Ácido α-(2-oxoborn-3-iliden)-toluen-4-sulfónico y sus sales	Ácido benciliden canfor sulfónico	6 %[d]	83/574/CEE^{II} 94/32/CEE$^{II \rightarrow I}$
2.25	3-(4'-metilbenciliden) canfor	4-metilbenciliden canfor	6 %	83/574/CEE^{II} 98/62/CE$^{II \rightarrow I}$
2.26	3-benciliden canfor	3-benciliden canfor	6 %	83/574/CEE^{II} 98/62/CE$^{II \rightarrow I}$
2.27	Ácido α-cyano-4-metoxicinamico y su éster hexílico	Hexil ciano metoxicinamato	5 %	83/574/CEE^{II} 89/174/CEE†
2.28	4-isopropil-dibenzoilmetano	Isopropil dibenzoilmetano	5 %	83/574/CEE^{II} 94/32/CEE†
2.29	Salicilato de 4-isopropilbencilo	Isopropilbencil salicilato	4 %	83/574/CEE^{II} 2000/6/CE†

2.30	4-metoxicinamato de ciclohexilo	Ciclohexil metoxicinamato	1 %	83/574/CEE^{II} 89/174/CEE†
2.31	4-tert-butil-4'-metoxidibenzoilmetano	Butil metoxidibenzoilmetano	5 %	83/574/CEE^{II} 93/47/CEE$^{II \rightarrow I}$

[a] Número de orden otorgado según las sucesivas directivas de la Unión Europea.
[b] INCI *(International Nomenclature for Cosmetic Ingredient)* elaborada por CTFA y COLIPA. En su defecto se nombrará por analogía con los nombres de compuestos similares cuya nomenclatura INCI sí existe.
[c] Directivas europeas que modificaron la ubicación de los filtros UV, pasando de la *Primera Parte* a la *Segunda Parte* (I→II) o al contrario (II→I), o bien fueron eliminados (†) (y por tanto prohibido su uso como filtros UV) del *Anexo VII de la Directiva Europea sobre productos cosméticos.*
[d] Expresado como ácido.

En posteriores directivas, se fueron eliminando parte de los compuestos inicialmente permitidos, por considerar que constituían un peligro para la salud e incorporando nuevos compuestos. En la Tabla 1.2 se recogen estas modificaciones y se muestran los compuestos que pueden emplearse en la actualidad como filtros UV de acuerdo con la legislación vigente dentro del marco de la Unión Europea (UE). Algunos de los compuestos que están actualmente permitidos pueden ser retirados del mercado a medio plazo, como por ejemplo el Et-PABA, que causa problemas dermatológicos [16] y sigue estando permitido hasta un 5% en la legislación europea y otros que están en estudio como BP4, BP3, EHMC o HMS [16]. Hay que puntualizar que esta legislación es únicamenta aplicable a protectores solares, por lo que otros PCPs pueden contener otros compuestos para protección UV.

La normativa española no aplica ningún cambio específico a la europea. Cabe mencionar que existen otras normativas como la de Estados Unidos (*FDA, 1999*) que permite tan sólo 14 sustancias y la Japonesa (*Japanese SCI, 1985*) que permite 22. Hay que resaltar la gran divergencia tanto en los niveles permitidos como en los UV-F autorizados por las diferentes normativas.

Tabla 1.2. Listado actualizado de compuestos que pueden ser empleados como UV-F en los productos cosméticos para la protección solar. (Anexo VI: Regulation (EU) 1223/2009).

UE[a]	Nombre químico	Nomenclatura INCI[b]	Conc máx. autorizada	Modificaciones[c]
Primera Parte. Lista de los filtros UV admitidos que podrán contener los productos cosméticos				
1.1	Ácido 4-aminobenzoico	Paba	5 %	83/574/CEE^{I}
1.2	Metil sulfato de N,N,N-trimetil-4-(2-oxoborn-3-ilidinemetil) anilinio	Canfor benzalconio metosulfato	6 %	83/574/CEE^{I}
1.3	Salicilato de 3,3,5-trimetil ciclohexilo *(homosalato)*	Homosalato	10 %	83/574/CEE^{I}
1.4	2-hidroxi-4-metoxibenzofenona *(oxibenzona)*	Benzofenona-3	10 %	83/574/CEE^{I}
1.6	Ácido 2-fenilbencimidazol-5-sulfónico y sus sales de K^+, Na^+, TEA	Ácido fenilbencimidazol sulfónico	8 %[d]	83/574/CEE^{I}
1.7	Ácido 3,3'-(1,4-fenilendimetileno) bis (7,7-dimetil-2-oxobiciclo-[2.2.1]hept-1- ilmetano sulfonico) y sus sales	Ácido tereftaliden dicanfor sulfónico	10 %[d]	91/184/CEE^{I}
1.8	4-tert-butil-4'-metoxidibenzoilmetano	Butil metoxidibenzoilmetano	5 %	83/574/CEE^{II} 93/47/CEE$^{II\rightarrow I}$
1.9	Ácido α-(2-oxoborn-3-iliden)-toluen-4-sulfónico y sus sales	Ácido benciliden canfor sulfónico	6 %[d]	83/574/CEE^{II} 94/32/CEE$^{II\rightarrow I}$
1.10	2-ciano-3,3-difenilacrilato de 2-etilhexilo	Octocrileno	10 %[d]	95/34/CEE^{I}
1.11	Polimero de N-{(2 y 4)-[(2-oxoborn-3- iliden)metil]bencil} acrilamida	Poliacrilamidometil benciliden canfor	6 %	94/32/CEE^{II} 96/41/CE$^{II\rightarrow I}$
1.12	4-metoxicinamato 2-etilhexilo	Octil metoxicinamato	10 %	83/574/CEE^{II} 97/45/CE$^{II\rightarrow I}$
1.13	4-aminobenzoato de etilo etoxilado	PEG-25 PABA	10 %	83/574/CEE^{II} 98/62/CE$^{II\rightarrow I}$
1.14	4-metoxicinamato de isoamilo (mezcla de isómeros)	Isoamil metoxicinamato	10 %	83/574/CEE^{II} 98/62/CE$^{II\rightarrow I}$
1.15	2,4,6-trianilina-(p-carbo-2'-etillhexil-1'-oxi)-1,3,5-triazina	Octil triazona	5 %	89/174/CEE^{II} 98/62/CE$^{II\rightarrow I}$
1.16	2-(2H-benzotriazol-2-il)-4-metil-6-[2-metil-3-(1,3,3,3-tetrametil-1-((trimetilsilil)oxi)disiloxanil)propi) fenol	Drometrizol trisiloxano	15 %	98/62/CE^{I}
1.17	4,4'-[[6-[[[(1,1-dimetiletil)amino] carbonil]fenil]amino]-1,3,5-triazina-2,4- diil]diimino]bis-bis(2-etilhexil) benzoate	Dioctil butamido triazona	10 %	98/62/CE^{I}
1.18	3-(4'-metilbenciliden) canfor	4-metilbenciliden canfor	4 %	83/574/CEE^{II} 98/62/CE$^{II\rightarrow I}$
1.19	3-benciliden canfor	3-benciliden canfor	2 %	83/574/CEE^{II} 98/62/CE$^{II\rightarrow I}$
1.20	Salicilato de 2-etilhexilo	Octil salicilato	5 %	83/574/CEE^{II} 98/62/CE$^{II\rightarrow I}$

1.21	4-dimetilaminobenzoato de 2-etilhexilo	Octil dimetil PABA	8 %	83/574/CEE[II] 2000/6/CE[II→I]
1.22	Ácido 2-hidroxi-4-metoxibenzofenona-5-sulfónico *(sulisobenzona)*	Benzofenona-4	5 %[d]	83/574/CEE[II] 2000/6/CE[II→I]
1.23	2,2'-metilen-bis-[4-(1,1,3,3-tetrametil butil)-6-(2H-benzotrizol-2-il) fenol]	Metilen bisbenzotriazolil tetrametilbutilfenol	10 %	2000/6/CE[I]
1.24	Ácido 2,2'-(1,4-fenilen) bis-1H-bencimi-dazol-4,6-disulfónico (sal monosódica)	Ácido fenildibencimidazol tetrasulfónico	10 %[d]	2000/6/CE[I]
1.25	2,2'-[6-(4-metoxifenil)-1,3,5 triazina-2,4-diil]bis[5-[(2-etilhexil)oxi]fenol	Bis-etilhexiloxifenol metoxifenil triazina	10 %	2000/6/CE[I]
1.26	Metil 3-[4-[2,2-bis(etoxicarbonil)etenil] fenoxi]propenil dimetil polisiloxano	Polisilicona-15	10 %	2002/34/CE[I]
1.27	Dióxido de titanio	Dióxido de titanio	25 %	2002/34/CE[I]
1.28	Benzoic acid,2-[4-(diethylamino)-2-hydroxybenzoyl]-, hexyl ester	UvinulA Plus	10 %	2005/9/CE
Segunda Parte. Lista de los filtros UV que podrán contener provisionalmente los productos cosméticos				
Actualmente no se incluye ningún compuesto en esta lista				

[a] Número de orden otorgado según las sucesivas directivas de la Unión Europea

[b] INCI *(International Nomenclature for Cosmetic Ingredient)* elaborada por CTFA y COLIPA

[c] Directivas europeas que admitieron el uso de estos compuestos en los productos cosméticos para la protección solar y por tanto aparecieron en la *Primera Parte del anexo VII* (I) o bien fueron admitidos provisionalmente (II), y posteriormente pasaron de la de la *Segunda Parte* a la *Primera Parte* (II→I).

[d] Expresado como ácido.

1.4. PRESENCIA Y NIVELES DE FILTROS UV EN EL MEDIO AMBIENTE

El intervalo de propiedades fisico-químicas de los UV-F es muy amplio, sin embargo, en general tienen un marcado carácter lipofílico. Estos compuestos tienden a acumularse en matrices sólidas como sedimentos o lodos de EDAR, donde alcanzan niveles similares a los PCBs o PAHs, y también a bioacumularse en organismos vivos como peces. A continuación se resumen los datos de presencia ambiental de los UV-F disponibles hasta la realización de la presente memoria.

Niveles en agua:

Los niveles de UV-F en muestras acuosas dependen de la época del año en que se toman las muestras. En verano, las concentraciones determinadas son significativamente más elevadas en todos los tipos de aguas, dado el incremento que se produce en el uso de productos que incluyen estos compuestos. No obstante, en los meses de otoño e invierno también se han detectado UV-F en concentraciones relevantes, ya que no solamente se utilizan en cremas para la protección solar, sino en una gran variedad de PCPs y en la fabricación de muchos materiales.

En la Tabla 1.3 se resumen los estudios que han evaluado la presencia de estos compuestos en muestras acuosas en el medio ambiente. En la Tabla se pueden apreciar los intervalos de concentración detectados para cada compuesto. BP3, 4MBC, EHMC y OC han sido los más estudiados, aunque en los últimos estudios se incluye BP4 y algunos productos de transformación de BP3 como BP1.

Los primeros resultados sobre la presencia de UV-F en el medio ambiente los obtuvo Balmer et al. [3], en un estudio que muestra la amplia presencia de algunos de estos compuestos en las aguas de lagos suizos, con valores entre 2 y 35 ngL^{-1} para BP3, 4MBC, EHMC y OC. Posteriormente se han llevado a cabo varios estudios referidos a la presencia de estos compuestos en ríos [1, 5, 27, 30-42] y lagos [3, 6, 37, 43, 44]. Las concentraciones en estas matrices son muy variadas, pero existe una gran diferencia, remarcada por varios autores, entre las aguas donde se realizan actividades acuáticas recreativas, con altos niveles de UV-F y en las que no tienen lugar este tipo de actividades (niveles mucho menores) [3, 45].

En menor medida se ha estudiado la presencia de UV-F en agua de mar, recogida cerca de la costa. Los compuestos derivados de la benzofenona fueron los más ampliamente

detectados [1, 6, 42, 46, 47], con concentraciones que llegan a sobrepasar los 3000 ngL^{-1} para BP3, en muestras recogidas en zonas de baño recreativo. El OC también ha sido detectado en agua de mar [1, 42].

De acuerdo con los balances de masa que relacionan las cantidades de UV-F entre diferentes matrices [3, 48], existen mecanismos de eliminación de estos compuestos de la fase acuosa; sin embargo, estos procesos aún no se han podido caracterizar. Los valores relativamente altos de K_{oc} (carbón orgánico (OC)-coeficiente de distribución) indican que estos compuestos se asocian con la materia particulada, especialmente cuando hay un alto contenido de materia orgánica, por lo que pueden ser eliminados del agua mediante adsorción o sedimentación. Sin embargo, datos en tiempo real sugieren que otros mecanismos pueden ser responsables de su eliminación en aguas ambientales. Uno de los escasos estudios al respecto demuestra que el EHMC sufre una rápida degradación en aguas superficiales a pesar de sus altos valores de Log K_{ow} y Log K_{oc} (constante de partición entre el carbono orgánico y el agua). En cursos de agua que reciben efluentes de EDARs, la tasa de pérdida de EHMC ha sido estimada en 1,03 h^{-1}, con su correspondiente vida media de 0,67 h, que fue seis veces mayor que la disminución de la demanda biológica de oxígeno (BOD) en el mismo curso de agua [49]. Sin embargo, el 4MBC que es conocido por ser químicamente estable y no fácilmente biodegradable, exhibió tasas de eliminación en aguas de lago que no pueden ser explicadas solo por dilución y adsorción [3, 6]. Estos datos sugieren que otros mecanismos de eliminación son efectivos en aguas ambientales y que probablemente son de origen fotoquímico.

El estudio llevado a cabo por Balmer et al. también detectó residuos de UV-F en aguas de entrada y salida de EDARs, en concentraciones mucho más altas (500–19000 ngL^{-1} para aguas de entrada y 10-2700 ngL^{-1} para aguas tratadas)[3]. A partir de aquí ha habido más estudios en aguas residuales [1, 4, 5, 31, 32, 34, 35, 39, 42, 43, 45, 47, 50-52], con niveles en un intervalo de concentración similar. Destacan los altos valores para el compuesto más hidrofílico de esta familia, BP4, que supera los 4000 ngL^{-1} en algunas aguas de entrada [31]. En general, los niveles que se encuentran en las aguas tratadas son mucho menores que en las aguas de entrada, aunque dependen en gran medida del compuesto. Algunos UV-F, como por ejemplo el EHMC, no se detectan prácticamente en las aguas de salida mientras otros, como BP4 o 4MBC, permanecen en concentraciones similares a las aguas de entrada. La diferencia de concentraciones

entre el influente y el efluente, en general, no proporciona una estimación rigurosa de la eficiencia de las EDARs.

La práctica totalidad de los estudios no tienen en cuenta la adsorción de estos compuestos en los lodos. Con su marcado carácter lipofílico, los UV-F tienden a acumularse en estos lodos en grandes concentraciones siendo ésta una de las principales vías de eliminación de los UV-F de las aguas residuales.

Por otra parte, estos métodos solo tienen en cuenta los UV-F originales, no sus productos de transformación. Esto significa que puede darse el caso de que un compuesto parezca que se degrade totalmente cuando en realidad ha sufrido alguna pequeña transformación en su estructura (por ejemplo una hidroxilación), la cual podría incrementar su biodisponibilidad e incluso aumentar su toxicidad. La determinación de los productos de transformación en aguas residuales es de gran importancia y un tema a considerar seriamente en el futuro. Por otra parte, para poder calcular los porcentajes de eliminación de una manera rigurosa hay que tener en cuenta en los muestreos los tiempos de retención hidráulicos, para asegurarnos así que el agua que analizamos en la salida de la EDAR proviene del agua de entrada que también hemos analizado. Los estudios llevados a cabo no han tenido en cuenta este aspecto. Así, estos estudios no proporcionan información rigurosa sobre la eficiencia de degradación pero sí permiten tener una idea de los compuestos más biodegradables por los procesos que se llevan a cabo, así como saber qué concentraciones tienen los efluentes que van a parar al ecosistema acuático.

Como ya se ha comentado, la presencia de los UV-F en el medio acuático tiene efectos nocivos sobre los seres vivos, especialmente del tipo disruptor endorcrino. Se han determinado efectos adversos inducidos por UV-F en experimentos tanto *in vivo* como *in vitro* que afectan a la reproducción y al desarrollo de los individuos, como una reducción de la fecundidad o alteraciones en los órganos así como efectos en el eje hipotálamo-pituitario-tiroide. Las implicaciones ecotoxicológicas de estos compuestos en el ecosistema acuático se explican detalladamente en la publicación científica #2, en la sección "*Ecotoxicological considerations*".

Tabla 1.3. Niveles de UV-F en agua según datos de la bibliografía.

UV-F	Agua de río (ngL^{-1})	Agua de lago (ngL^{-1})	Agua de mar (ngL^{-1})	Agua de entrada de EDAR (ngL^{-1})	Agua de salida de EDAR (ngL^{-1})	Referencia
Derivados de la benzofenona						
BP1	47					[30]
	6-9			306	32	[31]
				47-155	11	[50]
	24			31-148	11-13	[5]
	37			131-265	41	[34]
	0.9-29			43-448	12	[35]
			280			[46]
	221					[32]
BP2	4			25	1	[31]
	1.8-6.7			35-93	14	[35]
BP3			2			[53]
		5.125				[6]
		10-35		700-7800	10-700	[3]
	23					[33]
				97-722		[4]
	114					[36]
	27			31-168	16	[1]
	30	17-55			42-54	[37]
	28-37			971	143	[31]
	14					[27]
	85					[38]
				300-2300	1-13	[52]
	54-87			184-429	77-84	[5]
	52			216-462	13-44	[34]
		40		234	3-45	[43]
				11-286	20-100	[50]
	6-28					[39]
	8-42			17-222	10-58	[45]
	59					[41]
	47			195-518	96	[35]
			1340-3300			[46]
		32				[44]
	3-69		7-13	32-551	5-20	[42]
			70	100	200	[47]
	5-107				8-167	[32]
				6-163	5-28	[51]
BP4	849		38-138	237-1441	376-1359	[1]
	10-227			5790	4309	[31]
	20-416			1237-1596	765-1028	[5]
	51-1980			2120-5130	105-572	[35]
	14-952				41-2032	[32]
DHMB				55	59-185	[39]
Derivados del canfor						
4MBC		2-82				[6]
		2-28		600-6500	60-2700	[3]
				475-2128		[4]
				122	23-51	[1]
	5-15	25-148			38	[37]
		1140		278	30-62	[43]
	10					[41]
	7-1132			7-153	66-94	[45]

Cinamatos						
EHMC		2-26				[6]
		3-7		500-19000	10-100	[3]
	21	20-33			11-23	[37]
		3009		1732		[43]
	19-813			51-124		[45]
	10			23		[42]
				23-68		[51]
				54-116		[4]
IAMC					59	[1]
		51			3	[37]
		146		66		[43]
Crilenos						
OC		2-27				[6]
		5		100-12000	100-270	[3]
				34-153		[4]
	16	10-250			10-18	[37]
				36	20	[1]
		4381		5322	179	[43]
	18-4256			66-440	15-59	[45]
	29					[41]
		1710				[44]
	11-112		19-32	28-463	13	[42]
				12-390	4-126	[51]
PBSA	34		42	181-2503	109-2679	[1]
	48-3240			275-3890	316-1820	[35]
Derivados del ácido p-aminobenzoico (PABA)						
OD-PABA	3	2-5			2-7	[37]
				103	19	[50]
				25	55	[39]
				4		[51]
BM-DBM		2431		407	29	[43]
Salicilatos						
HMS	5	4-5			8-9	[37]
	18-124			11-20		[45]
EHS		51				[37]
				28	7.5	[34]
		748		753		[43]
	8					[41]
	12-62			6-32		[45]

Niveles en sedimentos y lodos de EDAR:

Los UV-F son en general lipofílicos y excepto los derivados de la benzofenona tienen $LogK_{ow}$ bastante altos, por lo que es de esperar que se acumulen en sedimentos, materia particulada y lodos de EDAR. No obstante, los datos existentes sobre la presencia de estos compuestos en matrices sólidas son escasos. La Tabla 1.4. muestra las concentraciones determinadas para UV-F en muestras de sedimentos y lodos de EDAR.

Algunos estudios han determinado la presencia de BP3 y algunos de sus derivados en sedimentos [30, 47, 54, 55], en concentraciones que van desde 0.3 a 47 ngg^{-1}. Solo dos estudios a parte de los llevados a cabo en esta Tesis, han determinado otros UV-F más lipofílicos. Rodil et al. determinó EHMC y OC en el intervalo 14 y- 93 ngg^{-1} [56] y Ricking et al. determinó EHMC a una concentración de 4 ngg^{-1} [55].

Los niveles determinados en lodos de EDAR son mucho más altos. El primer estudio en esta matriz lo llevó a cabo Plagellat et al. [57] para los compuestos 4MBC, EHMC y OC, que presentaron concentraciones por encima de los 4900 ngg^{-1} para el 4MBC y por encima de 18000 ngg^{-1} en el caso del OC. A partir de entonces se han llevado a cabo otros estudios en los que además del 4MBC, EHMC y OC se incluyeron BP3, IAMC, OD-PABA, EHS o HMS [58-60]. Los niveles de concentración fueron muy variables, entre 5 y 3900 ngg^{-1}, siendo otra vez máximos para los compuestos 4MBC y OC. Zhant et al. y Wick et al. [35, 54] estudiaron la presencia de derivados de la benzofenona en lodos. Estos compuestos se determinaron en concentraciones entre 0.3 y 29 ngg^{-1} a excepción de BP3, que es el más lipofílico de la familia de los derivados de la benzofenona, y alcanzó niveles por encima de los 100 ngg^{-1}.

Tabla 1.4. Niveles de UV-F en sedimentos y lodos de EDAR según datos de la bibliografía

UV-F	Lodo de EDAR (ngg^{-1})	Sedimento (ngg^{-1})	Referencia
Derivados de la benzofenona			
BP1	5.1		[35]
	4.41-91.6	0.259-0.607	[54]
BP2	11		[35]
		2.65	[54]
BP3		0.7-4	[30]
	10-20		[59]
	6.6-29		[60]
	132		[35]
	2.05-13.3	0.272-4.66	[54]
	93-295		[58]
		47	[47]
BP4	29		[35]
4HB	2.66-10.1	0.312-0.951	[54]
DHMB		0.133-0.796	[54]
		0.5-4	[30]
Derivados del canfor			
4MBC	150-4980		[57]
	73-3893		[60]
	97-1579		[58]

Cinamatos			
EHMC	10-390	4 14-34	[55] [57] [56]
	35-127		[60]
	100-3287		[58]
IAMC	5-20		[60]
Crilenos			
OC	320-18740	61-93	[57] [56]
	700-1842		[59]
	585-2479		[60]
	377-3263		[58]
Derivados del ácido p-aminobenzoico (PABA)			
OD-PABA	132-170		[59]
	1.4-1.9		[60]
Derivados del Dibenzoilmetano			
BM-DBM	144-517		[60]
Salicilatos			
HMS	22-331		[60]
	110-401		[58]
EHS	49-280		[60]
	13-270		[58]
Derivados de la triazina			
DBT	54-136		[60]
EHT	928-1433		[60]

Niveles en biota: Los UV-F son potencialmente bioacumulables, especialmente, en los tejidos lipídicos de los organismos. Hasta ahora se ha confirmado su acumulación en diversos organismos entre los que se incluyen macroinvertebrados acuáticos, peces y aves que basan su dieta en el pescado [2, 3, 7, 61-66]. Algunos estudios sugieren una posible biomagnificación de estos compuestos a través de la cadena trófica [7]. Los niveles para este tipo de matriz y consideraciones sobre la bioacumulación y biomagnificación de los UV-F se explican detalladamente en la publicación científica #2.

1.5. FILTROS SOLARES ORGÁNICOS CONSIDERADOS EN ESTA TESIS

La Tabla 1.5 presenta de forma detallada las principales características fisicoquímicas de los UV-F y productos de transformación que se analizan en los diferentes capítulos de esta Tesis Doctoral.

Se incluyen siete derivados de la benzofenona. Estos compuestos contienen la estructura de la benzofenona con diferentes sustituyentes (hidroxi y metoxi) en distintas posiciones de los anillos aromáticos. La legislación europea permite el uso de 2-hidroxi-4-metoxibenzofenona (BP3) y el ácido 2-hidroxi-4-metoxivenzofenona-5-sulfónico (BP4) en cremas solares. No obstante, otras legislaciones menos restrictivas como la japonesa también permiten el uso de 2-4-dihidroxibenzofenona (BP1) y 2,2',4,4'-tetrahidroxibenzofenona (BP2) en estos productos. BP1 es el principal producto de transformación de BP3. Este compuesto ha sido detectado como metabolito en orina humana [30] junto con 4-hidroxibenzofenona (4HB) y 4,4'-dihidroxibenzofenona (4DHB). BP1 también ha sido identificado como metabolito de BP3 en el proceso de degradación mediante el hongo *T. versicolor* [67], o como producto de transformación de BP3 en estudios de ozonación [68], como se verá en los próximos capítulos de esta memoria. El compuesto 2,2'-dihidroxi-4-metoxibenzofenona (DHMB), además de ser un producto de transformación de BP3 [30], se incluye como fotoestabilizador en otros PCPs y productos como pinturas, textiles o plásticos [30, 69].

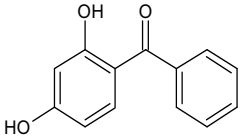
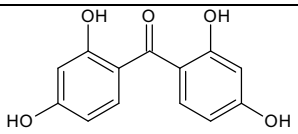
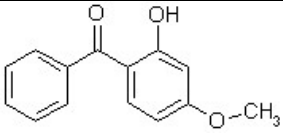
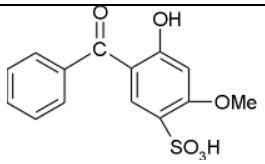
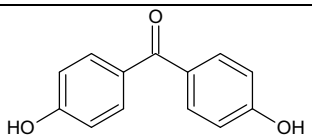
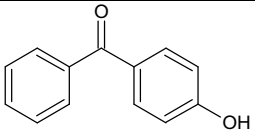
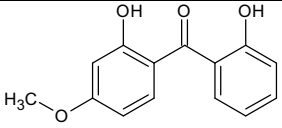
Con la excepción de BP4, este grupo de compuestos son moderadamente polares y ligeramente ácidos, con valores de pK_a entre 7 y 7.6. En cambio, BP4 es un compuesto muy soluble en agua y con fuerte carácter ácido.

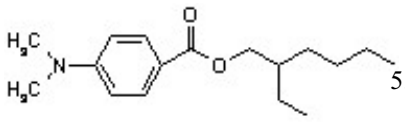
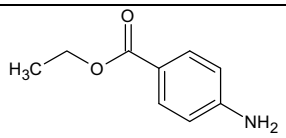
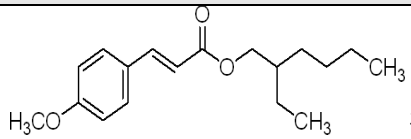
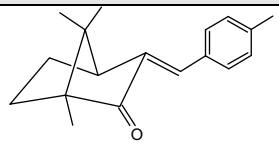
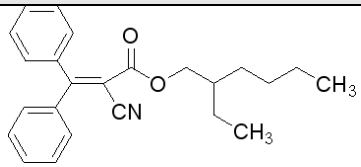
También se estudiaron dos derivados del ácido p-aminobenzoico (PABA), que se usan tanto en cremas solares como en productos industriales. Se ha consideró el 2-etilhexil-4-dimetilaminobenzoato (octil dimetil PABA, OD-PABA), compuesto muy lipofílico y el 2-etilhexil-4-dimetilaminobenzoato (etil PABA, Et-PABA), que es moderadamente polar.

Otros compuestos lipofílicos (LogKow entre 4.95 – 6.88) que se incluyen en estos estudios son el metoxicinamato 2-etilhexil-p-metoxicinamato (octil metoxicinamato, EHMC), el 4-metilbenciliden canfor (4MBC) y el octocrileno (OC). Estos compuestos

son ampliamente utilizados en protectores solares y también tienen aplicaciones industriales.

Tabla 1.5. Estructuras y propiedades fisicoquímicas (25 ºC) para los filtros solares orgánicos considerados en esta Tesis.

Analito (nomenclatura INCI)[a]	CAS Nº	Estructura y peso molecular (g/mol)	Log K_{ow}	Solubilidad (g/L, pH7)	Presión de vapor[c] (mTorr)	pK_a[c]
Benzofenonas						
Benzofenona-1 (BP1)	131-56-6	 214.22	3.15[c]	0.39[c]	0.012	7.6 ± 0.5
Benzofenona-2 (BP2)	131-55-5	 246.22	2.78[b]	0.98[c]	6.69E^{-9}	7.0 ± 0.4
Benzofenona-3 (BP3)	131-57-7	 228.24	3.79[b]	0.10[c]	0.056	7.6 ± 0.4
Benzofenona-4 (BP4)	4065-45-6	 308.31	0.993[c]	11[c]	-	-0.7 ± 0.5
4,4'-Dihidroxi benzofenona (4DHB)	611-99-4	 214.22	2.19[b]	0.6[c]		
4-Hidroxibenzofenona (4HB)	1137-42-4	 198.2	2.92[c]			
2,2'-Dihidroxi-4-metoxibenzofenona (DHMB)	131-53-3	 244			0.0373	7.0 ± 0.4

Derivados del ácido *p*-aminobenzoico						
2-Etilhexil-4-dimetilaminobenzoato (OD-PABA)	21245-02-3	 277.4	5.412[c]	4.7 x 10^{-3}[c]	0.0345	2.4 ± 0.1 (amino)
Etil-PABA (Et-PABA)	94-09-7	 165.19	1.86[b]	1310[b]	0.00457	2.5 ± 0.1 (amino) 4.9 ± 0.1 (carboxilo)
Cinamatos						
2-Etilhexyl-p-metoxicinamato (EHMC)	5466-77-3	 290.4	5.8[b]	6.4 x 10^{-3}[c]	$8.89 \cdot 10^{-4}$	-
Derivados del canfor						
4-Metilbenziliden canfor (4-MBC)	36861-47-9	 254.37	4.95[b]	0.017[c]	$9.99 \cdot 10^{-3}$	-
Crilenos						
Octocrileno (OC)	6197-30-4	 361.49	6.88[b]	3.6 x 10^{-4} [d]	$2.56 \cdot 10^{-6}$	-

[a] INCI *(International Nomenclature for Cosmetic Ingredient)* elaborada por CTFA y COLIPA.
[b] Valores experimentales, obtenidos a partir de la base de datos de propiedades fisico-químicas. Syracuse Research Corporation: http://www.syrres.com/esc/physdemo.htm.
[c] Calculados usando Advanced Chemistry Development (ACD/Labs) Software V11.02 (©1999-2011 ACD/Labs).

1.6 METODOLOGÍAS ANALÍTICAS PARA LA DETERMINACIÓN DE FILTROS UV EN EL MEDIO AMBIENTE

Como se ha venido explicando, los UV-F son contaminantes emergentes que se encuentran generalmente a nivel traza en las muestras medioambientales, por lo que es necesario disponer de métodos muy sensibles y selectivos para el estudio de estos compuestos. Por otra parte, dada la complejidad de las matrices ambientales, es necesario un proceso de tratamiento de muestra intensivo. Las metodologías analíticas desarrolladas hasta el momento generalmente se basan en un proceso de extracción y purificación, tanto para matrices acuosas como para matrices sólidas, y análisis por cromatografía líquida o de gases, acoplada a espectrometría de masas o espectrometría de masas en tándem. Los métodos que utilizan detección mediante espectroscopia UV-visible son adecuados para el análisis de estos compuestos a altas concentraciones, por ejemplo en cremas solares, pero no permiten una buena determinación a nivel traza en matrices ambientales. La mayoría de métodos desarrollados hasta el momento para el análisis de UV-F en este tipo de muestras se basan en GC-MS, que permite obtener una buena sensibilidad al mismo tiempo que el efecto matriz no es crítitico. No obstante, estos métodos están límitados a substancias volátiles y muchas veces se requiere una etapa de derivatización, con los inconvenientes que comporta. La LC-MS/MS, en cambio, permite el análisis simultáneo de compuestos con un amplio intervalo de propiedades fisicoquímicas. En el inicio de esta Tesis Doctoral había una falta de este tipo de metodologías, que permiten entre otras cosas incluir en los métodos analíticos productos de transformación sin necesidad de derivatizar. No obstante, en estos casos es necesaria una purificación previa eficaz para reducir el efecto matriz. También existía una falta de metodologías automatizadas que permiten reducir drásticamente tanto el volumen de muestra necesario, el consumo disolventes y el tiempo de trabajo en el laboratorio.

En general, para muestras acuosas la etapa repurificación/preconcentración se lleva a cabo mediante SPE, aunque también hay metodologías desarrolladas basadas en microextracción en fase sólida (SPME), microextracción con barras agitadoras (SBSE), microextracción mediante sorbentes empaquetados (MEPS), microextracción con gota suspendida (SDME), microextracción líquido-líquido con membranas o microextracción en fase líquida con fibra hueca (HF-LPME). Para matrices sólidas las etapas básicas del proceso de extracción y purificación son la desorción de los analitos

de los sitios activos de la matriz, la difusión de los analitos a través de la matriz, la solubilización de los analitos en el extractante y la recolección de los extractos con los analitos de interés. Para el análisis de UV-F en muestras sólidas ambientales las técnicas de extracción más utilizadas son la extracción con líquidos presurizados (PLE), Soxhlet y la extracción líquido-sólido mediante sonicación (USE). Teniendo en cuenta que la interacción entre los analitos y su matriz es generalmente más intensa en el caso de muestras sólidas que en el caso de las matrices líquidas, es necesario el uso de técnicas de extracción más enérgicas, lo cual disminuye la selectividad del proceso y hace necesaria una etapa posterior de purificación [14], como por ejemplo mediante SPE.

Las dos publicaciones científicas que siguen a continuación describen detalladamente el estado del arte del análisis de UV-F en el medio ambiente.

Metodologías analíticas para la determinación de filtros UV en el medio ambiente

Publicación científica #1:

"Liquid chromatography-tandem mass spectrometry for the multi-residue analysis of organic UV-Filters and their transformation products in the aquatic environment"

por:

Pablo Gago-Ferrero, M. Silvia Díaz-Cruz, Damià Barceló

en "Analytical Methods (2013, 5:355-366)"

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Liquid chromatography-tandem mass spectrometry for the multi-residue analysis of organic UV filters and their transformation products in the aquatic environment†

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UV-absorbing compounds are environmental pollutants of recent concern and the requirements for analytical methods are mainly driven by the low concentrations found in aqueous and solid environmental samples. In the current article, a review of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) based methods published so far for the determination of organic UV filters in the environment is presented. UV filters included in this overview belong to different families and are benzophenones, camphor derivatives, cinnamates, crylenes, benzimidazole derivatives, *p*-aminobenzoic acid and derivatives, dibenzoyl methane derivatives, salicylates and triazines, as well as their main transformation products. Advanced aspects of current LC-MS/MS methodology, including sample preparation and matrix effects, for the analysis of water, sludge, sediments and biota samples are discussed.

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Introduction

UV filters (UV Fs) are recent emerging environmental pollutants for which there is currently a lack of knowledge about their occurrence, fate and effects on the ecosystems.[1] UV Fs constitute a large and heterogeneous group of chemicals that are important ingredients in personal care products to protect skin and hair from the deleterious effects of sunlight, but also in a variety of plastic products, such as building materials, automobile components, wax, paint, glasses, plastic packaging and some sports equipment, to prevent yellowing and degradation of polymers and pigments.[2] These compounds are continually released into the environment, thus becoming pseudo-persistent contaminants irrespective of their degradation half-lives. Benzophenones are one of the most used families among UV Fs, since they can be used as photo initiators in UV-curing

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applications such as inks, imaging, and clear coatings in the printing industry. They prevent ultraviolet light from damaging scents and colors in products such as perfumes and soaps. Benzophenone and derivatives can also be added to the plastic packaging as UV blockers. Their use allows manufacturers to package the product in clear glass or plastic. Without them, opaque or dark packaging would be required.

Recently, the occurrence of UV filters in the environment has been reported.[3–5] Table 1 lists the concentrations in environmental matrices and biota reported in the LC-MS based studies published so far. Concentrations of benzophenone 3 (BP3) and 4-methylbenzylidene camphor (4MBC) in surface waters ranged from a few to several thousands of ng L^{-1}, being the higher concentrations observed in raw wastewater. In particular, the concentration of one of the main derivatives of BP3, BP4, was quite high, with values up to 1980 ng L^{-1}, but the highest levels determined in river water corresponded to PBSA, which reached a maximum value of 3240 ng L^{-1}.[6] BP3 and its derivatives, which are used as sunscreens and stabilizers, are of particular interest, due to their estrogenic and antiandrogenic activities in both *in vitro* and *in vivo* bioassays.[7] Despite these high levels of UV F residues in continental waters, groundwater appeared to be preserved since any of the investigated UV Fs was detected. Likely due to the dilution effect lower concentrations of UV Fs were observed in seawater (7–138 ng L^{-1}).[8,9]

Zenker *et al.* demonstrated that polar, semi-polar and lipophilic UV Fs accumulate in passive sampling devices by using a polar organic chemical integrative sampler (POCIS) deployed in surface water.[2] This study reported a maximum value of 1344 ng per POCIS for BP4 and lower values for other compounds such as BP3, 4MBC or EHMC (Table 1). Through the use of the passive samplers it was possible to compare integrated UV F concentrations from different sites and times concluding that wastewater treatment plants (WWTPs) are the major input sources under a noticeable seasonal variability.

Due to the high lipophilicity and poor biodegradability of most UV Fs they are expected to end up in sewage sludge during wastewater treatment. High concentrations of more lipophilic UV filters, octyldimethyl-*p*-aminobenzoic acid (OD-PABA), octocrylene (OC), ethylhexyl-methoxycinnamate (EHMC), and 4MBC, in the range 1.4–9170 ng g^{-1} (ref. 10–13) were detected in sewage sludge WWTPs, suggesting, as in other studies, that WWTPs are a major source of these compounds in the aquatic environment. This theory was also reinforced by the presence of UV F residues in reclaimed wastewater. The presence of UV F residues in biota has scarcely been studied as compared to water. Several fish species, macroinvertebrates and even fish eating birds have been investigated. The current knowledge on the environmental occurrence and analytical methodology of UV filters in biota, most of them analyzed by GC-MS, is reviewed by Gago-Ferrero *et al.*[5] Considerable concentrations of BP3, 4MBC, EHMC and OC, between 193 and 300 ng g^{-1} lipid weight, were determined by LC-MS/MS,[14] however, considerable concentrations were observed in freshwater fish, with reported levels greater than 190 ng g^{-1} on a lipid weight basis. Fent *et al.*[15] detected EHMC in crustaceans and molluscs at concentrations between 22 and 150 ng g^{-1} lipid. BP3 and EHMC were detected in different fish species at concentrations up to 337 ng g^{-1} lipid and in birds (*Phalacrocorax* sp.) at levels above 700 ng g^{-1} lipid. These results suggest that biomagnification occurs through the food web. Lower concentrations of the selected UV Fs were detected in river sediments, with the exception of OC, whose maximum concentration was 2400 ng g^{-1}.[16]

Because of the recent awareness of the potentially dangerous consequences of the occurrence of UV Fs in the environment, the analytical methodology for their determination in complex environmental matrices is still evolving and the number of methods described in the literature has steadily grown. So far, most of the analytical methods reported in the literature for UV F residue analysis were based on gas chromatography-mass spectrometry (GC-MS),[3,17–26] which often requires derivatization of acidic compounds and their polar metabolites and other transformation products.

However, in the last few decades, liquid chromatography-mass spectrometry (LC-MS) and LC-tandem MS have experienced an impressive progress, both in terms of technology development and application. Currently, LC-MS/MS may be considered the technique of choice to assay polar and semi-polar UV-absorbing compounds and their transformation products, and is especially suitable for environmental analysis because of its selectivity. This paper reviews the state-of-the-art in the LC-tandem MS analysis of main classes of UV Fs (listed in Table 2), including benzophenones, camphor derivatives, cinnamates, crylenes, benzimidazole derivatives, *p*-aminobenzoic acid and derivatives, dibenzoyl methane derivatives, salicylates and triazines in aqueous and solid environmental samples and biota. Various aspects of the current LC-MS/MS methodology, including sample contamination and preparation, and matrix effects, are discussed.

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Analytical methodology

Sample contamination remarks

Due to the extended use of UV Fs, background contamination was revealed as a common problem in the determination of

Table 1 Occurrence of UV F residues in environmental samples and biota[a]

Compound	River water (ng L^{-1})	Seawater (ng L^{-1})	Raw water (ng L^{-1})	Reclaimed water (ng L^{-1})	POCIS (ng per POCIS)	Sewage sludge (ng g^{-1})	River sediment (ng g^{-1})	Biota (ng g^{-1} lipid)	Reference
Benzophenone derivatives									
BP1	24		31–148	11–13					27
	0.9–29		43–448	12		5.1			6
						4.41–91.6	0.259–0.607		13
BP2	1.8–6.7		35–93	14		11			6
							2.65		13
BP3	27		31–168	16					8
	54–87		184–429	77–84					27
	47		195–518	96		132			6
	3–69	7–13	32–551	5–20					9
					96				2
						10–20			10
						6.6–29			11
						2.05–13.3	0.272–4.66		13
						790			12
							2.7–27		16
								193–525 (fish)	14
								91–151 (fish)	15
BP4	849	38–138	237–1441	376–1359					8
	20–416		1237–1596	765–1028					27
	51–1980		2120–5130	105–572		29			6
					272–1344				2
4HB						2.66–10.1	0.312–0.951		13
						150			12
4DHB						40–620			12
							12–21		16
DHMB							0.133–0.796		13
Camphor derivatives									
4MBC			122	23–51					8
					64				2
						73–3893			11
						730–3800			12
								214	14
Cinnamates									
EHMC	10		23						9
					18–27				2
						35–127			11
						610–3350			12
							5.3–42		12
								414	14
								22–150 (macrozoobenthos); 9–337 (fish); 16–701 (bird)	15

Table 1 (*Contd.*)

Compound	River water (ng L^{-1})	Seawater (ng L^{-1})	Raw water (ng L^{-1})	Reclaimed water (ng L^{-1})	POCIS (ng per POCIS)	Sewage sludge (ng g^{-1})	River sediment (ng g^{-1})	Biota (ng g^{-1} lipid)	Reference
IAMC				59					8
						5–20			11
Crylenes									
OC			36	20					8
	11–112	19–32	28–463	13					9
						700–1842			10
						585–2479			11
						1060–9170			12
						18–2400			12
								300	14
Benzimidazole derivatives									
PBSA	34	42	181–2503	109–2679					8
	48–3240		275–3890	316–1820					6
***p*-Aminobenzoic acid and derivatives**									
OD-PABA						132–170			10
						1.4–1.9			11
							0.8–5.2		16
Dibenzoyl methane derivatives									
BM-DBM						144–517			11
Salicylates									
HMS						22–331			11
EHS						49–280			11
Triazines									
DBT						54–136			11
EHT						928–1433			11

[a] BP1: benzophenone-1; BP2: benzophenone-2; BP3: benzophenone-3; BP4: benzophenone-4; BP6: benzophenone-6; 4HB: 4-hydroxybenzophenone; 4DHB: 4,4′-dihidroxybenzophenone; DHMB: 2,2′-dihydroxy-4-methoxybenzophenone; 4MBC: 4-methylbenzylidene camphor; 3BC: 3-benzylidene camphor; EHMC: ethylhexyl methoxycinnamate; IAMC: isoamyl methoxycinnamate; OC: octocrylene; PBSA: 2-phenylbenzimidazole-5-sulfonic acid; OD-PABA: octyldimethyl-*p*-aminobenzoic acid; Et-PABA: 4-*p*-aminobenzoic acid; BM-DBM: butylmethoxydibenzoylmethane; HMS: homosalate; EHS: ethylhexyl salicylate; DBT: diethylhexyl butamido triazone; EHT: ethylhexyl triazone.

Table 2 Survey of LC-MS/MS methods used for the analysis of UV Fs in environmental samples[a]

Matrix	Analytes	Sample amount	Sample preparation	LC technique, ionization mode, chromatographic column and mobile phases	Recovery (%)	MLOD	Reference
Water							
River water Seawater Reclaimed wastewater Raw water	PBSA, PDT, BP4, BP3, 4MBC, IAMC, OC, OD-PABA, BM-DBM	200 mL	SPE (Oasis HLB 60 mg). Elution with MeOH	HPLC-ESI-MS/MS, Symmetry Shield RP18 (150 mm × 2.1; 3.5 μm) (Waters); H_2O and MeOH (both 5 mM CH_3COONH_4)	74–102 66–91 55–108 29–93	4–46 ng L^{-1}	8
River water	BP1, BP2, BP3, BP4, BP8, DHMB	500 mL	SPE (Oasis HLB 60 mg). Elution with MeOH	HPLC-ESI-MS/MS, Kromasil C18 (100 mm × 2.1 mm; 5 μm) (Sugelbor); H_2O and MeOH (both 5 mM CH_3COONH_4)	84–105	0.4–8 ng L^{-1} (LOQ)	27
Reclaimed wastewater		300 mL			91–104	0.7–14 ng L^{-1} (LOQ)	
Raw water		200 mL			83–101	1–32 ng L^{-1} (LOQ)	
Groundwater	BP1, BP2, BP3, BP4, PBSA	1000 mL	SPE (Oasis HLB 60 mg). Elution with MeOH and acetone	HPLC-ESI-MS/MS and HPLC-APCI-MS/MS, Synergi Fusion-RP 80 A (150 mm × 3 mm; 4 μm) (Phenomenex); PI: H_2O (10 mM CH_3COONH_4 pH: 3.2) and ACN (0.1% FA); NI: H_2O (0.1% FA) and ACN	23–106 (ESI), Nd-263 (APCI)	0.5–5 ng L^{-1} (ESI) (LOQ)	6
River water		1000 mL			6–93 (ESI), Nd->1000 (APCI)	0.5–5 ng L^{-1} (ESI) (LOQ)	
Reclaimed wastewater		200 mL			6–89 (ESI), Nd->1000 (APCI)	2.5–25 ng L^{-1} (ESI) (LOQ)	
Raw wastewater		100 mL			9–89 (ESI), Nd->1000 (APCI)	5–50 ng L^{-1} (ESI) (LOQ), Nd-50 ng L^{-1} (APCI) (LOQ)	
River water Seawater Reclaimed wastewater Raw water	BP3, EHMC, EHS, HMS, OC, OD-PABA	50 mL	SBSE (750 rpm, 5 h) and LD (1 mL MeOH, 30 min)	HPLC-APCI-MS/MS, Hypersil Gold column C18 (30 mm × 3 mm; 1.9 μm) (Thermo Scientific); H_2O (0.1% FA) and MeOH	64–85	0.9–114 ng L^{-1}	9
POCIS	BP1, BP2, BP3, BP4, 4DHB, Et-PABA, EHMC, 4MBC, 3BC	28 days	Content of the POCIS (placed in the field for 28 days) washed with 20 mL H_2O in the SPE tubes. Elution with MeOH and MeOH–toluene–DCM	HPLC-ESI-MS/MS, Zorbax SB-C18 column (150 mm × 3.0 mm; 3.5 μm) (Agilent Technologies); H_2O and ACN (both 0.1% FA)	76–99	158–990 ng per POCIS	2
Sludge							
Sewage sludge	BP3, BP1, OC, OD-PABA	1 g	PLE (MeOH, MeOH–H_2O)	UPLC-ESI-MS/MS, Zorbax (50 mm × 4.6 mm; 1.8 μm) (Agilent Technologies); H_2O (acetic acid, pH: 3) and MeOH	30–108	1.5–3.5 ng g^{-1}	10
Sewage sludge	BP3, IAMC, 4MBC, OC, BM-DBM, OD-PABA, EHMC, EHS, HMS, DBT, EHT	0.5 g	PMALE with LDPE and PLE (AcET/hexane)	HPLC-APPI-MS/MS, Eclipse XDB C8 (150 mm × 4.6 mm; 5 μm) (Agilent Technologies); H_2O and MeOH	10–60	0.3–25 ng g^{-1}	11

Table 2 (*Contd.*)

Matrix	Analytes	Sample amount	Sample preparation	LC technique, ionization mode, chromatographic column and mobile phases	Recovery (%)	MLOD	Reference
Sewage sludge	BP1, BP2, BP3, BP4, PBSA	0.2 g	PLE (H_2O–MeOH) and further SPE (Oasis HLB 60 mg). Elution with MeOH and acetone	HPLC-ESI-MS/MS and HPLC-APCI-MS/MS, Synergi Fusion-RP 80 A (150 mm × 3 mm; 4 μm) (Phenomenex); PI: H_2O (10 mM CH_3COONH_4 pH: 3.2) and ACN (0.1% FA); NI: H_2O (0.1% FA) and ACN	18–93 (ESI), Nd-236 (APCI)	2.5–25 ng g^{-1} (ESI), 5–50 ng g^{-1} (APCI)	6
Sewage sludge	BP3, BP2, BP1, 4HB, DHMB	0.1 g	SLE (MeOH) and further SPE (Oasis HLB 500 mg). Elution with MeOH–AcEt	HPLC-ESI-MS/MS, Betasil C18 (100 mm × 2.1 mm; 5 μm) (Thermo); H_2O and MeOH	38–115	0.41–0.67 ng g^{-1}	13
Sewage sludge	BP3, BP1, 4HB, 4DHB, 4MBC, EHMC, OD-PABA, OC	1 g	PLE (MeOH, MeOH–H_2O) cell purification with alumina	UPLC-ESI-MS/MS, STAR_HR R-18 ec. (50 mm × 2.0 mm, 2 μm) (Merck); H_2O and ACN (both 0.3% FA)	30–102	0.2–60 ng g^{-1}	12
Sediments							
River sediments	BP3, BP1, 4HB, 4DHB, 4MBC, EHMC, OD-PABA, OC	1 g	PLE (MeOH, MeOH–H_2O) in-cell purification with alumina	UPLC-ESI-MS/MS, STAR_HR R-18 ec. (50 mm × 2.0 mm, 2 μm) (Merck); H_2O and ACN (both 0.3% FA)	58–125	0.5–15.5 ng g^{-1}	16
River sediments	BP3, BP2, BP1, 4HB, DHMB	1 g	SLE (MeOH) and further SPE (Oasis HLB 500 mg). Elution with MeOH–AcEt	HPLC-ESI-MS/MS, Betasil C18 (100 mm × 2.1 mm; 5 μm) (Thermo); H_2O and MeOH	38–115	0.041–0.067 ng g^{-1}	13
Biota							
Muscle fish	4MBC, BP3, EHMC, OC	10 g	Homogenized with sodium sulphate, Soxhlet extracted (*n*-hexane–acetone) and further GPC (Biobeads S-X3), Florisil	HPLC-ESI-MS/MS, Perfect Sil 120 ODS-2 (125 mm × 3 mm, 3.5 μm) (MZ Analysentechnik); H_2O and MeOH (both 0.05% acetic acid)	86–108	2.4 ng g^{-1} muscle	14
Whole fish	BP4, 4DHB, BP1, BP2, Et-PABA	4 g	SLE using ethyl acetate, *n*-heptane and HPLC water and further RP-HPLC (RP Spherisorb ODS2 column 4.6 mm × 150 mm, 5.0 μm)	HPLC-ESI-MS/MS, Zorbax SB-C18 (150 mm × 3.0 mm, 3.5 μm) (Agilent Technologies); H_2O and ACN (both 0.1% FA)	76–99 (BP4 no extracted)	78–205 ng g^{-1} lipid	2
		1 g	SLE with MeOH–ACN and syringe filtration		80–99	1.8–10.7 ng kg^{-1} body weight	

[a] BP1: benzophenone-1; BP2: benzophenone-2; BP3: benzophenone-3; BP4: benzophenone-4; BP6: benzophenone-6; 4HB: 4-hydroxybenzophenone; 4DHB: 4,4′-dihydroxybenzophenone; DHMB: 2,2′-dihydroxy-4-methoxybenzophenone; 4MBC: 4-methylbenzylidene camphor; 3BC: 3-benzylidene camphor; EHMC: ethylhexyl methoxycinnamate; IAMC: isoamyl methoxycinnamate; OC: octocrylene; PBSA: 2-phenylbenzimidazole-5-sulfonic acid; OD-PABA: octyldimethyl-*p*-aminobenzoic acid; Et-PABA: 4-*p*-aminobenzoic acid; BM-DBM: butylmethoxydibenzoylmethane; HMS: homosalate; EHS: ethylhexyl salicylate; DBT: diethylhexyl butamido triazone; EHT: ethylhexyl triazone; MLOD: method limit of detection; LOQ: limit of quantification; SPE: solid phase extraction; PI: positive ionization mode; NI: negative ionization mode; ACN: acetonitrile; FA: formic acid; DCM: dichloromethane; ESI: electrospray ionization; APCI: atmospheric-pressure chemical ionization; APPI: atmospheric-pressure photoionization; SBSE: stir bar sorptive extraction; LD: liquid desorption; POCIS: passive integrative sampling with the polar organic chemical integrative sampler; PLE: pressurised liquid extraction; PMALE: pressurized membrane-assisted liquid extraction; LDPE: low density polyethylene; SLE: solid–liquid extraction; GPC: gel permeation chromatography; RP: reverse phase.

these compounds at environmentally relevant concentrations. In order to prevent this problem, basic precautions include avoiding the use of personal care products containing UV Fs and the use of gloves for sample handling were taken. All glassware used must be previously washed and heated overnight at 400 °C, and after this, sequentially rinsed with different organic solvents, such as acetone, methanol, dichloromethane, and HPLC grade water. High purity solvents should be used. In addition, a set of operational blanks should be processed to monitor for contamination from the laboratory environment and any other sources.

Sample pre-treatment

Water. To remove particulate matter and to avoid clogging of the sorbent used for the extraction step (mainly solid phase extraction (SPE)), filtration of water samples was suggested prior to the concentration procedure. However, together with suspended solids, filtration also removes the fraction of target compounds sorbed to particulates. It is advisable to analyze this fraction in order to have a complete picture of the occurrence. Although this process is increasingly common for some families of pollutants, so far there are no studies for UV Fs. For their analysis, the water is usually filtered through 0.45 μm nitrocellulose filters[8] or glass fibre filters[6,27] and stored in the dark at 4 °C.

Sludge and sediments. The sampling of sludge and sediment often results in a solid sample as well as a liquid phase, so that separation may be necessary. Most methods carry out this process by freeze-drying followed by grinding and sieving. Eventually, a centrifugation step prior to freeze-drying is added.[6]

Biota. Most studies have focused on fish, a representative matrix of the aquatic environment assumed to be able to retain and bioaccumulate UV Fs because of the lipophilicity of some compounds. The most usual biotic sample analyzed is fish muscle, with low lipid content in comparison to other tissues.

In the analytical methods published so far, the selected fish tissues were homogenized by blending and freeze-dried before extraction. These methods have also been applied to the analysis of macroinvertebrates (using the whole individuals) and birds.[2]

Sample extraction and preparation

Water samples. A survey of LC-MS/MS based methods developed for the analysis of UV Fs in environmental aqueous and solid samples and biota is given in Table 2 and summarized in Fig. 1. UV filters can more efficiently be preconcentrated by SPE using several reversed-phase cartridges such as polymeric or classical silica bonded C18. UV Fs and derivatives constitute a group of chemicals with quite different physicochemical properties, and therefore, the copolymer of divinylbenzene and vinylpyrrolidone, known as Oasis HLB (Waters), is currently the most commonly used SPE sorbent for the simultaneous extraction of multiclass UV Fs.[6,8,27] The elution step using this cartridge is usually performed with methanol (MeOH) or with a mixture of MeOH–acetone.

Different sample volumes (100–1000 mL) were SPE-extracted depending on the type of water to be analyzed (groundwater, river water, treated or raw wastewater) and proper adjustment of the sample pH may be necessary to avoid protonation of basic compounds and to achieve reproducible and efficient recoveries. On-line SPE is emerging as an effective technique; both coupled online with an LC system or as a fully automated system for most emerging contaminants at trace levels, but these methods still have not been applied in the UV F environmental determination.

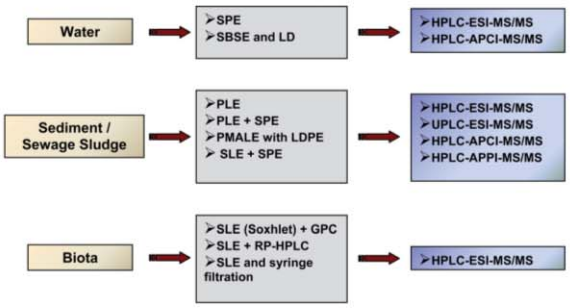

Fig. 1 Scheme of the analytical methods applied for the determination of UV filters in different types of environmental matrices. SPE: solid phase extraction; ESI: electrospray ionization; APCI: atmospheric-pressure chemical ionization; APPI: atmospheric-pressure photoionization; SBSE: stir bar sorptive extraction; LD: liquid desorption; PLE: pressurised liquid extraction; PMALE: pressurised membrane-assisted liquid extraction; LDPE: low density polyethylene; SLE: solid–liquid extraction; GPC: gel permeation chromatography; RP: reverse phase.

In addition to the classical off-line SPE, more advanced extraction techniques have also been used. For instance, Magi *et al.*[9] developed a method based on stir bar sorptive extraction followed by liquid desorption (SBSE-LD) for the determination of six UV Fs in different water matrices. Each stir bar was placed in a vial containing 2.5 mL MeOH and 50 mL of water sample at pH 6. The extraction was performed at room temperature at 750 rpm (5 h). Desorption was carried out with 1 mL of MeOH stirring at 750 rpm (30 min).

Passive sampling devices have also been employed for both sampling and extraction of UV Fs from water samples. Zenker *et al.*[2] analyzed UV Fs by using POCIS. These devices consisted of two polyethersulphone membranes filled with 200 mg of triphasic sorbent mixture (Isolute ENV+ and Ambersirb 572 dispersed on S-X3 Bio Beads). The passive samplers were placed in the river for 28 days, which lies in the linear uptake phase, and not reaching equilibrium. In this method the sorbent was eluted with MeOH and a mixture of MeOH, toluene and dichloromethane (DCM).

Sludge and sediments. Most UV Fs are lipophilic compounds, and therefore, tend to accumulate in solid matrices such as sewage sludge and sediments. As a consequence, to allow correct evaluation of the ecological impact of these substances, evaluation of their prevalence in solid matrices is important. Furthermore, the application of sewage sludge as a fertilizer to agricultural land may introduce these compounds in the soil. As in the case of water samples, the analytical approaches reported so far for the LC-MS/MS analysis of environmental solid samples are summarized in Table 2.

A method for the determination of eleven UV Fs based on the use of non-porous polymeric membranes in combination with pressurized liquid extraction (PLE) using ethyl acetate (AcEt)/hexane as a solvent extractor was developed by Rodil *et al.*[11] In PLE pressure is applied to allow the use of extraction solvents at temperatures higher than their boiling point. The increase of the extraction temperature can promote higher analyte solubility by increasing both solubility by decreasing the viscosity and mass transfer rate.[28,29] Most of the developed methodologies for the analysis of UV Fs in sewage sludge and sediments are based on this technique, since PLE presents several advantages over other extraction techniques such as better reproducibility, less solvent consume and reduced time for sample pre-treatment. Using this technique, Nieto *et al.* and Gago-Ferrero *et al.* developed methods for the determination of selected UV filters, in sewage sludge and sediments. These methods used MeOH and a combination of MeOH–H_2O for the PLE extraction and alumina to perform in-cell purification to avoid a further clean-up step in the method.[10,12,16] Wick *et al.* also used MeOH–H_2O for PLE solvent extraction for the determination of five UV Fs in sewage sludge.[6] Further clean-up of the extracts was performed by SPE using Oasis HLB cartridges and MeOH and acetone as elution solvents.

Despite the predominant application of PLE, solid–liquid extraction (SLE) is still applied. Recently Zhang *et al.*[13] developed a method for the determination of five benzophenone derivatives in sewage sludge. The selected analytes were SLE-extracted from the sludge with MeOH, and the extracts were purified by SPE on Oasis HLB cartridges by eluting with a mixture of MeOH–AcEt.

Biota. For the extraction of UV F residues in biota samples, only most classical techniques, SLE such as Soxhlet extraction, have been applied so far. Following the extraction step, a preconcentration and clean-up of extracts are usually needed. In the method developed by Meinerling and Daniels[14] for the analysis of four UV Fs in fish muscle, Soxhlet extraction with *n*-hexane–acetone was followed by GPC (Bio Beads SX-d) with cyclohexane–EtAc as an eluent solvent. In a further clean-up step, a Florisil column was used to remove polar compounds prior to the LC-MS/MS analysis.

A method for simultaneous determination of nine UV filters, from polar to lipophilic, in fish has been reported by Zenker *et al.*[2] Semi-polar and lipophilic UV filters were extracted from homogenized tissue by SLE with ethyl acetate–*n*-heptane–H_2O and further purified by RP-HPLC. The fraction containing polar and semi-polar UV filters was analyzed by HPLC-MS/MS, whereas the fraction containing the lipophilic ones was determined by GC-MS (out of the scope of this review). Polar and semi-polar UV filters were extracted with a mixture of MeOH–ACN. The same method proved to be suitable for the analysis of macrozoobenthos and fish eating bird samples.[15]

Liquid chromatography-tandem mass spectrometry analysis

Determination of UV filters in the aqueous environment is mainly performed by GC-MS after a preconcentration step carried out mainly by SPE[3,17–19] or LLE.[20,21] In general, matrix effects are not critical for the ionization modes typically used in GC-MS (electron impact or chemical ionization) and good method detection limits are achieved. On the other hand, all these methods are limited to those substances that are volatile or can be derivatized (where differences in matrix components may result in quite different derivatization efficiencies which may affect both precision and accuracy of the analysis) for GC determination.

When the objective of the study is to perform the simultaneous determination of several UV Fs, with a wide range of polarities and acid–base properties, liquid chromatography is the technique of choice. In particular, it is the most appropriate when analyzing metabolites and other transformation products, which usually have higher polarity than the parent compounds. There are only a few studies using LC-UV for the determination of these compounds in the aquatic environment[3,38] likely because this technique lacks sensitivity and selectivity (especially for the analysis of complex matrices). LC-MS/MS is an instrumental technique that allows the analysis of a wide range of compounds and significantly increases the possibility of analysis of transformation products without the need of derivatization. In addition to HPLC, ultra performance liquid chromatography (UPLC) is being used increasingly. UPLC uses analytical columns packed with smaller particles, which offers the advantages of increasing speed, improving sensitivity and mostly selectivity and specificity compared to conventional HPLC analysis. The higher efficiency of small particles enables shorter columns, reducing analysis time and solvent consumption. Although complete chromatographic separation is not necessary for the selective MS/MS detection, it generally improves detectability and reduces the ion suppression effect.

Reverse phase C18 HPLC columns were the most used by far in the analysis of UV Fs by LC-MS/MS. Most of the works here presented use MeOH and water as mobile phases.[9–11,13,14,27,30] However, the use of acetonitrile and water is also very common and has been employed in several works.[2,6,12,16] In order to obtain satisfactory ionization and improved sensitivity ammonium acetate,[6,8,27] formic[2,6,9,12,16] or acetic acid[10,14] is often included as a mobile phase additive, at concentrations typically between 0.05% and 0.3% (see Table 2).

Nieto *et al.*[10] developed an UPLC-MS/MS method for personal care products, including four UV Fs. Water with acetic acid (pH 3) and MeOH were used as mobile phases. All the compounds eluted in 9 min. Gago-Ferrero *et al.*[12,16] also used UPLC-MS/MS for the determination of eight UV Fs in sediments and sewage sludge. Fig. 2 shows an example of SRM chromatograms obtained by UPLC-ESI(+)-MS/MS of a treated sewage sludge sample where five compounds were detected. A STAR_HR R-18 ec. (50 mm × 2.0 mm, 2 μm) column was used for chromatographic separation and acetonitrile and water (each with 0.3% of formic acid) as mobile phases.

In the MS/MS detection of UV F residues, electrospray ionization (ESI),[2,6,8,10,12–16,27] atmospheric-pressure chemical ionization (APCI)[6,9] and atmospheric-pressure photoionization (APPI)[11] were employed as ionization techniques, being ESI (positive or negative ionization mode) the widest used technique by far. It achieves efficient ionization for a great variety of

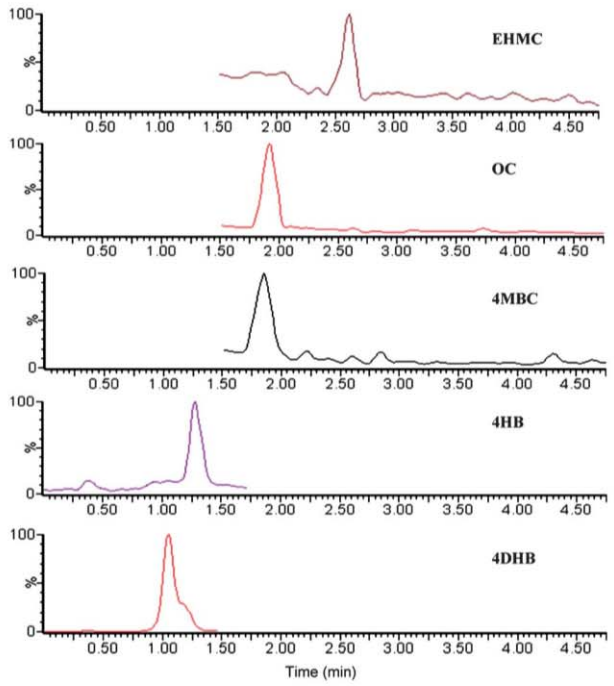

Fig. 2 Example of SRM chromatograms obtained by UPLC-ESI(+)-MS/MS of a treated sewage sludge sample.

analytes but can lead to problems due to its susceptibility to matrix effects. Rodil *et al.*[30] made a comparison between ESI and APPI for eleven UV Fs. For most of the analyzed compounds APPI generated a similar response to that of ESI but with signal to noise (S/N) ratios 1.3–60 times higher. BP4 and PBSA, the most polar analyzed compounds, were more efficiently ionized by ESI. APPI was, however, less susceptible to ion suppression than ESI when real samples were injected. APPI offered better detection limits, mainly because of the higher S/N ratios and thus it is feasible to use this technique to analyze UV Fs in environmental samples.

In all the studies reported here data acquisition was performed in SRM mode. A summary of the SRM transitions and proposed ions used for LC-MS/MS determination of UV filters in the aquatic environment is shown in Table 3.

Method performance

Matrix effect. The most outstanding limitation of LC-MS/MS is the susceptibility of atmospheric pressure ionization (API) interfaces to co-extracted matrix components, which can strongly vary with the matrix and result in poor analytical accuracy and reproducibility.[31–33] Despite ESI is the most susceptible to signal suppression and enhancement by matrix components, it is the most commonly used technique for trace analysis of UV Fs in environmental samples. It achieves efficient ionization of analytes and the matrix effect can be compensated using isotopically labelled compounds. APCI may be an alternative ionization interface to ESI since it has been reported to be generally less sensitive to the matrix effect.[34–36] Wick *et al.*[6] compared ESI and APCI sources in the analysis of thirty-six emerging organic pollutants, including five UV Fs (benzophenone-1 (BP1), benzophenone-2 (BP2), benzophenone-3 (BP3), benzophenone-4 (BP4) and 2-phenylbenzimidazole-5-sulfonic acid (PBSA)) in aqueous matrices and activated sludge. Ion suppression using the ESI source significantly reduced absolute recoveries for the studied UV Fs, and the use of appropriate labelled surrogate standards was necessary to achieve acceptable relative recoveries. APCI was shown to be less susceptible to ion suppression but this positive effect was overweighed by the negative effect of a decreased sensitivity. APCI also needed the use of surrogate standards for some analytes to compensate for significant ion enhancement and revealed similar or lower S/N ratios for all the UV Fs analyzed. According to Wick *et al.*,[6] in case ion suppression is significantly lower in comparison to ESI and no significant increase of the background noise occurs, APCI should be preferred to ESI if sensitivity is comparable or higher to ESI and/or no appropriate surrogates are available and ion enhancement does not occur. A comparison between ESI and APPI carried out by Rodit *et al.*[30] revealed that, due to the selective ionization process, APPI was less susceptible to matrix compounds which are often not ionisable by photons at only 10 eV.[37] The analyte responses even in raw wastewater extracts were less affected by matrix compounds when APPI was used for ionization and higher S/N ratios were obtained.

In general, the strategy to diminish matrix effects should take into account the variability of the matrix within the set of samples to be analyzed (*e.g.* river water, raw and treated wastewater, sewage sludge extracts, *etc.*) and should be tested for each type of matrix. The LC-ESI-MS/MS analysis of UV Fs in different water matrices[8,27] revealed a low matrix effect for groundwater, river water and treated wastewater (except for BP2, which presented high ion suppression) and important effects in raw wastewater. In this case both studies used the standard addition method to obtain a reliable quantification of the target compounds. Recently Magi *et al.*,[9] using APCI as the ionization source, verified the absence of a significant matrix effect for river water, seawater, treated wastewater and even raw wastewater.

The use of appropriate internal standards to compensate for the signal suppression or enhancement was followed by Zenker *et al.*,[2] who used the isotopically labelled benzoic-d_5 acid when analyzing the POCIS and fish tissue extracts, by HPLC-ESI-MS/MS. Similarly, the effect of co-extracted matrix components from fish muscle was assessed by Meinerling and Daniels[14] through standard addition experiments concluding that matrix effects were negligible.

Sewage sludge is also a complex matrix, which can lead to important matrix effects. Gago-Ferrero *et al.*[12] conducted a study by UPLC-ESI-MS/MS where results evidenced different possible behaviours: signal suppression, signal enhancement and the absence of matrix effects. Fig. 3 shows these three behaviours, for BP3 (Fig. 3a) and BP1 signal enhancement was observed, for 4MBC (Fig. 3c), OC, OD-PABA and EHMC strong signal suppression occurred. For 4,4′-dihydroxybenzophenone (4DHB) (Fig. 3b) and 4-hydroxybenzophenone (4HB) no significant differences were found. To overcome this problem, internal

Table 3 SRM transitions and proposed ions used for LC-MS/MS determination of UV filters in the aquatic environment[a]

Compound	Precursor ion	Product ion (1)	Product ion (2)	Reference
BP1	213 $[M - H]^-$	91 $[C_6H_3O]^{\cdot -}$	109 $[C_6H_5(OH)O]^-$	13 and 27
	213 $[M - H]^-$	135 $[C_6H_3(O)_2C{=}O]^{\cdot -}$	91 $[C_6H_3O]^{\cdot -}$	6
	213 $[M - H]^-$	213 $[M - H]^-$		2
	213 $[M - H]^-$	135 $[C_6H_3(O)_2C{=}O]^{\cdot -}$	169	10
	215 $[M + H]^+$	137 $[M - C_6H_5]^+$	105 $[C_6H_5{=}O]^+$	12 and 16
BP2	245 $[M - H]^-$	135	109	6 and 27
	245 $[M - H]^-$	245 $[M - H]^-$		2
	245 $[M - H]^-$	91		13
BP3	229 $[M + H]^+$	151 $[M - C_6H_5]^+$	105 $[C_6H_5{=}O]^+$	2,6,8–12,14,16 and 27
	227 $[M - H]^-$	211		13
BP4	307 $[M - H]^-$	80 $[SO_3]^-$	211 $[M - H{-}SO_3{-}CH_4]^-$	8
	307 $[M - H]^-$	210 $[M - H{-}SO_3{-}OH]^-$	227 $[M - H{-}SO_3]^-$	27
	307 $[M - H]^-$	211 $[M - H{-}SO_3{-}CH_4]^-$	227 $[M - H{-}SO_3]^-$	6
	307 $[M - H]^-$	307 $[M - H]^-$		2
BP6	273 $[M - H]^-$	123	108	27
4HB	199 $[M + H]^+$	121 $[M - C_6H_5]^+$	105 $[C_6H_5{=}O]^+$	12 and 16
	197 $[M - H]^-$	92		13
4DHB	213 $[M - H]^-$	213 $[M - H]^-$		2
	215 $[M + H]^+$	121 $[M - C_6H_5OH]^+$	93 $[C_6H_4OH]^+$	12 and 16
DHMB	243 $[M - H]^-$	93 $[C_6H_5O]^-$	123 $[C_6H_4(OMe)O]^-$	10,13 and 27
4MBC	255 $[M + H]^+$	105 $[MeC_6H_4CH_2]^+$	212 $[M + H{-}C_3H_7]^+$	8,12,14 and 16
	255 $[M + H]^+$	237 $[M - H_2O]^+$		2
	255 $[M + H]^+$	105 $[MeC_6H_4CH_2]^+$	97	11
EHMC	291 $[M + H]^+$	161 $[M + H{-}C_8H_{16}{-}H_2O]^+$	179 $[M + H{-}C_8H_{16}]^+$	2,9,11,12,14 and 16
IAMC	249 $[M + H]^+$	161 $[M + H{-}C_5H_{10}{-}H_2O]^+$	179 $[M + H{-}C_5H_{10}]^+$	8 and 11
OC	362 $[M + H]^+$	250 $[M + H{-}C_8H_{16}]^+$	232 $[M + H{-}C_8H_{16}{-}H_2O]^+$	8,9,11,12,14 and 16
	384 $[M + Na]^+$	272	228	10
PBSA	273 $[M - H]^-$	193 $[M - H{-}SO_3]^-$	80 $[SO_3]^-$	8
	273 $[M - H]^-$	194	166	6
	275 $[M + H]^+$	194	211	10
OD-PABA	278 $[M + H]^+$	151 $[i\text{-}BuC_6H_4C{=}O]^+$	166 $[M + H{-}C_8H_{16}{-}H_2O]^+$	8–12 and 16
Et-PABA	166 $[M + H]^+$	138 $[M - C_2H_4]^+$		2
BM-DBM	311 $[M + H]^+$	161 $[i\text{-}BuC_6H_4C{=}O]^+$	135 $[MeOC_6H_4C{=}O]^+$	8 and 11
HMS	263 $[M + H]^+$	139	121	9
	261 $[M - H]^-$	93	137	11
EHS	251 $[M + H]^+$	139	121	9
	249 $[M - H]^-$	93	137	11
DBT	767 $[M + H]^+$	307	468	11
EHT	824 $[M + H]^+$	308	325	11
3BC	241 $[M + H]^+$	223 $[M - H_2O]^+$		2

[a] BP1: benzophenone-1; BP2: benzophenone-2; BP3: benzophenone-3; BP4: benzophenone-4; BP6: benzophenone-6; 4HB: 4-hydroxybenzophenone; 4DHB: 4,4′-dihydroxybenzophenone; DHMB: 2,2′-dihydroxy-4-methoxybenzophenone; 4MBC: 4-methylbenzylidene camphor; 3BC: 3-benzylidene camphor; EHMC: ethylhexyl methoxycinnamate; IAMC: isoamyl methoxycinnamate; OC: octocrylene; PBSA: 2-phenylbenzimidazole-5-sulfonic acid; OD-PABA: octyldimethyl-*p*-aminobenzoic acid; Et-PABA: 4-*p*-aminobenzoic acid; BM-DBM: butylmethoxydibenzoylmethane; HMS: homosalate; EHS: ethylhexyl salicylate; DBT: diethylhexyl butamido triazone; EHT: ethylhexyl triazone.

standard quantification with matrix-matched standards was used. Rodil *et al.*[11] performed quantification by standard addition while for Nieto *et al.*,[10] the matrix effect was less than 15% and therefore it was not considered.

When analysing sediments by LC-ESI-MS/MS, all studies[13,16] reported the absence of significant matrix effects.

Method recovery and limits of detection

Despite significant differences in the physicochemical properties of UV Fs lead to a wide range of recovery rates in multi-residue methods, high recovery rates were achieved for most of the compounds in most of the methods and matrices here reported (see Table 2).

In water samples, high recovery rates and low limits of detection (LODs) were achieved for almost all compounds, especially in the cleaner matrices as groundwater, river water or seawater. Reclaimed wastewater, and especially raw water, often presented low recoveries and higher LODs. Rodil *et al.*[8] obtained good recoveries for the analysis of nine UV Fs in different water matrices, all above 63% except for butylmethoxydibenzoylmethane (BM-DBM) in treated (55%) and raw wastewater (29%). LODs were between 7 and 46 ng L^{-1}. Negreira *et al.*[27] obtained high recoveries (83–105%) for six benzophenone UV Fs. Limit of quantification (LOQ) values were between 0.4 and 8 ng L^{-1} (river water), 0.7 and 14 ng L^{-1} (treated wastewater) and 1 and 32 ng L^{-1} (raw water). Wick *et al.*[6] reported absolute recoveries mainly below 70% in river, treated and raw water (in groundwater are

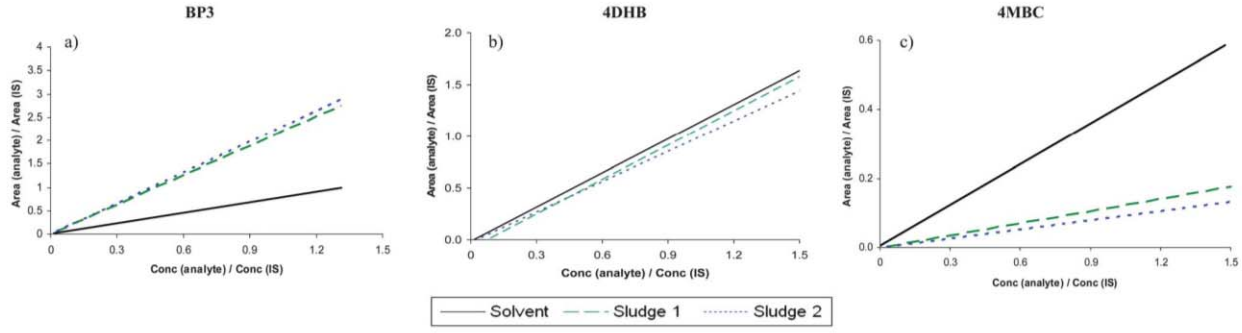

Fig. 3 Comparison of calibration curves obtained in acetonitrile and in PLE sewage sludge extracts for: (a) BP3, (b) 4DHB, and (c) 4MBC.

higher), revealing significant ion suppression by natural matrix components, as discussed in the "Matrix effect" section. For BP2 absolute recovery rates were below 23% in all cases. The use of surrogate standards allowed us to obtain high relative recoveries, in general between 53 and 130%. Nevertheless, for BP1 and BP2 the standard addition method had to be used for quantification in some matrices since the matrix effects could not be compensated by the surrogates tested. In these methods, LODs were between 0.5 and 5 ng L^{-1} in groundwater and river water, 2.5 and 25 ng L^{-1} in treated wastewater and 5 and 50 ng L^{-1} for raw wastewater. Magi *et al.*,[9] using stir bar sorptive extraction, obtained recoveries in different kinds of water matrices close to 85% for all the analytes except for BP3 (64%) and OC (76%). LODs ranged between 0.9 and 2.8 ng L^{-1} except for homosalate (HMS) (94 ng L^{-1}) and ethylhexyl salicylate (EHS) (114 ng L^{-1}). In the analysis of the POCIS Zenker *et al.*[2] achieved recoveries between 76 and 99% and LODs between 158 and 990 ng per POCIS.

Analysis of UV Fs in sewage sludge is a difficult task, as most studies shown being hard to achieve good recovery rates and low LODs for all compounds, especially taking into account the physicochemical differences between them. Authors often look for a compromise, seeking the conditions for obtaining good recoveries for most compounds. Nieto *et al.*[10] achieved recoveries between 79 and 108% except for BP1 (30%) and LODs between 1.5 and 3.5 ng g^{-1} dw for the four UV Fs analyzed. Gago-Ferrero *et al.*[12] achieved recovery rates between 70 and 102% for the eight UV Fs studied except for BP1 (30%). In this case the LODs ranged between 0.2 and 19 ng g^{-1} dw except for BP1 (60 ng g^{-1} dw). Rodil *et al.*[11] analyzed eleven UV Fs in sewage sludge using pressurised membrane-assisted liquid extraction (PMALE). The overall recovery was in the range 10–60% showing that the extraction was not quantitative. In this case, quantification was performed by standard addition. Reported LODs ranged from 0.3 to 25 ng g^{-1} dw. Zhang *et al.*[13] analyzed five benzophenone UV Fs in sewage sludge and sediments obtaining recovery rates between 70 and 115% except for BP3 (38%) and LODs between 0.41 and 0.67 ng g^{-1} dw (sludge) and 0.041–0.067 ng g^{-1} dw (sediments). Sediment is a clean matrix compared with sewage sludge and lower LODs were achieved in all studies. Gago-Ferrero *et al.*[16] analyzed eight UV Fs in sediments obtaining recovery rates between 58 and 125% and LODs in the range 0.5–15.5 ng g^{-1} dw.

So far there have been only a few methods for the analysis of UV Fs in biota samples, Meinerling and Daniels[14] analyzed four UV Fs in muscle fish obtaining recoveries between 86 and 108% and a LOD of 2.4 ng g^{-1} muscle. Zenker *et al.*[2] obtained recoveries between 76 and 99% and LODs in the range 78–205 ng g^{-1} lipid (except for BP4, not extracted) when using a mixture of ethyl acetate and *n*-heptane as an extraction solvent. Extraction of polar filter BP4 was feasible with ACN and MeOH. With this approach recovery rates between 80 and 99% were achieved and LODs in the range 1.8–10.7 ng kg^{-1} body weight for the studied UV Fs.

Conclusions

The application of LC-MS/MS technologies to environmental analysis has allowed the determination of a broad range of compounds and thus permitted a comprehensive assessment of environmental contaminants. For the analysis of UV Fs the LC-MS/MS method offers an improvement over GC-MS since the derivatization step is avoided for polar and semi-polar compounds and LODs in the low ng L^{-1} or ng g^{-1} can still be achieved. Currently, most efforts in environmental analysis have focused on the detection of parent compounds, whereas the analysis of transformation products is steadily increasing. Elimination of UV F residues during wastewater treatment is not completely satisfactory; and more research is needed to determine the breakdown pathways and to evaluate the fate of transformation products. Moreover, disinfection processes applied in water works (either chlorination or ozonation) potentially shift the assessment of the risk of human consumption of the parent compound to its degradation products. Consequently, development of generic analytical protocols that will permit simultaneous determination of parent compounds and their transformation products is recommended.

Researchers have used different analytical approaches for the environmental analysis of UV Fs; most of them are based on SPE for aqueous sample extraction and PLE or SLE for solid samples, mainly with further clean-up protocols. In the case of PLE the clean-up can be performed together with the extraction reducing laboriousness and time consumption. Finally, analyses were performed by both HPLC and UPLC attached to mass spectrometry using preferably ESI but also APCI or APPI. The

sensitivity and selectivity afforded are suitable for environmental trace analysis and recovery rates are also pretty good. Until now there have been no on-line coupling methodologies developed for the analysis of UV Fs in any environmental matrix.

Sediment, sewage and especially biota data so far available are quite limited. Differences in sample characteristics, target analytes and analytical methods used hinder the comparison among studies, which in turn also hinder making a reliable picture of the fate and effects of UV absorbing compounds in aquatic ecosystems.

Acknowledgements

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Metodologías analíticas para la determinación de filtros UV en el medio ambiente

Publicación científica #2:

"An overview of UV-absorbing compounds (organic UV-Filters) in aquatic biota"

por:

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REVIEW

An overview of UV-absorbing compounds (organic UV filters) in aquatic biota

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Abstract The purpose of this article is to summarize biological monitoring information on UV-absorbing compounds, commonly referred as organic UV filters or sunscreen agents, in aquatic ecosystems. To date a limited range of species (macroinvertebrates, fish, and birds), habitats (lakes, rivers, and sea), and compounds (benzophenones and camphors) have been investigated. As a consequence there is not enough data enabling reliable understanding of the global distribution and effect of UV filters on ecosystems. Both liquid chromatography and gas chromatography coupled with mass spectrometry-based methods have been developed and applied to the trace analysis of these pollutants in biota, enabling the required selectivity and sensitivity. As expected, the most lipophilic compounds occur most frequently with concentrations up to 7112 ng g^{-1} lipids in mussels and 3100 ng g^{-1} lipids (homosalate) in fish. High concentrations have also been reported for 4-methylbenzilidenecamphor (up to 1800 ng g^{-1} lipids) and octocrylene (2400 ng g^{-1} lipids). Many fewer studies have evaluated the potential bioaccumulation and biomagnification of these compounds in both fresh and marine water and terrestrial food webs. Estimated biomagnification factors suggest biomagnification in predator–prey pairs, for example bird–fish and fish–invertebrates. Ecotoxicological data and preliminary environmental assessment of the risk of UV filters are also included and discussed.

Keywords UV filters · Biota · Chromatography · Mass spectrometry · Bioaccumulation · Toxicity

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Introduction

UV filters, including both inorganic and organic sunscreen agents, constitute a group of emerging environmental pollutants, potentially hazardous compounds that have been receiving steadily growing attention over the last decade as society has become aware of the dangerous effects of UV solar radiation. These chemicals can be found not only in cosmetics but also in other personal care products, food packaging, pharmaceuticals, plastics, textiles, and vehicle-maintenance products to prevent photodegradation of polymers and pigments [1, 2].

Incomprehensibly, there are scarce data about, and limited understanding of, the environmental occurrence, fate, distribution and effects of many UV filters and their metabolites and other transformation products, despite their extensive use. According to market studies, sunscreen product sales were higher than half a billion US dollars in 2005, and it is estimated that 10,000 tons of UV filters are produced annually for the global market [3].

It is likely that usage of sunscreen agents is going to increase in the future, because of the recommendations of health authorities on the prevention of skin cancer. One of the main reasons for the scarcity of data was the lack of suitable analytical methods capable of detecting emerging

pollutants at trace levels in, usually, complex environmental matrices. However, as a consequence of increasing concern about the potential effects of sunscreens on ecosystems and humans, in last five years environmental analytical scientists have developed sensitive and selective analytical methods.

Eco-toxicological data on both UV filters and their degradation products is also missing. Despite the small amount of information available about their toxicity, the low environmental concentrations reported so far suggest a low potential risk. However, the long-term risk associated with the pseudo-persistence of these chemicals in the environment is largely unknown.

The purpose of this review is to summarize scarce and scattered information about the profiles of UV filters in aquatic organisms, analytical methods, bioaccumulation/biomagnification, ecotoxicity, and environmental analysis and risk assessment (ERA). Finally, the article identifies current gaps in our knowledge and potential future research needs in ERA.

Physicochemical properties

UV filters are substances with almost null absorption of visible radiation but important light absorption in the UVA (315–400 nm) and UVB (280–315 nm) ranges [4]. Sunscreens can be classified into organic (chemical) absorbers and inorganic (physical) blockers on the basis of their mechanism of action. Organic UV filters absorb UV radiation with excitation to a higher energy state. Excess energy is dissipated by emission of higher wavelengths or relaxation by photochemical processes, for example isomerization and heat release. They include camphors, benzophenones, cinnamates, triazines, among others. Inorganic sunscreens, i.e. titanium dioxide and zinc oxide, protect the skin by reflecting and scattering UV radiation.

The focus of this review is on organic UV filters. A feature common to all of these is the presence of an aromatic moiety with a side-chain with different degrees of unsaturation. Their structures and other physicochemical properties are listed in Table 1. Some, for example 4-methylbenzylidene camphor (4MBC), ethylhexylmethoxy cinnamate (EHMC), and octocrylene (OC), are chiral compounds. Although the enantiomers of these compounds are not expected to have different physicochemical properties, isomers and enantiomers may differ in biological behavior. Commercial formulations contain mainly geometrical (*E*) isomers, although some UV filters (e.g., methoxycinnamates) contain both the (*E*) and the (*Z*) isomers. Because of the high lipophilicity and poor biodegradability of many UV filters (mostly with log K_{ow} 4–8) they end up in sewage sludge during wastewater treatment [5–8], and accumulate in river sediments [9–12] and biota [13–20].

Analytical methodology

Sampling and sample preparation

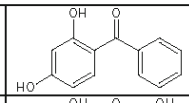
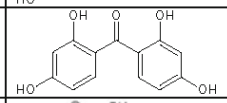
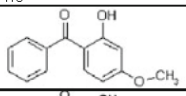
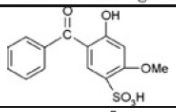
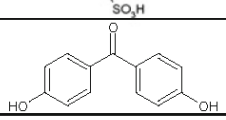
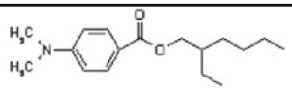
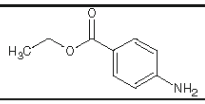
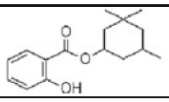
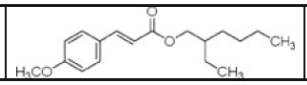
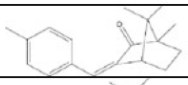
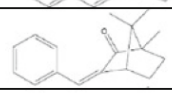
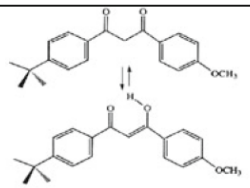
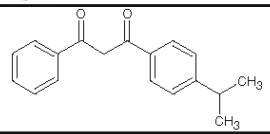
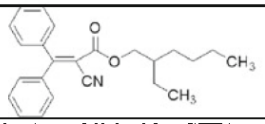
Sampling procedures for analysis of residues of UV filters in aquatic biota mainly involve traditional fishing, either by native fishers or by electric fishing, for which special permission is often needed. Unlike other matrices, there is the added difficulty of the availability of samples of the desired species, which often depends on external factors which are difficult to control. Moreover, the variability between individuals of the same species (size and living cycle) hinders comparison of results. Most studies have focused on fish, a representative matrix of the aquatic environment assumed to be able to retain and bioaccumulate UV filters because of the lipophilicity of the compounds. The most usual sample analyzed is muscle, probably because of its low lipid content in comparison with other tissues and because it is part of the human diet. Studies have also been conducted on macrozoobenthos, mussels, and birds. Selected tissues are homogenized by blending and often freeze-dried before extraction.

Extraction and clean up

Extraction of UV filters from tissues has been achieved by conventional Soxhlet extraction (which has become less attractive because of the time and solvent consumed) [16, 18], pressurized-liquid extraction (PLE) [14], solid–liquid extraction [13–15, 17, 19], and microwave-assisted extraction (MAE) [20]. These techniques lead to coextraction of a lipid fraction that must be removed before determination of the UV filters. Clean-up of biota sample extracts is usually a two-stage process. The sample extracts can first be subjected to gel-permeation chromatography (GPC), primarily to remove lipids, followed by adsorption chromatography on silica or Florisil columns. Quite often RP-HPLC has also been used for extraction and purification.

In the first work published on UV filter levels in biota [18], benzophenone-3 (BP3), 4MBC, homosalate (HMS), EHMC, ethylhexyl dimethyl PABA (OD-PABA), isopropyl dibenzoyl methane (IDM), and butyl methoxy dibenzoyl methane (BM-DBM) were extracted from fish tissue by Soxhlet extraction. The tissue was first homogenized and dried with sodium sulfate, then extracted with petroleum ether–ethyl acetate 2:1 (*v*/*v*). Lipids and other potential matrix interferences were removed by GPC (Bio Beads SX-3) with cyclohexane–acetone 3:1 (*v*/*v*) as mobile phase. For analysis of IDM and BM-DBM, CH_3I/NaH was added to half of the extract to form their derivatives for further GC–MS analysis. This half of the extract was then purified on a silica column (elution with hexane–ethyl acetate 7:3 (*v*/*v*)). The other half was also loaded on to a silica column and

Table 1 Physicochemical properties of the organic UV filters addressed in this review. In parentheses, the key system adopted herein

Name (INCI nomenclature)[a]	CAS no.	Structure	Molecular weight (g mol^{-1})	Log K_{ow}	Solubility (g L^{-1})[b]
Benzophenones					
Benzophenone-1 (BP1)	131-56-6		214.22	3.15[c]	0.39[c]
Benzophenone-2 (BP2)	131-55-5		246.22	2.78[d]	0.98[c]
Benzophenone-3 (BP3)	131-57-7		228.24	3.79[d]	0.10[c]
Benzophenone-4 (BP4)	4065-45-6		308.31	0.993[c]	11[c]
4,4′-Dihydroxybenzophenone (4DHB)	611-99-4		214.22	2.19[d]	0.6[c]
p-Aminobenzoic acid derivatives					
Ethylhexyldimethyl PABA (OD-PABA)	21245-02-3		277.4	5.412[c]	4.7×10^{-3}[c]
Ethyl-PABA (Et-PABA)	94-09-7		165.19	1.86[d]	1.31[d]
Salicylates					
Homosalate (HMS)	118-56-9		262.35	5.947[c]	0.021[c]
Cinnamates					
Ethylhexyl methoxycinnamate (EHMC)	5466-77-3		290.4	5.8	6.4×10^{-3}[c]
Camphor derivatives					
4-Methylbenzylidene camphor (4MBC)	36861-47-9		254.37	4.95	0.017
3-Benzylidene camphor (3BC)	15087-24-8		240.34	2.84	0.034
Dibenzoylmethane derivatives					
Butylmethoxydibenzoylmethane (BM-DBM)	70356-09-1		310.39	4.191	4.3×10^{-3}[c]
Isopropyldibenzoylmethane (IDM)	63250-25-9		266.33	4.382	0.027
Crylenes					
Octocrylene (OC)	6197-30-4		361.49	6.88	3.6×10^{-4}[d]

[a] INCI (International Nomenclature for Cosmetic Ingredient) established by CTFA and COLIPA

[b] In water at 25 °C

[c] Calculated by use of Advanced Chemistry Development (ACD/Labs) Software V11.02 (1999–2011 ACD/Labs)

[d] Experimental values from database of physicochemical properties; Syracuse Research Corporation: http://www.syrres.com/esc/physdemo.htm

the same solvent mixture in a different proportion (91:9 *v*/*v*) was used for elution.

A similar method was developed by Meinerling and Daniels [16] for analysis of 4MBC, BP3, EHMC, and OC in the muscle of rainbow trout. In this case Soxhlet extraction with *n*-hexane–acetone 9:1 (*v*/*v*) was followed by GPC (Bio Beads SX-3) with cyclohexane–ethyl acetate 1:1 (*v*/*v*) as eluent. In a further clean-up step, a Florisil column was used to remove polar compounds.

In the procedure followed by Balmer et al. [14] for analysis of 4MBC, BP3, EHMC, and OC, fish samples were homogenized with sodium sulfate and column extracted or PLE extracted with dichloromethane (DCM)–cyclohexane 1:1 (*v*/*v*). The extracts were then cleaned by GPC on a Biobeads S-X3 column with DCM–cyclohexane 35:65 (*v*/*v*) as eluent, followed by silica purification. Buser et al. [15] extracted 4MBC and OC by successive extraction with potassium oxalate (2 mL, 35 %), ethanol (100 mL), diethyl ether (50 mL), and *n*-pentane (70 mL). After extraction, matrix components were removed by GPC and silica purification.

The methods described above are only suitable for extracting UV filters with similar physicochemical properties. A method for simultaneous determination of nine UV filters, from polar to lipophilic, in fish has been reported by Zenker et al. [17]. Mid-polar and lipophilic UV filters were extracted from homogenized tissue by solvent extraction with ethyl acetate–*n*-heptane–water 1:1:1 (*v*/*v*) and further purified by reversed-phase RP-HPLC. The fraction containing mid-polarity UV filters was analyzed by HPLC–MS whereas the fraction containing the lipophilic ones was determined by GC–MS. Polar and medium-polarity UV filters were extracted with a mixture of methanol (MeOH) and acetonitrile (ACN), followed by HPLC–MS analysis. This is the procedure requiring the smallest amount of sample (4 g); good limits of detection are achieved for most compounds. The same method proved to be suitable for analysis of macrozoobenthos and bird samples also.

Bachelot et al. [20] developed a method for determination of EHMC, OC, and OD-PABA in marine mussels. MAE was performed with 25 mL acetone–heptane 1:1 (*v*/*v*). After extraction, the liner was rinsed with the same solvent mixture. The extracts were percolated through anhydrous sodium sulfate. Further purification was performed by RP-HPLC on a RP Spherisorb ODS2 column (4.6 mm×150 mm, 5.0 μm) following a procedure adapted from Zenker et al. [17].

GPC or column purification with silica or Florisil is useful whenever compounds with similar physicochemical properties must be separated from interfering matrix substances, for example lipids, present in the sample. When these methods are used for a mixture of compounds with different physicochemical properties they are less effective. RP-HPLC is a suitable alternative when UV filters with a large range of physicochemical properties must be analyzed.

Instrumental analysis

LC is the technique of choice for the analysis of UV filters in cosmetic products. In contrast, GC is preferred for their environmental analysis. Nevertheless, both techniques have been applied to the analysis of biological samples. The low concentration of the target analytes in biota samples requires high sensitivity and selectivity. Therefore, mass spectrometric (MS) detection is the most suitable technique for determination of these compounds in such complex matrices. Relevant data on analytical methods are summarized in Table 2.

GC–MS

UV filters are, with very few exceptions (e.g., octyl triazone (OT) and BM-DBM), amenable to GC. Matrix effects are not critical for the ionization modes, e.g. electron impact (EI) or chemical ionization (CI), typically used in GC–MS. As a consequence, method detection limits (MDL) are usually quite low [21]. On the other hand, this technique can only be successfully applied to a limited number of non-polar and volatile compounds. For more polar or thermally unstable compounds an additional derivatization step is required; here differences in matrix components may result in quite different derivatization efficiencies which may affect both precision and accuracy of the analysis.

Analysis has always been performed in electron-impact mode (GC–EI-MS). Quantification is achieved by operating in selected ion monitoring mode (SIM). The fragment ions usually selected for the quantification and confirmation of the analytes are listed in Table 2.

BM-DBM, IDM, 4MBC, OD-PABA, HMS, EHMC, and BP3 have been analyzed by GC–MS on a SE-54-CB column (50 m×55 mm, 0.25-μm film), working in SIM mode [18]. Balmer et al. [14] analyzed 4MBC, BP3, EHMC, and OC in fish by GC–EI-MS using two different columns a BGB-5 (30 m×0.25 mm; 0.25 μm) and an SE54 (25 m×0.32 mm; 0.25 μm). In that study $^{13}C_{12}$-PCB 77 was used as surrogate standard. Under the same GC–EI-MS conditions Buser et al. [15] analyzed 4MBC and OC in fish tissue, using $^{15}N_3$-musk xylene as internal standard. Zenker et al. and Fent et al. [17, 19] analyzed nine UV filters with a large range of physicochemical properties (log K_{ow} from 0.9 to 5.7) in fish, macrozoobenthos, and bird tissue. Four out of the nine UV filters investigated, BP3, 3BC, 4MBC, and EHMC (the most lipophilic) were detected by GC–EI-MS on an Optima-5-MS (50 m×0.2 mm; 0.35 μm) column. In this study benzophenone-d_{10} was used as surrogate standard.

Mottaleb et al. [13] analyzed 4MBC and OC with 10 other personal care products in fish tissue by both GC–EI-MS and GC–EI-IT (with an ion trap mass spectrometer). The GC–EI-MS analysis was carried out with a XTI-5 capillary column (30 m×0.25 mm; 0.25 μm) operating in

Table 2 Analytical methodology and occurrence data for the UV filters addressed in this review

Matrix	Specie	Tissue	UV filter	Sample amount	Extraction	Purification	Technique
Fish	Bluegill (*Lepomis macrochirus*)	Muscle	4MBC, OC	1 g	Rotatory extraction with acetone	Silica	GC-EI-MS
	Sonora sucker (*Catostomus insignis*)	Muscle, belly flap and skin	4MBC, OC	1 g	Sonication with acetone	GPC Silica	GC-EI-IT
Fish	White fish (*Coregonus sp.*)	Muscle	4MBC, BP3, EHMC, OC	5 g	ASE extraction: Homogenized with diatomaceous earth 3 cycles DCM/ cyclohexane (1:1, v/v) at room temperature	GPC (EnviroSep-ABC or Biobeads S-X3) Silica	GC-EI-MS
	Roach (*Rutilus rutilus*) Perch (*Perca fluviatilis*)			20 g	Homogenized with sodium sulphate Column extracted with DCM/cyclohexane (1:1, v/v))		
Fish	Brown trout (*S.Trutta fario*)	Muscle plus adipose tissue under the skin	4MBC, OC	10–25 g	Homogenized in 100 ml water with hand blender Solvent Extraction using potassium oxalate (2 mL, 35 %), ethanol (100 mL), diethyl ether (50 mL) and n-pentane (70 mL)	GPC (EnviroSep-ABC or Biobeads S-X3) Silica	GC-EI-MS
Fish	Rainbow trout (*Oncorhynchus mykiss*)	Muscle	4MBC, BP3, EHMC, OC	10 g	Homogenized with sodium sulphate Soxhlet extracted with n-hexane/acetone (9/1, v/v)	GPC (Biobeads S-X3) Florisil	LC-ESI-MS/MS
Fish	Barb (*Barbus barbus*) and Chub (*Leucisus cephalus*)	Muscle plus adipose tissue under the skin	4MBC, 3BC, BP1, BP2, 4DHB, BP3, BP4, EHMC, Et-PABA	4 g	Solvent extraction using ethyl acetate, n-heptane and HPLC water (1:1:1, v/v/v) or Solvent extraction with MeOH:ACN (1:1, v/v)	RP-HPLC (RP Spherisorb ODS2 column (4.6 mm × 150 mm, 5.0 μm)	LC-ESI-MS/MS and GC-EI-MS
	Barb (*Barbus barbus*) and Chub (*Leucisus cephalus*)	Muscle plus adipose tissue under the skin	BP4, 4DHB, BP1, BP2, Et-PABA	1 g	Solvent extraction with MeOH/ACN (1:1, v/v)	Syringe filtration	LC-ESI-MS/MS
Fish	Roach (*Rutilus rutilus*) Perch (*Perca fluviatilis*)	Muscle, offal, rest and whole fish	IDM, BM-DBM, 4MBC, OD-PABA, HMS, EHMC, BP3		Homogenized with sodium sulphate Soxhlet extracted with petroleum ether:Etyl acetate (1:1, v/v)	GPC (Biobeads S-X3)	GC-EI-MS

Table 2 (continued)

Matrix	Specie	Tissue	UV filter	Sample amount	Extraction	Purification	Technique
macrozoobenthos	Mussels (*Dreissena polymorpha*)	Whole macroinvertebrate	BP1, BP2, BP3, BP4, 4DHB, Et-PABA, EHMC, 4MBC, 3BC	4 g (fraction 1) and 1 g (fraction 2)	Fraction 1: Solvent extraction using ethyl acetate, n-heptane and HPLC water (1:1:1, v.v:v) or Solvent extraction with MeOH: ACN (1:1, v/v)	Fraction 1: RP-HPLC (RP Spherisorb ODS2 column (4.6 mm × 150 mm, 5.0 µm) Fraction 2: Syringe filtration	LC-ESI-MS/MS and GC-EI-MS
	Gammarus sp				Fraction 2: Solvent extraction with MeOH: ACN (1:1, v/v)		
Fish	Chub (*Leuciscus cephalus*) Brown trout (*Salmo trutta*) Barb (*Barbus barbus*) Eel (*Anguilla anguilla*)	Muscle plus adipose tissue under the skin					
Bird	Cormorants (*Phalacrocorax sp*)	Muscle					
Mussel[a]	*Mytilus edulis* and *Mytilus galloprovincialis*	Soft tissue	EHMC OC OD-PABA	3 g	MAE extraction with acetone:heptane (1:1, v/v)	Filtered (0.2 µm)) through 10 g anhydrous sodium sulphate RP-HPLC (RP Spherisorb ODS2 column (4.6 mm × 150 mm, 5.0 µm)	GC-EI-IT

Matrix	Chromatographic Column	MS/MS transition or SIM ions	Recovery (%)	MLOD	Concentrations (ng/g lipid)	Reference
Fish	XTI-5 capillary column (30 m × 0.25 mm; 0.25 µm)	4MBC: 115, 211, 254 OC: 177, 249, 361	98–99	5.3–17 ng/g	nd	13
	VF-5 MS capillary column (30 m × 0.25 mm; 0.25 µm)	4MBC: 211>169,155 OC: 250>248, 221	57–79	36–120 ng/g	nd	
Fish	BGB-5 (30 m × 0.25 mm; 0.25 µm) or SE54 (25 m × 0.32 mm; 0.25 µm)	4MBC: 254, 239 BP3: 228, 229 EHMC: 178, 290	93–115	7–380 ng/g lipid	72 (OC)	14
		OC: 249, 361		3–37 ng/g lipid	44–94 (4MBC), 66–118 (BP3), 64 (EHMC)	
				10–56 ng/g lipid	166 (4MBC), 123 (BP3), 25(OC)	
Fish	BGB-5 (30 m × 0.25 mm; 0.25 µm) or SE54 (25 m × 0.32 mm; 0.25 µm)	4MBC: 254, 239 OC: 249, 361	No data	5–20 ng/g lipid	50–1800 (4MBC) 40–2400 (OC)	15
Fish	PerfectSil 120 ODS-2 (125 mm × 3 mm, 3.5 µm)	4MBC: 255>105 EHMC: 291>161	86–108	2.4 ng/g muscle	214 (4MBC), 193–525 (BP3), 414 (EHMC), 300 (OC)	16

Table 2 (continued)

Matrix	Chromatographic Column	MS/MS transition or SIM ions	Recovery (%)	MLOD	Concentrations (ng/g lipid)	Reference
		BP3: 229>151 OC: 362>250				
Fish	Zorbax SB-C18 (150 mm × 3.0 mm, 3.5 μm) and OPTIMA-5-MS (50 m × 0.2 mm; 3.5 μm)	BP1: 213>213 BP2: 245>245 BP4: 307>307 4DHB: 213>213 Et-PABA: 166>138 EHMC: 178, 290 3BC: 240, 197 4MBC: 254, 237 BP3 : 228, 227	76–99 (BP4 no extracted)	8–205 ng/g lipid	45–700 (EHMC)	17
	Zorbax SB-C18 (150 mm+3.0 mm, 3.5 μm)	BP1: 213>213 BP2: 245>245 BP4: 307>307 4DHB: 213>213 Et-PAB: 166>138	80–99	1.8–10.7 ng/Kg body weigh		17
Fish	SE-54-CB (50 m × 0.55 mm; 0.25 μm)	IDM: 105, 147, 294 BM-DBM: 135, 161, 338 4MBC: 211, 239, 254 OD-PABA: 148, 165, 277 HMS: 109, 138, 262 EHMC: 161, 178,248 BP3: 165, 225, 242	89–106	50–90 ng/Kg body weigh	Muscle: 810 (4MBC), 310 (EHMC), 298 (BP3), 3100 (HMS); offal: 880 (4MBC), 283 (BP3), 185 (HMS); rest: 990 (4MBC), 50 (EHMC), 40 (BP3), 79 (HMS) whole fish: 930 (4MBC), 120 (EHMC), 150 (BP3), 791 (HMS); Muscle: 161 (4MBC), 41 (EHMC), 230 (BP3), 720 (HMS), 150(IDM); offal: 106 (4MBC), 270 (BP3), 970 (HMS), 210 (BM-DBM); rest: 60 (4MBC), 16 (EHMC), 22 (BP3), 41 (HMS), 9(IDM), 18 (TDM); whole fish: 78 (4MBC), 20 (EHMC), 78 (BP3), 237 (HMS), 29 (IDM), 44 (BM-DBM)	18
macrozoobenthos	Zorbax SB-C18 (150 mm+3.0 mm, 3.5 μm) and OPTIMA-5-MS (50 m × 0.2 mm; 0.35 μm)	BP1: 213>213 BP2: 245>245 BP4: 307>307 4DHB: 213>213 Et-PABA: 166>138 EHMC: 178, 290	70–105	6–50 ng/g lipid	22–150 (EHMC)	19

Table 2 (continued)

Matrix	Chromatographic Column	MS/MS transition or SIM ions	Recovery (%)	MLOD	Concentrations (ng/g lipid)	Reference
		3BC: 240, 197				
		4MBC: 254, 237				
		BP3: 228, 227				
Fish					91–133 (EHMC)	
					23–79 (EHMC)	
					91–151 (BP3), 11–173 (EHMC)	
					9–337 (EHMC)	
					<LOQ (BP3), 30 (EHMC)	
Bird					16–701 (EHMC)	
Mussel[a]	SGE-BPX5 capillary column (30 m × 0.25 mm, 0.25 μm)	EHMC: 178>121, 132, 161	89–116	2 ng/g dw	3–256 ng/g (EHMC)	20
		OC: 248>220, 219, 176			2–7112 ng/g (OC)	
		OD-PABA: 165>91, 118, 148				

[a] Concentrations expressed in ng/g (not ng/g lipid), *nd* not detected

SIM mode, and with benzophenone-d_{10} and $^{13}C_6$-*p*-*n*-nonylphenol as surrogates. Analysis performed on samples of bluegill (*Lepomis macrochirus*) muscle, with only 0.4 % lipid content, provided good results. When this method was applied to samples of sonora sucker (*Catostomus insignis*), with an average of 4.9 % lipid content, GC–EI-MS failed to provide acceptable results. Analysis of spiked sample extracts that had not previously been subjected to GPC purification resulted in substantially compromised chromatographic performance. In this case, addition of a GPC step was essential. This additional purification enabled continuous analysis of sonora sucker samples with no compromise in chromatographic performance compared with GC–EI-MS analysis of spiked bluegill tissue. However, dramatic increases in background signal and/or reduction in analyte sensitivity were observed for several analytes. As a consequence some compounds, including 4MBC, were indistinguishable from the background. To increase the sensitivity and reduce the background signal observed, the method was improved by the application of tandem mass spectrometric detection. GC–EI-IT analysis was performed with a VF-5 MS capillary column (30 m×0.25 mm; 0.25 μm) under the same chromatographic conditions and with detection in selected reaction monitoring mode (SRM). The optimized transitions used are listed in Table 2. Nevertheless, this approach only slightly improved detection of 4MBC and a few other compounds, but to much less an extent than expected (this aspect will be further discussed in the section "Limits of detection").

Similarly, GC–EI-IT with an ion-trap mass spectrometer was used by Bachelot et al. [20] for determination of EHMC, OC and OD-PABA. In this work an SGE-BPX5 capillary column (30 m×0.25 mm, 0.25 μm) was used for the separation. Data were acquired in SIM mode for the isotopically labeled internal standard chrysene-d_{12} and in SRM mode for the UV filters.

LC–MS

This technique enables analysis of a wide range of compounds and significantly increases the possibility of analysis of metabolites, which are usually more polar that the parent compounds, without the need for derivatization.

For ionization of the UV filters three different techniques have been used—electrospray ionization (ESI) (which is by far the most commonly used for trace analysis of these pollutants in environmental samples), atmospheric-pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). All the approaches used for LC–MS analysis of UV filters in biota use ESI mode, which achieves efficient ionization of the analytes even though ESI is assumed to be susceptible to signal suppression or signal enhancement by components of sample matrix, as shown

by previous studies on UV filters in complex matrices such as sewage sludge [8]. Isotopically labeled compounds should be used as internal standards to compensate for the matrix effect. Quantification of UV filters in biota samples by external standard calibration is not recommended.

Meinerling and Daniels [16] developed an LC–MS–MS method for analysis of 4MBC, OC, BP3, and EHMC. Chromatographic separation was performed on a PerfectSil 120 ODS-2 (125 mm×3 mm) column with MeOH and water (each containing 0.05 % acetic acid) as mobile phase. Analysis was performed with a triple-quadrupole mass spectrometer fitted with electrospray interface operated in positive mode under SRM conditions (LC–ESI(+)–MS–MS). In this study external standard calibration was used.

Zenker et al. [17] analyzed nine UV filters by LC–ESI–MS–MS. Chromatographic separation was achieved on a Zorbax SB-C18 column (150 mm×3.0 mm, 3.5 μm) with a binary gradient prepared from a mixture of 0.1 % (*v*/*v*) formic acid in HPLC-grade water and 0.1 % (*v*/*v*) formic acid in ACN. Data acquisition was performed in SRM mode. Benzoic-d_5 acid was used as internal standard.

Method performance

Method recovery

High recovery was achieved in all the methods reported, especially when the lipid content of the biological sample analyzed was low.

Nagtegaal et al. [18] achieved recovery from 89 % to 96 % when extracting compounds with a Soxhlet-based procedure. Most studies analyzing lipophilic UV filters used solvent extraction and further clean up by GPC, and usually achieved good recovery (86–115 %). Mottaleb et al. [13] analyzed 4MBC and OC in fish tissue with low lipid content, with recovery of 98 and 99 %. In analysis of samples with higher lipid content lower recovery (57–79 %) was achieved.

Zenker et al. [17] used a mixture of ethyl acetate and *n*-heptane as extraction solvent; this enabled efficient extraction of eight of the nine UV filters with a wide range of polarity. Average recovery ranged from 76 to 99 % (SD from 0.3 to 4 %). However, extraction of the polar filter BP4 was feasible only with ACN and MeOH. Bachelot et al. [20] achieved even higher recovery, from 89 to 116 %, in analysis of lipophilic UV filters in mussel soft tissue extracted by MAE then further RP-HPLC purification.

Limits of detection

Method limits of detection were calculated by extraction of samples of fish spiked with low concentrations of analytes which can be detected in the presence of possible matrix effect. For biota samples MLODs are in the sub-ng g^{-1} range, although authors normalize their results differently, depending on the matrix, and express them in ng g^{-1} lipid or simply ng g^{-1}. The presence of UV filters in blanks is eventually reflected by higher MLODs.

MLODs are highly dependent on the matrix analyzed. Biological matrices may be quite different depending on the organism selected, the species, and the tissue chosen; even so, there is still great variability. As an example, Balmer et al. [14], in analysis of four lipophilic UV filters, obtained three significantly different MLODs ranges, 3–37, 10–56, and 7–380 ng g^{-1} lipid, as a function of the fish species analyzed. To compare MLODs between different methods and for different matrices is complicated. MLODs are usually lower when analysis is performed by GC–MS because matrix effects are usually smaller. Table 2 summarizes the MLODs obtained in each study.

Zenker et al. [17] developed a method for analysis of nine UV filters by GC–EI–MS and LC–ESI–MS–MS. In the first of these the limits of detection ranged between 8 and 36 ng g^{-1} lipid. For UV filters analyzed by LC–MS–MS limits of detection were between 86 and 205 ng g^{-1} lipid. These different MLODs are because of the greater matrix effect in analysis using electrospray interfaces, which can affect analyte ionization.

Mottaleb et al. [13] analyzed bluegill tissue (with low lipid content, 0.4 %) by GC–EI-MS and sonora sucker tissue (high lipid content, 4.9 %) by GC–EI-IT, which is, a priori, a more sensitive and selective technique. MLODs for most compounds in the GC–EI-IT study were higher than those obtained for bluegill tissue by use of GC–EI-MS (especially for 4MBC, 23-fold higher). Differences in detectability between the two approaches cannot be explained solely by differences in extraction efficiency. The authors suggest inefficient fragmentation of precursor ions in the ion trap. Because all MS–MS precursor ions are produced by EI, a relative hard ionization technique, it is likely that generation of product ions via collision-induced dissociation may eventually be problematic (precursor ions may be sufficiently stable, which makes further fragmentation unlikely). The MLODs afforded by GC–EI-IT exceeded the corresponding environmentally relevant concentration range identified in the literature. OC and 4MBC were not detected in any sample in the study.

Critical aspects in the analysis of UV filters

Background contamination is a common problem in the determination of UV filters at environmentally relevant levels. Therefore, several measures must be taken to prevent this problem. All glassware should be carefully cleaned. A typical procedure consists in washing and heating at 380 °C, then sequentially rinsing with different high-purity organic

solvents. Furthermore, gloves should be worn during sample preparation; separate solvents and only previously unopened packages of solvents, chemicals and other supplies, and glassware should be used.

In addition, a set of at least two operational blanks should be processed together with each batch of samples. Because many of the compounds analyzed undergo photodegradation, stock standard solutions should always be covered with aluminium foil and stored in the dark.

The presence of matrix effects has the potential to lead to compromised results, so precautions should be taken to minimize this effect. Measurements are further hindered by the lack of appropriate commercially available reference standards. Currently, only BP3-d_5 and 4MBC-d_4 are commercially available; none of the studies reported herein used these. Other isotopically labeled compounds, namely benzophenone-d_{10}, $^{15}N_3$-musk xylene, and $^{13}C_6$-*p*-*n*-nonylphenol were used for quantification. Development and further marketing of a wider range of isotopically labeled compounds for use as surrogate and internal standards is an important need for analysis of sunscreen agents in complex matrices.

Biota levels

UV filters enter the aquatic environment directly, as a result of swimming and other recreational activities or indirectly via wastewater treatment plants (WWTPs). Thus, it is expected that, because of the lipophilic properties of these compounds, they can reach and accumulate in tissues of aquatic organisms. Several fish species have been investigated together with, although to a lesser extent, mollusks, crustaceans, and birds. Table 2 summarizes UV filter occurrence data in biota.

A study carried out by Nagtegaal et al. [18] provided the first data on the occurrence of UV filters in fish. Perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) from Maarfelder Lake (Eifel, Germany) were analyzed and the presence of seven UV filters with total concentrations of approximately 2000 ng g^{-1} lipid and 500 ng g^{-1} lipid, respectively, in whole fish were reported. This early study, besides providing the first data on bioaccumulation of UV filters in fish, reported prevalence profiles in different fish tissues (muscle, offal, the rest, and whole fish). Results indicated that 4MBC and HMS can be selectively accumulated depending on the species; perch accumulates 4MBC in muscle and HMS in offal. In contrast, roach had higher levels of 4MBC in offal and of HMS in muscle. In contrast, EHMC and BP3 had similar bioaccumulation profiles in both species.

In Switzerland similar concentrations were found in lake fish. 4MBC, BP3, EHMC, and OC were detected in white fish (*Coregonus sp.*), roach and perch in the range 25–166 ng g^{-1} lipid, and from 45 to 700 ng g^{-1} lipid for EHMC in barb (*Barbus barbus*) and chub (*Leucisus cephalus*) [17]. Meinerling et al. [16] reported concentrations from 193 to 525 ng g^{-1} lipid in rainbow trout (*Oncorhynchus mykiss*).

Higher levels for 4MBC and OC (up to 1800 and 2400 ng g^{-1} lipid, respectively) were found in fish (brown trout, *S. Trutta fario*) from rivers downstream from a WWTP discharge [15], revealing its impact on the ecosystem. Buser et al. [28] demonstrated that the enantiomeric composition of 4MBC in perch was much different from that observed in the surrounding lake water. In contrast, the enantiomeric composition of 4MBC in roach was similar to that of the lake water indicating that bioconcentration or metabolism of a compound can be quite different from one species to another. The factors responsible for the differences in the enantiomeric composition of 4MBC found in fish remain unclear.

Concerning organisms other than fish, Fent et al. detected EHMC in crustaceans (*Dammarus sp.*) and mollusks (*Dreissena polymorpha*) at concentrations between 22 and 150 ng g^{-1} lipid. EHMC was also detected in different fish species at concentrations up to 337 ng g^{-1} lipid and in cormorants (*Phalacrocorax sp.*), at levels above 700 ng g^{-1}. BP3 was also detected, but at lower concentrations, in brown trout (*Salmo trutta*) and eel (*Anguilla anguilla*). These results suggest that biomagnification occurs through the food web; this aspect will be further discussed in the section "Bioaccumulation and biomagnification") [19].

Bachelot et al. proved the presence of UV filters residues in marine mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) of the Mediterranean French coast [20]. In that study all mussel samples contained EHMC, at concentrations up to 256 ng g^{-1} dw. In addition, 55 % of the samples contained OC also. In particular one of these samples had the highest concentration reported so far for an UV filter in biota, 7,112 ng g^{-1} dw.

Besides WWTP discharges, another important factor affecting accumulation in aquatic biota samples is the season of the year, with summer being the period when peak concentrations of sunscreens are observed. As an example, the concentrations reported for OC in fish in September were found to be three to five-fold higher than those corresponding to May, before the swimming period [15]. Similarly, a study carried out by Fent et al. showed that concentrations in freshwater mussels collected in a lake with recreational activity were higher after the summer [19].

UV filter 4MBC and other benzotriazole compounds have been analyzed in a great variety of aquatic species, for example tidal flat organisms, fish, coastal birds, and even hammerhead sharks of the Japan coast, by Nakata et al. [22]. 4MBC was not detected in any of the samples analyzed, even though this common sunscreen agent has been detected in samples in different studies in Europe. The authors of that

study suggested these results were evidence of the different production and usage profiles of UV filters among countries.

As shown in Table 2, of all the sunscreen agents investigated, EHMC is the most frequently found, but at lower concentrations that those usually observed for UV filters of similar log K_{ow}, e.g. HMS (log K_{ow} 6.16) and OC (log K_{ow} 4.95). On the other hand, other sunscreens with log K_{ow} in the same range, OD-PABA (log K_{ow} 6.15) and 3BC (log K_{ow} 4.49) were never detected, suggesting fast and effective metabolism.

Bioaccumulation and biomagnification

The net accumulation of a chemical by an organism from its combined exposure to water, food, and sediment is known as bioaccumulation. Species higher in the food web can be exposed to all the chemicals that lower-order species accumulate (biomagnification). Bioaccumulation models are useful tools for understanding the biomagnification of such substances [23]. The extent of biomagnification for a given contaminant is expressed in terms of the biomagnification factor (BMF), the ratio of the concentration of the contaminant in a predator to its concentration in prey. A BMF above 1 indicates biomagnification of the contaminant. However, the most conclusive evidence of the accumulation of chemicals by organisms and biomagnification in food webs is expressed by the trophic magnification factor (TMF) [23, 24], which is used to estimate the accumulation of contaminants through trophic levels of different food webs, for instance between fresh water and marine water systems.

Because of the lipophilic character (low water solubility) of most UV filters they may be expected to accumulate in biota and in humans and be stored rather faster than they are metabolized or excreted. A recent study by León-González et al. [25] revealed that the metabolites of OD-PABA were detected in human urine after 8 days of single cutaneous application of a cream, indicating slow metabolism. Despite this, only one field-based study has examined biomagnification through food webs [19]. EHMC bioconcentration was proven in macrozoobenthos, fish from different trophic levels, and cormorants (*Phalacrocorax sp.*) a species of fish-eating birds, in Switzerland. For example, estimated BCF for fish were far above unity, in the range 167–1500. Biomagnification was also assessed, but higher concentrations of EHMC in cormorants than in fish were not statistically significant. Despite this, estimated BMF, occasionally slightly higher than unity, were indicative of possible positive trophic magnification of EHMC. Nevertheless, a possible explanation of this finding may be found in feeding ecology; because birds and mammals are homeotherms their rates of feeding higher than for fish and invertebrates (poikilotherms) [26]. Higher BCFs were, however, reported by Balmer et al. [14] for 4-MBC in fish from Lake Zürich. In particular, BCFs for roach ranged from 2,300 to 9,700.

Biological and chemical factors, for example size, sex, age, life cycle, and metabolic activity, are important when assessing bioaccumulation and trophic transfer of contaminants in food webs. The lipid and protein content of tissue, which vary according to season, reproduction, migration, feeding rate, diet composition, growth rate, and food chain length, should be taken into account in analysis of UV filters [27].

The phenomenon of chirality exists in all biological systems. All proteins, enzymes, and carbohydrates are chiral. Because biological processes may be stereoselective (favor one structural form over the other), enantiomers of chiral compounds, both parent compounds and transformation products (metabolites, products of photodegradation, and disinfection byproducts) must be investigated separately to obtain reliable information about their bioavailability and biomagnification through the food web. It must be taken into account that metabolites of achiral compounds can also be chiral. Buser et al. [28] investigated the enantiomeric pattern of 4-MBC in lake fish. This compound exists as two stereoisomers, (*Z*) and (*E*), as a consequence of an exocyclic C=C bond. Both are chiral compounds with two stereocenters provided by the camphor moiety of their chemical structure. First, HPLC separation of the (*E*) and (*Z*) isomers of 4MBC was performed on a Nucleosil 120–5 C-18 reversed-phase column (250 mm×4 mm) with ACN–water 60:40 (*v*/*v*) as mobile phase. Enantiomeric separation of 4MBC was subsequently achieved by GC on a laboratory-prepared column containing 2,6-bis-(*tert*-butyldimethylsilyl)-γ-cyclodextrin in 70 % PS086. Detection was performed in full scan and SIM (GC–MS) or SRM (GC–EI-IT) modes.

Although the study did not reach a sound conclusion, the isomer ratio observed seemed to indicate preferential accumulation of the (*E*) isomer. With regard to potential enantioselective fish metabolism of 4-MBC, results were completely different for roach and perch; roach accumulates both enantiomers whereas perch is unable to metabolize the [1*R*,4*S*-(*E*)-4-MBC] stereoisomer only.

Ecotoxicological considerations

The ecotoxicological implications of exposure of biota to sunscreens have been addressed quite frequently. Despite this, the sparse studies available are conclusive [29]. Fish have long been regarded as tracers for assessing the extent of lipophilic contamination of aquatic ecosystems. As a consequence most ecotoxicological studies on the effect of UV filters have been conducted on different fish species for "in vivo" testing. Several sunscreens have been found to

have estrogenic hormonal activity, in particular, two which are extensively used 4-MBC and OMC; moreover, some have been shown to have multiple endocrine-disrupting activity, for example androgenicity or antiestrogenicity. Adverse effects on fecundity and reproduction have also been observed for BP3, benzophenone 2 (BP2), and 3 benzylidene camphor (3BC). Analysis of vitellogenin (VTG) in rainbow trout and Japanese medaka (*Oricias latipes*) after aqueous exposure to BP3 indicated, however, that high effective concentrations in the range 620–749 μg g^{-1} were needed to induce these effects [30]. These concentrations are greater than the reported level (19 ng L^{-1}) of BP3 in estrogenic fractions of effluent wastewater extracts [31]. When a similar test was conducted on 4MBC and EHMC [32], 4MBC had high estrogenic potency.

In male fathead minnows (*Pimephales promelas*), concentrations of BP2 of 1.2 mg L^{-1} and higher were found to induce VTG, modify gonad histology, and emasculate secondary sex characteristics; oocyte production in female fish was also inhibited significantly [33, 34]. In the same fish species, 3BC had high estrogenic potency, inducing VTG at doses of 435 μg L^{-1} and higher [35]; at concentrations near predicted environmental levels (3 μg L^{-1}) significant VTG induction, loss of secondary sexual characteristics, and inhibition of spermatogenesis were observed for male specimens. Loss of gender-specific mating behavior and cessation of milt production were, moreover, observed at 74 μg L^{-1} and 285 μg L^{-1}, respectively [34, 35]. In females, at the same concentrations, first oogenesis was inhibited, followed by cessation of egg production and release of mature oocytes.

Effects on algae have also been assessed. In a test on inhibition of reproduction of the green alga *Scenedesmus vacuolatus* OC and 4MBC had no activity whereas EHMC, BP3, and OD-PABA at predicted no-effects concentrations (PNEC; calculated from EC_{50} with a safety factor of 1000) in the range 0.17–0.76 μg L^{-1} were found to significantly inhibit algal growth [36]. In a similar study, exposure to BP3, 3BC, 4MBC, and EHMC resulted in inhibition of the growth of *Desmodesmus suspicatus*, with 72-h EC10 values in the range 0.21–0.56 mg L^{-1} [37]. Potential endocrine and toxic effects of BM-DBM, EHMC, and OC on infaunal and epibenthic invertebrates and zebra fish (*Danio rerio*) embryos was recently investigated by the same authors [38]. Test results revealed toxicity of these sunscreens was low (Table 3), with effect concentrations far higher than those reported in the environment. In particular, EHMC was found to have a toxic reproductive effect on *Potamopyrgus antipodarum* and on *Melanoides tuberculata* with no-observed effect concentrations (NOECs) of 0.08 mg kg^{-1} and 2 mg kg^{-1}, respectively; it also had sub-lethal effects on zebra fish with even higher NOEC, 100 mg kg^{-1}. Toxic effects on reproduction of the crustaceans *Daphnia magna* [19] and *Acartia tonsa* [39] have also been reported for BP1, BP3, and BP4.

In an early study by Donavaro and Corinaldesi [40] the affect of sunscreen agents on marine ecosystems was also demonstrated. These authors observed that UV filters increased virus production via prophage induction in marine bacterioplankton. Most recently the same authors also provided scientific evidence of the effect of these chemicals on hard corals and their symbiotic algae in the Celebes Sea, the Caribbean Sea, the Andaman Sea, and the Red Sea, by inducing rapid and complete coral bleaching even at extremely low concentrations [41]. Coral bleaching, the loss of intracellular endosymbionts (symbiodinium, also known as zooxanthellae, which impart specific colors, depending on the particular clade) as a result of expulsion under stress situations, has a negative effect on biodiversity and functioning of the great reef ecosystems of tropical seas.

Despite studies are mainly focused on solely one chemical, an organism is exposed not to single environmental chemicals but to mixtures of many. According to the literature, assessment of the effects of mixtures of chemicals has attracted increasing attention in recent decades. With regard to UV filters additive effects of mixtures are largely unknown, and are an important concern in environmental studies, because these substances are usually formulated as complex mixtures to achieve the high sun protection factors (SPF) currently demanded. Taking into account the large number of sunscreens used, and other endocrine-disrupting compounds, hormonally-active UV filters, may act additively. Indeed, cumulative interactions have been shown in a few studies [34, 42–44]; in particular, these papers report significant synergistic effects of combinations of UV filters mixed at NOECs of the individual compounds.

The lack of environmental occurrence and ecotoxicological data for most UV filters and matrices hinders reliable and integral environmental risk assessment for comprehensive protection of the environment. Moreover, for complete risk assessment metabolites produced by the organisms should also be considered and their prevalence and their ecotoxicity be. Nevertheless, preliminary ERA has recently been conducted by Fent et al. [34, 45] using the limited data available. According to calculated hazard quotients, a potential risk to aquatic ecosystems may be posed by 3BC, 4MBC, and EHMC.

Conclusions and future research perspectives

The biomonitoring data so far available have been provided by a limited number of research groups. Different sample characteristics, for example location, species, season, tissue, target analytes, and the analytical methods used, hinders comparison among studies, which in turn also hinders

Table 3 Summary of toxicity data available in the literature for UV filters

UV filter	Fish LOEC (mg L^{-1})	Aquatic invertebrates EC_{50} (mg L^{-1})	Algae EC_{50} (mg L^{-1})	Ref.
BP1	–	0.49–1.5[a] (AT)	–	[38]
	0.005[f]	–	–	[34]
BP2	0.001[f]	–	–	[34]
BP3	–	1.67 (DM)	–	[37]
	–	1.9 (DM)	–	[45]
	0.75[d]	–	–	[30]
	0.62[e]	–	–	[30]
	–	–	0.36 (EV)	[36]
BP4	–	50 (DM)	–	[45]
EHMC	–	0.57 (DM)	–	[37]
	–	–	0.19 (EV)	[36]
	–	0.29 (DM)	–	[45]
	9.87[e]	–	–	[32]
3BC	–	3.61 (DM)	–	[37]
	–	26.9–5.95[b,c] (LV)	–	[48]
	0.003[f]	–	–	[34]
4MBC	–	0.80 (DM)	–	[37]
	–	4.6[b] (PA)	–	[46]
	–	0.56 (DM)	–	[45]
	9.9[e]	–	–	[32]
IAMC	–	–	0.76 (EV)	[36]
OD-PABA	–	–	0.17 (EV)	[36]
Et-PABA	0.004[f]	–	–	[34]

[a]Under different experimental conditions

[b]μmol L^{-1}

[c]Different endpoints

[d]Vitellogenin induction in rainbow trout

[e]Vitellogenin induction in medaka

[f]Vitellogenin induction in fathead minnow

DM, *Daphnia magna*; AT, *Acartia tonsa*; EV, *Scenedesmus vacuolatus*; LV, *Lumbriculus variegatus*; PA, *Potamopyrgus antipodarum*; LOEC, lowest observed effect concentration

reliable assessment of the fate and effects of UV-absorbing compounds in aquatic ecosystems. Expression of the data in different units (body weight, lipids-basis) should be standardized, enabling comparison among similar studies. Researchers have used quite different analytical approaches, although most are based on solvent extraction, GPC clean-up, and analysis by GC–MS. The sensitivity and selectivity afforded are suitable for environmental trace analysis and recovery is also very good, with values close to 100 % when the lipid content of tissues is not high. Fish and, specifically, muscle has been the preferred sample for analysis, despite results which seem to indicate that individual compounds are selectively accumulated in muscle or offal, depending on the species.

Besides smart experimental design, for accurate comparison of contamination levels among different tissues, species, and locations, reporting of range and median values may be quite useful. Moreover, reporting of sunscreen concentrations in a specific organ, instead of a whole burden estimate approach, may lead to overestimation of BMFs and TMFs. Stable isotope analysis should be conducted to properly identify the trophic position of every species for further biomagnification considerations.

Ecotoxicological assessment of exposure to UV filters is a challenging task. Despite being scattered and limited, current ecotoxicological data indicate that the potential risk posed by these widely used chemicals requires further investigation. The estrogenic activity of most of the commonly used sunscreen agents is in the range of other well-characterized estrogenic chemicals. Findings indicate that some UV filters have endocrine-disrupting activity in, and/or affect reproduction of several species, although at concentrations higher than those measured in the environment. However, a propensity for rapid accumulation and temporal effects at environmentally relevant concentrations and the potential of mixture effects indicate the need for further studies to evaluate the effects of long-term exposure of biota to UV filters. Moreover, ecotoxicological studies indicate the need to consider multicomponent mixtures when evaluating hormonal activity of UV filters in aquatic organisms, for use in risk assessment to consider potential synergistic and/or antagonistic effects.

There should be greater emphasis on measurement of ecological, biological, and physicochemical variables in field studies conducted to analyze contaminants in species, and, more importantly, when comparing data between studies. In particular, the chemical characteristics of the UV filters should be considered on the basis of their different structural forms, including isomers and enantiomers. Preliminary findings indicate that stereochemical aspects of sunscreens should be included in future environmental and

toxicological research for proper characterization of their global prevalence in the environment and for elucidation of the processes of biodegradation of theses contaminants, because these properties may result in different potential for accumulation, as observed for other contaminants (perfluorinated compounds, halogenated flame retardants, etc).

Marine and, particularly, terrestrial environments should be more widely investigated in future studies to better understand the fate and effects of UV filters. The effect of climate change should also be considered, because seasonal changes in ice formation, temperature, drought–flood episodes, or food webs might have important effects on bioaccumulation and/or biomagnification of contaminants. For UV filters this is especially relevant, because higher levels of sunlight radiation, a consequence of increased depletion of the ozone layer, would increase the use of such chemicals.

Combining monitoring field studies with work on species biology, behavioral science, and exposure biomarkers, among others, would significantly contribute to improving our knowledge about these compounds.

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CAPÍTULO II

DESARROLLO DE METODOLOGÍAS ANALÍTICAS Y EVALUACIÓN DE LA PRESENCIA DE FILTROS SOLARES EN EL MEDIO AMBIENTE

Desarrollo de metodologías analíticas y evaluación de la presencia de filtros solares en el medio ambiente

En el Capítulo I se describe la problemática ambiental que representan los UV-F, el constante incremento en el uso de estos productos que conlleva una presencia creciente en el ecosistema acuático. Uno de los mayores problemas a día de hoy es la falta de métodos analíticos suficientemente sensibles y precisos para el análisis de estos compuestos y sus productos de transformación en el sistema agua/sedimento/biota, así como en otras matrices relevantes y complejas como aguas residuales y lodos de EDAR. El análisis de UV-F en todas estas matrices es de gran importancia para tener un conocimiento profundo sobre la problemática de esta familia de contaminantes emergentes y los posibles riesgos ambientales que puede comportar su presencia en el ecosistema acuático.

En este capítulo se presentan y discuten las metodologías analíticas desarrolladas para el análisis de UV-F y algunos de sus productos de transformación en diferentes matrices ambientales, así como nuevos datos sobre su presencia medioambiental.

Las metodologías desarrolladas se basan en el análisis por LC-MS/MS, que permite obtener una gran sensibilidad y selectividad, lo cual es básico para el tipo de muestras que se pretenden analizar. Se ha optado por LC en lugar de GC ya que permite analizar un intervalo muy amplio de compuestos, con propiedades fisicoquímicas muy diferentes, como ocurre con la familia de los UV-F y sus productos de transformación, sin necesidad de etapas previas de derivatización.

Objetivos

El principal objetivo de este capítulo fue desarrollar metodologías analíticas sensibles, selectivas, precisas y robustas para determinar UV-F en diferentes matrices ambientales. Así, se decidió desarrollar un método *on line,* SPE-LC-MS/MS, para el análisis de estos compuestos en aguas subterráneas, aguas superficiales y aguas residuales. Hasta la fecha no había desarrollada ninguna metodología *on-line* para estos compuestos. La automatización presenta la ventaja de minimizar la manipulación de las muestras, lo que afecta positivamente a la precisión y repetibilidad. También reduce muy

significativamente la carga de trabajo y por tanto el tiempo de análisis. Así mismo, se han desarrollado métodos de análisis para matrices sólidas tales como sedimentos, lodos de EDAR y biota. En este caso las extracciones se basan en PLE, llevando a cabo un proceso de purificación en la misma celda de extracción.

El desarrollo de estas metodologías incluye la optimización de las condiciones experimentales de detección mediante espectrometría de masas, tanto para la ionización de los analitos (UV-F y productos de transformación), como para su fragmentación y seguimiento en modo *monitorización de reacciones seleccionadas* (*selected reaction monitoring*, SRM). Es necesario también optimizar la separación cromatográfica, así como el pretratamiento de las muestras sólidas (mediante PLE) y de las muestras acuosas (mediante SPE). Todas las metodologías desarrolladas han sido validadas en términos de linealidad, precisión, sensibilidad y exactitud. También se ha evaluado el efecto matriz, de gran importancia en matrices tan complejas como las analizadas cuando se trabaja con ionización mediante electrospray (ESI).

Otro de los objetivos de los trabajos recogidos en este capítulo fue aportar datos sobre la presencia de estos compuestos en las matrices ambientales, poco estudiadas en relación a estos compuestos. De esta forma se quiso contribuir al conocimiento del estado actual de la presencia de estos compuestos en el ecosistema acuático, así como también evaluar la efectividad de los procesos de depuración que se llevan a cabo en las EDARs. Hasta la fecha no hay prácticamente estudios en lodos de EDARs, principalmente debido a la complejidad de esta matriz. Ha sido especialmente interesante proporcionar nuevos datos sobre su presencia en biota, ya que hasta el momento no se dispone de información suficiente sobre su bioacumulación y posible biomagnificación.

Estructura

El presente capítulo incluye cuatro publicaciones científicas que describen el desarrollo y validación de metodologías analíticas para el análisis de UV-F en aguas naturales y residuales, sedimentos, lodos de EDAR y organismos acuáticos. En cada uno de estos estudios los métodos son aplicados en muestras reales para evaluar la presencia de estos compuestos en el medio ambiente.

Publicación ciéntífica #3: Fully automated determination of nine UV-Filters and transformation products in natural waters and wastewaters by on-line solid phase extraction-liquid chromatography-tandem mass spectrometry (Journal of Chromatography A (2013) En prensa DOI:10.1016/j.chroma.2013.04.037).

Publicación ciéntífica #4: Occurrence of multiclass UV-Filters in treated sewage sludge from wastewater treatments plants (Chemosphere (2011) 84:1158-1165).

Publicación ciéntífica #5: Fast pressurized liquid extraction with in-cell purification and analysis by liquid chromatography-tandem mass spectrometry for the determination of UV-Filters and their degradation products in sediments (Analytical and Bioanalytical Chemistry (2011) 400:2195-2204).

Publicación ciéntífica #6: Multi-residue method for trace level determination of UV-Filters in fish based on pressurized liquid extraction and liquid chromatography-quadrupole-linear ion trap-mass spectrometry (Journal of Chromatography A (2013) 1286:93-101).

Publicación ciéntífica #7: First determination of UV-Filtres in marine mammals. Octocrylene levels in Franciscana dolphins (Environmental Science & Technology (2013) Aceptado).

A continuación se discuten los aspectos más destacados de las metodologías desarrolladas, así como posibles tendencias futuras en el análisis de estos compuestos en los ecosistemas acuáticos. Posteriormente se comentan los resultados obtenidos en los estudios de vigilancia ambiental y sus implicaciones. Se hace especial hincapié en el estudio de la bioacumulación en peces y se evalúa la presencia del octocrileno, el UV-F con más presencia ambiental, en delfines.

2.1. ANÁLISIS DE FILTROS UV Y PRODUCTOS DE TRANSFORMACIÓN EN MUESTRAS ACUOSAS

Publicación científica #3:

"Fully automated determination of nine UV-Filters and transformation products in natural waters and wastewaters by on-line solid phase extraction-liquid chromatography-tandem mass spectrometry"

por:

Pablo Gago-Ferrero, Nicola Mastroianni, M. Silvia Díaz-Cruz, Damià Barceló

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Fully automated determination of nine ultraviolet filters and transformation products in natural waters and wastewaters by on-line solid phase extraction–liquid chromatography–tandem mass spectrometry

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ABSTRACT

This paper describes the development and validation of the first fully automated method, based on on-line solid phase extraction–liquid chromatography–tandem mass spectrometry (on line-SPE–LC–MS/MS), for the determination of UV filters (UV F) and transformation products, with a wide range of physicochemical properties. The developed method was validated and applied to the analysis of the selected compounds in river water (RW), groundwater (GW), influent and effluent wastewater (IWW and EWW). Comparing with the off-line methodologies published so far the presented method provides several advantages including a shorter extraction time, lower sample volume and minimum manipulation. Low limits of detection were achieved for all the studied matrices, <3 ng/L (GW), <3.5 ng/L (RW), <4 ng/L (EWW) and <10 ng/L (IWW), allowing a reliable and accurate quantification of the analytes at trace level. SPE optimization and critical aspects associated with the trace level determination of the target compounds (*e.g.* matrix effects) have been also considered and discussed. The method was successfully applied to the analysis of the UV F in five real water samples of each considered matrix, being the first time that these compounds were determined in GW.

1. Introduction

UV filters (UV F, sunscreens) constitute an heterogeneous group of chemicals used to protect against the harmful effects of UV solar radiation. They are widely used in a wide variety of personal care products such as lotions, shampoos or sunscreens. They are extensively used as additives in polymeric materials that have to be protected from sunlight-initiated disruption, in pharmaceutical or vehicle maintenance products, and other industrial goods. Over the last few decades, UV F have been increasingly used. These emerging pollutants are considered as persistent pollutants because they are continuously released into the aquatic ecosystems through two pathways; direct inputs as consequence of recreational water activities, and mainly, indirect inputs from wastewater treatment plants (WWTPs) as result of the domestic use of personal care products, as well as industrial waste.

The presence of UV F was determined in different water matrices as swimming pool waters [1,2] open bathing waters (lakes) [3,4], surface waters [2,5–9], wastewaters [3,4,6,8–12], seawater [9,13] and drinking water [14]. Relevant concentrations of these compounds have also been found in other environmental matrices as sediments [7,15], sewage sludge [16–18] or biota [19], indicating that bioaccumulation of UV F is a fact. Besides, Fent et al. [5] suggested that biomagnification may occur through the food web.

Several studies have revealed their potential toxicity and endocrine disrupting capacity, including benzophenone-1 (BP1), benzophenone-2 (BP2), benzophenone-3 (BP3), benzophenone-4 (BP4), 4,4′-dihydroxybenzophenone (4DHB), 4-methylbenzylidene camphor (4MBC) and ethyl-PABA (Et-PABA) [20–23]. Dermal and oral administration of BP3 to rats and mice has shown alterations in liver, kidney, and reproductive organs [21]; BP2 showed adverse effects on fecundity and reproduction [24] and mixtures of UV F showed synergistic interactions *in vitro* [25] and antagonistic activity *in vivo* [26]. Recently, a study performed by Kunisue et al. [27] indicates that exposure to high levels of benzophenone type UV F compounds may be associated with oestrogen-dependent diseases such as endometriosis in women.

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So far, most of the developed analytical methods for the analysis of UV F in waters were based on gas chromatography–mass spectrometry (GC–MS) after a preconcentration step, mainly by solid phase extraction (SPE) but also with other methodologies such as liquid–liquid extraction, solid phase microextraction or stir-bar sorptive extraction [2–4,6,7,9,10,20,28–31]. The main drawback of GC–MS is that this technique is limited to those substances that are volatile or can be derivatized (with the consequent inconveniences in precision and accuracy, and long sample preparation time). To perform the simultaneous analysis of several UV F and their transformation products, with a wide range of physicochemical properties, liquid chromatography coupled to tandem-mass spectrometry (LC–MS/MS) is the technique of choice. The state of the art of LC–MS/MS applied to the analysis of UV F in the environment has been recently reviewed by Gago-Ferrero et al. [32].

Fully automated methodologies based on LC–MS/MS, which have been proved to be a powerful tool for the trace determination of other families of organic emerging pollutants in water, such as pharmaceuticals or drugs of abuse [33,34], have not been developed so far for the analysis of UV F. Fully automated methods offer several advantages: the time of the overall analysis is shortened, the sample manipulation is minimized, and the sample volume required is extremely reduced, just a few millilitres are required against the higher volumes required for the off-line procedure (typically from 100 to 500 mL). Despite the low sample volume extracted sensitivity is not compromised but enhanced, because the whole sample, instead of an aliquot of the final extract, enters the LC–MS/MS system.

The aim of this work was to develop and validate a highly sensitive automated multi-residue method based on on-line SPE–LC–MS/MS for the analysis of nine UV filters with a wide range of physicochemical properties, namely benzophenone derivatives (BP1, BP2, BP3, BP4, 4-Hydroxybenzophenone (4HB), 4DHB and 2,2′-dihydroxy-4-methoxybenzophenone (DHMB)), 4MBC, and Et-PABA in waters including river water (RW), groundwater (GW), effluent wastewater (EWW) and influent wastewater (IWW).

The suitability of the proposed method was tested through the trace determination of selected UV F in real samples of the four studied matrices. UV F residues were observed in high concentration in wastewaters, but also at significant levels in RW and GW, constituting the first evidence of the presence of these compounds in GW.

2. Materials and methods

2.1. Standards and reagents

Structures and CAS numbers of the target compounds are summarized in Table 1.

BP3, BP1, 4-hydroxybenzophenone (4HB), BP4, 4DHB and Et-PABA, were of the highest purity (>99%) and were obtained from Sigma–Aldrich (Steinheim, Germany); 4MBC (99% purity) was supplied by Dr. Ehrenstorfer (Augsburg, Germany); and benzophenone-2 (BP2) and 2,2′-dihydroxy-4-methoxybenzophenone (DHMB) (99%) by Merck (Darmstadt, Germany). The isotopically labelled compounds 2-hydroxy-4-methoxy-2′,3′,4′,5′,6′-d_5 (BP3-d_5) and 3-(4-methylbenzylidene-d_4)camphor, used as internal standards (>99%), were obtained from CDN isotopes (Quebec, Canada). Methanol (MeOH), acetonitrile (ACN) and HPLC grade water (Lichrosolv), as well as formic acid (98% purity) were provided by Merk. N_2 and Ar purchased from Air Liquide (Barcelona, Spain) were of 99.995% purity. Glass fibre filters (1 μm) and nylon membrane filters (0.45 μm) were obtained from Whatman International Ltd (Maidstone, England).

Individual stock standard solutions as well as the isotopically labelled internal stock standard solution were prepared on a weight basis in MeOH at 200 mg/L. The solutions were stored in the dark at −20 °C. A mixture standard solution at 20 mg/L in MeOH of each compound was prepared weekly. Working solutions were prepared daily by appropriate dilution of the mixture stock standard solution in MeOH.

2.2. On-line trace enrichment

Preconcentration of the samples and its chromatographic separation was performed using an automated on-line SPE–LC instrument Symbiosis™ Pico from Spark Holland (Emmen, The Netherlands). The base of the Symbiosis™ Pico system is a high-end HPLC system with a high performance injector that handles sample volumes from 10 μL up to 5 mL fully automated. This equipment also counts with the AliasTM autosampler that includes positive headspace pressure, extensive wash routines for minimal carry over and 2 injection modes, off-line and on-line SPE. Off-line mode was only used in the optimization procedure to assess the recovery by comparing the peak areas obtained in the on-line analyses of spiked water samples with those obtained from the injection of standard mixtures of the analytes in the mixture ACN/HPLC water (25:75, v/v) at equivalent concentrations.

On-line SPE preconcentration of all samples (previously filtered), aqueous standard solutions and blanks was performed by loading 5 mL of the corresponding solutions at 1 mL/min through a PLRP-s cartridge previously conditioned with 1 mL of MeOH, 1 mL of ACN and 1 mL of HPLC water (flow rate 5 mL/min). After sample loading and prior to elution, the cartridges are washed with 0.5 mL of HPLC water at a flow rate of 5 mL/min to complete transfer of the sample and remove interferences such as inorganic salts.

After completion of each SPE sampling load, which takes place in the left clamp of the Symbiosis Pico system, the cartridge is moved to the right clamp where the trapped analytes are eluted to the LC column with the chromatographic mobile phase. Meanwhile, a new cartridge is placed in the left clamp, where preconcentration of the next sample in a sequence is simultaneously performed. A scheme of the function of the on-line SPE system is included in the Supporting Information. This kind of configuration allows short cycle times, which in our approach are 20 min, the duration of the chromatographic run time.

2.3. LC–ESI-(QqLIT) MS/MS analysis

Analyses were performed by liquid chromatography–tandem mass spectrometry using a 4000 Q TRAP™ MS/MS system from Applied Biosystems-Sciex (Foster City, CA, USA). The chromatographic separation was achieved on a Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 5 μm) from Merck, preceded by a guard column of the same packaging material. In the optimized method, elution of the trapped analytes to the LC system was performed with the chromatographic gradient. For the analyses under positive ionization (PI) mode the mobile phase consisted of a mixture of HPLC grade water and ACN, both with 0.1% formic acid. In the negative ionization (NI) mode, the mobile phase consisted of HPLC grade water containing 5 mM of ammonium acetate (pH 6.8). The adopted elution gradient started with 5% of ACN, increasing to 75% in 7 min, and then to 100% in the following 3 min. Pure organic conditions were kept constant for 5 min and finally initial conditions were reached in the next 2 min. The total run time for each injection was 23 min. The mobile phase flow rate was set to 0.3 mL/min.

MS/MS detection was performed in PI and NI electrospray (ESI) ionization mode under selected reaction monitoring (SRM) mode. Two major characteristic fragments of the precursor molecular

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Table 1
Selected UV filters, experimental conditions used in their HPLC–ESI-MS/MS determination and proposed product ions formed.

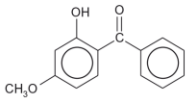
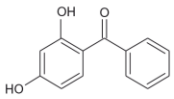
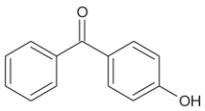
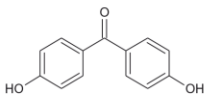
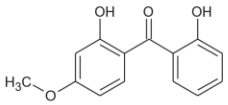
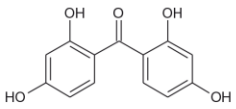
Target compound (INCI nomenclature)[a]	CAS number	Chemical structure	Precursor ion	Retention time (min)	Internal Standard	Transition	Cone voltage (V)	Collision energy (eV)	Proposed product ion
Benzophenone 3 (BP3)	131-57-7		$[M+H]^+$	9.51	BP3-d_5	229 > 151 229 > 105	40	25 27	$[M-C_6H_5]^+$ $[C_6H_5C{=}O]^+$
2,4-Dihydroxybenzophenone (BP1)	131-56-6		$[M+H]^+$	7.70	BP3-d_5	215 > 137 215 > 105	40	27 29	$[M-C_6H_5]^+$ $[C_6H_5C{=}O]^+$
4-Hydroxybenzophenone (4HB)	1137-42-4		$[M+H]^+$	7.12	BP3-d_5	199 > 121 199 > 105	40	25 27	$[M-C_6H_5]^+$ $[C_6H_5C{=}O]^+$
4,4′-Dihydroxybenzophenone (4DHB)	611-99-4		$[M+H]^+$	5.67	BP3-d_5	215 > 121 215 > 93	45	27 45	$[M-C_6H_5OH]^+$ $[C_6H_4OH]^+$
2,2′-Dihydroxy-4-methoxybenzophenone (DHMB)	131-53-3		$[M+H]^+$	8.31	BP3-d_5	245 > 151 245 > 121	40	27 29	$[M-C_6H_5OH]^+$ $[C_6H_4(OH)C{=}O]^+$
Benzophenone-2 (BP2)	131-55-5		$[M+H]^+$	6.12	BP3-d_5	247 > 137 247 > 109	46	25 45	$[M-C_6H_4(OH)_2]^+$ $[C_6H_3(OH)_2]^+$

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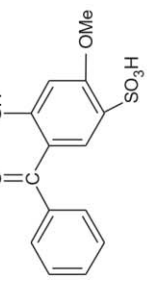
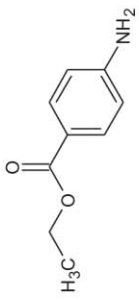
Table 1 (*Continued*)

Target compound (INCI nomenclature)[a]	CAS number	Chemical structure	Precursor ion	Retention time (min)	Internal Standard	Transition	Cone voltage (V)	Collision energy (eV)	Proposed product ion
Benzophenone-4 (BP4)	4065-45-6		$[M+H]^-$	4.98	BP3-d_5	307 > 227 307 > 211	−80	−32 −32	$[M-H-SO_3]^-$ $[M-H-SO_3-CH_4]^-$
4-Methylbenzylidene camphor (4MBC)	36861-47-9		$[M+H]^+$	11.01	4MBC-d_4	255 > 212 255 > 105	61	29 41	$[M+H-C_3H_7]^{\bullet+}$ $[MeC_6H_4CH_2]^+$
Ethyl-PABA (Et-PABA)	94-09-7		$[M+H]^+$	6.51	BP3-d_5	166 > 138 166 > 120	41	20 25	$[M+H-C_2H_4]^+$ $[M+H-C_2H_5OH]^+$

[a] INCI (International Nomenclature for Cosmetic Ingredient) elaborated by CTFA and Cosmetics Europe (former COLIPA).

ion ($[M+H]^+$ or $[M-H]^-$) were monitored per analyte to enhance method sensitivity and selectivity. The most abundant transition was used for quantification, whereas the second most abundant was used for confirmation. Fragmentation voltage and collision energy were optimized for each transition. For the PI mode, ESI conditions were obtained as a compromise using the optimum values for most compounds. Optimum conditions were: capillary voltage, 5000 V; source temperature, 700 °C; curtain gas, 30 psi; ion source gas 1, 50 psi, ion source gas 2, 60 psi; entrance potential 10 V. For the NI mode, ESI condition were as follows: capillary voltage, −4000 V; source temperature 500 °C, curtain gas 20 psi, ion source gas 1, 50 psi, ion source gas 2, 60 psi; entrance potential −10 V.

This procedure was in compliance with the European Council Directive 2002/657/EC, that although it was initially conceived for food residue analysis, it has been accepted by the scientific community for environmental analysis. Chromatographic retention times (t_R), SRM transitions, cone voltages, collision energies and the proposed ions for the transitions are shown in Table 1. Instrument control and data acquisition and evaluation were performed with Analyst 1.4.2 software from Applied Biosystems/MDS Sciex and the Symbiosis from the Symbiosis Pico for Analyst software.

2.4. Sample collection

All samples were taken in amber glass bottles, vacuum filtered through 1 μm glass fibre filters, followed by 0.45 μm nylon membrane filters, and stored in the dark at −20 °C.

Five river waters were collected from the Llobregat River (NE Spain) in October 2011, Samples LLO1, LLO2, and LLO3 correspond to the upper part of the river while LLO4 and LLO5 to the lower river. Five GW were collected from different sites in the Barcelona metropolitan area in May 2011. Twenty-four-hour composite water samples were taken from influents and effluents of five WWTPs in Catalonia (NE, Spain) in October 2011. These plants received different amounts of wastewater with flow rates varying from 21.103 to 267.103 m^3/d and corresponding to population from 154.103 to 1.142.103 equivalents inhabitants. Selected WWTPs operate with conventional secondary treatment using activated sludge (CAS) with the exception of WWTP1 and WWTP2 that apply also a tertiary treatment.

3. Results and discussion

3.1. Sample preparation and preservation

Background contamination is a common problem in the determination of UV filters at environmental levels. Therefore, several measures were taken in order to prevent this problem. All glassware used was previously washed and heated overnight at 380 °C, and further sequentially rinsed with a collection of organic solvents and HPLC grade water, and immediately used. Furthermore, gloves were worn during sample preparation; separate solvents and only previously unopened packages of solvents, chemicals and other supplies, and glassware were used. Since many of the compounds analyzed undergo photodegradation stock standard solutions and samples were always covered with aluminium foil and stored in the dark.

In this fully automated methodology, sample handling is limited to the filtration step, performed to remove the particulate matter present in the water samples, and the addition of the internal standard mixture. Samples are stored in the freeze at −20 °C, halting any biological activity and avoiding the risk of contamination or alteration of the sample nature due to the addition of preserving agents until analysis.

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3.2. Optimization of LC–MS/MS conditions

LC separation was based on the previously developed method by Gago-Ferrero et al. [35] for the determination of UV F in fish samples. As regards MS/MS detection, for the PI mode, ACN and HPLC water, both with 0.1% formic acid, were used as mobile phase. The formic acid significantly improved the peak shape of most of the analytes, in particular BP3 and its transformation products. Due to the strong acid character of BP4 its analysis could only be performed in NI mode. This compound was undetectable under acidic conditions, however, they were strictly necessary in order to obtain good peak shapes and sufficient sensitivity for most of the rest of compounds. Taking into account the relevance of BP4, which is one of the most used UV F and the most present by far in water samples, justify the individual analysis of this compound. Reproducible results in term of retention time were obtained by the use of ammonium acetate (5 mM, pH 6.8).

The optimized values of the MS/MS operational parameters for the target compounds and the proposed product ions are summarized in Table 1. The optimization for BP3, BP1, 4HB, 4DHB and 4MBC were recently performed by Gago-Ferrero et al. [35]. For the new compounds, MS/MS parameters were optimized by using UV F individual standard solutions at 0.5 mg/L. DHMB followed the typical pattern of benzophenone transformation products. The collision-induced dissociation (CID) occurred through the loss of 94 Da, which corresponds to the $[M+H]^+ \rightarrow [M-C_6H_5OH]^+$ transition. The complementary fragment of the molecule, $[C_6H_4(OH)C{=}O]^+$, was also observed and was selected as confirmation transition. A similar fragmentation pathway was found for BP2. In this case there was a loss of 110 Da corresponding to the transition $[M+H]^+ \rightarrow [M-C_6H_4(OH)_2]^+$. Being BP2 a symmetric compound with two hydroxyl groups on each side of the ketone, we selected for confirming the transition 247 → 109 ($[C_6H_3(OH)_2]^+$).

Et-PABA was ionized at the ester group and the CID occurred through the loss of the alkane leaving the charge at the carboxylic group ($[M+H-C_2H_4]^+$). Subsequently the formed cation lost OH leaving the positive charge in the keto group ($[M+H-C_2H_5OH]^+$).

BP4 is an aromatic sulfonic acid, with very strong acidic character and solely can be ionized under NI mode [8]. CID fragmentation is dominated by the loss of neutral SO_3 ($[M-H-SO_3]^-$, $[M-H-SO_3-CH_4]^-$).

Data acquisition was performed in SRM mode, and different collision energies were tested to obtain the optimum response. Two transitions per compound (including internal standards), for quantification and confirmation, were selected.

3.3. SPE optimization

Sample preconcentration and purification of the target analytes are critical and contribute most significantly to the final performance of the analytical method. Four different disposable trace enrichment cartridges were evaluated for their efficiency in the on-line SPE of the target UV F from water: Oasis HLB (macroporous copolymer of divinylbenzene and N-vinylpyrrolidone, 30 μm particle size) from Waters Corporation (Milford, MA, USA), PLRP-s (cross-linked styrenedivinylbenzene polymer, 15–25 μm particle size) from Spark Holland (Emmen, The Netherlands), HySphere Resin GP (polydivinyl-benzene, 5–15 μm particle size) from Spark Holland and Hysphere C18 EC (endcapped octadecyl phase, 8 μm particle size,) also from Spark Holland.

In order to evaluate which of these four cartridges yielded higher recoveries of target compounds, GW water was spiked with 100 ng/L of each target compound. The extraction efficiency was estimated by comparing the peak areas obtained for each target analyte (blank subtracted) when loading 5 mL of the spiked GW solution ($n = 3$) with the peak areas of unextracted standards at the same concentration (off-line analysis, no SPE step). As it can be observed in Fig. 1, absolute recoveries higher than 50% were obtained for all the studied compounds with PLRP-s cartridges. Hysphere Resi GP provided comparatively low recovery rate for Et-PABA, whereas the use of Oasis HLB cartridge negatively affected peak shape and chromatographic separation of BP2. The C18 cartridge showed the best recovery efficiency for 4MBC, the most lipophilic compound, but lower recovery rates for other compounds, in particular BP3, BP1 and BP4. Besides the efficiency of the extraction, the peak shape, affecting limit of detection and quantification accuracy, was also a criteria for the selection. Based on the obtained results, PLRP-s was selected for further optimization.

In comparison with off-line extraction procedures, where sample volumes are typically about 100 mL, the present on-line method requires smaller volumes since the whole extract is introduced into the analytical column. In this study three sample volumes were tested (1, 2.5 and 5 mL). A sample volume of 5 mL was selected as the optimum as a compromise between sensitivity and matrix effects. Small sample volumes may decrease the sensitivity but large sample volumes may also affect the method sensitivity due to ionization suppression effects. In the case of IWW matrix, the sample was diluted three times with HPLC grade water in order to minimize matrix effect. In this case, the highest sensitivity was obtained by loading 5 mL of the diluted IWW water.

3.4. Method performance

The performance of the method was evaluated under the optimized conditions in terms of linearity range, sensitivity, accuracy, repeatability, reproducibility and matrix effects.

Instrumental analytical parameters including linearity ranges, correlation coefficients (r^2), instrumental limits of detection (ILOD) and quantification (ILOQ) and inter-day and intra-day precisions expressed as relative standard deviation (RSD) are summarized in Table 2.

For all the compounds, wide linearity ranges were obtained for both the quantification and the confirmation SRM transitions. Seven to ten points' calibration curves were constructed, using least-squares linear regression analysis, from application of the whole method to 5 mL aliquots of HPLC grade water spiked with the analytes, typically from 0.5 to 500 ng/L with $r^2 > 0.9991$ for all compounds. ILOD, defined as the lowest analyte concentration with a signal to noise ratio (S/N) of 3, and ILOQ, defined as the concentration with S/N ratio of 10 and imprecision lower than 20% were evaluated by injecting 5 μL of diluted UV F solutions. ILODs ranged from 0.2 to 14 pg injected and ILOQs from 0.7 to 47 pg injected. The intra-day instrumental precision (determined by analysing seven replicates of a UV F mixture standard solution at 20 μg/L within a given day) was in the range 3–5% (RSD); and the inter-day instrumental precision (estimated by analysing seven replicates of the same solution on seven different days) showed RSD values from 5% to 7%.

The robustness of the method, despite it is not strictly required if method transfer is not intended, was not systematically tested during the development of the method. Slight changes in the temperature and/or in the modifiers' concentration of the mobile phase did not influence significantly the retention times and sensitivities. As regards the effect of the injected volume, it was discussed in the previous section. Lastly, the fact that different operators performed the validation without affecting negatively the results can also be used as indicator of the robustness of the analytical procedure.

Quantification was based on peak areas and was performed by the IS method. The corresponding or the most similar deuterated compound (in terms of chemical structure and chromatographic

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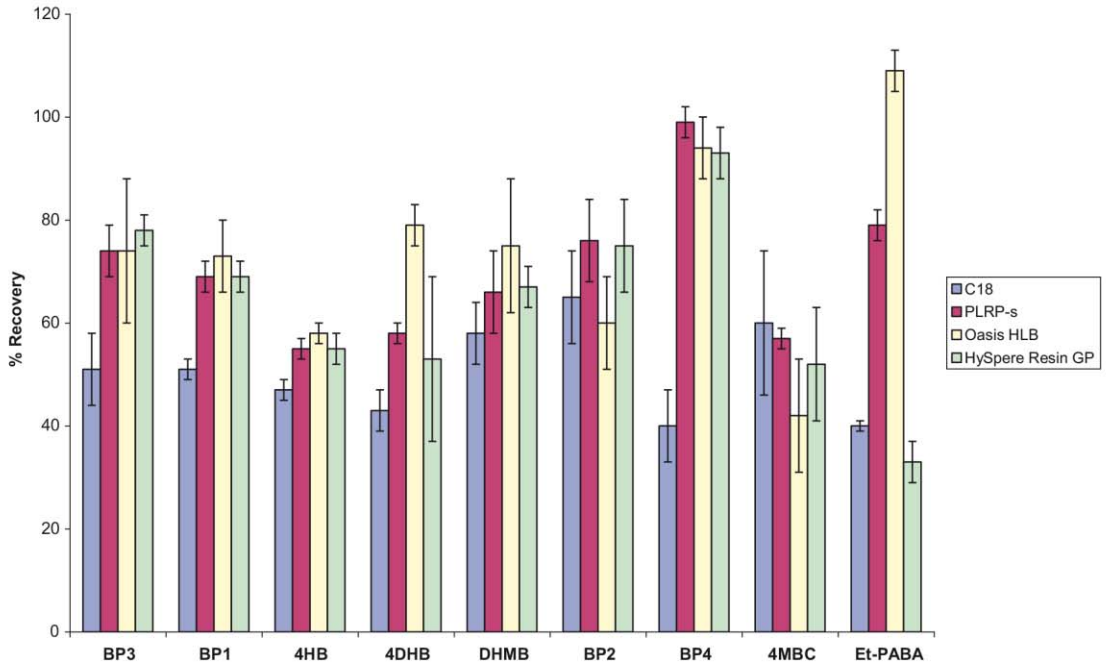

Fig. 1. Extraction efficiency for the cartridges tested.

retention time) was used as IS for each compound. The identification and confirmation criteria for the analysis of the target compounds were based on the Commission Decision 2002/657/EC. Retention times of UV F in standards and in the samples were compared at a tolerance of 2.5% and the relationship between the two transitions was compared with the relative ion intensities of UV F standards. A difference of less than 20% was considered acceptable according to the EU directive.

Sensitivity is one of the method parameters usually enhanced in on-line systems. MLODs (lowest analyte concentration with S/N ratio of 3) and MLOQs (concentration with S/N ratio of 10 and imprecision lower than 20%) were estimated in the four tested water matrices: RW, GW, IWW and EWW. Results are summarized in Table 3. In GW and RW, MLODs and MLOQs were calculated from the recovery experiments at the lowest concentration, spiked at 20 ng/L. In sewage waters, considering the high variability from sample to sample, these limits have been calculated as the average of LODs and LOQs estimated for each of the sewage waters evaluated (for positive samples). LODs and LOQs for compounds that have not been detected in these samples have been calculated also from spiked wastewater samples. As shown in Table 3, pretty low MLODs and MLOQs were achieved for all matrices. In GW and RW values are similar, in the range 0.3–1.8 ng/L (MLOD) and 1–6 ng/L (MLOQ) except for 4MBC, with values of 3–3.5 and 10–11.7 ng/L, respectively. In EWW, MLODs and MLOQs were in the range of 1–4 ng/L and 3.3–13.3 ng/L, respectively; and 5–10 and 16.7–33.3 ng/L for IWW. These higher values are a consequence of the increased suppression effect, which is especially significant in IWW.

Because of these MLODs and MLOQs values are below the reported concentrations in the literature, the developed method allowed a reliable and accurate quantification of UV F in environmental waters and wastewaters.

The precision of the method was evaluated for $n = 5$ extracts of HPLC water spiked at three different concentrations *i.e.* 20, 100 and 500 ng/L. RSDs values were always below 13%. This satisfactory repeatability is possible with automated procedures such as that described here where manipulation of the sample is minimized.

Extraction efficiency was determined for all different matrices by spiking samples ($n = 3$) at three different levels: 20, 50 and 100 ng/L for GW and RW and 20, 50 and 200 ng/L for

Table 2
Instrumental quality parameters of the HPLC-ESI-MS/MS method for the analysis of UV F.

	Linearity range (ng/L)	r^2	ILOD (pg)	ILOQ (pg)	Precision[a] (%RSD), $n = 7$	
					Intra-day	Inter-day
BP3	0.5–500	0.9998	4	13	3	5
BP1	0.5–500	0.9998	10	33	3	6
4HB	0.5–500	0.9999	6	20	4	5
4DHB	0.5–500	0.9997	14	47	3	5
DHMB	0.5–500	0.9995	8	27	4	6
BP2	1–500	0.9996	14	20	5	7
BP4	0.2–500	0.9993	4	13	5	7
4MBC	1–500	0.9995	6	20	3	6
Et-PABA	0.1–200	0.9991	0.2	0.7	5	7

[a] Injections of 100 pg.

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Table 3
Performance of the HPLC–ESI-MS/MS developed method for the analysis of UV F in water samples.

	Conc. (ng/L)	Rec. (%) ± RSD	Conc. (ng/L)	Rec. (%) ± RSD	Conc. (ng/L)	Rec. (%) ± RSD	ME (%)	MLOD (ng/L)	MLOQ (ng/L)
Groundwater									
BP3	20	104 ± 1	50	107 ± 1	100	103 ± 6	101	0.5	1.7
BP1	20	98 ± 2	50	104 ± 2	100	100 ± 6	93	1.0	3.3
4HB	20	91 ± 1	50	92 ± 3	100	89 ± 3	95	0.8	2.7
4DHB	20	90 ± 2	50	96 ± 3	100	93 ± 8	86	1.5	5.0
DHMB	20	93 ± 3	50	100 ± 4	100	99 ± 9	94	0.8	2.7
BP2	20	88 ± 1	50	94 ± 1	100	90 ± 5	91	1.0	3.3
BP4	20	110 ± 2	50	114 ± 4	100	112 ± 5	99	0.3	1.0
4MBC	20	99 ± 3	50	100 ± 3	100	99 ± 11	97	3.0	10.0
Et-PABA	20	110 ± 1	50	112 ± 1	100	109 ± 4	101	1.0	3.3
River water									
BP3	20	99 ± 3	50	100 ± 3	100	97 ± 4	83	0.7	2.3
BP1	20	104 ± 1	50	103 ± 6	100	100 ± 5	79	1.0	3.3
4HB	20	82 ± 1	50	84 ± 2	100	81 ± 6	85	1.1	3.7
4DHB	20	83 ± 2	50	82 ± 3	100	82 ± 5	65	1.8	6.0
DHMB	20	95 ± 2	50	94 ± 6	100	98 ± 5	75	1.0	3.3
BP2	20	90 ± 1	50	91 ± 4	100	91 ± 7	71	1.2	4.0
BP4	20	107 ± 3	50	110 ± 5	100	111 ± 6	89	0.5	1.7
4MBC	20	101 ± 0	50	102 ± 1	100	100 ± 8	75	3.5	11.7
Et-PABA	20	113 ± 1	50	111 ± 1	100	112 ± 1	83	1.5	5.0
Effluent wastewater									
BP3	20	111 ± 5	50	101 ± 2	200	101 ± 8	52	1.5	5.0
BP1	20	100 ± 3	50	90 ± 3	200	86 ± 8	45	2.5	8.3
4HB	20	92 ± 4	50	77 ± 5	200	77 ± 7	50	1.5	5.0
4DHB	20	80 ± 3	50	70 ± 4	200	70 ± 7	37	3.5	11.7
DHMB	20	78 ± 3	50	74 ± 7	200	75 ± 9	43	1.5	5.0
BP2	20	85 ± 1	50	84 ± 5	200	84 ± 5	48	3.0	10.0
BP4	20	111 ± 3	50	109 ± 5	200	108 ± 8	64	1.0	3.3
4MBC	20	100 ± 3	50	99 ± 4	200	103 ± 5	63	4.0	13.3
Et-PABA	20	110 ± 3	50	109 ± 3	200	105 ± 2	58	2.5	8.3
Influent wastewater									
BP3	20	96 ± 4	50	98 ± 5	200	106 ± 5	25	5.0	16.7
BP1	20	105 ± 10	50	104 ± 4	200	101 ± 12	24	8.0	26.7
4HB	20	76 ± 1	50	70 ± 2	200	71 ± 5	27	8.0	26.7
4DHB	20	88 ± 2	50	70 ± 4	200	70 ± 6	18	9.0	30.0
DHMB	20	81 ± 4	50	84 ± 5	200	87 ± 10	28	7.0	23.3
BP2	20	80 ± 2	50	74 ± 6	200	70 ± 12	24	7.0	23.3
BP4	20	105 ± 5	50	103 ± 6	200	102 ± 8	40	6.0	20.0
4MBC	20	102 ± 4	50	98 ± 2	200	99 ± 2	34	10.0	33.3
Et-PABA	20	112 ± 2	50	110 ± 6	200	110 ± 3	32	5.0	16.7

Conc: concentration; Rec: recovery; RSD: relative standard deviation; ME: matrix effects; MLOD: method limit of detection; MLOQ: method limit of quantification.

wastewaters. As spiked waters already contained target compounds, blanks (non-spiked samples) were analyzed to determine their concentrations, which were afterward subtracted from the spiked waters. Results for each matrix and for each concentration level are presented in Table 3. Relative recoveries, determined from the absolute recoveries for each compound as percentages of the absolute recoveries of the associated surrogates were between 70 and 110%, with good reproducibility (expressed as RSD, Table 3). Although only two deuterated surrogate standards were used because there were not more commercially available deuterated UV F, both were useful to correct for potential losses during extraction, as well as for matrix effects.

The repeatability of the method was also evaluated in the four aforementioned matrices, and the results obtained showed good repeatability, with RSDs values always below 15%.

ESI source is highly susceptible to other components present in the matrix, which may result in a signal suppression or enhancement. These may influence sensitivity, linearity, accuracy and precision of quantitative LC–MS/MS analysis. Natural organic matter, salts, ion-paring agents, non target contaminants have shown to be responsible for ion suppression.

The extent of matrix effects was estimated during analysis. They were determined by comparing the analytical response given by a standard in pure solvent and in real water samples at the different spike levels (after subtracting the peak areas corresponding to the native analytes present in the sample). In the absence of matrix effects, analyte peak areas should be similar in both HPLC and target waters. However, when matrix effects occur the signal intensity for the analytes can decrease (signal suppression) or increase (enhancement). Table 3 summarized the matrix effects observed for the different studied matrices. Values of 100% are obtained when no matrix effect occurs whereas values below 100% indicate ion suppression.

Results showed that matrix effects were practically inexistent in GW (86–101%) and low in RW (65–89%). However, in other more complex matrices such as EWW, a significant reduced response was observed (37–63%). The reduction in the efficiency of the ionization of the target species in the more complex matrix, IWW, varied between 18 and 40%, even when these samples have been previously diluted 3-fold. Therefore, the use of isotopically labelled compounds for I.S. quantification is nearly indispensable in order to obtain accurate results in complex matrices.

As an example, Fig. 2 shows the reconstructed ion chromatograms showing the SRM transitions for the target UV F in spiked river water at 50 ng/L.

Blank tests were carried out to rule out possible contamination from the sampling, storage or instrumentation. In order to comply with internal quality control procedures, two control spiked samples, two solvent injections and two procedural blanks were included into each experimental batch made up of six samples.

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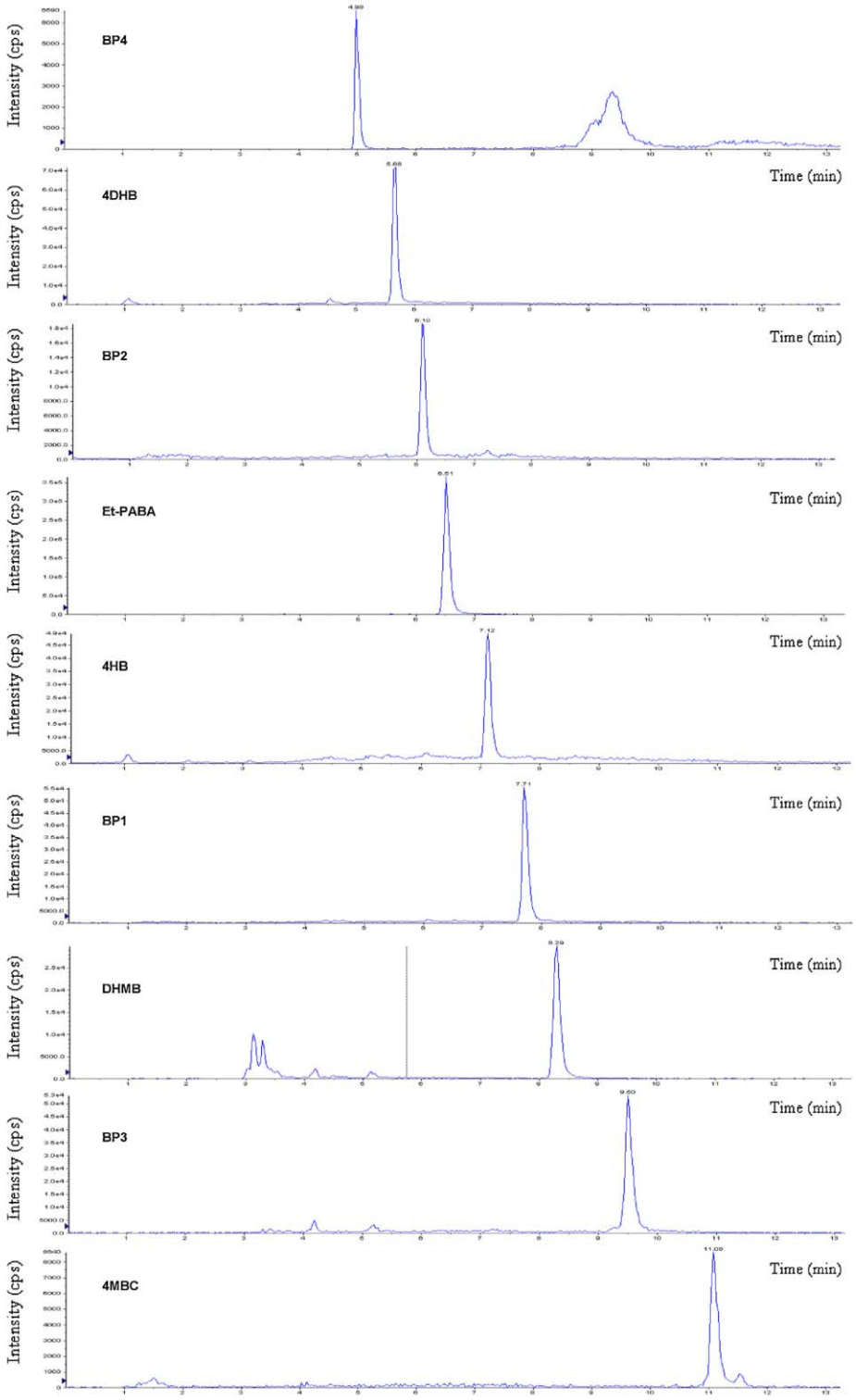

Fig. 2. Reconstructed ion chromatograms showing the SRM transitions selected for the studied UV F in a river water sample spiked at 50 ng/L.

The individual values obtained for the control solutions were plotted on a process-behaviour chart during the entire duration of the study to establish if the analysis was in a state of statistical control.

3.5. Application to real samples

The occurrence of the selected UV F in, RWs from the Llobregat river, GWs of Barcelona city and in IWWs and EWWs

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Table 4
Concentrations of UV filters in water samples (ng/L).

Sample	BP3	BP1	4HB	4DHB	DHMB	BP2	BP4	4MBC	Et-PABA
Groundwater									
GW1	34	15.6	n.d.	<LOQ	n.d.	n.d.	n.d.	<LOQ	n.d.
GW2	4.36	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GW3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	21.3	n.d.	n.d.
GW4	n.d.	19.4	n.d.	n.d.	n.d.	n.d.	36.6	n.d.	n.d.
GW5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
River water									
LLO1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	no data	n.d.	n.d.
LLO2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	62.3	n.d.	n.d.
LLO3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	30.4	12.6	n.d.
LLO4	4.84	4.22	n.d.	n.d.	n.d.	n.d.	61.6	n.d.	n.d.
LLO5	37.8	7.54	n.d.	n.d.	n.d.	n.d.	862	n.d.	n.d.
Effluent water									
EWW1	8.53	2.89	n.d.	n.d.	n.d.	n.d.	366	<LOQ	n.d.
EWW2	34	12.9	n.d.	n.d.	n.d.	n.d.	1200	n.d.	n.d.
EWW3	19.6	31.1	n.d.	n.d.	n.d.	n.d.	1420	23.8	n.d.
EWW4	7.71	n.d.	n.d.	n.d.	n.d.	n.d.	624	n.d.	n.d.
EWW5	15.6	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent water									
IWW1	75.6	222.9	n.d.	n.d.	n.d.	n.d.	738	<LOQ	17.22
IWW2	182.4	283.2	n.d.	n.d.	n.d.	n.d.	1548	48.3	36
IWW3	148.2	152.4	n.d.	n.d.	n.d.	n.d.	1329	<LOQ	120.9
IWW4	306	351	n.d.	n.d.	n.d.	n.d.	876	<LOQ	37.2
IWW5	214.6	722	n.d.	n.d.	n.d.	n.d.	912	<LOQ	31.35

n.d.: not detected; LOQ: limit of quantification.

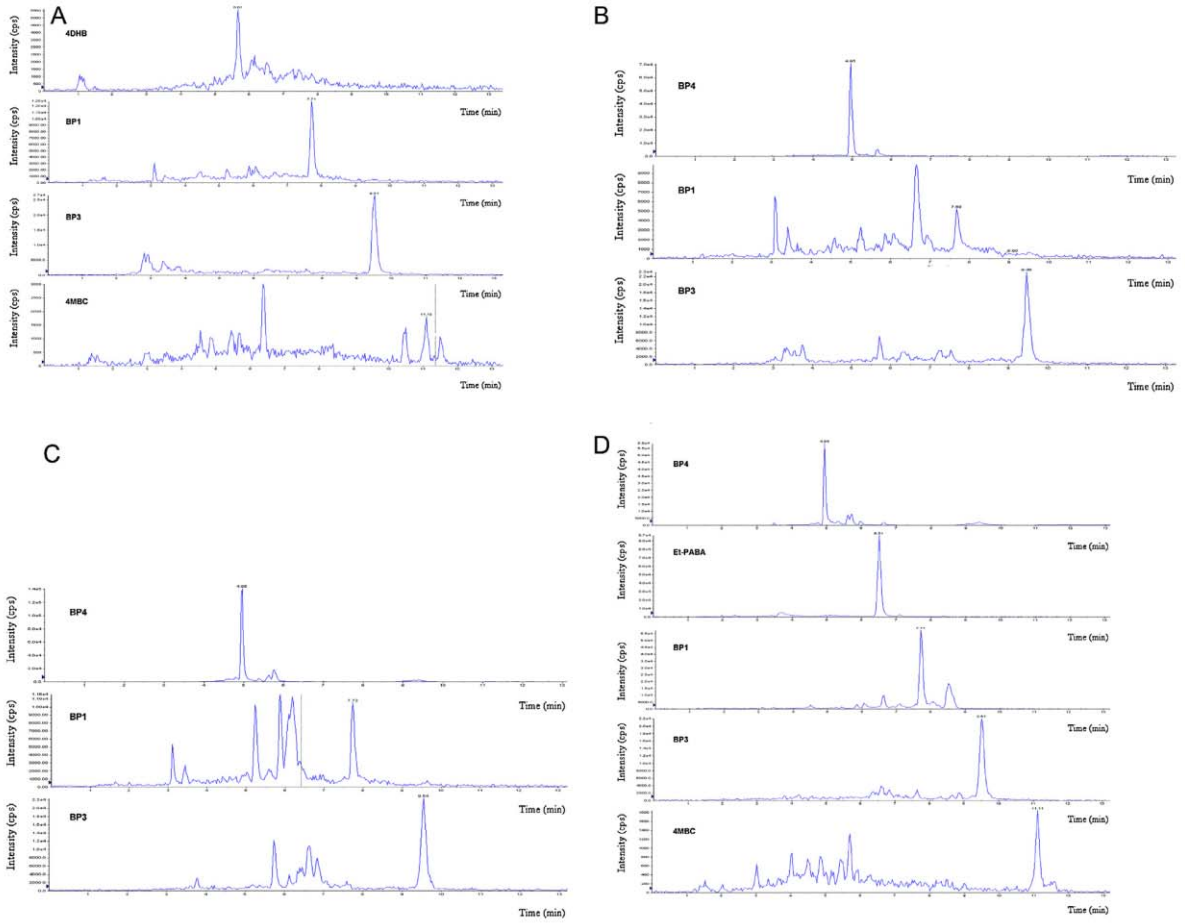

Fig. 3. Reconstructed ion chromatograms of (A) a groundwater sample (GW1), (B) a river water (LLO4), (C) an effluent wastewater (EWW2), and (D) an influent wastewater (IWW2).

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from five WWTPs located in Catalonia were evaluated using the multi-residue method described above. A summary of the UV F concentrations observed is displayed in Table 4. Four out of the five analyzed GWs contained UV F residues, including BP3. BP1, 4DHB, BP4 and 4MBC, at concentrations in the range 4–37 ng/L. Presence of UV F in GW may be due to water leaks in the part of the plumbing system which collects wastewater, making that part of the domestic water reach the aquifer. As example, Fig. 3a shows the chromatogram for the detected UV F in GW1 sample. As far as we know these is the first data on the occurrence of UV filters in groundwaters.

UV F residues were also detected in the surface waters from the Llobregat River analyzed. Four out of the nine compounds were present, including BP3, BP1, BP4 and 4MBC. Samples collected in the lower part of the river were much more contaminated than the ones sampled at the upper part. This trend is in agreement with that observed in other studies performed in the same river for other emerging pollutants such as pharmaceuticals or pesticides [36–38]. Fig. 3b shows the chromatogram of the detected UV F in the sample LLO5.

Higher levels of UV F were determined in EWWs and IWWs. BP3, BP1, BP4, 4MBC and Et-PABA were found in all the IWWs. The higher concentrations corresponded to BP3, BP1 and in particular to BP4, reaching concentrations up to 1548 ng/L. Et-PABA, which was detected in IWWs in the range 17–121 ng/L, was not observed in EWW, which suggests its efficient elimination during the treatment. BP3 and BP1 were significantly removed (EWW concentrations always below35 ng/L). Fig. 3c and d shows the chromatogram for the detected UV F in EWW2 and IWW2, respectively.

Draws attention the high levels observed for BP4 even in EWW, showing that this compound is not effectively removed by the applied treatments. This may explain the high concentrations determined in RW for this compound.

BP4 is highly soluble in water and frequently used as UV-absorber in cosmetics but also as colour protector in products whose package is translucent. Despite its frequent detection in the aquatic environment potential effects on aquatic life are largely unknown. A recent study, however, indicates that BP4 alters transcripts of genes involved in hormonal pathways in zebrafish eleuthero-embryos and adult males exposed to concentrations in the range 30 and 3000 μg/L [39].

On the other hand 4HB, DHMB and BP2 were not detected in any of the samples analyzed.

4. Conclusions

A highly sensitive analytical method based on on-line SPE–LC–MS/MS has been developed, allowing the simultaneous multi-analyte determination of nine UV F and transformation products. To the authors' knowledge this method constitutes the first fully automated method for the determination of these compounds in water samples. The on-line methodologies allowed considerably shortening the overall analysis time and reduced the sample volume required, providing the same sensitivity if not better achieved with off-line SPE using higher sample volumes. It should be remarked that the small size of sample needed, 5 mL, relieves the storage problems so usual in analytical laboratories. The developed method was proved to be a powerful tool for the analysis of UV F and their transformation products in waters, allowing determining concentrations down to the ng/L level in several water samples (groundwater, river water, effluent and influent wastewater). The use of isotopically labelled standards for quantification is essential to ensure accurate and reliable results.

Application of the developed method to real water samples showed the widespread occurrence of several UV F in the aquatic environment. UV F residues were detected in all the matrices. The higher concentrations corresponded to wastewaters, however, also relevant concentrations were observed in river water and groundwater. BP4 was identified as the most important in concentration among the UV filters studied, particularly in wastewater (738–1548 ng/L). These results points out that WWTPs are the major sources of sunscreen residues in the aquatic ecosystem when they are not efficient at removing selected UV F, as for instance BP4.

The application of the method allowed reporting levels of UV F in groundwater samples for the first time, and contributed to improve the knowledge on the fate of selected UV F in the aquatic environment.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.04.037.

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2.2. ANÁLISIS DE FILTROS UV Y PRODUCTOS DE TRANSFORMACIÓN EN LODOS DE EDAR

Publicación científica #4:

"Occurrence of multiclass UV-Filters in treated sewage sludge from wastewater treatments plants"

por:

Pablo Gago-Ferrero, M. Silvia Díaz-Cruz, Damià Barceló

en "Chemosphere" (2011, 84:1158-1165)

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Occurrence of multiclass UV filters in treated sewage sludge from wastewater treatment plants

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ABSTRACT

Many substances related to human activities end up in wastewater and accumulate in sewage sludge. So far, there is only one extensive survey on the occurrence of UV filter residues in sewage sludge. However, more data are required to draw a reliable picture of the fate and effects of these compounds in the environment. This study attempts to fill this gap through the determination of selected UV filters and derivatives namely 4-methylbenzylidenecamphor, benzophenone-3, octocrylene, ethylhexylmethoxycinnamate, ethylhexyldimethyl PABA, 4-hydroxybenzophenone, 2,4-dihydroxybenzophenone, and 4,4′-dihydroxybenzophenone in treated sewage sludge.

The target compounds were extracted using pressurized liquid extraction and after this, determined by ultra high resolution liquid chromatography–tandem mass spectrometry. The determination was fast and sensitive, affording limits of detection lower than 19 ng g^{-1} dry weight (dw) except for 2,4-dihydroxybenzophenone (60 ng g^{-1} dw). Good recovery rates, especially given the high complexity of sludge matrix (between 70% and 102% except for 2,4-dihydroxybenzophenone (30%)) were achieved.

The application of developed method allowed reporting for the first time the occurrence of two major degradation products of benzophenone-3 that have estrogenic activity in sewage sludge: 4,4′-dihydroxybenzophenone (in 5/15 WWTPs) and 4-hydroxybenzophenone (in 1/15 WWTPs). Results revealed the presence of UV filters in 15 wastewater treatment plants in Catalonia (Spain) at concentrations ranging from 0.04 to 9.17 μg g^{-1} dw.

1. Introduction

Organic UV filters are used as additives in formulations to absorb solar radiation, and therefore, to protect against their harmful effects. These compounds are extensively used in personal care products and are also present in a wide variety of industrial goods such as paints, plastics or textiles to prevent degradation of polymers and pigments (Lowe et al., 1997). According to an exhaustive report on personal care products, UV filters experienced the highest sales growth, 13%, in Europe from 2002 to 2003 reaching around 1000 millions US $ mill (ACNielsen Global Services, 2004).

In this work analytes were selected according two criteria, the extent of their use and the toxicological effects they may cause. The five selected UV filters have presented estrogenic activity, some of them also anti-androgenic activity and interactions with the thyroid axis (Matsumoto et al., 2005; Schlumpf et al., 2008a,b). 4-Methylbenzylidene camphor (4MBC) was found to interfere with development of reproductive organs, brain and behaviour in rats (Schlumpf et al., 2008a,b; Faass et al., 2009). Moreover, 4MBC is known to cause adverse effects on reproduction for some invertebrates at concentrations of 1.7 mg kg^{-1} sediment (Schmitt et al., 2008). For ethylhexyl-methoxy cinnamate (EHMC), long-term effects on reproduction in adult ovariectomized rats (Seidlová-Wuttke et al., 2006) and on expression of genes involved in hormonal pathways in fathead minnows (Pimephales promelas) (Christen et al., 2011) have been described. Besides, these compounds tend to bioaccumulate even being detected in human milk (Schlumpf et al., 2008b, 2010). Concerning the benzophenone-3 (BP3) derivatives, 2,4-dihydroxybenzophenone (BP1) and 4-hydroxybenzophenone (4HB), both showed the highest estrogenicity (below 1 mg L^{-1} exposure) in a recombinant yeast assay with rainbow trout ERα (Kunz et al., 2006). Additional data on environmental concentrations and toxicity have been reviewed by Fent et al. (2008) and Brausch and Rand (2011).

UV filters enter the aquatic environment directly as a result of water recreational activities when they are washed off from the skin and/or clothes, or indirectly through wastewater resulting

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Table 1
Characteristics of the selected wastewater treatment plant (WWTP).

WWTP	Type of treatment	HRT (d)	SRT (d)	Equivalents inhabitants	Influent flow rate (m^3 d^{-1})	Sludge treatment	Disposal	Dry matter (t y^{-1})	% Organic matter
Girona	Biological with P and N removal + Tertiary	12.5	–	147 000	37 000	Anaerobic digestion/centrifuge	Soil application (97%) Composting (2%) Land reclamation (1%)	3162	63
St. Feliu de Llobretat	Biological + Tertiary	0.36	12	198 000	46 000	Anaerobic digestion/centrifuge	Thermal drying (51%) Soil application (43%) Landfill (6%)	3789	48
Rubí	Biological with N removal	0.3	4	142 000	22 000	Anaerobic digestion	Thermal drying (84%) Landfill (16%)	1845	59
Granollers	Biological with P and N removal	4.45	–	182 000	22 000	Anaerobic digestion/centrifuge	Thermal drying (89%) Soil application (11%)	2325	64
Reus	Biological	18.96	–	124 000	17 000	Anaerobic digestion/filter press	Soil application (79%) Composting (21%)	1954	55
Montcada	Biological	0.3	–	229 000	52 000	–	Dehydratation (100%)	6155	–
Llagosta	Biological with P removal	0.5	–	205 000	30 000	Anaerobic digestion/centrifuge	Thermal drying (93%) Landfill (7%)	2142	53
Lleida	Biological	0.16	4–6	177 000	58 000	Anaerobic digestion/centrifuge	Soil application (100%)	2045	54
Vilafranca	Biological with P and N removal	7.5	9	87 000	12 000	Anaerobic digestion/centrifuge	Soil application (99%) Composting (1%)	992	61
Mataró	Biological	0.23	–	154 000	29 000	Anaerobic digestion/centrifuge	Thermal drying (63%) Soil application (37%)	2797	63
Prat de Llobregat	Biological with P and N removal + Tertiary	0.5	–	1 142 000	267 000	Anaerobic digestion/centrifuge	Thermal drying (76%) Soil application (24%)	14 955	51
Terrassa	Biological	29.9	17	165 000	42 000	Anaerobic digestion/centrifuge	Soil application (98%) Composting (2%)	3787	52
Gavà	Biological	0.44	10	272 000	41 000	Anaerobic digestion/centrifuge	Soil application (100%)	2750	62
Teià	Biological	0.6	–	84 000	15 000	Centrifuge	Composting (99%) Thermal drying (1%)	1512	77
Vic	Biological with P and N removal	1.27	16	399 000	21 000	Anaerobic digestion/filter press	Thermal drying (99%) Landfill (1%)	2865	53

HRT: hydraulic retention time; SRT: solid retention time; Influent flow rate: average inflow over the year. (–): data not available.

Table 2
Recovery rates (RR) and relative standard deviation (RSD) of UV filters as a function of the extraction solvent and temperature (T).

Solvent	RR ± RSD (%)								
	T (°C)	4MBC	OCT	EHMC	OD-PABA	BP3	BP1	4HB	4DHB
3 cycles MeOH	50	45 ± 12	60 ± 10	35 ± 21	40 ± 6	30 ± 21	6 ± 41	47 ± 21	44 ± 10
	75	75 ± 7	71 ± 21	75 ± 8	61 ± 14	41 ± 18	3 ± 66	49 ± 22	50 ± 12
	100	80 ± 8	66 ± 5	78 ± 6	69 ± 12	55 ± 9	20 ± 22	77 ± 32	79 ± 21
3 cycles MeOH/H_2O (1:1)	50	12 ± 11	14 ± 5	3 ± 4	11 ± 5	39 ± 22	15 ± 7	38 ± 12	53 ± 4
	75	41 ± 5	13 ± 7	15 ± 43	22 ± 6	40 ± 6	32 ± 3	40 ± 14	60 ± 5
	100	33 ± 25	35 ± 7	31 ± 5	35 ± 10	41 ± 4	22 ± 14	46 ± 12	66 ± 8
2 cycles MeOH + 2 cycles MeOH/H_2O (1:1)	50	55 ± 5	65 ± 12	52 ± 9	65 ± 12	49 ± 10	20 ± 8	66 ± 5	75 ± 12
	75	82 ± 8	60 ± 10	81 ± 12	80 ± 5	72 ± 8	24 ± 23	96 ± 17	110 ± 4
	100	102 ± 6	70 ± 7	90 ± 6	85 ± 4	70 ± 6	30 ± 16	95 ± 8	96 ± 9
3 cycles DCM/hexane (1:1)	50	50 ± 7	43 ± 12	23 ± 5	33 ± 6	33 ± 5	–	15 ± 15	5 ± 8
	75	59 ± 11	55 ± 7	65 ± 5	55 ± 11	41 ± 4	–	12 ± 4	19 ± 13
	100	51 ± 4	55 ± 6	61 ± 12	49 ± 5	32 ± 5	–	18 ± 6	12 ± 4

4-Methylbenzylidene camphor (4MBC); Octocrylene (OCT); Ethylhexyl methoxycinnamate (EHMC); Ethylhexyl dimethyl PABA (OD-PABA); Benzophenone-3 (BP3); 2,4-Dihydroxybenzophenone (BP1), 4-Hydroxybenzophenone (4HB); 4,4′-Dihydroxybenzophenone (4DHB); methanol (MeOH); dichloromethane (DCM), (–): not detected.

from the use of personal care products, washing clothes and industrial discharges, among others. Residues of more polar organic UV filters have been found in all kind of water matrices (Balmer et al., 2005; Rodil et al., 2008; Fent et al., 2010; Tarazona et al., 2010). Due to the significant efforts carried out in recent years in terms of improving water quality, the new treatment plants (built to meet the requirements of European Council Directives 91/271/EEC and 98/15/EEC by 2005), have led to a substantial increase in sewage sludge loads. Data collected in the 1999 Report on the Implementation of Legislation on Waste, estimated a value of 7.2 Mt of dry matter production (dry weight (dw)) in European Union countries. The use of sludge in agriculture is the most important outlet in Europe, accounting for about 50% of sludge production (Schowanek et al., 2004).

The European Union is currently working on new legislation on the quality of biosolids (Working Document on Sludge, 3rd Draft, 2000). In most cases, direct risk would currently be considered to have adverse effects to consumers of crops (humans and animals) by virtue of uptake by crops or contamination of crops. However, also an important risk at heavily amended sites is that of groundwater pollution. The 3rd Draft proposes limit values for concentrations of heavy metals and also for organic compounds, such as PAHs and PCBs. This fact should restrict the use of sewage sludge in agriculture if the limits are exceeded. In Spain, the quality criteria of the sludge generated in wastewater treatment plants (WWTPs) for possible use in agriculture focused on its metal content (Spanish Directive 1310/1990, 10–29th-1990 on the use of sewage sludge in agriculture).

Due to the high lipophilicity and poor biodegradability of most UV filters they are expected to end up in sewage sludge during wastewater treatment as reported for other personal care products (Shek et al., 2008; Chen et al., 2009). Despite that, current knowledge on concentrations of UV filters in sewage sludge is scarce. To the author's knowledge there are only three studies on the analysis of UV filters in sewage sludge (Plagellat et al., 2006; Nieto et al., 2009; Rodil et al., 2009).

Thus, the aims of the present study were: (i) to develop and validate a selective and sensitive multi-residual analytical methodology based on pressurized liquid extraction (PLE) and ultra high resolution liquid chromatography–electrospray-tandem mass spectrometry (UPLC–ESI-MS/MS) for the determination of UV filters and derivatives having quite different polarities (log Kow from 2.55 to 7.53) in sewage sludge, and (ii) to perform a survey of multiclass UV filters and some of the major degradation products of BP3 in the 15 main WWTPs of Catalonia (Spain).

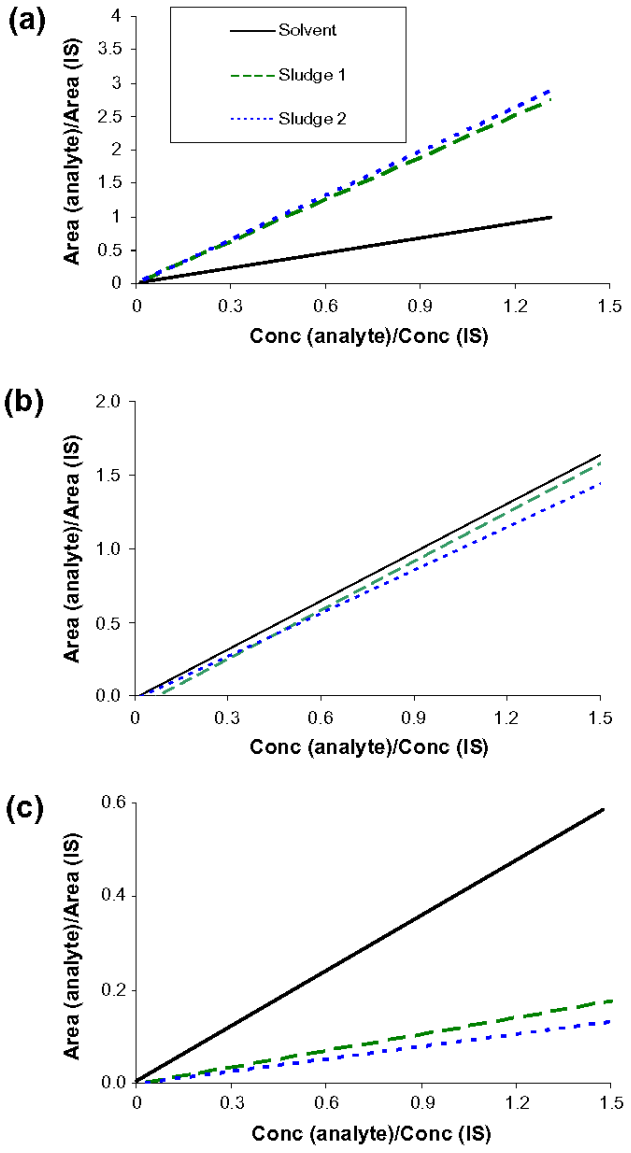

Fig. 1. Comparison of calibration curves obtained in acetonitrile and in PLE sewage sludge extracts for: (a) Benzophenone-3 (BP3), (b) 4,4′-Dihydroxybenzophenone (4DHB), and (c) 4-Methylbenzylidene camphor (4MBC). Conc: Concentration; IS: Internal standard.

2. Materials and methods

2.1. Chemicals and materials

BP3, octocrylene (OCT), OD-PABA, BP1, 4HB, 4,4′-dihydroxybenzophenone (4DHB) and the isotopically labelled compound benzophenone-d_{10} were used as internal standard (IS). They were of high purity and obtained from Sigma–Aldrich (Steinheim, Germany); 4MBC was supplied by Dr. Ehrenstorfer (Augsburg, Germany); and EHMC by Merck (Darmstadt, Germany). Methanol, acetone, dichloromethane (DCM), acetonitrile and HPLC grade water (Lichrosolv), as well as formic acid (98% purity), aluminium oxide and hydromatrix were provided by Merck. N_2 and Ar (purchased from Air Liquide (Barcelona, Spain)) were of 99.995% purity. The syringe and the PLE cellulose filters used were obtained from Whatman (London, UK) and from Dionex Corporation (Sunnyvale, CA, USA), respectively. Isolute C18 Cartridges used for solid phase extraction (SPE) were obtained from Biotage (Uppsala, Sweden).

Individual UV stock standard solutions and an isotopically labelled internal stock standard solution were prepared on a weight basis in methanol at 200 mg L^{-1}. The solutions were stored in the dark at −20 °C. A mixture standard solution at 20 mg L^{-1} in methanol of each compound was prepared weekly. Working solutions were prepared daily by appropriate dilution of the mixture stock standard solution in methanol.

2.2. Sample collection and pre-treatment

Treated sewage sludge samples were collected in June 2009 from 15 full-scale WWTPs in Catalonia (Spain). Table 1 summarizes the main characteristics of the selected facilities. These plants are the biggest ones in Catalonia in terms of equivalent inhabitants (Eq-Inh), and in terms of dry matter tonnes generated (amount of stabilized sewage sludge produced).

Dehydrated sludge samples, about 500 g dw, were collected in clean brown glass containers and shipped in refrigerated coolers to the laboratory. Then, they were freeze-dried in a LyoAlfa 6–50 (Telstar S.A., Barcelona, Spain) and stored in the dark at −20 °C until analysis.

2.3. Contamination and photo-degradation considerations

Background contamination is a common problem in the determination of UV filters at environmental levels. Therefore, several measures were taken in order to prevent this problem. All glassware used was previously washed and heated overnight at 380 °C, and after this sequentially rinsed with different organic solvents and HPLC grade water. Then it was immediately used. Furthermore, gloves were worn during the sample preparation; separate solvents and only previously unopened packages of solvents, chemicals and other supplies, and glassware were used. In

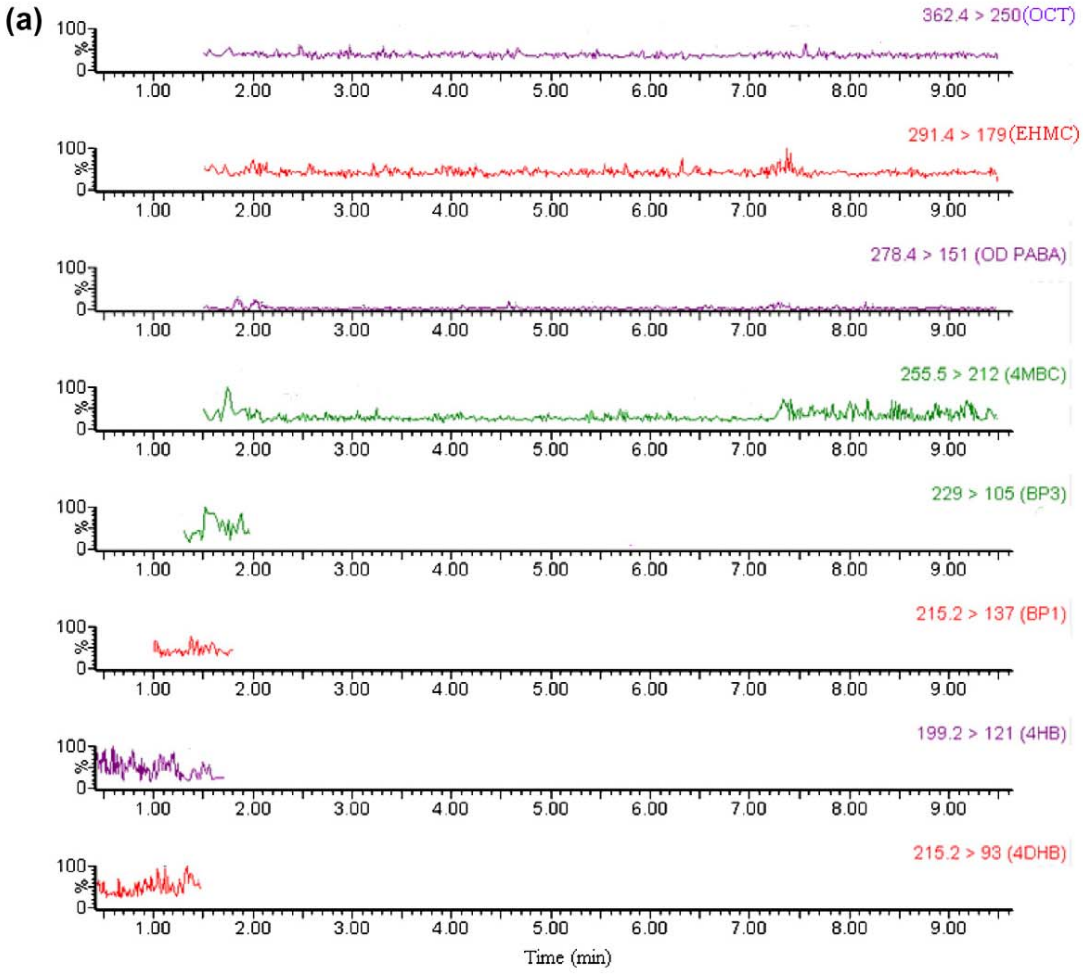

Fig. 2. Selected reaction monitoring (SRM) chromatograms obtained by UPLC–ESI(+)-MS/MS of (a) an operational blank sample and (b) the treated sewage sludge sample corresponding to the wastewater treatment plant of Granollers. 2,4-Dihydroxybenzophenone (BP1); 4,4′-Dihydroxybenzophenone (4DHB); 4-Hydroxybenzophenone (4HB); Benzophenone-3 (BP3); 4-Methylbenzylidene camphor (4MBC); Octocrylene (OCT); Ethylhexyl dimethyl PABA (OD-PABA); Ethylhexyl methoxycinnamate (EHMC).

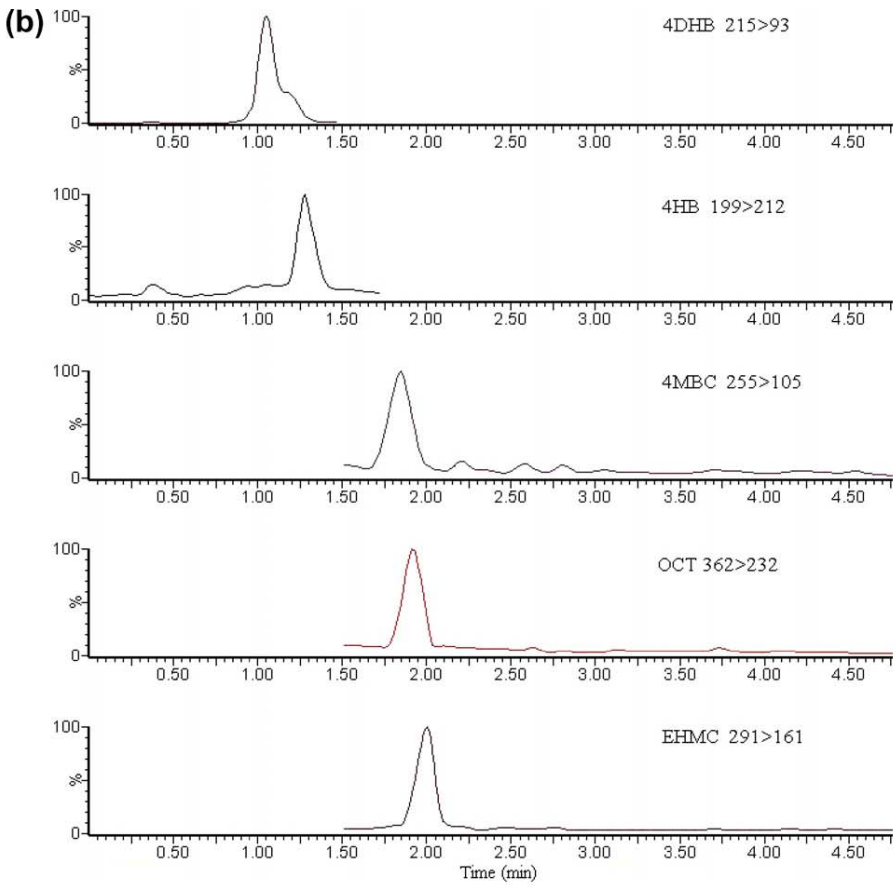

Fig. 2 (*continued*)

addition, a set of at least two operational blanks were processed together with each batch of samples. Since many of the compounds analyzed undergo photodegradation, stock standard solutions and samples were always covered with aluminium foil and stored in the dark.

2.4. Sample extraction and purification

Samples were extracted by PLE using an automatic extractor ASE 200 (Dionex Corporation, Sunnyvale, CA, USA). One gram of freeze-dried and grinded sludge samples were mixed in the extraction cells with aluminium oxide (alumina, previously activated by heating at 130 °C for 24 h) in order to perform the purification step within the cell (in-cell purification). The PLE optimised parameters were as follows: preheating of 5 min, two static cycles of 5 min using methanol as extraction solvent followed by other two static cycles of 5 min using methanol/water (1/1, v/v), temperature of 100 °C, pressure of 10 000 kPa, flush volume of 40% of cell and 60 s of nitrogen purge. The PLE extract obtained was brought to 25 mL with methanol. A 2 mL aliquot of this solution was passed through a syringe filter of 0.45 μm to a LC-vial. The filter was washed three times with 1.5 mL of methanol. All extracts were joint and evaporated to dryness. The residue was further reconstituted with 1 mL of acetonitrile and the internal standard was added before LC–MS analysis.

2.5. UPLC–ESI-MS/MS analysis

Analyses were performed on an Acquity UPLC chromatograph attached to a TQD mass spectrometer (Waters). A Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 2 μm) column (Merck) was used. The solvent flow rate was set to 0.4 mL min^{-1} and the column temperature was kept at 50 °C. The sample volume injected was 10 μL.

In order to optimize chromatographic separation several mobile phases and additives were tested. The use of formic acid at different concentration levels was evaluated, whilst methanol, acetonitrile and mixtures of both solvents were tested for the organic phase using methanol and acetonitrile as organic component and different concentrations of formic acid as modifier. The best results were obtained using as mobile phase HPLC grade water (A) and acetonitrile (B), both with 0.3% formic acid (which significantly improved the peak shape of BP3 and its derivatives). The adopted elution gradient started with 5% of eluent B, increasing to 95% in 1.20 min, kept constant for 2.30 min, and rising to 100% in the following 0.5 min. During the next 2.5 min the elution gradient was kept constant, and then back to initial conditions in 3 min.

MS/MS was operated in selected reaction monitoring (SRM) mode. ESI ionization was tested in both positive and negative modes. For all analytes a significant improvement of sensitivity was observed working in positive mode. Fragmentation voltage and collision energy were evaluated for each transition. The optimized parameters were obtained by flow injection analysis of single analyte standard solutions at 500 ng L^{-1}, and they were as follows: desolvation gas (nitrogen), 750 L h^{-1}; collision gas (Ar), 0.19 mL min^{-1}, nebulisation gas (nitrogen), 90 L h^{-1}; ion spray voltage 3.35 kV; source temperature, 130 °C and desolvation temperature, 450 °C. For each compound, two characteristic fragments of the protonated molecular ion $[M+H]^+$ were recorded. The most

abundant transition was used for quantification, whereas the second most abundant was used for confirmation.

3. Results and discussion

3.1. Analytical aspects

In the optimization of the extraction and purification process, aliquots of 1 g of sample were placed in scintillation vials, covered completely with acetone and spiked with the appropriate volume of the UV filter mixture stock standard solution. The acetone was allowed to evaporate at room temperature until sludge was dry. In order to homogenize the suspension the mixtures were frequently shacked during the process. For recovery experiments samples were spiked at 2500 ng g^{-1}. A high level of spike was necessary, as sewage sludge pollutants are usually found in high concentrations. As shown in Table 2, recovery rates obtained were in the range 70–102% for all compounds except for BP1 (30%) (see Materials and Methods) under the optimized experimental conditions. The low recovery obtained for BP1 is consistent with the value reported by Nieto et al. (2009).

For extraction and purification another approach was evaluated. The method was based on PLE extraction, where the samples were mixed in the extraction cells with hidromatrix. Next, the extracts were purified using Isolute C18 SPE cartridges. In the evaluation, a number of extraction pure and binary mixture solvents and several temperatures were tested. The best results were obtained at 75 °C using a mixture of DCM/hexane (1/2 v/v) as extraction solvent. However, recoveries achieved were below 50%.

The performance of LC–ESI-MS/MS methods is strongly affected by the ionisable impurities coming from the matrix that can interfere with the ionization processes. In the present study matrix effects were evaluated. Results evidenced three different behaviours as represented in Fig. 1; for 4MBC, OCT, OD-PABA and EHMC, signal suppression was observed, for BP3 and BP1, the opposite effect occurred, and for the rest of compounds no significant differences were found. Therefore, internal standard quantification with matrix-matched standards was finally used. With each set of samples two operational blanks were processed. Fig. 2a shows the chromatogram of a blank where it can be observed that no contamination occurs. The quality parameters of the whole method are compiled in Table 3.

3.2. Occurrence of UV filters in treated sewage sludge

As reported in Table 4, all sewage sludge samples analysed contained at least two out of the eight UV filters investigated. As an example, Fig. 2b shows the chromatogram of a sludge sample where five UV filters were found.

Table 3
Performance of the UPLC–ESI(+)-MS/MS developed method for the analysis of UV filters in sewage sludge.

	Instrumental						Method		
	Linearity range (ng L^{-1})	r^2	ILOD (pg)	ILOQ (pg)	Precision (RSD%) $n = 5$		RR[a] (RSD%, $n = 5$)	MLOD (ng g^{-1})	MLOQ (ng g^{-1})
					Intraday	Interday			
4MBC	20–900	0.998	13.11	43.70	6	10	102 (6)	12	40
OCT	20–900	0.997	2.53	8.44	4	9	70 (7)	18	60
EHMC	10–500	0.993	7.53	25.08	5	10	90 (6)	19	63
OD-PABA	1–300	0.994	0.07	0.22	7	7	85 (4)	0.2	0.7
BP3	1–300	0.994	1.66	5.53	5	9	70 (10)	1.0	3.3
BP1	5–300	0.994	2.01	6.70	9	14	30 (16)	60	200
4HB	5–300	0.992	3.09	10.29	4	11	95 (8)	5.0	17
4DHB	1–300	0.996	3.35	11.17	3	6	96 (9)	5.0	17

[a] Samples spiked at 2500 ng g^{-1} (dw). ILOD: instrumental limit of detection; ILOQ: instrumental limit of quantification; RSD: relative standard deviation; RR: recovery rates; MLOD: method limit of detection; MLOQ: method limit of quantification; 4-Methylbenzylidene camphor (4MBC); Octocrylene (OCT); Ethylhexyl methoxycinnamate (EHMC); Ethylhexyl dimethyl PABA (OD-PABA); Benzophenone-3 (BP3); 2,4-Dihydroxybenzophenone (BP1); 4-Hydroxybenzophenone (4HB); 4,4′-Dihydroxybenzophenone (4DHB).

Table 4
Concentration of the target UV filters in sewage sludge (μg g^{-1} dw). In parentheses: daily mass loads (mg (day × 1000 Eq-Inh) $^{-1}$).

WWTP	BP1	4DHB	4HB	BP3	4MBC	OCT	OD-PABA	EHMC
Girona	n.d	<LOQ	n.d	n.d	1.63 (96.4)	2.60 (153.8)	n.d	n.d
St Feliu de Llobregat	n.d	n.d	n.d	n.d	1.61 (84.2)	2.87 (150.6)	n.d	0.75 (39.1)
Rubí	n.d	0.07 (2.3)	n.d	n.d	3.83 (136.4)	9.17 (326.9)	n.d	1.22 (43.6)
Granollers	n.d	0.04 (1.2)	0.15 (5.1)	n.d	1.52 (53.4)	2.61 (91.6)	n.d	0.78 (27.3)
Reus	n.d	n.d	n.d	n.d	2.98 (128.1)	5.39 (231.8)	n.d	1.91 (82.5)
Montcada	n.d	n.d	n.d	n.d	3.17 (232.9)	4.15 (304.8)	n.d	1.09 (80.1)
Llagosta	n.d	0.62 (17.8)	n.d	n.d	1.79 (51.4)	4.49 (128.78)	n.d	n.d
Lleida	n.d	n.d	n.d	n.d	0.73 (23.2)	2.86 (90.6)	n.d	n.d
Vilafranca	n.d	n.d	n.d	n.d	1.53 (47.6)	2.16 (67.3)	n.d	0.61 (18.9)
Mataró	n.d	n.d	n.d	n.d	1.76 (87.5)	3.63 (180.5)	n.d	1.08 (53.6)
Prat de Llobregat	n.d	n.d	n.d	n.d	1.84 (66.1)	6.60 (236.9)	n.d	3.35 (120.3)
Terrassa	n.d	n.d	n.d	n.d	1.34 (84.2)	2.25 (141.2)	n.d	n.d
Gavà	n.d	n.d	n.d	n.d	2.84 (78.6)	3.86 (106.8)	n.d	2.09 (57.7)
Teià	n.d	n.d	n.d	0.79 (9.18)	0.81 (40.0)	3.00 (148.9)	n.d	2.01 (99.8)
Vic	n.d	<LOQ	n.d	n.d	0.89 (17.5)	1.06 (20.8)	n.d	n.d
Mean over positive samples	–	0.24 (7.1)	0.15 (5.1)	0.79 (9.18)	1.88 (81.8)	3.78 (158.8)	–	1.49 (62.3)

WWTP: wastewater treatment plant; 2,4-Dihydroxybenzophenone (BP1); 4,4′-Dihydroxybenzophenone (4DHB); 4-Hydroxybenzophenone (4HB); Benzophenone-3 (BP3); 4-Methylbenzylidene camphor (4MBC); Octocrylene (OCT); Ethylhexyl dimethyl PABA (OD-PABA); Ethylhexyl methoxycinnamate (EHMC); n.d: not detected; <LOQ: below the limit of quantification.

As expected, more lipophilic compounds were present in higher quantities, with the exception of OD-PABA, which was not detected in any of the samples. This fact may be attributed to decreased use of this compound as previously reported for PABA. In addition, degradation processes of OD-PABA in the sewer and during wastewater and/or sludge treatment cannot be ruled out. Moreover, the more polar compounds, when detected, were found at low concentrations, as was the case with BP3.

Determined range and mean concentrations (corrected for recoveries) were as follows: 4MBC (0.73–3.83, 1.88 $\mu g\,g^{-1}$ dw), OCT (1.06–9.17, 3.78 $\mu g\,g^{-1}$ dw) and EHMC (nd-3.35, 1.49 $\mu g\,g^{-1}$ dw). These values are comparable to those found by Plagellat et al. (2006) (4MBC (0.15–4.98, 1.78 μg^{-1} g dw), OCT (0.32–18.74, 4.84 $\mu g\,g^{-1}$ dw), EHMC (0.01–0.39, 0.11 $\mu g\,g^{-1}$ dw)). In the second study on sludge samples, Nieto et al. (2009) analyzed three sludge samples from the municipal WWTP of Tarragona city (Spain) reporting lower mean concentrations of OCT, BP3, and OD-PABA (1.45, 0.017, and 0.15 $\mu g\,g^{-1}$ dw, respectively). In the third study Rodil et al. (2009) analyzed eleven UV filters in two spot sludge samples, one from Geel (Belgium) where, among others, OCT, BP3, 4MBC and OD-PABA were determined (2.48, 0.007, 3.89, and 0.001 $\mu g\,g^{-1}$ dw, respectively); and the other from a WWTP from Leipzig (Germany) reporting also lower levels for these compounds (OCT (0.59 $\mu g\,g^{-1}$ dw), BP3 (0.029 $\mu g\,g^{-1}$ dw), 4MBC (0.073 $\mu g\,g^{-1}$ dw) and OD-PABA (0.002 $\mu g\,g^{-1}$ dw)).

Concentration levels found for 4MBC, OCT and EHMC are notoriously high, reflecting that there is no effective elimination of such compounds through the treatments performed to the sewage sludge. In addition to the selected UV filters, we determined two major BP3 degradation products 4HB and 4DHB, both exhibiting endocrine disrupting activity (Kunz and Fent, 2006) in four samples at concentrations in the range 0.04–0.62 $\mu g\,g^{-1}$ dw, 4DHB being the most frequently found. It is noteworthy that these results constitute the first data on the occurrence of these derivatives in sewage sludge.

Considering the lipophilicity of UV filters, an attempt to correlate concentrations detected with the organic matter of the sludge was made, however no correlation was found. Further, normalized concentrations of the target compounds to the content of organic matter were calculated (data not shown). Again, no correlation could be established.

In order to compare the WWTPs investigated, the specific loads (mg (day × 1000 Eq-Inhab)$^{-1}$) were estimated for each compound (Table 4). The WWTP that generally showed higher concentrations were those with higher specific loads. The WWTPs of Rubí, Montcada and Prat de Llobregat, (with a big industrial contribution) were those with the highest specific loads. In contrast, the lower specific load corresponded to Vic, an area where the main economic activity is agriculture/livestock.

According to the 2008 Annual Report of the Catalan Water Agency (ACA), the sludge generated in most WWTPs with high concentrations of UV filters were usually applied as fertilizer to agricultural soils. This common practice can lead to certain environmental issues such as: an excess and/or imbalance of nutrients, the spread of human, animal and plant pathogens, and the introduction of inorganic contaminants such as heavy metals and organic pollutants (including UV filters). UV filters have scarcely been studied in sludge. However, if more experience and monitoring results (on sludge and soil) are gathered, a decision about submitting these compounds under legislation might be reached.

4. Conclusions

A multi residue analytical methodology based on ultra high resolution liquid chromatography separation and detection by electrospray-tandem mass spectrometry (UPLC–ESI-MS/MS) was developed and validated for the determination of UV filters in sewage sludge. The method was fast (chromatographic run time of 9.5 min), sensitive (LODs below 19 ng g^{-1}) and selective with good recovery rates (70–102%).

The widespread occurrence of UV filter residues in the 15 major WWTPs in Catalonia (Spain) was evidenced. Both, the frequency of detection and the mean concentrations found for lipophilic compounds (4MBC (100%, 1.88 $\mu g\,g^{-1}$ dw), OCT (100%, 3.78 $\mu g\,g^{-1}$ dw) and EHMC (66.6%, 1.49 $\mu g\,g^{-1}$ dw)) were notoriously high. In contrast, BP3 were rarely detected (1/15 WWTPs) and BP1 and OD-PABA were not detected in any sample. Moreover, two major BP3 degradation products 4DHB and 4HB, both well-known endocrine disrupting compounds, were determined in sewage sludge for the first time. More information on UV filters consumption, degradability and WWTPs technology is needed to provide a reliable explanation to the estimated occurrence data. It should be pointed out that the treated sludge generated in most WWTPs is used as fertilizer in agriculture. Therefore, in light of these results, a greater knowledge over the presence and potential effects of UV filters in the environment is needed to ensure a safer management of the generated biosolids.

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2.3. ANÁLISIS DE FILTROS UV Y PRODUCTOS DE TRANSFORMACIÓN EN SEDIMENTOS

Publicación científica #5:

"Fast pressurized liquid extraction with in-cell purification and analysis by liquid chromatography-tandem mass spectrometry for the determination of UV-Filters and their degradation products in sediments"

por:

Pablo Gago-Ferrero, M. Silvia Díaz-Cruz, Damià Barceló

en "Analytical and Bioanalytical Chemistry" (2011, 400:2195-2204)

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ORIGINAL PAPER

Fast pressurized liquid extraction with in-cell purification and analysis by liquid chromatography tandem mass spectrometry for the determination of UV filters and their degradation products in sediments

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Abstract This paper presents the development of a fast and sensitive analytical method for the simultaneous determination of UV filters and degradation products having quite different polarities (log Kow 2.19–6.88) in sediment, by means of pressurized liquid extraction (PLE) with in-cell purification and analysis by ultra-performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS). Analytes were simultaneously concentrated and purified by placing aluminium oxide as clean-up sorbent in the extraction cell for a faster sample pre-treatment. Under optimized conditions, quantitative recoveries (only one compound below 80%) and satisfactory precision (RSD, 5–15%) were obtained. Low limits of detection were achieved of 0.5–15 ng/g dry weight (dw). The use of PLE extraction and purification and UPLC technology enabled all the compounds to be separated chromatographically in less than 9 min, and with a total chromatographic analysis time of 18 min. This method significantly decreased the overall time of analysis as compared to those of previously developed. Finally, the optimized methodology was applied to investigate the occurrence of the target UV filters in sediment samples collected along the Ebro river basin (Spain). UV filters were detected in 95% of the sediment samples analysed. Results revealed a widespread presence of octocrylene (OC), reaching concentrations up to 24×10^2 ng/g dw, the highest reported so far. Ethylhexyl dimethyl PABA (OD-PABA) and benzophenone-3 (BP3) were also frequently detected (60–65%), but at lower concentrations (4.4–27 ng/g dw). 4DHB (an estrogenic degradation product of BP3) was present in three samples at concentrations between 12 and 21 ng/g dw. These results constitute the first data on the occurrence of OD-PABA and 4DHB in sediments.

Keywords UV filters · River sediments · UPLC-MS/MS analysis · Pressurized liquid extraction · In-cell purification

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Introduction

Nowadays, society depends on a large number of chemical compounds. Once discharged from industrial and urban sources, they may ultimately enter wastewater. Most of them may be to some extent eliminated during water treatment. However, those not removed may be present in residual concentrations in the receiving aquatic ecosystem. Through the water/sediment equilibrium and depending on their lipophilicity properties, they may accumulate in sediments and biota. Among them, organic UV absorbing compounds are chemicals of particular interest due to the raised concern about harmful effects of sunlight, they have been increasingly used in the last decades. These compounds are worldwide used in many personal care products and in other goods such as furniture, clothes, and varnishes,

paints and polymeric materials used in vehicle cabins and buildings to enhance their light stability [1].

There is still limited information about the fate of UV filters in the environment, although several of these compounds, such as 4-methylbenzylidene camphor (4MBC) and ethylhexyl methoxycinnamate (EHMC), are known to display estrogenic activity [2–4]. Moreover, it is documented that UV filters can be bioaccumulated in organisms and concentrated on solid matrices [5].

Reported UV filter levels in water, sediments, sewage sludge and biota vary greatly depending on geographic area, season, etc. UV filters have been detected in surface and wastewater samples [6–9], in the range 2–350 ng/L level (surface water), 10–2,700 ng/L (effluent water) and 100–19,000 ng/L (influent water).

Occurrence of UV filters in solid matrices has received much less attention as compared to that given to waters. Few studies have investigated them in sewage sludge [10–12] and biota [5, 6]. River sediments are unique in providing historical contamination and constitute suitable monitoring tools to assess pollution episodes. In addition, river sediments reflect the quality of surface water. Specifically in sediments, existing data on UV filters is scarce; in Germany, EHMC and BP3 were detected in river and lake sediments at concentrations ranging from 0.5 to 4 ng/g [13]. In Korea, benzophenone and three derivatives, namely, 4-hydroxybenzophenone (4HB), 2,2′-dihydroxy-4-methoxybenzophenone (DHMB) and benzhydrol (BH), were also found in river sediments at concentrations from 0.5 ng/g for benzophenone to 19 ng/g for HBP [14]. More recently, Rodil and Moeder reported levels from 14 to 34 ng/g of EHMC and from 63 to 128 ng/g of octocrylene (OC) in sediments also from Germany [15]. The extraction methods used in these works were based on solid–liquid extraction of the freeze-dried sediment sample with different organic solvents, and analyses were performed by gas chromatography-mass spectrometry (GC-MS), with chromatographic analysis times higher than 60 min, and requiring a derivatization step for the most polar compounds.

The objective of the present work was to develop a fast, selective and sensitive multi-residue analytical methodology for the determination of a group of UV filters of increased interest together with some of their major metabolites in sediments. The aim was to explore the possibilities of combining pressurized liquid extraction (PLE) as the sample preparation technique because of its low solvent consumption, its high throughput and the ability to integrate extraction and purification in a single step with the improved chromatographic resolution, increased peak capacity and rapid elution of an ultra-performance liquid chromatography (UPLC) separation. To the best of our knowledge, this is the first time that UPLC-MS/MS has been used for the analysis of UV filters in sediment samples. In addition, the established method was applied to provide a first survey of the occurrence of widely used UV filters in sediment of the Ebro river basin (Spain). Two of the compounds investigated have been found for the first time in sediments.

Materials and methods

Chemicals and materials

Analytical standards of BP3 (99% purity), OC (98% purity), ethylhexyl dimethyl PABA (OD-PABA; 98% purity), benzophenone-1 (BP1; 99% purity), 4HB (98% purity), 4,4′-dihydroxybenzophenone (4DHB; 99% purity) and the isotopically labelled compound benzophenone-d_{10} (BP-d_{10}; 99% purity), used as internal standard (IS), were obtained from Sigma-Aldrich (Steinheim, Germany); 4MBC (99% purity) was supplied by Dr. Ehrenstorfer (Augsburg, Germany) and EHMC (98% purity) by Merck (Darmstadt, Germany). Analyte names, abbreviations, structures and other relevant physicochemical data of the above compounds are given in Table 1. As listed, all selected UV filters show moderate to high lipophilicity character in contrast to the three metabolites also analyzed.

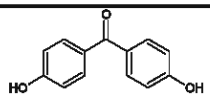
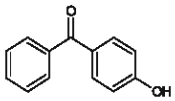
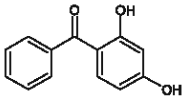
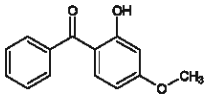
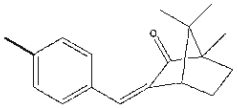
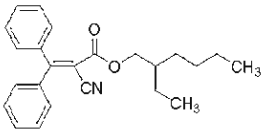
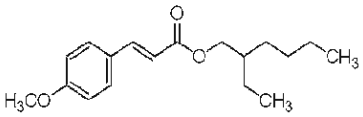
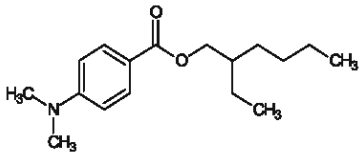
The organic solvents (>99.8% purity) methanol, acetone, acetonitrile and HPLC grade water (Lichrosolv), as well as formic acid (98% purity) and aluminium oxide (99% purity), were provided by Merk. N_2 and Ar purchased from Air Liquide (Barcelona, Spain) were of 99.995% purity. Syringe filters used (0.45 μm) were obtained from Whatman (London, UK). PLE cellulose filters were purchased from Dionex Corporation (Sunnyvale, CA, USA).

Both stock standards and the isotopically labelled compound were prepared in methanol at a concentration of 200 mg/L. Standards solutions were stored in the dark at −20 °C. From these solutions, a mixture standard solution of all UV filters in methanol at a concentration of 20 mg/L of each compound was prepared weekly. Mixed working solutions were prepared daily by appropriate dilution of the mixed stock standard solution in methanol.

Description of study areas

The Ebro river basin (NE Spain) drains an area of 85,362 km^2, which equals to 17.3% of the surface area of Spain being the largest river basin in the country. The Ebro River, of 910 km, receives waters from several tributaries, which altogether represent 12,000 km of the waterway network. Sediments were sampled in December 2009 at 20 locations covering the whole Ebro river basin, including ten sites at the Ebro River and ten at the main tributaries. Their

Table 1 Physicochemical properties, chromatographic retention time and MS/MS transitions of UV filters studied

INCI name[a]	Structure	CAS	Log K_{ow}	Retention Time (min)	Transition[b]
4,4'-Dihydroxybenzophenone (4DHB)		611-99-4	2.19[c]	2.58	215 > 121 215 > 93
4-Hydroxybenzophenone (4HB)		1137-42-4	2.92[d]	3.06	199 > 212 199 > 105
2,4-Dihydroxybenzophenone (BP1)		131-56-6	3.17[d]	3.26	215 > 137 215 > 105
Benzophenone 3 (BP3)		131-57-7	3.79[c]	4.13	229 > 151 229 > 105
4-Methylbenzylidene camphor (4MBC)		36861-47-9	4.95[c]	5.90	255 > 105 255 > 212
Octocrylene (OC)		6197-30-4	6.88[c]	7.15	362 > 250 362 > 232
Ethylhexyl methoxycinnamate (EHMC)		5466-77-3	5.80[c]	7.75	291 > 179 291 > 161
Ethylhexyl dimethyl PABA (OD-PABA)		21245-02-3	5.41[d]	7.91	278 > 166 278 > 151

[a] INCI (International Nomenclature for Cosmetic Ingredient) elaborated by CTFA and COLIPA

[b] All compounds were determined with electrospray in positive mode. Parent ion corresponds in all cases to [M+H]+

[c] Experimental values, from database of physicochemical properties. Syracuse Research Corporation: http://www.syrres.com/esc/physdemo.htm

[d] Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (©1999-2011 ACD/Labs)

specific locations are shown in Fig. 1 and are numbered from 0 to 19 from north to south. "R" indicates sites on the Ebro River, whereas "T" indicates tributary sites. All sampling points selected are sites included in the network control of dangerous substances from the Ebro Hydrographic Confederation (CHE) authority. Table 2 lists the locations and coordinates of each sampling point, the corresponding river and the total organic carbon (TOC) values determined. Analyzed sediments had TOC values from 0.39% to 4.40%. This broad spectrum indicates that sampling area comprises quite uncontaminated (TOC<1) and highly contaminated (TOC>3) sites.

Sample collection and preparation

Surface sediment samples (0–2 cm) were collected from the middle river bed using a hand held Van Veen sediment grab. In sites with high flow, samples were collected using the grab coming a few meters from the shore. Samples were transferred to pre-cleaned (with HPLC water and river water) amber glass containers and shipped to the laboratory in portable fridges. Upon receipt, samples were immediately processed to prevent degradation of target compounds; sediments were freeze-dried at −50 °C and 0.044 bar vacuum in the laboratory. For

Fig. 1 Location map of sampling points of sediments in the Ebro river basin

this procedure, a LyoAlfa 6 50 (~230 V, 50 Hz, 1.5 KW) from Telstar S.A. (Barcelona, Spain) was used. Then samples were sieved through 120-μm sieve and, finally, stored in the dark at −20 °C until analysis.

Samples and standards handling

Background contamination in the laboratory is a common problem observed in the determination of UV filters at

Table 2 Description of sediment samples

Site	Sampling name	Basin	Lat.	Long.	TOC (%)
R0	Reinosa (Cantabria)	Ebro	42.999 N	4.153 O	1.85
R1	Miranda de Ebro (Burgos)	Ebro	42.684 N	2.951 O	0.96
T2	Audinaka (Álava)	Zadorra	42.884 N	2.486 O	1.21
R3	Haro (La Rioja)	Ebro	42.589 N	2.842 O	0.42
T4	Najera (La Rioja)	Najerilla	42.418 N	2.733 O	0.52
R5	Logroño (La Rioja)	Ebro	42.470 N	2.444 O	1.20
T6	Estella (Navarra)	Ega	42.669 N	2.031 O	0.39
R7	Tudela (Navarra)	Ebro	42.067 N	1.601 O	0.69
T8	Alsasua (Navarra)	Araquil	42.895 N	2.135 O	3.44
T9	Puente de la Reina (Navarra)	Arga	42.671 N	1.819 O	0.44
T10	Grisen (Zaragoza)	Jalon	41.734 N	1.175 O	4.40
T11	Zaragoza (Zaragoza)	Huerva	41.614 N	0.915 O	2.94
T12	Caldearenas (Huesca)	Gallego	42.402 N	0.499 O	0.61
T13	San Mateo de Gállego (Zaragoza)	Gallego	41.823 N	0.785 O	0.64
R14	Presa de Pina (Zaragoza)	Ebro	41.567 N	0.691 O	1.94
R15	Sástago (Zaragoza)	Ebro	41.320 N	0.340 O	1.73
T16	Alcolea de Cinca (Huesca)	Cinca	41.725 N	0.136 E	1.67
R17	Flix (Tarragona)	Ebro	41.229 N	0.552 E	0.66
R18	Amposta (Tarragona)	Ebro	40.715 N	0.581 E	1.10
R19	Delta del Ebre (Tarragona)	Ebro	40.714 N	0.714 E	0.76

environmental trace levels. As suggested by many authors [8, 10, 16], several measures were taken in the current study in order to prevent this problem. All glassware used was previously washed and heated overnight at 380 °C and further sequentially rinsed with HPLC grade water, ethanol and acetone, and immediately used. Furthermore, gloves were worn during sample preparation; separate solvents and only previously unopened packages of solvents, chemicals and other supplies, and glassware were used. Since many of the compounds analyzed undergo photo-degradation and the samples may suffer the exposure to light during the procedure, all samples and stock standard solutions were always covered with aluminium foil and stored in the dark. Moreover, a set of at least two operational blanks were processed together with each batch of samples.

Sample extraction and purification

The sediments were extracted and purified by PLE using an automatic system ASE 200 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA, USA) using 11 ml capacity stainless steel extraction cells. Aliquots of 1 g of aluminium oxide (previously heated at 130 °C, for 24 h, and then allowed to cool down in a dissicator before use) were placed at the outflow side of each cell onto two cellulose filters. Under optimized conditions, aliquots of 1 g of freeze-dried and sieved sediments were mixed in the PLE extraction cells with aluminium oxide in order to perform the purification step within the cell in a single step (in-cell purification). Finally, the PLE extract (ca. 20 mL) was brought to 25 mL with methanol. A 2-mL aliquot of this solution was passed through 0.45-μm syringe filter to a LC-vial, evaporated to dryness under a gentle stream of nitrogen in a TurboVap LV evaporator from Zymark (Zymark, Hopkin, MA, USA), and dried samples were reconstituted in 250 μl of acetonitrile containing the I.S.

UPLC-MS/MS determination

Chromatographic separation

Chromatographic separations were performed on a Hibar Purospher® STAR® HR R-18 ec. (50×2.0 mm, 2 μm) LC column supplied by Merck with an Acquity UPLC chromatograph (Waters). The column temperature was kept at 50 °C. Separation was performed with a binary mobile phase at a flow rate of 0.4 mL/min. The optimized separation conditions were as follows: solvent A consisted of HPLC grade water and solvent B acetonitrile, both with 0.3% formic acid. The gradient elution started with 5% eluent B, increasing to 80% in 2 min and raising to 100% in the following 9 min, kept constant for 2 min, then return to initial conditions in 2 min and, finally, three additional minutes to allow the equilibration of the column. The sample volume injected was 10 μL.

Mass spectrometry detection

The UPLC instrument was coupled to a triple quadrupole TQD mass spectrometer (Waters). Acquisition was achieved in ESI positive mode (ESI(+)) using selected reaction monitoring (SRM) mode recording two transitions for each compound for enhanced sensitivity and selectivity. For each compound, two characteristic fragments of the protonated molecule $[M+H]^+$ were monitored (see Table 1). The most abundant transition was used for quantification, while the second most abundant was used for confirmation. This procedure was in compliance with the European Council Directive 2002/657/EC that although it was initially conceived for food residue analysis, it has been accepted by the scientific community for environmental analysis.

In BP3 and derivatives, except 4DHB, we observed the loss of 77 u, which corresponds to $[M\text{-}C_6H_5]^+$, and other signal related to the fragment $[C_6H_5C{=}O]^+$. The 4DHB structure, with one symmetric hydroxyl group on each side of the ketone, made the 93 u, associated to $[C_6H_4OH]^+$, a characteristic loss.

OC, EHMC and OD-PABA contain a relatively long chain branched alkyl group at the ester group, and hence, their collision-induced dissociation mainly occurs through a McLafferty rearrangement, where the corresponding alkenes are lost, leaving the charge back at the ester group. Subsequently, the formed cation continues losing mainly H_2O.

Quantification was carried out considering internal standard calibration based on peak areas. The isotopically labelled compound BP-d_{10} was used with this purpose since even knowing that it was not the ideal compound for the quantification of all analytes, it was used because it was found to properly correct the signal variations produced by the matrix (see matrix effect section). However, since isotopically labelled standards were not commercially available at that time for each compound, some analytes were subject to a certain level of inaccuracy.

Results and discussion

Optimization of the sample preparation

An extraction procedure previously developed by the authors for the analysis of sewage sludge [12] was taken as the basis for the isolation of the target compounds from sediments.

Optimization of extraction parameters included the selection of solvent, temperature, time and number of extraction cycles, pressure and flush volume. A blank was performed for each extraction conditions tested.

To optimize the extraction procedure, aliquots of 1 g of sediment were placed in scintillation vials, covered completely with acetone and spiked with the UV filters mixture standard solution. Finally, the acetone was allowed to evaporate at room temperature until the sediment was dry. Also, unfortified sediments were processed for each method as blanks, and their signal was subtracted from the spiked samples to calculate recovery rates.

In addition to testing the previously optimized extraction method for the analysis of sludge samples, which implements two cycles of methanol followed by two cycles of methanol/water (1:1), we evaluated other conditions since in this case, sediments are a cleaner matrix than sludge. The possibility of further reducing solvent consumption and sample preparation time by reducing the number of extraction cycles was considered. The number of cycles and type of solvent evaluated as well as the recoveries obtained in each case are provided in Table 3. In all experiments, the pressure was maintained at 10,000 kPa and the temperature set to 100 °C.

Results showed that extraction using two cycles of methanol was not suitable for more polar compounds, yielding recoveries in some cases below 50%. A third cycle of methanol did not significantly improve recoveries. However, by using both methanol and methanol/water (1/1 *v*/*v*) as extraction solvent, the extraction became more efficient (especially for more polar compounds). It is unusual to use binary mixtures with water for the extraction of lipophilic compounds; however, it must be taken into account that the dielectric constant of water drops as the temperature increases, which can favour these processes [17]. By raising the temperature, the viscosity decreases, and thus, the extracting agent can interact better with the sample allowing higher recovery rates.

In the light of these results, it appears that it is not possible to reduce the number of cycles or to use a single type of solvent. As a consequence, we applied the same extraction strategy used for sludge samples, i.e. two cycles of methanol followed by two cycles of methanol/water (1:1). However, as sediments are cleaner than sludge, the dry extract was reconstituted solely in 250 μL of a solution of the I.S. in acetonitrile.

The optimal experimental conditions were as follows: a preheating period of 5 min, a heating period of 5 min, static time of 5 min, flush volume of 40% of cell volume with 90 s of nitrogen purge, 2 methanol and 2 methanol/water (1/1) static cycles, pressure 10,000 kPa and temperature of 100 °C. Recoveries obtained under these conditions were higher than 58%. These values are better than those obtained for sewage sludge, improving especially in the case of BP1 for which values did not exceed 30%.

Quality parameters and quantification

The performance of electrospray MS is strongly affected by the ionizable impurities coming from the matrix (e.g. natural organic matter, salts, ion-pairing agents and non-target contaminants) that can interfere in the ionization processes. This may result in a signal suppression or enhancement leading to low sensitivity and inaccurate results. Thus, in the present study, we evaluated potential matrix effect as a part of the validation of the method to ensure the reliability of the results obtained.

In order to identify potential matrix effects, calibration curves in pure solvent were compared with those in extract matrix solution; no significant differences between the curves obtained by both methods were observed for any analyte in contrast to what happened with sludge, where both signal enhancement and suppression were observed,

Table 3 Recovery rates of UV filters as a function of the extraction solvent and number of cycles at 100 °C

Solvent	*R*%±SD (*n*=3)							
	4MBC	OC	EHMC	OD-PABA	BP3	BP1	4HB	4DHB
2 cycles MeOH	54±12	78±5	84±8	75±16	44±9	30±17	40±6	58±12
3 cycles MeOH	58±5	78±6	80±7	85±23	50±6	27±21	45±5	55±16
2 cycles MeOH/H_2O (1:1)	24±9	15±7	18±11	25±24	40±21	33±8	21±13	48±11
2 cycles MeOH+2 cycles MeOH/H_2O (1:1)	89±6	85±7	90±6	120±4	125±10	58±16	80±8	120±9
MLOD (ng/g)	8.0	2.2	1.6	0.5	0.8	15.5	2.4	2.8
MLOQ (ng/g)	27	7.3	5.3	0.8	2.7	52	8.0	9.3

Samples spiked at 0.10 μg/g dw

4,4′-Dihydroxybenzophenone (DHB), 4-hydroxybenzophenone (4HB), 2,4-dihydroxybenzophenone (BP1), benzophenone 3 (BP3), 4-methylbenzylidene camphor (4MBC), octocrylene (OC), ethylhexyl methoxycinnamate (EHMC) and ethylhexyl dimethyl PABA (OD-PABA)

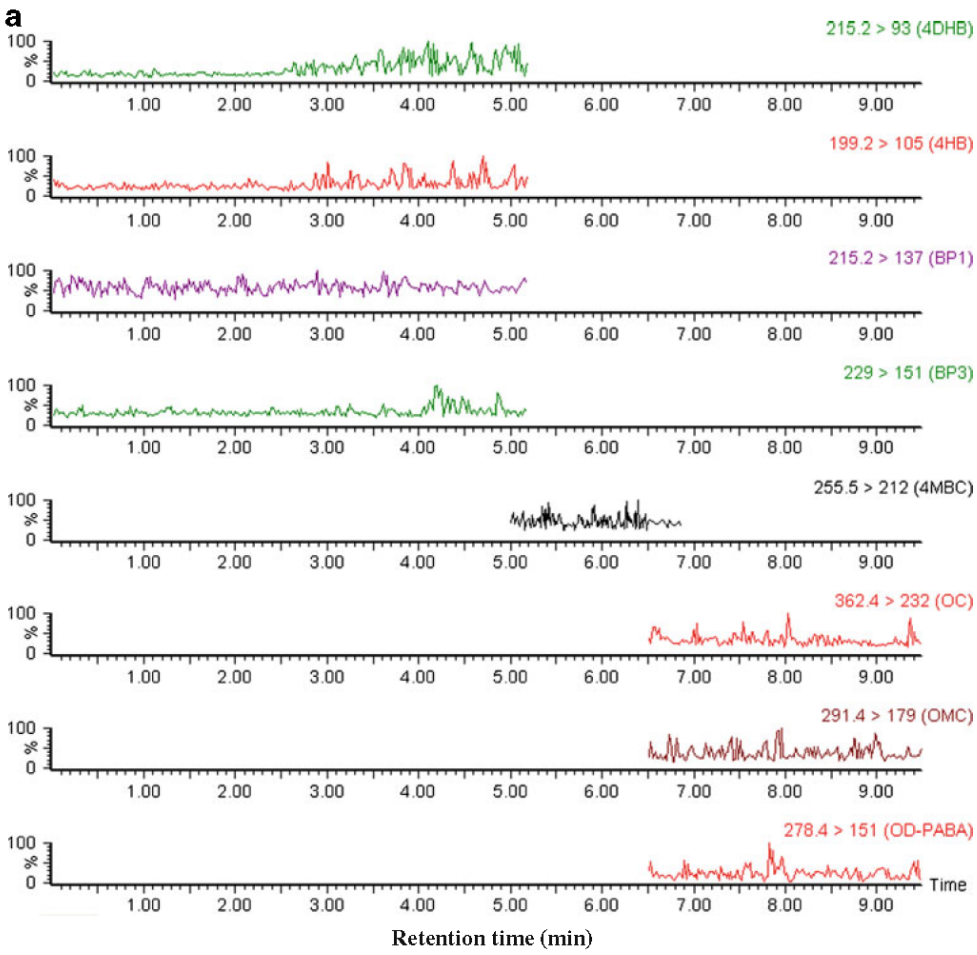


Fig. 2 Chromatogram of a procedural blank (**a**). Chromatogram of a sediment sample from Zaragoza city (**b**)

thus requiring the use of matrix-matched standards for quantification. Therefore, for the analyses, standard solutions in methanol were used.

As previously mentioned, contamination problems are common in the analysis of UV filters. With each set of samples, we processed two operational blanks. Figure 2a shows the chromatogram of a blank where the absence of contamination problems can be observed.

The quality parameters of the whole method are listed in Table 3. The linear response range was tested by using increasing concentrations of standard solutions in the range 0.1–750 ng/L. The method exhibited good linearity with squared correlation coefficients (r^2) higher than 0.995 for all compounds. The intra-day instrumental precision was determined by analysing five replicates of a mixture standard solution at 100 ng/L within a given day. Inter-day instrumental precision was also estimated by analysing five replicates of the same solution on five different days. Good precision was obtained, with values of relative standard deviation (RSD) in the ranges 3–9% (intra-day) and 6–14% (inter-day). The overall precision of the proposed method was evaluated by analysing a spiked sample at 100 ng/g (three replicates). RSD values obtained were in the range 4–16%.

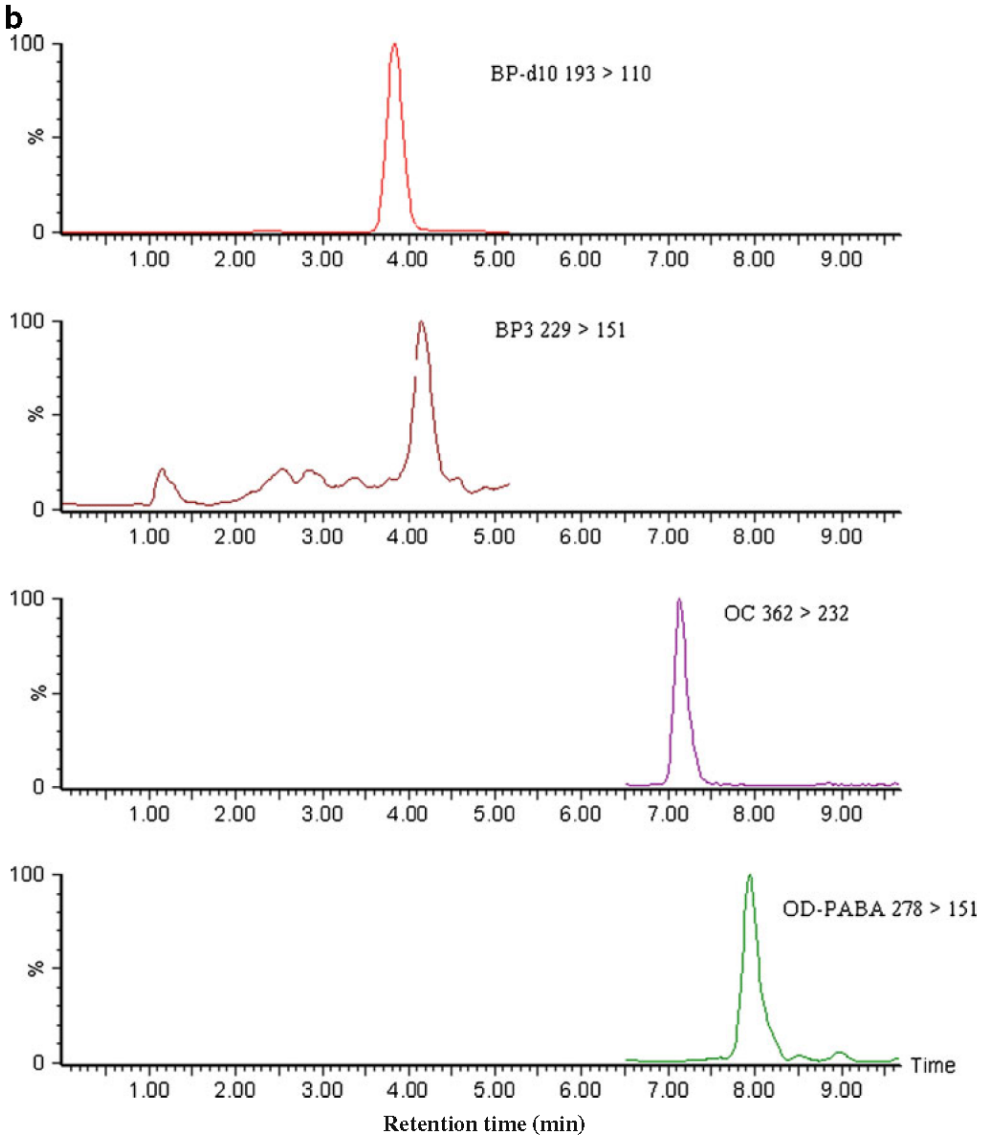
Because there are no reference sediments for the analysis of UV filters, we accomplished recovery studies with actual spiked sediment samples. Taking into account the environmental levels reported in the literature [13–15], samples were spiked at 100 ng/g. Three blanks were analyzed and their signal subtracted from the corresponding spiked samples. Table 3 shows the recoveries and standard deviations obtained.

The recoveries varied from 80% to 125%, with the exception of BP1 (58%). The relatively low value and two other ones higher than 100% may be a problem of quantification with solely one internal standard for all the analytes.

Reported concentrations were corrected for recoveries.

The method detection (MLOD) and quantification (MLOQ) limits were calculated as follows: MLOD=(C×3)/(S/N) and MLOQ=(C×10)/(S/N), where C was the concentration of the spiked samples and S/N the signal-to-noise ratio. Limits of detection were quite low for all species, in the range 0.5–15.5 ng/g dw. MLOQs achieved were between 0.8 and 52 ng/g dw.

Fig. 2 (continued)



Occurrence of UV filters in river sediments

The occurrence of UV filters along the Ebro river basin (including its main tributaries) was evaluated using the multi-residue method described above.

The concentrations of the target compounds in the 20 sediment samples analyzed are summarized in Table 4.

UV filters residues were detected in all processed sediments except for that taken at the tributary Cinca, in Huesca city (53,000 inhabitants in 2010). Five out of the eight compounds investigated could be determined. As an example, Fig. 2b shows the chromatogram corresponding to the sediment sample collected in the city of Zaragoza (747.377 inhabitants in 2010), where three out of the eight target compounds were found.

OC was the most ubiquitous compound with a 95% frequency of detection and with the highest concentrations in the analyzed samples. The relatively high values of this compound must be underlined; the site where its concentration was higher 24×10^2 ng/g dw corresponds to sample T11. This sampling site is inside the city of Zaragoza, just before the Huerva River reaches the Ebro River. Huerva River receives the effluents from many industrial areas around the city of Zaragoza. Furthermore, also an important level of OC, 87×10^1 ng/g dw, was determined in the sediments gathered at a sampling site situated downstream Zaragoza, in the main Ebro River, which receives the effluents from its wastewater treatment plant. OC is a highly lipophilic compound with a log Kow of 6.88, therefore, tending to adsorb upon sediment organic matter. It is not water-soluble (making lixiviation not possible), highly stable and resistant to sunlight degradation. Moreover, it should be highlighted that there is evidence that OC can trigger the production of potentially harmful free

Table 4 UV filters concentrations (ng/g dw) in sediments from the Ebro river basin analyzed by PLE UPLC-ESI(+)-MS/MS

Sample point	4MBC	OC	OD-PABA	EHMC	BP3	BP1	4DHB	4HB
R0	nd	13×10^1	4.4	42	17	nd	nd	nd
R1	nd	30×10^1	nd	nd	nd	nd	nd	nd
T2	nd	35	<LOQ	nd	4.9	nd	21	nd
R3	nd	13×10^1	<LOQ	nd	nd	nd	nd	nd
T4	nd	37	nd	nd	<LOQ	nd	nd	nd
R5	nd	17×10^1	<LOQ	nd	nd	nd	nd	nd
T6	nd	39	<LOQ	<LOQ	5.6	nd	nd	nd
R7	nd	54×10^1	<LOQ	25	nd	nd	nd	nd
T8	nd	70	nd	<LOQ	nd	nd	nd	nd
T9	nd	49	<LOQ	<LOQ	<LOQ	nd	nd	nd
T10	nd	18	<LOQ	nd	nd	nd	nd	nd
T11	nd	24×10^2	4.4	<LOQ	27	nd	nd	nd
T12	nd	34	nd	nd	<LOQ	nd	nd	nd
T13	nd	34	nd	nd	<LOQ	nd	12	nd
R14	nd	87×10^1	5.2	nd	18	nd	nd	nd
R15	nd	60	nd	nd	<LOQ	nd	nd	nd
T16	nd	nd	nd	nd	nd	nd	nd	nd
R17	nd	64	<LOQ	nd	<LOQ	nd	20	nd
R18	nd	84	<LOQ	7.9	6.8	nd	nd	nd
R19	nd	32	nd	nd	<LOQ	nd	nd	nd
LOQ	27	7.3	0.8	5.3	2.7	52	9.3	8.0

nd not detected, *<LOQ* below the limit of quantification

4,4′-Dihydroxybenzophenone (4DHB), 4-hydroxybenzophenone (4HB), 2,4-dihydroxybenzophenone (BP1), benzophenone 3 (BP3), 4-methylbenzylidene camphor (4MBC), octocrylene (OC), ethylhexyl methoxycinnamate (EHMC), ethylhexyl dimethyl PABA (OD-PABA)

radicals when it releases the absorbed energy. The widespread occurrence of this compound, as well as its high concentrations found in the sediments analyzed, is supposed to be associated with its extensive use in formulations, especially personal care products, because both protects in UVA and UVB regions, and because it augments the absorbing capacity of other organic UV filters.

OD-PABA and BP3 were also frequently detected in 60% and 65% of the processed samples, respectively. For both compounds, lower concentrations were measured from 4.4 to 5.2 ng/g dw for OD-PABA and between 4.9 and 27 ng/g dw for BP3. EHMC and the degradation product 4DHB were found less frequently, 35% and 15% of the sediments investigated, respectively, with concentrations in the range 7.9–42 ng/g dw for EHMC and from 12 to 21 for 4DHB. The compounds 4MBC, BP1 and the degradation product 4HB were not detected in any sample.

Considering the lipophilicity of UV filters, an attempt to correlate concentrations detected with the TOC values of the sediments was made; however, no correlation was found. Further, normalized concentrations of the target compounds to the content of organic carbon were calculated (data not shown). Again, no correlation could be established.

In the sampling sites where the highest levels of UV filters were detected, also high concentrations of other organic pollutants, such as PAHs, pesticides and alkyl phenols, were found in previous studies on sediments of the Ebro river basin [18].

These results indicate that Ebro River is more polluted by UV filters than its tributaries, except for Jalón River and Gallego River, close to Zaragoza city, likely due to the highest population and increased industrial activities located along the main river of the basin and despite its higher dilution potential. It is also notorious the contribution of the Huerva tributary to the load of OC of Ebro River. As such, the concentration of OC and OD-PABA increased from 54×10^1 ng/g dw and below LOQ, respectively (sampling site R7), before its contribution up to 87×10^1 and 5.2 ng/g dw, respectively, after they join.

The data reported here provide the first evidence of the occurrence of OD-PABA and the estrogenic degradation product 4DHB [3] in sediments. On the other hand, the levels found for OC are in some cases much larger than those identified in a previous study (14–93 ng/g dw) on lake sediment samples with very low TOC values (between 0.02% and 0.22%) [15].

Conclusions

A fast analytical method has been developed for the simultaneous determination of eight UV filters and degradation products having quite different polarities in sediment. The proposed approach combines PLE and UPLC-

MS/MS. The use of an automated PLE-based procedure for sample preparation allowed the fast and efficient extraction of analytes and removal of interfering species in the same step. Furthermore, the use of UPLC technology has also made possible a fast separation (18 min chromatographic run time), decreasing the overall time of analysis in comparison to that of the methods developed so far, together with a significant cost reduction in terms of solvent consumption. This method provided good LODs, linearity and recovery for all the compounds, permitting the use of the multi-residue methodology to monitor some of the more widely used sunscreen agents in river sediment. Therefore, the benefits of PLE with in-cell purification combined with UPLC for the trace determination of UV filters in the environment have been demonstrated.

Finally, the applicability of the method developed was evaluated by analyzing river sediment samples (n=20) collected along the Ebro river basin. The analytes and the degradation products identified revealed the ubiquity and high point source contamination by OC (in 19 out of the 20 samples investigated) with levels as high as 24× 10^2 ng/g dw. OD-PABA and BP3 were also frequently detected (60–65%), but at lower concentrations (4.4–27 ng/g dw). 4DHB (an estrogenic degradation product of BP3) was present in three samples at concentrations between 12 and 21 ng/g dw. The results achieved constitute the first report on the occurrence of OD-PABA and 4DHB in sediments.

These findings suggest that further research should be performed to evaluate potential effects of OC on fish and terrestrial organisms since its extremely low solubility in water, high lipophilicity and low degradation make it a good candidate to persist long time in sediment and soil.

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2.4. ANÁLISIS DE FILTROS UV Y PRODUCTOS DE TRANSFORMACIÓN EN PECES

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"Multi-residue method for trace level determination of UV-Filters in fish based on pressurized liquid extraction and liquid chromatography-quadrupole-linear ion trap-mass spectrometry"

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Pablo Gago-Ferrero, M. Silvia Díaz-Cruz, Damià Barceló

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Multi-residue method for trace level determination of UV filters in fish based on pressurized liquid extraction and liquid chromatography–quadrupole-linear ion trap-mass spectrometry☆

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ABSTRACT

So far, the very few studies addressing the occurrence of UV filters (UV F) in biota showed important limitations in the analysis of the so complex biological matrices. In order to improve the knowledge on the bioaccumulation of UV F by fish, a simple and highly sensitive method was successfully developed and validated for the simultaneous determination of eight extensively used UV F and transformation products with a wide range of physicochemical properties. The present study demonstrated that liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) using a QqLIT mass analyser was applicable to the simultaneous analysis of UV F in fish. Pressurized liquid extraction (PLE) was chosen for the sample pretreatment due to the good extraction efficiency provided. An additional SPE clean-up step was added in order to minimize matrix effects and to improve the sensitivity. The method allowed recovery efficiencies in the range 70–112% for most compounds at the three spike levels. The low limits of detection (MLOD) achieved (0.1–6.0 ng/g dw) allowed the reliable quantification of UV F residues in fish samples. The developed methodology was applied to assess the occurrence of UV F in different fish species from the Guadalquivir river basin (Spain). Results confirmed the bioaccumulation of benzophenone-3 (BP3), ethylhexyl methoxycinnamate (EHMC) and octocrylene (OC) in the fish samples. The maximum concentration of 240 ng/g dw corresponded to EHMC, which was also the most ubiquitous compound. The reported concentrations constitute the first occurrence data of UV F residues in fish from Iberian rivers.

1. Introduction

UV filters (UV F) or sunscreen agents are chemical compounds that mitigate the deleterious effects of sunlight. This group of additives is worldwide used in many personal care products as well as in many industrial goods to protect skin from chronic (skin cancer) or acute (photoaeging, sunburn) exposure to UV radiation [1] and to protect products from photodegradation (yellowing) [2].

UV F enter the aquatic environment continuously through two principal pathways: by direct inputs from aquatic recreational activities, and mainly by indirect inputs through sewage waters. Once discharged from industrial and urban sources, they ultimately enter surface waters and, as they are only partly removed in wastewater treatment plants (WWTPs), act as pseudo-persistent pollutants.

The increasing use of UV F may constitute a potential risk for the environment since most of them have multiple hormonal activities in fish [3], even at environmentally relevant concentrations [4], and in rodents. [5]. Besides, a recent study indicates that exposure to benzophenone type UV F may be associated with oestrogen-dependent diseases such as endometriosis in women [6].

Because of the high lipophilicity and poor biodegradability of many UV filters they have been detected at high concentrations in sewage sludge [7–11]. UV F have also been observed in surface water [12–17], seawater [15,17–19] and wastewater [13,15–17,20,21] and in sediments [11,22–24]. UV F also accumulate in humans being detected in milk [25,26], semen [27] and placental tissues [28].

The accumulation of UV F residues in biota has scarcely been studied. The current knowledge on the bioaccumulation and analytical methodology applied for the determination of UV F in

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biota was recently reviewed by Gago-Ferrero et al. [29]. Fish are important bioindicators of the occurrence of persistent lipophilic contaminants. Values from 9 to 2400 ng/g lipids have been reported in fish samples in a few studies [2,13,30–33] and concentrations over 7000 ng/g were detected in mussels [34]. Fent et al. [2] detected EHMC in crustacean and mollusks in the range 22–50 ng/g lipids, and in fish at values up to 337 ng/g lipids. The higher concentration, above 700 ng/g lipids, was reported for fish-eating birds (*Phalacrocorax* sp.), which suggests that biomagnification occurs through the food web.

Determination of UV F in the aqueous environment has been mainly performed by gas chromatography coupled to mass spectrometry (GC–MS). Matrix effects are not critical for the ionization modes typically used and good method limits of detection (MLODs) are achieved. However, these methods have important limitations. They solely can be applied to substances that are volatile and of low polarity or can be derivatized (where differences in matrix components may result in quite different derivatization efficiencies which compromise precision and accuracy of the analysis). If the objective is to perform the simultaneous determination of several UV F, with a wide range of physicochemical properties, liquid chromatography (LC) offers better features than GC. LC allows the analysis of a wide range of compounds and significantly increases the potential for the analysis of transformation products and metabolites, which are usually more hydrophilic than the parent compounds, without the need of derivatization. Thus, LC coupled to tandem mass spectrometry (LC–MS/MS) is the technique of choice for a multiclass UV F determination in environmental samples. Gago-Ferrero et al. have recently reviewed the LC–MS/MS methods published up to date for the determination of UV F in the environment [35]. So far there are only two approaches for the LC–MS/MS determination of UV F in biota. In the first one, Meinerling and Daniels developed a method for the analysis of four UV F in the muscle of rainbow trout. Soxhlet extraction was used followed by gel permeation chromatography (GPC) and by a clean-up step with a Florisil column [31]. In the second one, a method for the simultaneous determination of nine UV F was reported by Zenker et al. [32]. Mid-polar and lipophilic UV F were extracted by solvent extraction and further purified by reversed phase chromatography (RP-HPLC). The fraction containing mid-polarity UV F was analysed by HPLC–MS, whereas the fraction containing the lipophilic ones was determined by GC–MS.

The present study aims to develop and validate a robust, simple, fast, environmentally friendly, sensitive and selective analytical method based on HPLC–MS/MS for the quantitative determination of eight UV F in fish. Pressurized liquid extraction (PLE) was chosen as the extraction method because it is an automated technique, highly reproducible, and of low solvent and time consumption, especially compared with other conventional methods such as Soxhlet or ultrasound extraction.

Analyte identification and confirmation were performed using a hybrid quadrupole-linear ion trap-mass spectrometer (HPLC–QqLIT-MS/MS). The new method was applied to the determination of UV F in fish samples collected along the Guadalquivir river basin (Spain), constituting the first study on UV F bioaccumulation in fish from Iberian rivers.

2. Materials and methods

2.1. Standards and reagents

The most commonly used UV F were selected for the study covering a wide range of physicochemical properties. Table 1 shows their structures, CAS numbers and other properties.

Benzophenone-3 (BP3), octocrylene (OC), 2-ethylhexyl 4-dimethylaminobenzoate (OD-PABA), 2,4-dihydroxybenzophenone (BP1), 4-hydroxybenzophenone (4HB), 4,4′-dihydroxybenzophenone (4DHB) and the isotopically labelled compound benzophenone-C_{13} (BP-C_{13}, were of the highest purity (>99%) and were obtained from Sigma–Aldrich (Steinheim, Germany); 4-methylbenzylidenecamphor (4MBC, 99% purity) was supplied by Dr Ehrenstorfer (Augsburg, Germany); and EHMC (98%) by Merck (Darmstadt, Germany). The isotopically labelled compounds 2-hydroxy-4-methoxy-2′,3′,4′,5′,6′-d_5 (BP3-d_5) and 3-(4-methylbenzylidene-d_4)camphor(4MBC-d_4, used as internal standards (>99%), were obtained from CDN isotopes (Quebec, Canada). Methanol (MeOH), acetone, dichloromethane (DCM), acetonitrile (ACN), ethyl acetate (AcEt) and HPLC grade water (Lichrosolv), as well as formic acid (98% purity), aluminium oxide and Florisil were provided by Merck. N_2 and Ar purchased from Air Liquide (Barcelona, Spain) were of 99.995% purity. Pressurized liquid extraction cellulose filters used were obtained from Dionex Corporation (Sunnyvale, CA, USA). Isolute C18 (500 mg, 3 mL) and Isolute aluminium oxide AL-N (500 mg, 6 mL) cartridges used for solid phase extraction (SPE) were obtained from Biotage (Uppsale, Sweden). Cartridges Oasis HLB (200 mg, 3 mL) were obtained from Waters Corporation (Milford, MA, USA), and basic alumina cartridges (5 g, 25 mL) were obtained from Interchim (Montluçon Cedex, France).

Individual stock standard solutions as well as the isotopically labelled internal stock standard solution were prepared on a weight basis in MeOH at 200 mg/L. The solutions were stored in the dark at −20 °C. A mixture standard solution at 20 mg/L in MeOH of each compound was prepared weekly. Working solutions were prepared daily by appropriate dilution of the mixture stock standard solution in MeOH.

2.2. Sample collection

Fish samples analysed in this study were collected along the Guadalquivir river basin (south of Spain) in 2010. Fish of the species *Luciobarbus sclateri* and *Cyprinus carpio* were captured at the selected sampling points. Fish were sampled by DC electric pulse. Next, the fish were killed, frozen, thawed, homogenized and lyophilized. The samples were made up from a pool of each fish species from each sampling point. The lyophilized samples were stored in sealed containers at −20 °C until analysis.

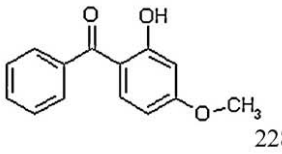
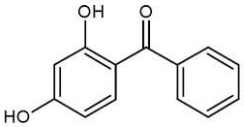
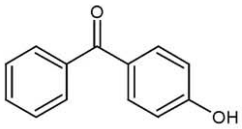
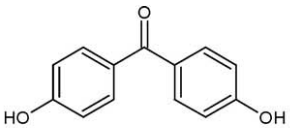
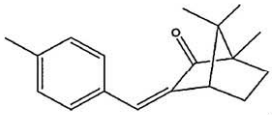
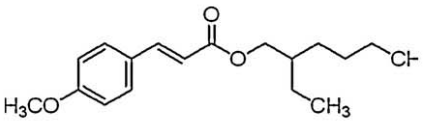
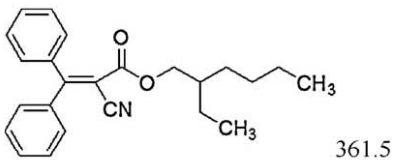
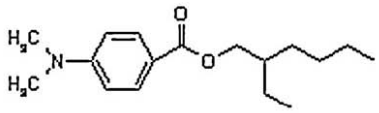
2.3. Sample preparation

Aliquots of the sample (1 g) were placed in different beakers, completely covered with acetone and spiked with 50 ng of the surrogate standard solution of BP-C_{13}. The acetone was left to evaporate at room temperature until the fish samples were dry.

Background contamination is a common problem in the determination of UV filters at environmental levels. Therefore, several measures were taken in order to prevent this problem. All glassware used was previously washed and heated overnight at 380 °C, and further sequentially rinsed with a collection of organic solvents and HPLC grade water, and immediately used. Furthermore, gloves were worn during sample preparation; separate solvents and only previously unopened packages of solvents, chemicals and other supplies, and glassware were used. Since many of the compounds analysed undergo photo-degradation stock standard solutions and samples were always covered with aluminium foil and stored in the dark.

Lipid content in fish was determined by PLE following the method developed by Spiric et al. [36].

Table 1
UV F abbreviations, structures and other relevant data.

Abbreviation	Name (INCI nomenclature)[a]	CAS no.	Structure and molecular weight (g/mol)	Log K_{ow}
BP3	Benzophenone-3	131-57-7	 228.24	3.79[b]
BP1	Benzophenone-1	131-56-6	 214.22	3.15[c]
4HB	4-Hydroxybenzophenone	1137-42-4	 198.2	2.92[c]
4DHB	4,4′-Dihydroxy benzophenone	611-99-4	 214.22	2.19[b]
4MBC	4-Methylbenzylidene camphor	36861-47-9	 254.37	4.95[b]
EHMC	Ethylexyl methoxycinnamate	5466-77-3	 290.4	5.8[b]
OC	Octocrylene	6197-30-4	 361.5	6.88[b]
OD-PABA	2-Ethylhexyl 4-dimethylaminobenzoate	21245-02-3	 277.4	5.412[c]

[a] INCI (International Nomenclature for Cosmetic Ingredient) elaborated by CTFA and Cosmetics Europe (former COLIPA).
[b] Experimental values from database of physicochemical properties. Syracuse Research Corporation: http://www.syrres.com/esc/physdemo.htm.
[c] Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (©1999–2011 ACD/Labs).

2.3.1. Extraction and clean up

Fish samples were extracted using an ASE 350 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA, USA) in 11 mL stainless steel cells. Solvent composition, adsorbent material, extraction temperature, extraction time and number of cycles of the PLE procedure were optimized. The experiments were conducted as follows: one cellulose filter followed by 1 g of Florisil (previously heated at 130 °C, for 24 h) was placed at the bottom of the cells. Aliquots of 1 g of freeze dried fish spiked with 50 ng of the surrogate standard BP-C_{13} were mixed in the extraction cells with Florisil in order to perform in-cell purification. Extraction was implemented in 2 cycles of 5 min of static time at 100 °C and 1500 psi using AcEt/DCM (1:1, v/v) as extracting solvent.

The PLE extract obtained (~25 mL) was diluted to 200 mL with HPLC water (MeOH < 5%), and further purified by solid phase extraction (SPE) using Isolute C18 (500 mg, 3 mL) cartridges, from Biotage. The cartridges were conditioned with 5 mL of AcEt/DCM (1:1, v/v) followed by 5 ml of MeOH and 5 mL of HPLC water at neutral pH. Then the PLE diluted extract was loaded onto the cartridges using a Baker vacuum system (J.T. Baker, The Netherlands). Finally, the compounds were eluted sequentially with 7 mL of AcEt/DCM (1:1, v/v) and 2 mL of DCM at 1 mL/min flow rate. Finally, the SPE extracts

were evaporated under a gentle nitrogen stream and reconstituted with 1 mL of ACN containing the isotopically labelled internal standards at a concentration of 20 ng/mL. Analysis was carried out by triplicate.

2.4. LC–MS/MS analysis

Analyses were performed by liquid chromatography–tandem mass spectrometry using a 4000 Q TRAP™ MS/MS system from Applied Biosystems–Sciex (Foster City, CA, USA). The chromatographic separation was achieved on a Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 5 μm) from Merck, preceded by a guard column of the same packaging material. In the optimized method, the mobile phase consisted of a mixture of HPLC grade water and ACN, both with 0.15% formic acid. The adopted elution gradient started with 5% of ACN, increasing to 75% in 7 min, and then to 100% in the following 3 min. Pure organic conditions were kept constant for 2 min and finally initial conditions were reached in the next 2 min. The total run time for each injection was 20 min, the injection volume was set to 20 μL and the mobile phase flow-rate to 0.3 mL/min.

MS/MS detection was performed in positive (PI) electrospray (ESI) ionization mode under selected reaction monitoring (SRM) mode. Two major characteristic fragments of the protonated molecular ion $[M+H]^+$ were monitored per analyte to enhance method sensitivity and selectivity. The most abundant transition was used for quantification, while the second most abundant was used for confirmation. Fragmentation voltage and collision energy were optimized for each transition. The optimized values were selected as a compromise using the optimum values for the majority of the analytes. This procedure was in compliance with the European Council Directive 2002/657/EC, that although it was initially conceived for food residue analysis, it has been accepted by the scientific community for environmental analysis. Chromatographic retention times (t_R), SRM transitions, cone voltages, collision energies and the proposed product ions for the transitions are shown in Table 2. The mass spectrometer was controlled by Analyst 1.4.2 software from Applied Biosystems/MDS Sciex and the Symbiosis from the Symbiosis Pico for Analyst software.

2.5. Validation

The developed method was evaluated under the optimized conditions in terms of linearity range, sensitivity, accuracy, repeatability, reproducibility and matrix effects.

Blank tests were carried out to rule out possible contamination from the sampling, storage or instrumentation. In order to comply with internal quality control procedures, two control spiked samples, two solvent injections and two procedural blanks were inserted into each analytical batch made up of six samples. The individual values obtained for control samples were plotted on a process-behaviour chart during the entire duration of the study to establish if the analysis was in a state of statistical control.

3. Results and discussion

3.1. Optimization of LC–MS/MS

According to the literature, for the efficient separation of UV F organic solvents such as MeOH and ACN with buffers and other organic modifiers are commonly used. In the current study, several combinations were tested using MeOH and ACN as organic phase and different concentrations of formic acid as organic modifier to determine the mobile phase that offered short retention time and sufficient resolution with little if any signal suppression. The best results were obtained using water and ACN, both with 0.15% formic acid. The formic acid significantly improved the peak shape of BP3 and its derivatives, whereas the other compounds showed equal or slightly better performance.

MS/MS operational parameters were optimized by using UV F individual standard solutions at 0.5 mg/L. ESI conditions were obtained as a compromise using the optimum values for most compounds. For all target UV F, ESI operating under positive conditions showed the best performance. Optimum conditions were: capillary voltage, 5000 V; source temperature, 700 C; curtain gas, 30 psi; ion source gas 1, 50 psi, ion source gas 2, 60 psi; entrance potential 10 V. Cone voltage was optimized for each compound in order to obtain maximum response for the protonated molecular ion $[M+H]^+$ and to prevent in-source fragmentation. Data acquisition was performed in SRM mode, and different collision energies were tested to obtain the optimum response. Two transitions per compound (including internal standards), for quantification and confirmation, were selected.

Table 2 summarizes the optimized values of MS/MS parameters for the target compounds and the proposed product ions. For BP3 and its derivatives the loss of 77 amu occurs, which corresponds to the $[M+H]^+ \rightarrow [M-C_6H_5]^+$, transition. We observed also another fragment corresponding to the complementary fragment of the molecule $[C_6H_5C{=}O]^+$. In the case of 4DHB, with one symmetric hydroxyl group on each side of the ketone, besides the loss of 77 amu, we considered the transition 215 → 93, which corresponds to $[C_6H_4OH]^+$.

Other compounds, including EHMC, OC and OD-PABA contain a relatively long chain branched alkyl group at the ester group and hence their collision induced dissociation mainly occurs through a McLafferty rearrangement, where the corresponding alkenes are lost, leaving the charge back at the ester group. Subsequently, the formed cation continues losing other fragments mainly H_2O [15].

3.2. Optimization of the extraction

3.2.1. PLE extraction

The optimization of the PLE conditions was performed by analysing fish samples spiked at 100 ng/g dw. Different blanks of fish were analysed by LC–MS/MS under the initial conditions and the chromatograms showed some peaks of target analytes at the same retention time. In each experiment a blank and three spiked samples were analysed and the signal of the blank was subtracted.

Fish is a complex matrix which can contain high percentages of fats that may hinder the analysis leading to strong matrix effects. Considering these facts and previous works conducted with UV F in complex environmental matrices [8,10,23], a simultaneous in-cell clean-up step was incorporated by including adsorbents other than diatomaceous earths together with the sample in the extraction cell. Aluminium oxide and Florisil were tested in order to obtain cleaner extracts to facilitate further analysis. The combination of solvent, temperature, number and time of extraction cycles were investigated in order to determine optimum extraction conditions for the target analytes.

The initial conditions were selected from our previous study [10,23], and were as follows: temperature of 100 °C, pressure of 1500 psi, 5 min of static extraction time, two cycles, 90 s of purge time, 30% of flush volume and 1 g of lyophilized fish sample. Extraction pressure was set up to 1500 psi for all PLE experiments since no significant impact on the extraction efficiency was expected [37].

The use of Florisil showed better results than aluminium oxide in terms of extraction efficiency, extract cleaning and chromatographic peak shape under all the tested conditions.

Since UV F constitutes a family of compounds with a wide range of physicochemical properties it was necessary to reach a compromise which provided good recovery rates for most compounds. The tested solvents and mixture solvents were DCM/AcEt (1/1, v/v),

Table 2
SMR experimental conditions used in the HPLC–MS/MS determination of UV F and proposed products ions.

Compound	Retention time (min)	SRM transition[a]	Cone (V)	Collision energy (eV)	Proposed ion
4DHB	5.65	215 → 121 215 → 93	45	27 45	$[M-C_6H_5OH]^+$ $[C_6H_4OH]^+$
4HB	7.04	199 → 121 199 → 105	40	25 27	$[M-C_6H_5]^+$ $[C_6H_5{=}O]^+$
BP1	7.63	215 → 137 215 → 105	40	27 29	$[M-C_6H_5]^+$ $[C_6H_5{=}O]^+$
BP3	9.27	229 → 151 229 → 105	40	25 27	$[M-C_6H_5]^+$ $[C_6H_5{=}O]^+$
4MBC	10.92	255 → 212 212 → 105	61	29 41	$[M+H-C_3H_7]^{\bullet+}$ $[MeC_6H_4CH_2]^+$
OC	11.56	362 → 250 362 → 232	71	15 27	$[M+H-C_8H_{16}]^+$ $[M+H-C_8H_{16}-H_2O]^+$
EHMC	11.81	291 → 161 291 → 179	51	25 13	$[M+H-C_8H_{16}-H_2O]^+$ $[M+H-C_8H_{16}]^+$
OD-PABA	12.00	278 → 166 278 → 151	86	27 43	$[M+H-C_8H_{16}-H_2O]^+$ $[i\text{-}BuC_6H_4C{=}O]^+$

[a] All compounds were determined in positive electrospray mode. Precursor ions correspond in all cases to $[M+H]^+$.

AcEt, AcEt/MeOH (1/1, v/v), MeOH and MeOH/H_2O (1/1, v/v). Fig. 1 shows the recovery rates obtained for each compound as a function of the extracting solvent. H_2O was not suitable to extract the most lipophilic compounds, whereas the use of MeOH resulted in poorly clean extracts. Best conditions were observed using the organic mixture DCM/AcEt (1/1, v/v) which allowed good recovery rates for all the target compounds.

Temperature is a very important parameter in PLE extraction. Application of higher temperature in PLE decreases the viscosity of solvents, thus allowing their better penetration into the sample matrix [38]. Temperatures over 100 °C were discarded since a big increase in matrix effects and interferences were previously observed (data not shown). The recoveries obtained at low temperatures were lower for most of the analysed compounds, thus 100 °C was selected as the optimum temperature for the extraction. Each static cycle introduce fresh solvent, which is very useful for complex matrix as fish, whereas the longer the time of a cycle the better the diffusion of analytes into the extraction solvent. For a more exhaustive extraction process it is recommended to divide the extraction into more than one cycle [39]. To check this, the extracts of individual cycles of 5 min were collected as well as the extracts from 2 and 3 cycles. Results showed that two cycles were sufficient to satisfactorily extract the compounds.

Despite the Florisil in-cell purification, the direct injection of the PLE extracts leads to high matrix effects and interferences. LODs obtained were above 40 ng/g dw for most compounds, too

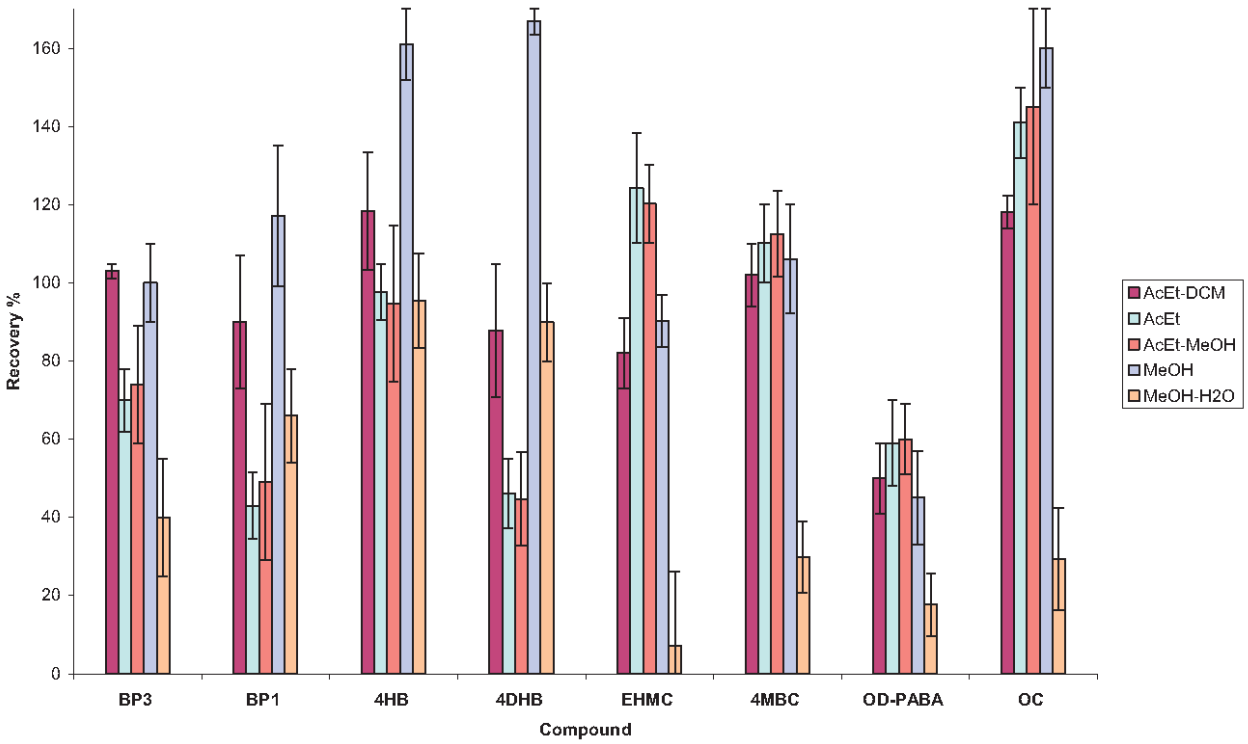


Fig. 1. The influence on the recovery efficiency of the different solvent combinations tested. AcEt, ethyl acetate; DCM, dichloromethane; MeOH, methanol; H_2O, water.

high for the determination of these compounds in fish samples. In consequence an additional clean-up step was found to be required.

3.2.2. SPE purification

The optimization of the SPE process was conducted by analysing the PLE extracts of fish samples spiked at 100 ng/g dw and diluted to 200 mL with HPLC grade water (MeOH < 5%). As in the optimization of the PLE conditions, in each experiment a blank and three spiked samples were analysed and the signal of the blank was further subtracted. The cartridge type and the sample extraction volume were optimized. Higher recovery rates and lower MLODs were the criteria applied in the optimization process. Fig. 2 compares the recovery rates achieved with the different cartridges tested. Polymeric cartridges Oasis HLB, silica bonded Isolute C18, alumina Isolute AL-N and a tandem combination of Isolute C18 and basic alumina Interchim ALB cartridges were the four approaches tested. SPE experiments were performed as described previously in Section 2.3.1. The tandem approach achieved good recoveries for the most lipophilic compounds, but could not retain BP3 and its transformation products. The alumina cartridge provided rather low recoveries for most compounds. The Oasis HLB cartridge achieved good results for the less lipophilic compounds, however for the most lipophilic ones were poor, in particular compared with the Isolute C18 cartridge. In conclusion, further experiments were carried out with Isolute C18 cartridges.

A breakthrough study was then carried out to determine the best sample volume. PLE extracts were diluted to 100, 200 and 500 mL with HPLC grade water. Differences among these volumes were not significant. Since low volumes may lead to clogging in the loading step when extracts are very dirty, 200 mL was selected as the optimum volume.

3.3. Validation

Instrumental analytical parameters, linearity ranges, correlation coefficients (r^2), instrumental limits of detection (ILOD) and quantification (ILOQ) and inter-day and intra-day precisions expressed as relative standard deviation (RSD) are summarized in Table 3. The calibration curves obtained for the SRM transitions were linear for all the compounds in a wide range of concentrations, typically from 0.5 to 500 ng/g with $r^2 > 0.9978$ for all compounds. ILODs and ILOQs were estimated for each compound. ILOD, defined as the lowest analyte concentration with a signal to noise ratio (S/N) of three, and ILOQ, defined as the concentration with S/N ratio of 10 and imprecision lower than 20% were evaluated by injecting 5 μL of diluted UV F solutions. ILODs ranged from 0.2 to 4 pg injected and ILOQs from 0.7 to 47 pg injected. The intra-day instrumental precision was determined by analysing seven replicates of a mixture standard solution at 20 μg/L within a given day. Inter-day instrumental precision was also estimated by analysing seven replicates of the same solution on seven different days. Good precision was obtained with RSD values in the range 3–5% (intra-day) and 5–8% (inter-day).

The identification and confirmation criteria for the analysis of the target compounds were based on the Commission Decision 2002/657/EC. Retention times of UV F in standards and in the samples were compared at a tolerance of 2.5% and the relationship between the two transitions was compared with the relative ion intensities of UV F standards. A difference of less than 20% was considered acceptable according to the EU directive.

MLODs (lowest analyte concentration with a signal to noise ratio (S/N) of three) and MLOQs (concentration with S/N ratio of 10 and imprecision lower than 20%) were evaluated by spiking fish samples, extracting and analysing several times. Low MLOD and MLOQ values, ranging from 0.1 to 6.0 ng/g dw and 0.3 to 20.0 ng/g dw, respectively were achieved as summarized in Table 4. MLODs were improved by almost one order of magnitude as a result of the addition of the SPE clean-up step. These MLOQs were below the reported concentrations in fish [35] and suitable to be used in routine screening and quantification of UV F in biota samples.

Since no certified reference materials were available for the analysis of UV F in fish, the accuracy assessment was performed with relatively uncontaminated fish. Seven fish samples were spiked with the target UV F and the surrogate standard at three levels (10 (or 20 when MLOQ > 10), 50 and 100 ng/g dw). The isotopically labelled internal standards BP3-d_5 and 4MBC-d_4 were added before the injection in order to compensate the instrumental variability and matrix effects. Different blanks of fish were processed by each tested procedure. The chromatograms showed some small peaks corresponding to the target analytes. In each experiment one blank and seven spiked samples were analysed and the signal of the blank was subtracted. Mean recovery rates were calculated for each analyte and for spike level. Table 4 shows that recovery rates were between 70% and 111% except for OD-PABA (36–42%) with RSD < 15%. The low extraction efficiency reported for OD-PABA can be the consequence of the high matrix effects observed at its retention time that could not be compensated by the internal standards. The surrogate standard should exceed 75% recovery to meet the quality criterion for an efficient extraction. Otherwise, the analysis was considered invalid and the sample was prepared and analysed again. Fig. 3 shows the extracted ion chromatograms for the studied UV F in a spiked fish sample at 50 ng/g dw.

The extent of matrix effects was estimated during analysis. They were determined by comparing the analytical response given by a standard in pure solvent and in fish extract at the different spike levels. Although the addition of a SPE clean-up step considerably decreased the matrix effects, their suppression values comprised between 15% and 65%. The use of the isotopically labelled internal standards compensated the matrix effects almost completely for all compounds except for OD-PABA, for which compensation was solely partial.

Considering the aforementioned, quantification was carried out following the internal standard calibration approach.

Finally, to ensure a good accuracy of the developed method, the samples analysed in Section 3.4 were further quantified by standard addition showing no significant differences in the obtained concentration values.

3.4. Application to real samples

To demonstrate the suitability of the developed method for the determination of UV F in biota, some fish samples from the Guadalquivir river basin were analysed. Table 5 summarizes the results of this study, showing solely the detected compounds. Three out of the eight target UV F: BP3, EHMC and OC, were present. The UV F detection frequency was about 80%. The highest levels (above 290 ng/g dw) were detected in fish of the species *L. sclateri*, endemic of the Iberian Peninsula. EHMC was the most ubiquitous and at the highest concentration. EHMC is extensively used in several personal care products and has shown estrogenic activity [3] and effects on the global gene expression in fish [4]. One factor to consider in the high levels found for this compound in *L. sclateri* fish, a predator species, is biomagnification. Fent et al. suggested that biomagnification occurs for EHMC in the aquatic environment [2]. In this study biomagnification is suggested in the predator/prey pairs cormorant and fish (barb, chub and brown trout) and between the omnivorous barb feeding on *Gammarus*. The herein reported values are in agreement with studies performed in other European river basins but constitute the first data on UV F bioconcentration in fish from Iberian rivers [2,13,31–33].

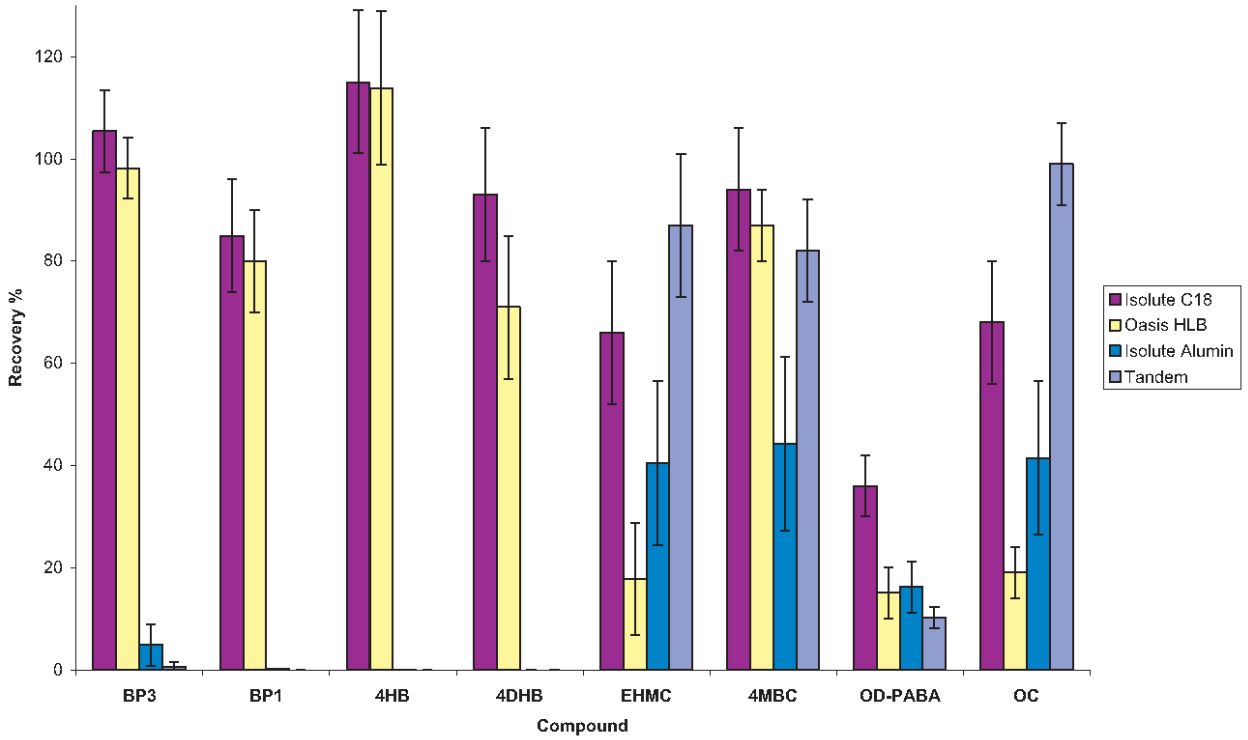


Fig. 2. The influence on the recovery efficiency of the different cartridges tested (Isolute C18, Oasis HLB, alumina Isolute AL-N and a tandem combination of Isolute C18 and basic alumina Interchim ALB).

Table 3
Instrumental quality parameters obtained for the LC–QqLIT-MS/MS method for the analysis of UV F in fish.

	Linearity range (μg/L)	r^2	ILOD (pg)	ILOQ (pg)	Precision[a] (%RSD) $n=7$	
					Intraday	Interday
BP3	0.5–500	0.9997	4	13	3	5
BP1	0.5–500	0.9999	10	33	3	6
4HB	0.5–500	0.9998	6	20	4	5
4DHB	0.5–500	0.9998	14	47	3	5
4MBC	0.5–500	0.9997	6	20	3	6
EHMC	0.5–500	0.9994	10	33	5	7
OC	2.5–500	0.9993	10	33	5	8
OD-PABA	0.1–100	0.9978	0.2	0.7	4	7

[a] Injections of 100 pg.

Table 4
Performance of the HPLC–ESI-MS/MS developed method for the analysis of UV filters in fish.

	Conc. (ng/g dw)	Rec. (%) ±RSD	Conc. (ng/g dw)	Rec. (%) ±RSD	Conc. (ng/g dw)	Rec. (%) ±RSD	MLOD (ng/g dw)	MLOQ (ng/g dw)
BP3	10	112 ± 14	50	106 ± 7	200	107 ± 6	1.2	4.0
BP1	20	91 ± 7	50	90 ± 7	200	92 ± 6	4.0	13.3
4HB	20	110 ± 12	50	112 ± 6	200	110 ± 5	6.0	20.0
4DHB	20	94 ± 10	50	92 ± 9	200	96 ± 8	5.0	16.7
4MBC	10	109 ± 10	50	99 ± 7	200	95 ± 5	0.7	2.3
EHMC	20	70 ± 10	50	72 ± 10	200	66 ± 7	5.0	16.7
OC	20	70 ± 11	50	80 ± 10	200	75 ± 9	6.0	20.0
OD-PABA	10	36 ± 12	50	40 ± 11	200	42 ± 11	0.1	0.3

Conc., concentration; Rec., recovery; RSD, relative standard deviation; MLOD, method limit of detection; MLOQ, method limit of quantification.

Table 5
Concentration of the detected UV F in fish (ng/g dw).

Sample	Common name	Scientific name	% lipid	BP3	EHMC	OC
F1	Andalusian Barbel	*Luciobarbus sclateri*	27	n.d.	n.d.	n.d.
F2			29	n.d.	19.0	<LOQ
F3			41	24.3	241.7	30.4
F4			34	16.5	63.0	n.d.
F5	Common Carp	*Cyprinus carpio*	9	11.2	<LOQ	n.d.

n.d., not detected.

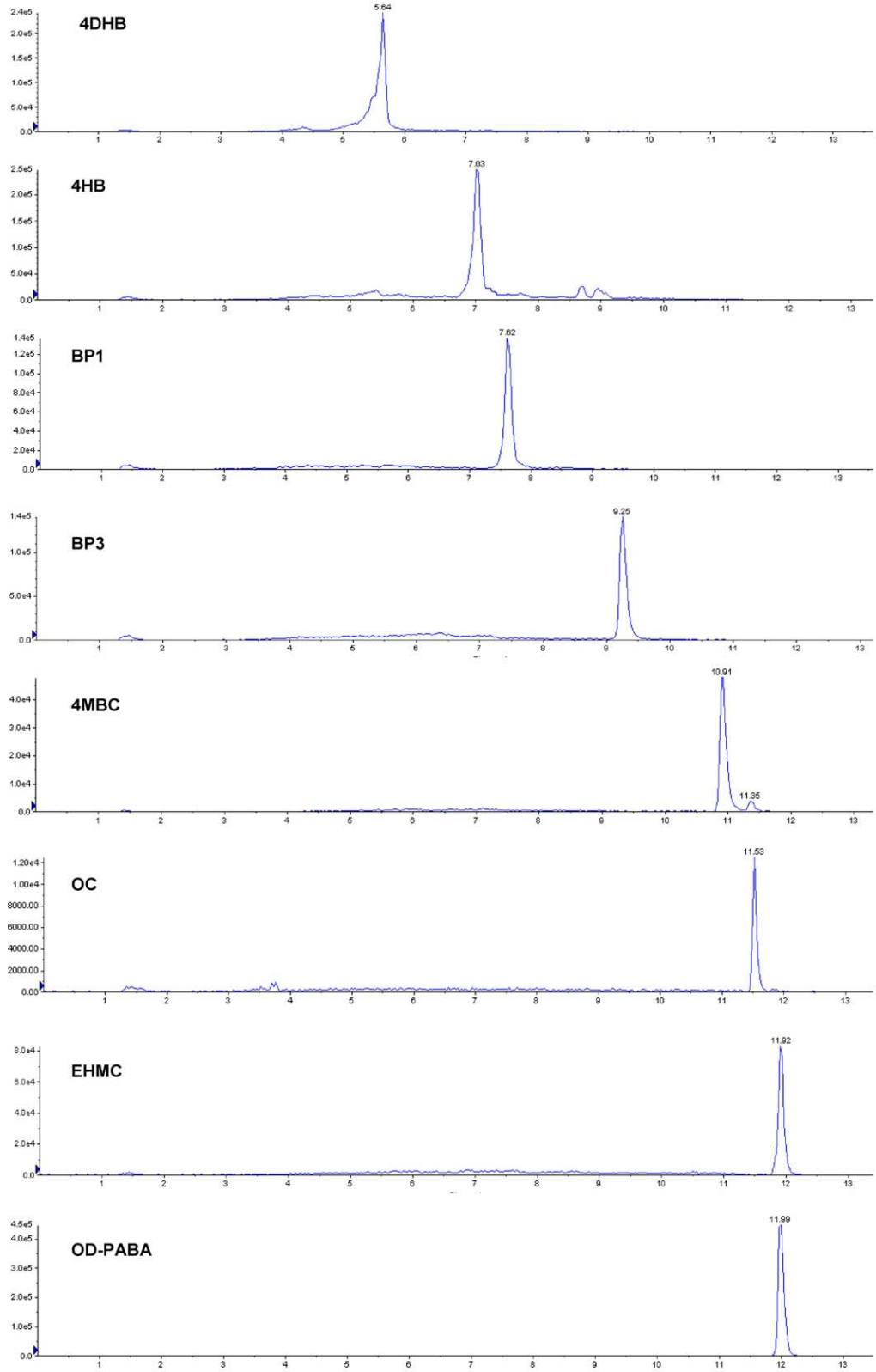


Fig. 3. Reconstructed ion chromatograms showing the SRM transitions for the studied UV F in a spiked fish sample at 50 ng/g dw.

4. Conclusions

This study involved the development and validation of a LC–MS/MS method for the simultaneous analysis of eight UV F compounds, including three BP3 transformation products, with a wide range of physicochemical properties, in fish. Especial efforts were devoted to optimize an efficient purification process together with an exhaustive extraction procedure. Results indicated that a two-step purification procedure, involving in-cell PLE and further SPE purifications, was required to obtain a clean fish extract.

The multi-residue method developed was efficient, with high sensitivity and accuracy allowing its use for monitoring the bioaccumulation potential of sunscreen agents in fish. In particular, the sample preparation step developed allowed a considerable reduction in time, solvents and personnel effort when analysing very complex sample matrix such as fish.

The performance of the proposed method was satisfactory in the determination of the target UV F in real fish samples from the Guadalquivir river basin (Spain). BP3, EHMC and OC were detected at high concentrations, up to 240 ng/g dw. These data constitute the first determination of UV F residues in fish from Iberian rivers.

The detection of these emerging pollutants also in fish from Spain evidences their widespread distribution in the environment, and therefore the need to carry out further studies in order to better understand their occurrence and fate. To carry out such research simple, fast and robust methodology with the appropriate sensitivity and selectivity as the one presented here is required.

Acknowledgments

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2.5. DETERMINACIÓN DE FILTROS UV EN MAMÍFEROS MARINOS

Publicación científica #7:

"First Determination of UV-Filters in Marine Mammals Octocrylene Levels in Franciscana Dolphins"

por:

Pablo Gago-Ferrero, Mariana B. Alonso, Carolina P. Bertozzi, Juliana Marigo, Lupércio Barbosa, Marta Cremer, Eduardo R. Secchi, Alexandre Azevedo, José Lailson-Brito Jr, Joao PM. Torres, Olaf Malm, Ethel Eljarrat, M.Silvia Díaz-Cruz, Damià Barceló

en "Environmental Science & Technology" (Aceptado)

First Determination of UV Filters in Marine Mammals. Octocrylene Levels in Franciscana Dolphins

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ABSTRACT

Most current bioexposure assessments for UV filters focus on contaminants concentrations in fish from river and lake. To date there is not information available on the occurrence of UV filters in marine mammals. This is the first study to investigate the presence of sunscreen agents in tissue liver of Franciscana dolphin (*Pontoporia blainvillei*), a species under special measures for conservation. Fifty six liver tissue samples were taken from dead individuals accidentally caught or found stranded along the Brazilian coastal area (six States). The extensively used octocrylene (2-ethylhexyl-2-cyano-3,3-diphenyl-2-propenoate, OC) was frequently found in the samples investigated (21 out of 56) at concentrations in the range 89-782 $ng.g^{-1}$ lipid weight. São Paulo was found to be the most polluted area (70% frequency of detection). Nevertheless, the highest concentration was observed in the dolphins from Rio Grande do Sul (42% frequency of detection within that area). These findings constitute the first data reported on the occurrence of UV filters in marine mammals worldwide.

KEYWORDS: UV filters, marine mammals, dolphins, UPLC-MS/MS analysis

INTRODUCTION

UV filters (UV F) are emerging environmental contaminants for which there is currently a lack of knowledge about their occurrence, fate and effects on the ecosystems (Richardson et al., 2010). UV F constitutes a large and heterogeneous group of chemicals that are ingredients in personal care products to protect skin and hair from the sunlight, and in other industrial goods such as paint, wax, plastic or textile to prevent photodegradation of polymers and pigments (Zenker at al. 2008).

These chemicals enter the aquatic environment either indirectly, via wastewater treatment plant effluents (urban and industrial) or directly, through human aquatic recreational activities. Previous studies have demonstrated the occurrence of UV F in water, sewage sludge, sediment, and biota (Fent et al., 2010; Gago-Ferrero et al., 2011a; Gago-Ferrero et al., 2011b). Many UV F are lipophilic compounds, therefore have the potential for bioaccumulation and biomagnification in aquatic ecosystems through the trophic chain (Fent et al., 2010). Works on biota were mainly focused on fish (Balmer et al., 2005; Buser et al., 2006; Zenker at al., 2008; Fent et al., 2010), but other organisms have been studied as well, such as fish eating birds and aquatic invertebrates (Fent et al., 2010). Several UV filters are known to have toxic effects on both aquatic and terrestrial organisms. Although the studies dealing with ecotoxicity of these compounds is scarce, they have been shown to act as environmental estrogens and antiandrogens, cause reproductive disruption and affect the thyroid axis (Fent et al., 2008; Brausch and Rand, 2011). So far, there is still even more limited information available about the fate and effects of these chemicals in marine ecosystems. High levels of multiclass UV F in seawater have been reported, with concentrations up to 799 ngL^{-1} of 4-methylbenzylidene camphor (4MBC) (Landford and Thomas, 2008; Tarazona et al., 2010). Recently, it has been documented that UV F caused harmful effects on coral reefs (coral bleaching) by promoting viral infections (Danovaro et al., 2008). As regards marine biota, the analysis of four benzotriazole UV stabilizers, namely UV-320, UV-326, UV-327 and UV-328, and the UV filter 4MBC in marine organisms from the Ariake Sea (Japan) revealed that the three benzotriazole stabilizers investigated bioaccumulated in all the species analyzed, from benthic invertebrates to several fish species,

including the hammerhead shark (Nakata et al., 2009).

Among UV filters, octocrylene (2-ethylhexyl-2-cyano-3,3-diphenyl-2-propenoate, OC) is of great concern since it is a highly lipophilic compound (log K_{ow} 6.88), stable, and resistant to sunlight degradation, but there is evidence that it can trigger the production of potentially harmful free radicals (reactive oxygen species) when it releases the absorbed energy. The widespread occurrence of this compound, as well as its high concentrations found in sewage sludge and sediments (Gago-Ferrero et al., 2011a, Gago-Ferrero et al., 2011b) appears to be associated with its extensive use in formulations, especially personal care products, because both protects in UVA and UVB regions, and augments the absorbing capacity of other organic UV filters, such as ethylhexylmethoxycinnamate (EHMC), avobenzone (AVB) and benzophenone-3 (BP3) (Gaspar and Maia Campos, 2006). Since maintaining the absorption capacity is important to prevent erythema and to reduce the subsequent risk of melanoma development, formulations containing OC had superior performance compared to other formulations that did not contain OC, and therefore, preferably used.

The goal of the present study was to contribute for a better understanding of the impact of the increasing use of UV filters in densely populated coastal areas on marine organisms. The study aimed at demonstrating the potential for biomagnification of the extensively used sunscreen agent OC on marine mammals, specifically on dolphin, since they occupy a higher trophic level in the marine food chain, and have relatively low metabolic activity, thus accumulating high levels of organic pollutants in their body (Tanabe, 2002). For this study Franciscana dolphin (*Pontoporia blainvillei)* was the selected species. It is a small cetacean with a distribution restricted to the southwest Atlantic Ocean. This is the most impacted cetacean off the eastern coast of South America (Secchi, 2010) and is listed as “vulnerable” in the Red Book of the International Union for Conservation of the Nature (IUCN). Franciscana was considered a species that needs particularly measures of conservation (Reeves et al., 2008) and is also included in the Index II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), that Argentina, Uruguay

and Brazil are undersigned. Their coastal distribution makes it particularly vulnerable to human activities such as incidental capture in fisheries and habitat degradation by anthropic contaminants (Ott et al., 2002; Alonso et al., 2012a; 2012b). Evidences suggest that the mortality rates are excessive and unsustainable (Secchi & Wang 2003).

To the best of our knowledge, this work provides the first evidence of UV absorbing compounds bioaccumulation in marine mammals.

EXPERIMENTAL SECTION

Chemicals and reagents

OC (>98% purity), and the isotopically labelled compound benzophenone-d_{10} (BP-d_{10} 99% purity), used as internal standard (IS), were obtained from Sigma-Aldrich (Steinheim, Germany). Organic solvents and HPLC grade water (Lichrosolv), as well as H_2SO_4, formic acid (98% purity) and hydromatrix were provided by Merck (Darmstad, Germany). Nitrogen and argon (purchased from Air Liquide, Barcelona, Spain) were of 99.995% purity. The syringe and the pressurized liquid extraction (PLE) cellulose filters used were purchased from Whatman (London, U.K.) and from Dionex Corporation (Sunnyvale, CA, USA), respectively. Isolute Alumina Cartridges used for solid phase extraction (SPE) were obtained from Biotage (Uppsala, Sweden).

The OC and BP-d_{10} stock standard solutions were prepared in methanol at 200 mg L^{-1}. The solutions were stored in the dark at -20 ºC. A diluted 20 mgL^{-1} stock standard solution was prepared weekly. Working solutions were prepared daily by appropriate dilution of the diluted stock standard solution.

Sampling area and sample collection

The Brazilian coastline has around 8500 km of length. The Southeast Brazilian region, historically, had turned into an important industrial centre of Brazil. Rio de Janeiro and São Paulo States are the most anthropogenically disturbed areas along the country shoreline.

zMassive metropolitan complex surrounds the estuaries and bays, which have been receiving discharges of chemical contaminants from domestic, industrial and agricultural wastewaters besides also they are impacted by overfishing, harbour activities and solid

trash (Bícego et al, 2006; Dorneles et al., 2010). Santos estuary, in São Paulo coast, is the most important Brazilian example of environmental degradation from aquatic and atmospheric pollution by industrial origin. The largest harbour in Latin America (the Port of Santos) and the largest industrial complex in Brazil are located in this area. Industrial activities began in the 1950s with the establishment of diverse factories (steel, oil, and agribusiness) and have turned this estuary into the final destination for toxic waste and contaminated effluents since then (Lamparelli *et al.*, 2001).

Collected samples were taken from individual dolphins found stranded dead at the beaches or incidental caught in fishing nets along the Brazilian coast, Southwestern Atlantic, from 1994 to 2009. Available information on the samples is given in Table 1. Sexual maturity is known to occur at different length depending on the coastal area. The individuals considered in this study included males and females, adult (sexually matured), juvenile (sexually immature > 100 cm length) and calves (sexually immature < 100 cm length) specimens.

Fifty six individual were analyzed, belonging from many States of Brazil: Espírito Santo (n = 12), Rio de Janeiro (n = 1), São Paulo (n = 10), Paraná (n = 3), Santa Catarina (n = 11) and Rio Grande do Sul (n = 19). Liver samples collected were placed in aluminium foil, frozen, and further lyophilized. Freeze-dried liver tissue was ground, homogenized and stored in brown glass sealed containers at –20^{o} C until analysis.

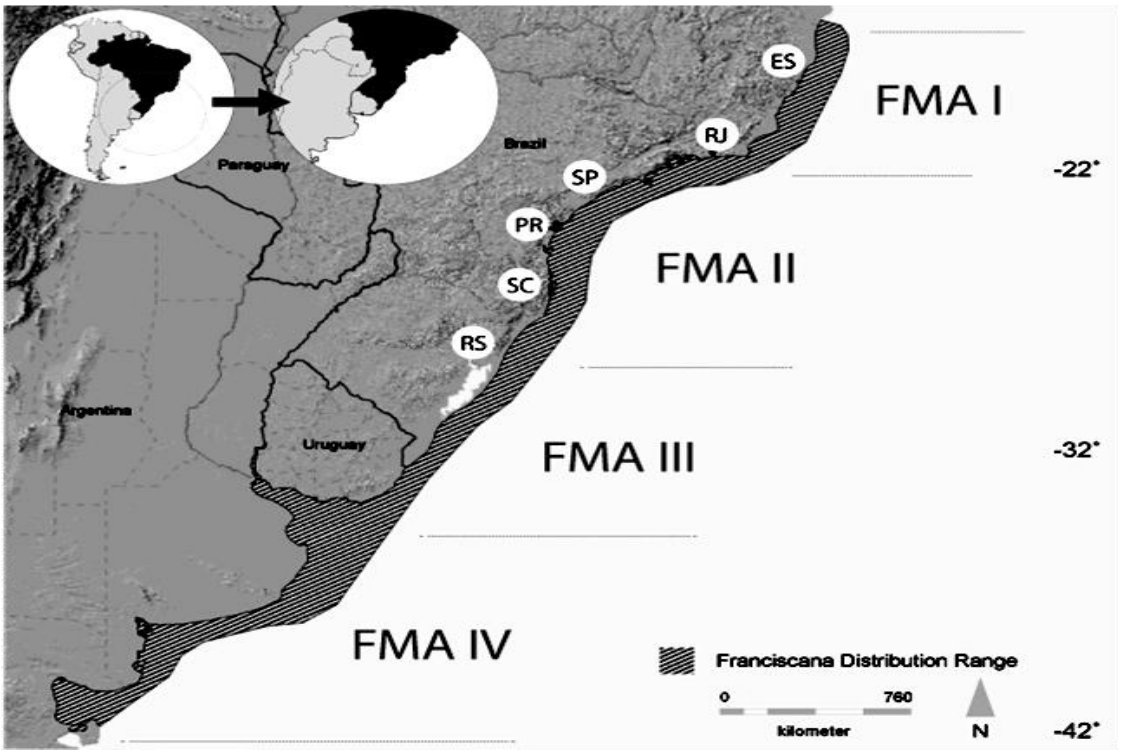

Figure 1: Study area map, Southeaster and Southern coast of Brazil. Brazilian States sampled: ES – Espírito Santo, RJ – Rio de Janeiro, SP - São Paulo, PR, Paraná, SC – Santa Catarina, RS – Rio Grande do Sul. FMA – Franciscana Management Areas (I – IV).

Analytical methods

In order to prevent contamination and photodegradation of samples and standard solutions all glassware used was previously washed and heated overnight at 380 ºC, and further sequentially rinsed with different organic solvents and HPLC grade water. Separate solvents and only previously unopened packages of solvents, chemicals and other supplies were used.

In addition, a set of at least two operational blanks were processed together with each batch of samples. Standard solutions and samples were always covered with aluminium foil and stored in the dark. Furthermore, gloves were worn during the sample preparation process.

Sample preparation

Samples were extracted by PLE using an automatic extractor ASE 200 (Dionex Corporation, Sunnyvale, CA, USA). One gram dry weight of freeze-

dried dolphin liver tissue was mixed in the extraction cells with hydromatrix. The PLE optimised parameters were as follows: preheating of 5 min, heating of 5 min, two extraction cycles of 10 min using dichloromethane/hexane as extraction solvent (1/1, v/v), temperature of 100 ºC, pressure of 10000 kPa, flush volume of 80 % of cell and 90 s of nitrogen purge. The PLE extract obtained was concentrated to 3 mL and then subjected to a purification step via acid attack with concentrated H_2SO_4 (95-97% purity) (4 × 2 mL). The extract was purified by SPE with alumina cartridges (5 g/20 mL), using 40 mL of hexane:dichloromethane (1:2). Finally, the extract was evaporated to dryness. The residue was further reconstituted with 0.1 mL of acetonitrile and the IS was added before LC-MS analysis.

Lipid content determination

The lipid content determination was performed by gravimetric analysis. After the extraction, the extracts were concentrated to incipient dryness, each vial was weighed and the difference between the initial weighing and weighing after the addition and evaporation rate was used to calculate the percentage of lipids.

Percentage lipid content was determined for each individual. Mean values were calculated for those specimens sampled in the same geographical area, which were in the range 4%-7%.

UPLC-ESI(+)-MS/MS analysis

Target analysis of OC was performed by ultra-high performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) using an Acquity UPLC chromatograph attached to a triple quadrupole detector (TQD) mass spectrometer (Waters). A Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 2 µm) column (Merck) was used. The solvent flow rate was set to 0.4 mL min^{-1} and the column temperature was kept at 50 ºC. The sample volume injected was 10 µL. The chromatographic separation was performed by using as mobile phase HPLC grade water (A) and acetonitrile (B), both with 0.3% formic acid. The adopted elution gradient started with 5% of eluent B, increasing to 95% in 1.20 min, kept constant for 2.30 min, and rising to 100% in the following 0.5 min. During the next 2.5 min the elution

gradient was kept constant, and then back to initial conditions in 3 min.

MS/MS was operated in selected reaction monitoring (SRM) and positive electrospray ionization mode (ESI+). The optimized parameters were as follows: desolvation gas (nitrogen), 750 L h^{-1}; collision gas (argon), 0.19 mL min^{-1}, nebulisation gas (nitrogen), 90 L h^{-1}; ion spray voltage 3.35 kV; source temperature, 130 ºC and desolvation temperature, 450 ºC. Two characteristic transitions of the protonated molecular ion $[M+H]^+$ (precursor ion) were recorded.

For the positive confirmation of OC in liver tissue samples, strict criteria had to be met in order to avoid false positives. Following the European Commission Decision 2002/657/EC (European Commission, 2002), that although it was initially conceived for food residue analysis, it has been accepted by the scientific community for environmental analysis, a minimum of three identification points (IPs) is required for this purpose. In our case, these 3 IPs corresponded to the precursor ion (m/z 362 amu) and to the two transitions recorded from the precursor ion ([M+H]+) to the product ions $[M+H-C_8H_{16}]^+$ and $[M+H-C_8H_{16}-H_2O]^+$ at m/z 250 and 232 amu, respectively. Besides, the chromatographic retention time of the analyte in the sample should not vary more than 2.5% in comparison to the calibration standards', and the relative abundance of the two SRM transitions monitored must also be compared to the standards' corresponding values, and range about ±20%. Figure 2 represents the chromatograms for OC corresponding to a standard solution at 40 ng mL^{-1}, and to a dolphin liver sample from an adult male from São Paulo. In this case, retention times were exactly the same and the difference in SRM ratios was solely 6%, and thus, confirming the identification

The described methodology probed to be precise and sensitive for the quantification of OC in dolphin liver samples affording method limits of detection (LOD) and quantification (LOQ) of 23 and 75 ng g^{-1} lipid weight (lw), respectively, and a relative standard deviation of 9%.

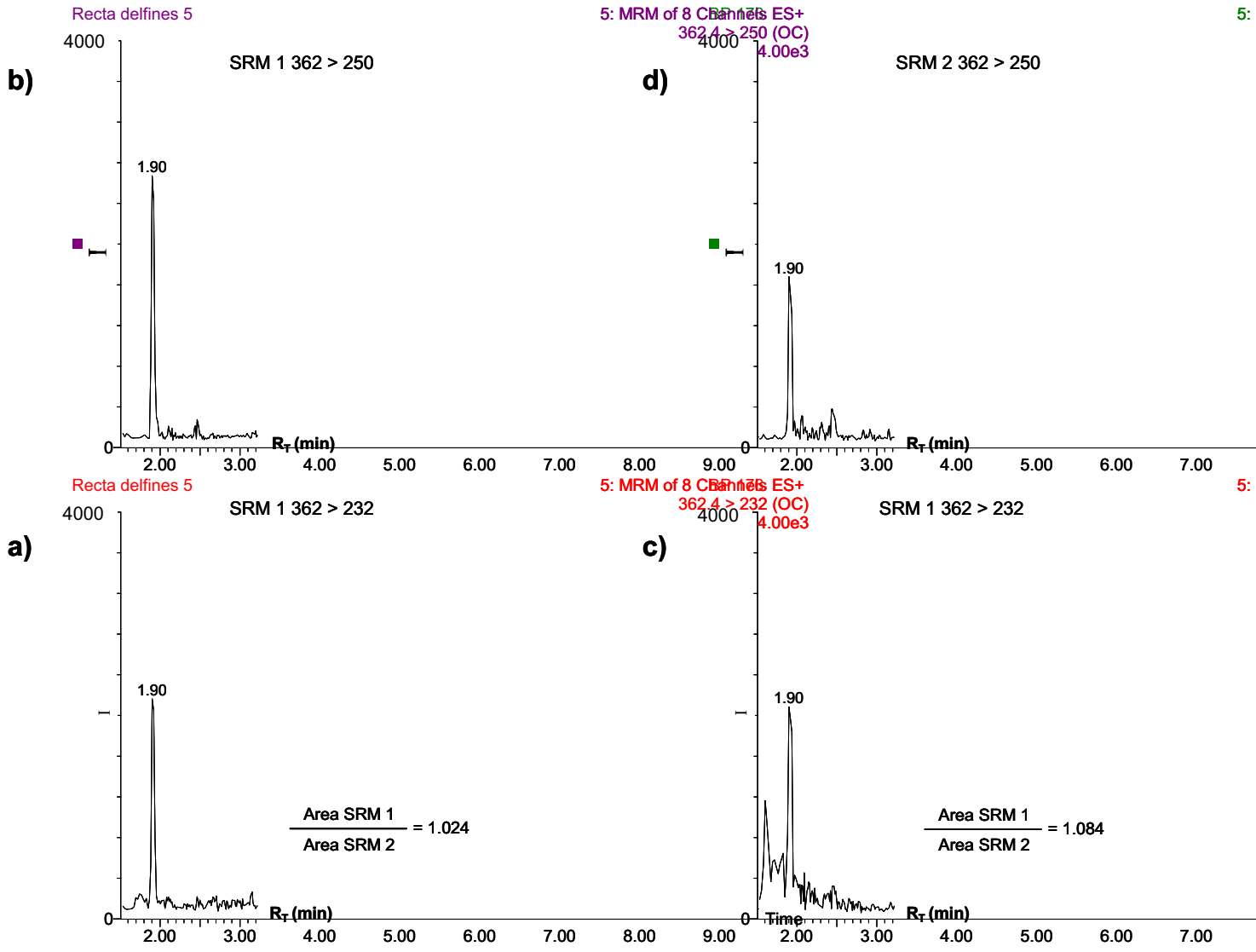

Figure 2: Reconstructed SRM reference chromatogram for octocrylene (OC) corresponding to a standard solution at 40 ng mL^{-1} (a and b), and a chromatogram corresponding to a dolphin liver sample from an adult male (sample code BP 176) from Sao Paulo (c and d).

RESULTS AND DISCUSSION

OC concentration in liver tissue

The analysis of the samples revealed that OC was present in 21 out of the 56 samples analyzed (38% frequency of detection) with concentrations in the range 89-782 ng g^{-1} lw (see Table 1). These concentrations are notoriously higher than that reported by Balmer et al. (Balmer et al., 2005) for OC in lake fish (25 ng g^{-1} lw). This outcome was expected, as with other organic pollutants that bioaccumulate and

biomagnify along the food chain, given the higher trophic level occupied by dolphins.

From the six sampling areas selected, Rio de Janeiro was one of the areas where we expected to find residues of the sunscreen because of its beach area with very active aquatic activities. However, in the only sample taken OC was not detected. Despite that, we cannot rule out its presence in the area since we were only able to analyze one sample. In contrast, the most contaminated zone was São Paulo, where OC was most frequently detected (70%), followed by Rio Grande do Sul, where the UV filter was observed in 8 out of the 19 dolphins sampled, and at the highest concentration, 782 ng g^{-1} lw. Nevertheless, the geographical distribution of positive samples, as depicted in Figure 3, indicated that the highest mean concentration (373 ng g^{-1} lw) was determined in the samples from Santa Catarina State. This sampling area is a partially enclosed estuarine receiving industrial and urban wastewater discharge, which could act as a sink for anthropogenic pollutants. In a recent study with polybrominated diphenyl ethers (PBDEs) in the same samples from this work, it was observed also the higher levels in the dolphins from this disturbed bay, in Santa Catarina (Alonso et al., 2012a). Similar mean concentrations were reported for OC and for the group of PBDEs. The comparison of the concentrations observed in the rest of areas evidenced a different source for OC and PBDEs anthropogenic emissions. The geographical distribution of mean concentrations (ng g^{-1} lw) for OC was: 373 (SC) > 310 (ES) > 298 (RS) > 241 (SP) >129 (PR), whereas for the group of congeners of PBDEs it was: 432 (SC) > 329 (SP) > 156 (PR) > 144 (ES) >37 (RJ) > 34 (RS).

Relationships between OC concentration and biological characteristics

The inclusion of individuals with varying biological characteristics in this study provided an opportunity to examine contaminant liver tissue concentrations in relation to lipid content, sex, and physical and sexual maturity. The trend of increased concentration in biota samples with increasing lipid content may be observed for a number of organic pollutants (Coat et al., 2011). In an attempt to assess the behavior of the lipophilic UV filter, the correlation

between liver lipid content and OC concentration was evaluated (data not shown), however, no correlation could be established based on these parameters. The reason for this differential behavior remains unclear.

Table 1: Sampling locations, biological information on the dolphins collected along the Brazilian coast, and concentrations of octocrylene (OC) in the liver samples. In parenthesis, the number of samples analyzed, nd: not detected, uk: unknown, na: not available, FT: percentage of positive samples within the area, Ca: Calves, Im: immature, Ma: mature, Ju: juvenile, Ad: adult.

Location Brazilian State	Sample code	Sex	Length	Sexual Maturity	Physical Maturity	Concentration OC (ng g^{-1} dw)
Espirito Santo (12) **FT: 25%**	PON 08	M	70	Im	Ca	Nd
	PON 12	M	73	Im	Ca	**129**
	PON 11	M	100	Im	Ju	Nd
	PON 02	M	112	Im	Ju	Nd
	PON 13	M	113	Im	Ju	Nd
	PON 06	M	114	Im	Ju	Nd
	PON 14	M	115	Ma	Ad	Nd
	PON 09	M	117	Ma	Ad	Nd
	PON 07	F	109	Im	Ju	Nd
	PON 15	F	115	Im	Ju	Nd
	PON 03	F	118	Im	Ju	**89**
	PON 10	F	136	Ma	Ad	**712**
Rio de Janeiro (1) **FT: 0%**	RJ 46	uk	na	na	na	nd
Sao Paulo (10) **FT: 70%**	BP 125	M	100	Im	Ju	**100**
	BP 120	M	103	Im	Ju	Nd
	BP 133	M	112	Ma	Ad	**380**
	BP 149	M	116	Ma	Ad	**144**
	BP 176	**M**	**122**	**Ma**	**Ad**	**141**
	BP 110	M	124	Ma	Ad	nd
	BP 108	F	94	Im	Ca	**524**
	BP 113	F	110	Im	Ju	**269**
	BP 151	F	138	Ma	Ad	**130**
	BP 140	F	110	Im	Ju	nd

Paraná (3) **FT: 33%**	PR 50	F	56.5	Im	Ca	**129**
	PR 53	F	98	Im	Ca	nd
	PR 01	F	140	Ma	Ad	nd
Santa Catarina (11) FT: 18%	PB 221	M	83.5	Im	Ca	nd
	PB 23	M	102	Im	Ju	nd
	PB 22	M	107	Im	Ju	nd
	PB 53	**M**	**87.3**	**Im**	**Ca**	**345**
	PB 62	**M**	102	Im	Ju	**401**
	PB 56	**M**	109	Im	Ju	nd
	PB 222	F	129	Ma	Ad	nd
	PB 30	F	133	Ma	Ad	nd
	PB 37	F	133.5	Ma	Ad	nd
	PB 162	uk	127.5	Ma	Ad	nd
	PB 44	uk	145	Ma	Ad	nd
Rio Grande do Sul (19) FT: 42%	CA 143	M	125.5	na	na	nd
	CA 32	M	129.5	Ma	Ad	nd
	CA 142	M	133.7	Ma	Ad	nd
	CA 36	M	137	Ma	Ad	**153**
	CA 156	M	137	Ma	Ad	nd
	CA 172	M	143	Ma	Ad	nd
	CA 152	F	107.5	Im	Ju	**142**
	CA 63	F	135.5	Im	Ju	nd
	CA 124	F	137	Im	Ju	nd
	CA 153	F	na	na	na	nd
	CA 33	M	131	na	na	473
	CA 108	F	157	na	na	nd
	CA 173	F	161	na	na	493
	CA 179	M	110	na	na	107
	CA 193	F	116	na	na	129
	CA 194	F	123	na	na	782
	CA 234	M	103	na	na	103
	CA 237	M	132	na	na	nd
	CA 255	M	106	na	na	nd

Total frequency: 100 * 21/56 = 38%

Calves: 100 * 4/7 = 5

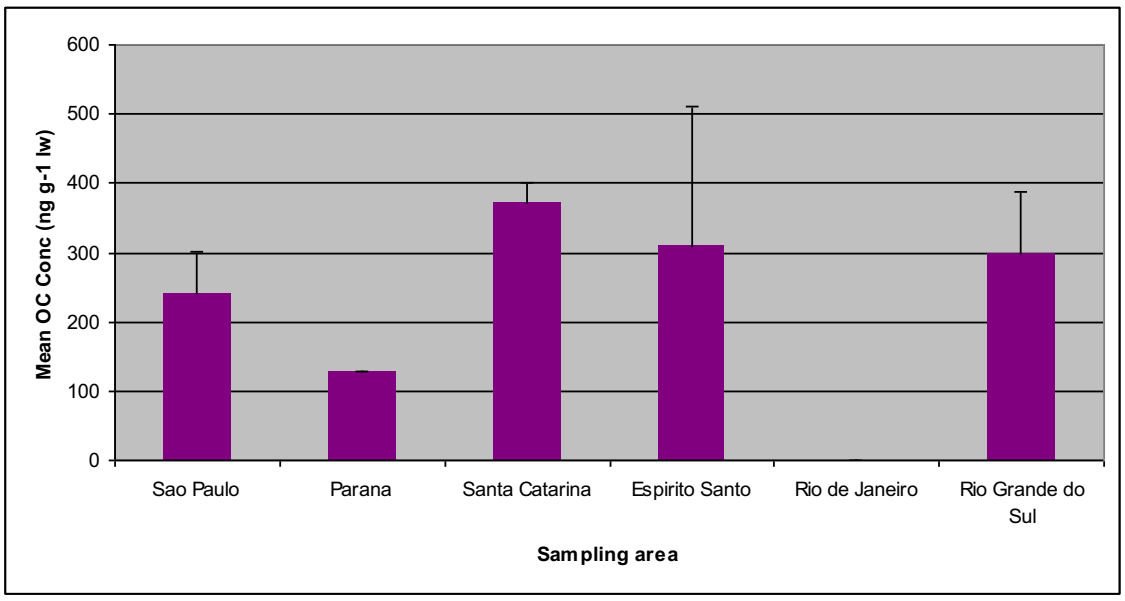

Figure 3: Distribution of OC mean concentrations in dolphin's liver (ng g^{-1} dw) and standard error, along the Brazilian coast sampling areas.

Many studies have reported gender-specific differences in the concentrations of persistent organic pollutants in marine mammals, showing the well-known high variation in the burden of lipophilic pollutants of females due to reproductive stage (Weijs et al., 2009; Alonso et al., 2012a). Therefore, the potential correlation between sex and OC concentration was also assessed; again no relationship could be observed. Nevertheless, potential maternal transfer can not be ruled out. In order to assess the mother-to-calf transfer of OC, a sample of placenta from one pregnant female dolphin was collected and analyzed, revealing that OC was present in both the placenta and the liver tissue at concentrations below LOQ (61 ng g^{-1} lw semiquantitative analysis) and 130 ng g^{-1} lw, respectively (liver sample reference BP151 in Table 1) being indicative of gestational transfer. This hypothesis, however, cannot be fully confirmed without data on a higher sample size of pregnant female dolphins. However, it must be taken into account the great difficulty in obtaining this kind of samples. On the other hand, contamination data on breast milk could also support the maternal transfer, specifically lactation transfer, of the bioaccumulated OC. This fact recently has already been characterized in humans. Reported levels of OC in women breast milk were in the range 4.70-135 ng g^{-1} lw, with 67% frequency of detection (Schlumpf et al., 2010). This transfer was consistent with the OC accumulation data obtained in the present study, where OC was found in 4 out of the 7 analyzed calves (57% frequency of detection) higher than the frequency estimated considering the complete set of individuals (38%), and at increasing concentrations (129-524 ng g^{-1} lw) as their length was greater. Nevertheless, it has to be highlighted that, obviously, the exposure pathways of human and dolphins are clearly different.

This lack of correlations found as regards OC bioaccumulated levels and biological features might be attributed in part to the different extent of use of UV filters during the interval time compressed between the collection of samples. From 1994 to 2009 there has been a steady growing use of UV filters as society has become aware of the dangerous effects of sunlight. These currently popular chemicals have shown to have a protective role against photoaging, photocarcinogenesis and photoimmunosuppression promoted by UV sun radiation (Whitmore &

Morison, 1995; Seite et al., 2000; Liardet et al., 2001).

Biomagnification

Several studies have probed that biomagnification can occur for lipophilic organic contaminants. In addition to high concentration levels, the process that produce biomagnification also results in age (or length)-specific patterns for adult male marine mammal. A different pattern has been reported for adult female mammals despite being exposed to contamination similarly to males. During gestation and lactation contaminant body burden deceased by transfer to calves (Wolkers et al., 2006; Alonso et al. 2012a).

In order to probe the potential biomagnification of OC, a full trophic analysis of this compound must be accomplished. However, as lower trophic level organisms on these areas were not available, we tried to provide preliminary evidence by comparing our results with published data on the terrestrial food chain. To date solely one study reported data on UV filter biomagnifications. Fent et al. (2010) recently investigated the accumulation in the terrestrial food chain of EHMC, an UV filter, having a similar lipophilicity (log K_{ow} 6.1) to that of OC. The differences in EHMC concentrations in cormorant, fish and macroinvertebrates suggested a trend for biomagnifications. Cormorants are migratory fish eating birds, representing a high terrestrial trophic level that we could consider comparable to the dophin's level in the aquatic food chain. Average concentrations of EHMC in five of these birds was 341 ng g^{-1} lw, with values in the range 16-701 ng g^{-1} lw, whereas decreased average concentrations were observed in lower trophic levels. This concentration range was comparable to that obtained for OC in the present study (89-782 ng g^{-1} lw).

In summary, these findings demonstrate for the first time that the extensively used sunscreen agent OC accumulates in liver of dolphins at high concentration levels (up to 782 ng g^{-1} lw) similar to those of anthropogenic organic persistent pollutants. This study also provides evidence that maternal transfer may occurs trough placenta and likely also through breast milk.

The results presented herein suggest that OC biomagnifies through the marine food web. In order to probe the biomagnification of this UV filter a full trophic level analysis of OC will be

further performed. The present study establishes the baseline levels for OC in dolphins from Brazilian coastal waters. Efforts should be directed toward the analysis of other marine organisms to assess the impact of OC as well as other extensively used UV filters and their transformation products on marine ecosystems.

ACKNOWLEDGEMENTS

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2.6. DISCUSIÓN SOBRE LAS METODOLOGÍAS ANALÍTICAS DESARROLLADAS:

Las metodologías analíticas desarrolladas en este capítulo permitieron la determinación de los principales UV-F seleccionados en esta Tesis Doctoral. También se han estudiado varios productos de transformación de BP3, y en muchos casos, detectado en muestras reales. BP3 es uno de los UV-F más ampliamente utilizados, y hay que señalar que tanto su presencia en concentraciones relevantes en el medio ambiente, como la de sus productos de transformación pueden afectar negativamente al ecosistema acuático. Ello se debe principalmente a su actividad disruptora endocrina, ya comentada en el Capítulo I. Los analitos estudiados tienen propiedades físicoquímicas muy diferentes, dada la heterogeneidad de esta familia de compuestos. Este hecho ha supuesto un reto a la hora de analizarlos simultáneamente. Así, los métodos desarrollados constituyen herramientas útiles para conseguir un conocimiento más amplio y global sobre el destino y la presencia de estos compuestos en el ecosistema acuático y de su comportamiento en EDARs.

La publicación #3 describe la única metodología completamente automatizada, basada en SPE *on-line* y LC-MS/MS, descrita hasta el momento para la determinación de UV-F en muestras acuosas. Este procedimiento permite ahorrar una gran cantidad de tiempo y trabajo de laboratorio, simplificando el proceso de análisis, reduciendo el volumen de muestra necesario de entre 100 y 500 mL a solo 5 mL, lo cual facilita el transporte de muestras y sobre todo permite almacenar un gran número de ellas en el laboratorio. Esto es imprescindible cuando el número de muestras es muy elevado por amplitud geográfica y/o temporal, como en los programas de vigilancia ambiental. La metodología descrita en esta publicación es la más sensible desarrollada hasta el momento para la mayoría de los compuestos considerados, con MLODs que van desde 0.5 - 3 ngL^{-1} para aguas subterráneas a 5 -10 ngL^{-1}para aguas de entrada de EDAR. El hecho de que al analizar volúmenes tan pequeños no se comprometa la sensibilidad en la determinación hacen de este método una herramienta muy poderosa para el análisis de UV-F. Dadas las ventajas que ofrecen las técnicas *on-line* para la determinación a nivel traza de contaminantes orgánicos, en los últimos años se ha visto una tendencia clara hacia una automatización mayor de los métodos analíticos desarrollados.

Como ejemplo, recientemente se ha publicado una metodología automatizada para el análisis de cuatro UV-F (tres derivados de la benzofenona y PMDSA). En este caso está basada en SPE y electroforesis capilar acoplada a espectrometría de masas (SPE-CE-MS/MS) [70]. La sensibilidad obtenida en este trabajo es sin embargo, inferior a la que se describe en la publicación #3 de esta memoria de Tesis. La CE ofrece ventajas como la simplicidad de uso, una gran eficiencia en la separación o un consumo muy bajo de disolventes. No obstante, esta técnica es muy sensible a pequeños cambios en las condiciones instrumentales de trabajo, siendo una técnica poco robusta. Otras desventajas que presenta es que solo se puede aplicar a un intervalo relativamente pequeño de compuestos y generalmente la sensibilidad es menor que la obtenida por LC-MS, como ocurre al compararla con nuestro método.

El análisis de UV-F en el medio ambiente tiende a una mayor automatización, no solo para las muestras de agua, como ya se ha discutido en el inicio de este apartado, sino también para las muestras de otros fluidos. Técnicas incipientes como el Turboflow que permiten la utilización de flujos turbulentos, pueden ser muy útiles especialmente para el análisis de muestras y extractos biológicos, como leche, miel o extractos de biota, ya que permiten separar mediante difusión moléculas grandes como proteínas que afectan muy negativamente a la ionización de los analitos en la espectrometría de masas. El avance en este tipo de técnicas puede facilitar y reducir muy significativamente el tiempo de preparación de muestras para matrices acuosas complejas.

El análisis de muestras sólidas es menos susceptible a ser automatizado. Generalmente la extracción y la purificación se pueden llegar a automatizar, pero su acoplamiento *on-line* LC-MS/MS está aún por llegar. En esta Tesis Doctoral, en las metodologías desarrolladas para matrices sólidas las extracciones se llevaron a cabo mediante PLE. Se trata de una técnica rápida (aproximadamente 15-20 minutos por muestra, en función del número de ciclos), de bajo consumo de disolvente, que precisa de poca cantidad de muestra y que permite operar automáticamente, lo que le hace preferible a otras técnicas como la extracción con ultrasonidos o el Soxhlet. Entre los principales parámetros a optimizar destaca la temperatura, la cual es crítica en la extracción mediante PLE. Las temperaturas altas disminuyen la viscosidad del solvente, obteniendo una mejor penetración en la matriz. Por el contrario, un exceso de temperatura puede dar lugar a la degradación térmica de algún analito así como una mayor extracción de otros compuestos presentes en la matriz, que pueden comprometer la selectividad y la

sensibilidad del análisis. En todos los métodos desarrollados en esta Tesis Doctoral la temperatura óptima ha sido de 100 ºC, ya que temperaturas inferiores disminuían la eficiencia en la recuperación de los analitos, mientras valores superiores aumentaban sensiblemente el efecto matriz. La elección del disolvente también es decisiva para la extración de los analitos de la matriz sólida. Para el caso de los lodos de EDAR y los sedimentos se obtuvieron los mejores resultados combinando ciclos de metanol con ciclos de metanol-agua (1:1 *v/v*). Aunque no es habitual el uso de mezclas binarias con agua para la extracción de compuestos lipofílicos, hay que considerar que a medida que se aumenta la temperatura la constante dieléctrica del agua disminuye, lo cual favorece el proceso de extracción. Para el análisis de muestras de pez, la mejor combinación fue el uso de diclorometano-acetato de etilo (1:1, *v/v*). En este caso el uso de metanol aumentaba muy sensiblemente el efecto matriz para algunos compuestos, lo que hizo descartar su uso.

Las publicaciones #4 y 5# describen metodologías para el análisis de UV-F en lodos de EDAR y en sedimentos, respectivamente. Estos métodos son parecidos y muestran solo pequeñas diferencias entre sí. Su principal ventaja radica en la inclusión de una etapa de purificación en la misma celda de extracción de PLE mediante la adición de alúmina, Este proceso permite reducir muy significativamente el tiempo de análisis y el consumo de disolventes ya que no precisan de una etapa de purificación después de la PLE, por lo que en este caso se automatiza la extracción y la purificación. Los LODs obtenidos son adecuados para el análisis de estos compuestos en las dos matrices seleccionadas.

Los lodos de EDAR son una matriz mucho más compleja que los sedimentos, con un porcentaje más elevado de materia orgánica y de otras interferencias. Por una parte, para el análisis de lodos de EDAR fue necesario trabajar con estándares en matriz (*matrix matched standards*) para corregir la variación de la señal debido a interferencias causadas por compuestos presentes en la matriz. Contrariamente, esto no fue necesario en el caso del análisis de sedimentos, donde el efecto matriz fue mucho menos pronunciado y el análisis se llevó a cabo con los estándares disueltos en metanol. Como era de esperar, los límites de detección obtenidos para los sedimentos fueron significativamente inferiores que los obtenidos para lodos.

En el caso del método para el análisis de UV-F en peces, descrito en la publicación #6, también se incluyó una etapa de purificación en la misma celda de extracción durante el

proceso de PLE. El adsorbente utilizado fue Florisil que, en este caso, permitió obtener mejores resultados que con la utilización de alúmina, en términos de eficiencia en las recuperaciones y limpieza de los extractos. Es posible seguir un procedimiento similar al descrito en los métodos de lodos de EDAR y sedimentos, analizando directamente los extractos obtenidos mediante PLE pero, en este caso, las interferencias debido al elevado efecto matriz incrementaron los MLODs por encima de los 40 ngg^{-1} d.w. para la mayoría de compuestos. Estos MLODs eran demasiado altos para la determinación de UV-F en muestras de pez por lo que fue necesario añadir una etapa adicional de purificación-preconcentración del extracto mediante SPE, que permitió reducir los MLODs a valores inferiores a los 6 ngg^{-1} d.w. para todos los compuestos. Aún así, esta metodología es la más rápida, simple y sensible desarrollada hasta el momento para el análisis de UV-F con un amplio intervalo de propiedades fisico-químicas en muestras de pez,

El uso de los patrones internos es un tema delicado en el análisis de UV-F. En el momento en que se realizaron los estudios en lodos y sedimentos, no había ningún compuesto comercial marcado isotópicamente que fuera idéntico a ninguno de los analitos. Este hecho dificultó mucho el análisis en matrices complejas, ya que como se describe en las correspondientes publicaciones, el efecto matriz, tanto de aumento de la señal como de disminución, es notable. En estos estudios se utilizó la benzofenona marcada isotópicamente con ^{13}C como marcador de extracción (*surrogate standard*). Este compuesto se añadía inicialmente a las muestras en una concentración conocida y se cuantificaba como un analito más. Si la recuperación de este compuesto estaba entre 70 y 100% se consideraba que el análisis era válido, si no, el análisis debía repetirse. Además, para corregir las variaciones de la señal producidas durante el análisis instrumental, básicamente por los efectos de la matriz, se utilizó la benzofenona deuterada (BP-d_{10}) como patrón interno. Este es el procedimiento que siguen la mayoría de estudios publicados sobre UV-F cuando la cuantificación externa, por razones de efecto matriz, no es posible y la adición estándar resulta demasiado laboriosa debido al gran número de muestras. Durante la realización de la Tesis se comercializaron dos UV-F marcados isotópicamente, BP3-d_5 y 4MBC-d_4. En la publicación referente al análisis de UV-F en peces (publicación #6) se describe el uso de estos compuestos, siendo el primer estudio que los utiliza como patrones internos. Debido a la gran diferencia de propiedades fisicoquímicas entre los compuestos, no se consideró adecuado utilizar

estos productos deuterados como *surrogates*, debido al comportamiento dispar que tienen los diferentes compuestos tanto en la etapa de PLE como en la de SPE. Se siguió utilizando un marcador de extracción (benzofenona-^{13}C) para asegurarnos que el proceso se ha llevado a cabo correctamente y se utilizó BP3-d_5 y 4MBC-d_4 como patrones internos para corregir los efectos de la matriz. No obstante, para garantizar una buena exactitud del método, se analizó una serie muestras mediante adición estándar. Los resultados de estas muestras no mostraron diferencias significativas con los obtenidos siguiendo el método de cuantificación con patrón interno.

En el caso de la metodología desarrollada para muestras acuosas, BP3-d_5 y 4MBC-d_4, sí que se utilizaron como *surrogate standars*, ya que se comprobó que se su comportamiento era muy similar al de los compuestos de interés a lo largo de todo el proceso analítico. En este estudio se obtuvieron recuperaciones relativas cercanas al 100% para todos los compuestos analizados. Solo hay un compuesto, Et-PABA, con una estructura muy diferente a cualquiera de los dos patrones internos utilizados. Sin embargo, comprobamos que BP3-d_5 era capaz de corregir la señal satisfactoriamente en todas las matrices y niveles de concentración con los que trabajamos. Hay que destacar, que esta metodología no incluye a los compuestos más lipofílicos, como OC, EHMC o OD-PABA, con estructuras y tiempos de retención muy diferentes, para que los que los compuestos marcados isotópicamente no corrigieron correctamente todas las variaciones que se dan en el proceso analítico y, por lo tanto, no fue posible una determinación precisa de sus concentraciones.

Como está descrito en la publicación #1, la inmensa mayoría de las metodologías desarrolladas para el análisis de UV-F mediante HPLC-MS/MS, incluídas las que se han desarrollado en esta Tesis doctoral, utilizan una fuente de ESI para la ionización de los compuestos. La ionización mediante esta fuente permite el análisis de un gran espectro de compuestos diferentes, pero tiene el inconveniente de ser bastante susceptible al efecto matriz. También existen estudios con ionización química a presión atmosférica (APCI), que no muestran ventajas relevantes con respecto a ESI. La fotoionización a presión atmosférica (APPI) puede ser una buena opción para el análisis de UV-F ya que estos compuestos absorben la radiación UV. En un estudio realizado por Rodil et al. [71] se compara APPI con ESI para el análisis de estas sustancias. Para la mayoría de los UV-F estudiados APPI mostró una respuesta similar a la obtenida mediante ESI, pero con una relación señal ruido (S/N) mucho más alta. En este estudio, incluso para

compuestos para los cuales la respuesta que ofrecen es más intensa trabajando con ESI, los límites de detección obtenidos en muestras reales son mejores con APPI debido a la mejor relación S/N. Otras ventajas que ofrece la ionización mediante APPI es la obtención de un intervalo lineal más amplio, así como una minimización en la formación de iones aductos como $[M+Na]^+$ o $[M+NH_4]^+$. Este hecho beneficia el proceso de cuantificación, ya que la formación de aductos diferentes a la molécula protonada reduce la sensibilidad y precisión del análisis debido a la naturaleza no reproducible del proceso de formación de aductos. A la vista de estos resultados sería interesante llevar a cabo más estudios utilizando APPI en el análisis de UV-F mediante LC-MS/MS.

En los estudios que se presentan en este capítulo se analizan diversos productos de transformación de BP3. Sin embargo, no se incluyen productos de transformación de otros compuestos y no hay estudios sobre el análisis y determinación de éstos en la literatura. En futuros estudios sería interesante profundizar más en el análisis de más productos de transformación, ya que la presencia de éstos en el medio ambiente es igual de relevante que la presencia de los propios UV-F. Estos compuestos pueden sufrir procesos de transformación química durante los tratamientos a los que son sometidos en las EDARs, ya sean bióticos o abióticos, y los productos resultantes pueden ser liberados al medio ambiente en cantidades relevantes. En el mismo ecosistema acuático también se dan procesos de transformación, como por ejemplo la fotodegradación de algunos contaminantes en aguas superficiales o la metabolización de algunos compuestos por seres vivos que pueden excretarlos.

El hecho de no incluir estos compuestos en los métodos analíticos supone una limitación importante a la hora de tener una visión amplia y más precisa sobre el problema ambiental que comporta una determinada familia de contaminantes. Por ejemplo, cuando evaluamos la calidad de las aguas de salida de EDARs con respecto a la presencia de UV-F, para tener información sobre si son efectivas o no en su eliminación, el hecho de no tener información sobre los productos de transformación que se forman no permite obtener una información completa. Se da el caso de eliminaciones negativas, cuando un compuesto entra en forma conjugada y no se detecta como tal en las aguas de entrada y en cambio sí en las de salida, debido a que se rompe el metabolito dando lugar al original [72-74]. Por tanto, un compuesto puede parecer que ha sido eliminado, al no determinarse en el agua de salida mientras en realidad,

puede que simplemente haya sufrido una pequeña modificación estructural, la cual puede incluso agudizar los posibles efectos nocivos que tuviese el compuesto original. Algo parecido ocurre en el análisis de biota, ya que puede darse el caso que no se detecten residuos de UV-F y sin embargo, la muestra esté contaminada por productos de transformación de éstos. La identificación y determinación de estos compuestos juega cada vez un papel mucho más importante, sobre todo desde la aparición de nuevas técnicas de espectrometría de masas de alta resolución de tipo Orbitrap, que facilitan la identificación de compuestos, así como el análisis no dirigido (non-target análisis).

2.7. DISCUSIÓN SOBRE LA PRESENCIA DE FILTROS UV EN LAS MATRICES ESTUDIADAS

En general, los estudios de evaluación de la presencia de UV-F que se muestran en las publicaciones revelan una presencia muy amplia de estos compuestos, tanto en el ecosistema acuático, como en aguas y lodos de EDARs. En todas las matrices estudiadas se han detectado residuos de UV-F en mayor o menor grado.

Por una parte se observó una fuerte presencia de estos compuestos en aguas de entrada de EDARs, donde algunos de los compuestos menos lipofílicos se detectaron en todas las muestras y en altas concentraciones, como en el caso de BP4. Entre las EDARs incluidas en el estudio, las hay que reciben mayoritariamente aguas urbanas y también que reciben aguas industriales, sin que se observe ninguna diferencia significativa en los niveles de UV-F detectados en sus aguas de entrada. Las concentraciones encontradas en los efluentes son menores que en las de entrada, pero siguen siendo muy relevantes, lo que muestra que la degradación de muchos de estos compuestos, no es del todo efectiva mediante los procesos bióticos convencionales que se aplican.

Esta hipótesis se ve reforzada por las altas concentraciones de UV-F (superior a los 8000 ngg^{-1} en algunos casos), sobre todo de los más lipofílicos, en lodos de EDAR. Estas concentraciones, acordes con las encontradas en otros estudios europeos [57-60], demuestran que la adsorción (y no la biodegradación) sería el factor principal de eliminación de estos compuestos. Las altas concentraciones en lodos de EDARs nos conducen al problema de la gestión de los propios lodos, que acaban teniendo usos

agrícolas en la mayoría de casos, y que pueden reintroducir grandes cantidades de contaminantes en el medioambiente e incluso llegar a alcanzar la cadena alimentaria.

Los UV-F llegan a las aguas superficiales principalmente a través de las EDARs. En las aguas del río Llobregat se encontraron residuos de BP3, BP1, BP4 y 4MBC. Cabe destacar que los niveles de UV-F aumentan a medida que bajamos en el curso del río y éste va recibiendo más aportaciones de industrias y de EDARs, siendo el punto más bajo analizado el más contaminado, con una concentración de BP4 superior a 800 ngL^{-1}. No obstante, se llevó a cabo la estimación de riesgo ambiental para los compuestos cuya EC_{50} en *Dapnia Magna* se ha estudiado [75], indicando que las concentraciones encontradas en aguas no suponen un riesgo para el medio ambiente. Hay que señalar la presencia de estos compuestos en aguas subterráneas, en concentraciones en algunos casos por encima de los 30 ngL^{-1}. Normalmente la presencia de contaminantes orgánicos en aguas subterráneas se debe o bien a fugas en el sistema de canalización de las aguas residuales, o bien a filtraciones de aguas superficiales, como por ejemplo provenientes de ríos. Las aguas subterráneas analizadas proceden de puntos muy densamente poblados (todos están dentro de la ciudad de Barcelona). Teniendo en cuenta este hecho y sumando el amplio uso que se hace de los UV-F en productos para el cuidado personal de uso diario, todo parece indicar que las fugas en el sistema de canalización de las aguas de deshecho urbanas permiten que estos compuestos se filtren hasta llegar al acuífero. Estudios anteriores han determinado la presencia de otras familias de contaminantes emergentes como fármacos o drogas de abuso en aguas subterráneas urbanas [76, 77], y proponen esta vía como principal factor. Estos resultados constituyen la primera evidencia de la presencia de UV-F en aguas subterráneas.

Las concentraciones halladas en los sedimentos, entre 1 y 40 ngg^{-1} para la mayoría de compuestos, resultaron ser significativamente mayores que las encontradas en las aguas. Este resultado era de esperar ya que la mayoría de estos UV-F son lipofílicos, y por tanto, tienden a acumularse en el sedimento. El OC fue el compuesto detectado con mayor frecuencia y el que está presente a niveles más altos, como en el caso de los lodos de EDAR. La elevada frecuencia de detección así como los altos niveles de concentración encontrados para este UV-F se pueden asociar al gran uso en formulaciones, especialmente, en productos para el cuidado personal resistentes al agua (bronceadores, maquillajes, bálsamo labial, etc), ya que tiene efecto protector en las

regiones UVA y UVB del espectro y porque aumenta la capacidad de absorción de la radiación de otros UV-F orgánicos. El uso del OC está permitido en todos los países y su proporción máxima en la formulación depende del país. En España, se puede aplicar en el máximo permitido por la legislación para un UV-F, un 10% del producto.

No existen hasta el momento datos toxicológicos que permitan estimar el riesgo ambiental de estos compuestos en sedimentos. No obstante, algunos estudios demuestran que los UV-F se bioacumulan, tanto en especies fluviales como marinas [2, 3, 7, 61, 66], como se discutirá más adelante, e incluso se han llegado a hallar a niveles considerables en leche materna humana [24] o semen humano [29].

Tanto en las aguas como en los sedimentos de los estudios llevados a cabo, los puntos más contaminados corresponden a zonas cercanas a grandes núcleos de población donde la presencia de residuos de UV-F se debería principalmente al uso doméstico de PCPs, hipótesis reforzada por las altas concentraciones de dichos compuestos en EDARs urbanas. No obstante, también se han detectado concentraciones importantes en puntos cercanos a núcleos industriales, evidenciando su amplio uso industrial.

También se evaluó la presencia de UV-F en sedimentos fluviales, marinos y de estuarios en muestras provenientes de Chile y Colombia [78]. En las muestras de Chile solamente se detectó un compuesto, BP3, en bajos niveles de concentración (1.05-2.96 ngg^{-1}). En las muestras procedentes de Colombia, además de BP3, se detectó 4MBC y EHMC. La frecuencia de detacción para EHMC fue del 38%, con los mayores niveles encontrados, por encima de 47 ngg^{-1} en algunos casos. 4MBC estuvo presente en el 23% de las muestras en el intervalo de concentración 7.9-17.2 ngg^{-1}. BP3 fue el compuesto más ampliamente detectado, con un 77% de frecuencia de detección y con niveles más altos que en las muestras de Chile, 1.05-5.38 ngg^{-1}. Llama la atención la no detección de OC, un compuesto muy lipofílico y que como ya se ha comentado suele ser el UV-F más detectado en matrices ambientales sólidas. Este hecho sugiere que la producción y el perfil de uso de los UV-F son diferentes entre paises.

Un factor a tener en cuenta en la evaluación de los niveles de estos contaminantes en aguas fluviales y sedimentos es el caudal de los ríos, el cual depende de las estaciones y los ciclos de sequía. Esto es especialmente importante en ríos de régimen mediterráneo

como el Llobregat o el Ebro. Cuando el caudal es muy bajo se reduce el efecto dilución y las concentraciones de contaminantes son más elevadas. En el estudio presentado en este capítulo, las muestras se recogieron en un periodo de caudal relativamente bajo. Los valores encontrados para aguas superficiales son del mismo orden que los encontrados en otros ríos europeos. Sin embargo, las concentraciones de estos compuestos en sedimentos son las más altas nunca detectadas hasta la fecha. No obstante, hay que tener en cuenta que los estudios de UV-F en sedimentos son realmente escasos.

Bioacumulación: La bioacumulación es la acumulación de una substancia por organismos vivos y se debe a diferentes factores como la exposición a un medio contaminado o la alimentación. Algunos estudios previos ya han demostrado el potencial de los UV-F para bioacumularse en organismos acuáticos [7], ya que en su mayoría son compuestos lipofílicos con valores de log Kow > 5, y se acumulan en los componentes lipídicos de los organismos. Si el compuesto no se metaboliza ni excreta al ritmo que se ingiere se acumula y se puede biomagnificar a través de la cadena trófica como muestra el estudio realizado por Fent et al. [7] para algunos UV-F.

La publicación #6 muestra la presencia de UV-F en muestras de peces del río Guadalquivir. Recientemente, este estudio se ha ampliado al análisis de estos compuestos en peces de las cuencas hidrográficas de los ríos Ebro, Llobregat, Júcar y Guadalquivir. Estos resultados se pueden consultar en anexo II, donde la Tabla A.1. recoge las concentraciones de los UV-F que han sido detectados en alguna muestra de la ampliación de este estudio.La Figura 2.1 muestra la localización de los puntos de muestreo, donde se tomaron peces de distintas especies. Se detectaron cuatro de los ocho UV-F estudiados: BP3, EHMC, 4MBC y OC, que son los cuatro más lipofílicos si excluimos OD-PABA. Los peces provenientes del Guadalquivir son los más contaminados del estudio, con una frecuencia de detección del 80%. Los niveles más altos se encontraron para la especie *Luciobarbus sclateri*, una especie de barbo endémica de la península Ibérica, con niveles que superan los 240 ngg^{-1} d.w. en algún caso.

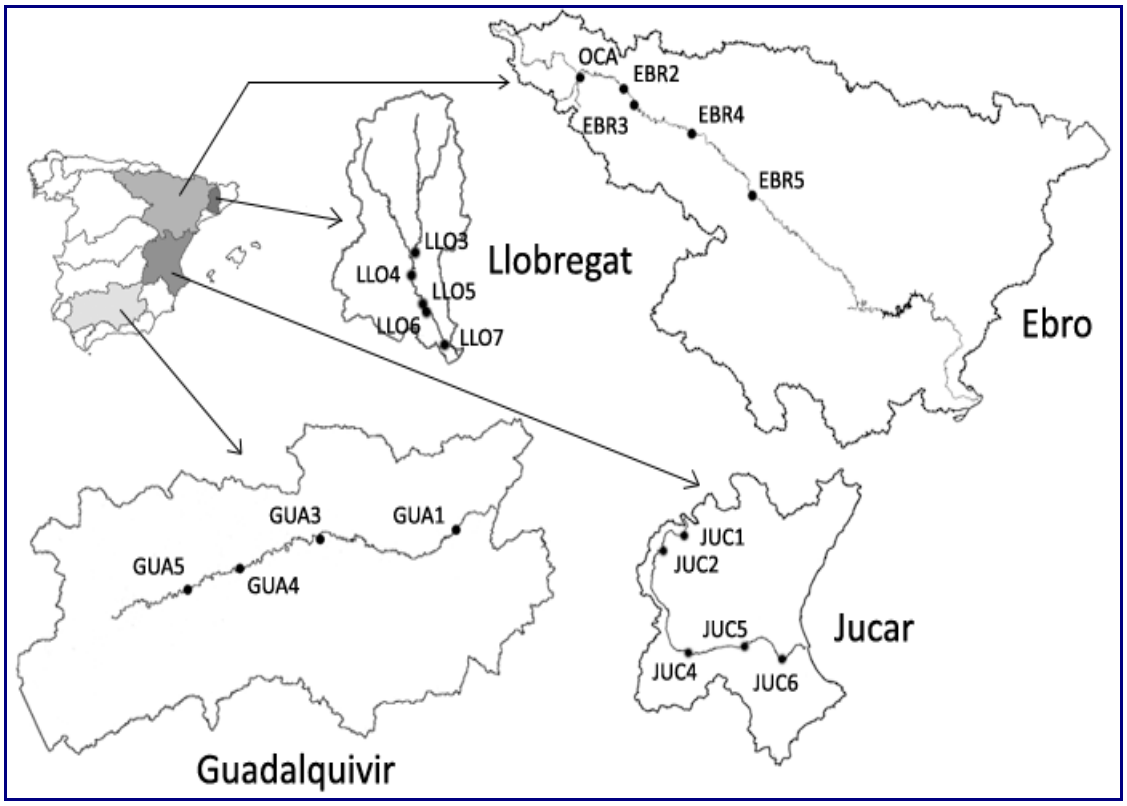

Figura 2.1. Localización de los puntos de muestreo.

Las localizaciones donde se detectaron mayores concentraciones de UV-F en peces se corresponden con los puntos con mayor presencia de estos compuestos en sedimentos [79], particularmente, en el punto *GUA 4*, que se encuentra aguas debajo de la EDAR de la ciudad de Córdoba y que muestra concentraciones por encima de 20 ngg^{-1} dw en sedimentos para EHMC y OC. En los peces provenientes del río Llobregat no se encontraron apenas residuos de UV-F, tan sólo en una muestra se determinó OC en concentraciones por debajo del LOQ. Este compuesto, que es el más frecuentemente detectado y el que presenta niveles más elevados tanto en lodos de EDAR, como en sedimentos [56-60], ha sido detectado en peces de todas las cuencas hidrográficas estudiadas, pero con frecuencias de detección relativamente bajas. El EHMC ha sido detectado en varias de las especies de pez analizadas (*Luciobarbus sclateri*, *Silurus glanis*, *Anguila anguila* y, en menor concentración, en Cyprinus carpio), las cuales muestran preferencias muy distintas en cuanto a habitat. Todas estas especies, a excepción de *Cyprinus carpio* (con niveles por debajo del LOQ), son depredadoras. Teniendo en cuenta estos datos, la biomagnificación podría jugar un papel importante en la presencia de UV-F en peces. Esto parece ser bastante relevante en las muestras del Ebro, donde solo los peces de la especie *Silurus Glanis*, un gran depredador, presentan concentraciones relevantes de UV-F. No obstante, la cantidad de muestras analizadas no

permite hacer afirmaciones rotundas sobre la biomagnificación de estos compuestos y serían precisos más estudios, focalizando en un área menor, con más especies de diferentes niveles tróficos y más individuos por especie para llegar a conclusiones firmes al respecto.

Por otra parte, aunque existen evidencias sobre las propiedades estrogénicas de los UV-F detectados, son necesarios más estudios par investigar las implicaciones ecotoxicológicas de la exposición de organismos acuáticos a este compuesto.

Los niveles de UV-F detectados en estas muestras son del mismo orden que los encontrados en estudios previos realizados en otras cuencas hidrográficas europeas (Suiza y Alemania) y de USA [3, 7, 63, 64, 66] y, constituyen los primeros datos de la presencia de UV-F en ríos ibéricos.

Bioacumulación en mamíferos marinos.

Hasta el momento, los estudios de bioacumulación de UV-F se han realizado casi exclusivamente en peces de agua dulce [2, 3, 7, 63, 64, 66], aunque también existen algunos datos en aves (que basan su dieta en pescado) e invertebrados acuáticos [7]. La información con respecto a la presencia y el comportamiento de estos compuestos en ecosistemas marinos es realmente escasa. Se han encontrado niveles relativamente altos de UV-F en aguas de mar (en la costa) (2-3300 ngg^{-1}) [46, 80]pero no se ha focalizado sobre la presencia de estos compuestos en organismos marinos. En el estudio que se presenta, se aporta información sobre la presencia de UV-F, concretamente del OC, en organismos marinos que viven en aguas costeras de zonas densamente pobladas, concretamente en delfines de diferentes áreas de Brasil. Este estudio constituye la primera evidencia de la presencia de UV-F en mamíferos marinos.

Los análisis del estudio muestran una amplia presencia del OC en los hígados de los delfines analizados. Estos resultados, así como los obtenidos anteriormente para peces, ponen de manifiesto el potencial de bioacumulación de estos compuestos. Dado que los delfines ocupan el nivel más alto de la cadena trófica en el ecosistema marino, la presencia del OC a concentraciones tan altas (89-782 ngg^{-1}) poría deberse a biomagnificación, aunque sería necesario el análisis de más especies con diferentes posiciones en la cadena trófica para poder obtener conclusiones sólidas en este sentido.

En general, el creciente aumento del uso de los UV-F, unido a la escasez de datos de presencia y toxicidad hacen necesarios más estudios en el tema para poder hacer una evaluación realista del riesgo ambiental que representan.

2.8 CONCLUSIONES

La principal conclusión del capítulo es que las metodologías que se describen, basadas en el análisis mediante LC-MS/MS, son válidas para la determinación de UV-F y algunos de sus productos de transformación en aguas naturales, aguas residuales, sedimento, lodos de EDARs y biota. Los métodos son sensibles y precisos, permitiendo la cuantificación de estos contaminantes a nivel traza en matrices complejas.

Con respecto a las metodologías analíticas desarrolladas podemos concluir:

- La técnica LC-ESI-MS/MS es adecuada para el análisis de UV-F y sus productos de transformación. Proporciona una elevada sensibilidad y selectividad. Trabajando en modo SRM, adquiriendo dos transiciones por compuesto, se consiguen los cuatro puntos de identificación requeridos por las normativas europeas (Decisión 2002/657/CE) concernientes al análisis de alimentos y sustancias prohibidas.

- El desarrollo de metodologías completamente automatizadas mediante *on-line* SPE-LC-MS/MS, permite el análisis de UV-F en aguas naturales y residuales con límites de detección muy bajos, desde 0.3-3 $ngmL^{-1}$ en agua subterránea a 5-10 $ngmL^{-1}$ en aguas de entrada de EDAR (la mayor sensibilidad descrita hasta ahora en la literatura para la mayoría de compuestos) reduciendo el volumen de muestra a solo 5 mL. Esto es posible gracias a que toda la muestra, en lugar de una alícuota como en el caso del análisis *off-line*, se transfiere al sistema cromatográfico. Este tipo de metodologías disminuyen al máximo la manipulación de la muestra y permiten un gran ahorro de tiempo de preparación de estas en el laboratorio, y también reducen el consumo de disolvente.

- El efecto matriz es un aspecto crucial para el análisis de estos contaminantes en matrices tan complejas como lo son las aquí estudiadas. Los patrones internos marcados isotópicamente, generalmente, son capaces de corregir estos efectos, especialmente en muestras acuosas. En lodos de EDAR es donde este efecto es más pronunciado y es necesaria una calibración en matriz para obtener buenos resultados.

- El uso de adsorbentes no inertes como Alúmina o Florisil para las extracciones mediante PLE (purificación en la misma celda) permite obtener extractos más

limpios y, en algunos casos, poder prescindir de una etapa extra de purificación sin prácticamente perder sensibilidad con el consecuente ahorro de tiempo y material.

- En el caso de muestras de biota se propone llevar a cabo una etapa de purificación (basada en SPE) posterior a la extracción mediante PLE, para conseguir unos límites de detección suficientemente bajos para analizar estos compuestos a los niveles en los que se encuentran en el medio ambiente. El resultado es un método robusto y rápido para el análisis de UV-F y algunos de sus productos de transformación con un intervalo muy amplio de propiedades fisico-químicas, especialmente si lo comparamos con los existentes en la literatura hasta el momento.

- La falta de UV-F comerciales marcados isotópicamente para su uso como patrón interno dificulta un correcto análisis de estos compuestos en matrices ambientales. Recientemente se han comercializado dos compuestos (BP3-d_5 y 4MBC-d_4) que han permitido mejoras en el análisis. No obstante, fue necesario en muchos casos analizar un porcentaje de muestras mediante adición estándar para asegurarnos el buen funcionamiento y exactitud del método.

Por otra parte, los estudios mostrados en este capítulo muestran una extensa presencia de estos compuestos en el ecosistema acuático. Con respecto a su presencia en el medio ambiente podemos concluir:

- Se determinaron concentraciones relevantes de UV-F en todas las matrices acuosas analizadas (aguas superficiales, aguas subterráneas y aguas residuales de entrada y salida de EDARs). Llaman la atención las altas concentraciones observadas para BP4 en todos los tipos de aguas, aunque también se detectó BP3 y su producto de transformación BP1. Estos estudios constituyen la primera evidencia de la presencia de estos compuestos en aguas subterráneas.

- Se determinaron concentraciones elevadas de UV-F, especialmente para los más lipofílicos, en sedimentos y sobre todo en lodos de EDAR, con concentraciones superiores a los 8000 ngg^{-1}.

- Los resultados en aguas residuales y en lodos de EDAR muestran que la eliminación de UV-F mediante los tratamientos biológicos convencionales no es del todo efectiva.

- Los resultados obtenidos en biota muestran que los UV-F se bioacumulan fuertemente. Estos compuestos se detectaron en diferentes especies de peces en áreas de régimen mediterráneo, así como también en delfines de diferentes áreas de la costa brasileña, siendo éstos los primeros resultados de bioacumulación en mamíferos marinos.

- El hecho de que los delfines se encuentran en la cima de la cadena trófica del ecosistema en el que viven sumado a que la presencia de estos compuestos fue mayor en especies depredadoras en los estudios llevados a cabo en peces de agua dulce, parece indicar que existe biomagnificación para estos compuestos. No obstante, serían necesarios más estudios incluyendo más niveles de la cadena trófica en áreas concretas para obtener conclusiones más sólidas en este sentido.

- Son necesarios más estudios sobre la presencia, destino y ecotoxicidad de estos compuestos en el medio ambiente para tener una visión completa y precisa sobre el problema ambiental que pueden representar los UV-F cuyo uso, ya de por sí muy elevado, se incrementa año a año.

CAPÍTULO III

EVALUACIÓN DE LA DEGRADACIÓN DE FILTROS UV POR LA ACCIÓN DEL HONGO TRAMETES VERSICOLOR

Evaluación de la degradación de filtros UV en lodos de depuradora y en medio acuso por la acción del hongo *Trametes versicolor*

La investigación sobre la presencia y el impacto de los contaminantes, tanto prioritarios como emergentes, en el ecosistema acuático es importante con el fin de alcanzar un medio ambiente más sostenible. Tanto la detección de las diferentes familias de contaminantes como el desarrollo y la implementación de tecnologías eficaces para remediar esta contaminación merecen un papel importante dentro de los estudios ambientales de los UV-F.

Los diferentes estudios existentes sobre la presencia de UV-F en efluentes de EDARs indican que los tratamientos convencionales que se aplican, básicamente tratamientos biológicos mediante lodos activados, no son completamente efectivos para eliminar estos compuestos. Los UV-F, que se encuentran en grandes concentraciones en aguas de entrada de depuradoras, siguen estando presentes en cantidades significativas en aguas de salida (especialmente los menos lipofílicos que quedan menos retenidos en los lodos), y en concentraciones muy elevadas en lodos de depuradora, como ya se ha descrito en el Capítulo II.

Teniendo en cuenta que incluso las EDARs más modernas no están diseñadas para eliminar muchos de los microcontaminantes orgánicos, especialmente los emergentes, en este capítulo se investigará sobre los procesos de tratamiento con hongos, en este caso el *Trametes versicolor (T. versicolor)* tanto en agua como en lodo de EDAR. Este tratamiento tiene un consumo energético muy bajo y es muy respetuoso con el medio ambiente.

Objetivos

El primero de los objetivos de estos estudios fue determinar cuantitativamente el potencial del hongo para la eliminación de los principales UV-F en lodos reales de EDAR.

Por otra parte, se quiso estudiar el proceso de eliminación de dos de los UV-F más importantes tanto en términos de utilización como de efectos nocivos para el medio ambiente, BP3 y 4MBC. Para estos compuestos se evaluó su biodegradación, así como se identificaron productos de transformación formados estos procesos y se determinaron sus porcentajes de eliminación.

Estructura

Este capítulo se inicia con una explicación sobre el estado del arte de la biodegradación de los UV-F, que da paso a una pequeña introducción sobre el hongo *T. versicolor* y su potencial para degradar compuestos orgánicos. Tras esto, se comenta la problemática actual en la gestión de los lodos de EDAR para dar paso al primer estudio, que trata sobre la eliminación de UV-F en lodos reales de EDAR. Posteriormente se describe la biodegradación de BP3, de su derivado BP1 y de 4MBC en medio acuoso así como la identificación de sus productos de transformación. Finalmente, se discuten los mecanismos de degradación que tienen lugar en estos procesos así como la aplicación de esta tecnología no convencional a una escala mayor.

Los resultados de estos estudios han sido plasmados en dos publicaciones, una de ellas incluida como publicación científica en esta tesis:

- Publicación ciéntífica #8: Evaluation of fungal- and photo-degradation as potential treatments for the removal of sunscreens BP3 and BP1 (Science of the Total Environment (2012) 427-428:355-363).

- Degradation of UV-Filters in sewage sludge and 4-MBC in liquid medium by the ligninolytic fungus *Trametes versicolor* (Journal of Environmental Management (2012) 104:114-120).

- Estos estudios se han llevado a cabo en colaboración con el grupo de "Biodegradación de contaminantes industriales y valorización de residuos", dirigido por Teresa Vicent, y perteneciente al Departamento de Ingeniería Química de la Universita Autònoma de Barcelona (UAB), dentro del marco del proyecto "Presencia de contaminantes orgánicos prioritarios y emergentes en lodos de EDAR y su biodegradación por hongos" (MMAMRM 010/PC08/3-04.1).

3.1. BIODEGRADACIÓN DE FILTROS UV

Los contaminantes prioritarios y los contaminantes emergentes tienen características físico-químicas muy diferentes entre sí, que influyen en la efectividad de los procesos de tratamiento para su eliminación. Esto mismo ocurre dentro de la familia de UV-F, con un gran intervalo de propiedades fisicoquímicas, incluyendo la polaridad o la solubilidad (Tabla 1.5.).

La biodegradación es el proceso más importante para la eliminación de contaminantes del medio ambiente y se define como el proceso de descomposición de una sustancia mediante la acción de organismos vivos. Los trabajos publicados sobre biodegradación de UV-F son realmente escasos, y principalmente tratan sobre su eliminación mediante lodos activos en EDARs. Los resultados de estos estudios no conducen a conclusiones determinantes, y en algunos casos son incluso contradictorios ya que se comparan diferentes concentraciones de efluentes y parámetros operacionales del tratamiento. No obstante, se detectan UV-F en cantidades muy significativas en prácticamente la mayoría de aguas de salida de depuradora, así como en lodos de EDAR (Tablas 1.3. y 1.4.), como se ha comentado en el capítulo anterior.

Uno de los pocos estudios sobre la biodegradación de UV-F evaluó la degradación microbiana de BP3 y 4MBC en filtros de suelo de flujo vertical [81]. La degradación de BP3 fue solo del 12 % mientras la del 4MBC llegó al 75%. Sin embargo, debido a su tendencia a ser adsorbidos, los porcentajes de eliminación fueron del 100 % en un experimento con carga baja y entre 82–86% (BP3) y 91-96% (4MBC) en experimentos con carga alta. Estos experimentos se llevaron a cabo en concentraciones de 3 ugL^{-1}. Hernández-Leal et al. [82] estudió la eliminación de UV-F, incluyendo 4MBC, BP3, OC) en sistemas biológicos diferentes bajo condiciones aeróbicas y anaeróbicas. Basándose en los altos valores de $logK_{ow}$, este estudio concluyó que las elevadas tasas de eliminación que se obtienen en condiciones aeróbicas y la alta variabilidad en los rendimientos que se observan tienen como principal motivo la adsorción, y no la biodegradación.

Las vías de degradación de los contaminantes orgánicos prioritarios por las bacterias aerobias y anaerobias son conocidas. No obstante, la biodegradación no siempre llega a la mineralización e incluso puede darse el caso de que los productos de transformación sean más tóxicos que los propios precursores (como por ejemplo el caso de la

degradación anaerobia de los disolventes clorados que pueden dar lugar a la formación de cloruro de vinilo, potente carcinógeno), casos en los que estaría desaconsejado el tratamiento. En general, los sistemas enzimáticos bacterianos son intracelulares y específicos, de manera que la degradación de contaminantes xenobióticos por una especie bacteriana concreta se ve limitada a unos pocos compuestos de similar estructura, y por la disponibilidad, toxicidad y concentración de estos.

Los hongos ligninolíticos, como el *T. versicolor*, tienen capacidad para degradar una gran variedad de contaminantes debido a la no especificidad de sus enzimas, lo cual les confiere un gran potencial para tratamientos de biodegradación.

3.2. HONGO TRAMETES VERSICOLOR

El género *Trametes* comprende un grupo de hongos ligninolíticos productores de pudrición blanca que poseen propiedades medicinales, son importantes en biotecnología y tienen aplicaciones ambientales. Una de las especies potencialmente más útil es *T. versicolor*, antes conocido como *Coriolus versicolor* o *Polyporus versicolor*. Es un hongo muy común perteneciente a las especies de la clase Basidiomicetes. Presenta una generalizada aplicación como hongo medicinal, y también es consumido como alimento y como infusión de té [83].

Debido a su capacidad para degradar compuestos ligninolíticos y fenólicos, se han estudiado recientemente diversas especies de Basidiomicetes, siendo el hongo *T. versicolor* uno de los más estudiados. Este tipo de hongos son los microorganismos de la naturaleza más efectivos en la degradación de la lignina, debido a procesos oxidativos no específicos que incluyen principalmente tres familias de enzimas ligninolíticas diferentes llamadas lacasas, lignina peroxidasas y manganeso peroxidasas. La no especificidad de estas enzimas (utilizan mecanismos de radicales libres para catalizar las degradaciones) es de gran importancia para degradar productos estructuralmente similares a la lignina, como hidrocarburos aromáticos policíclicos (PAHs), policlorobifenilos (PCBs) o bifenilos policromados entre otros [84-86]. Estos hongos son capaces de degradar también mezclas de contaminantes con niveles bajos de concentración [87-89], a la vez que el número de compuestos degradables está en continuo crecimiento, con bastantes investigaciones en marcha. Hay resultados que

muestran que la degradación no siempre se realiza en las mismas condiciones, sugiriendo la implicación de otro mecanismo de degradación diferente a las peroxidasas. Entre los posibles mecanismos enzimáticos alternativos que pueden utilizar los hongos se incluyen las monooxigenasas del citocromo P-450, que actuan de una manera similar al mecanismo que utilizan los mamíferos para metabolizar compuestos tóxicos. Los hongos, en función de las condiciones ambientales y nutricionales, pueden utilizar indistintamente las dos vías de degradación, tanto la ligninolíticoa como la del citocromo P-450 [90].

Debido a la gran capacidad de degradación de biopolímeros recalcitrantes, estos hongos tienen un gran potencial en aplicaciones biotecnológicas como bioremediación (quizá la más estudiada hasta el momento), pero también para su uso en el tratamiento de efluentes provenientes de industrias textiles o del papel entre otros [91-93].

El nivel de conocimiento sobre la degradación de contaminantes emergentes por hongos ligninolíticos es más bajo que sobre los contaminantes prioritarios. No obstante, hay trabajos publicados sobre la degradación por hongos o por enzimas ligninolíticas de disruptores endocrinos incluyendo el nonilfenol, el bisfenol A o el Triclosan [94-98]. Estos estudios muestran elevados porcentajes de eliminación pero también muestran aspectos negativos, los cuales deben ser estudiados rigurosamente, como la formación de metabolitos tóxicos en determinadas condiciones.

Demostrada la habilidad de los hongos ligninolíticos para degradar una gran variedad de contaminantes orgánicos, incluso llegando a la mineralización en algunos casos, parece interesante explotar su potencial de degradación y desarrollar tecnologías no convencionales de tratamiento de aguas residuales y lodos de depuradora.

3.3. DEGRADACIÓN DE FILTROS UV MEDIANTE EL HONGO *TRAMETES VERSICOLOR* EN MEDIO ACUOSO

3.3.1 EVALUACIÓN DE LA DEGRADACIÓN MEDIANTE HONGOS DE BP3 Y BP1 Y COMPARACIÓN CON SU FOTODEGRADACIÓN. IDENTIFICACIÓN DE SUS PRODUCTOS DE TRANSFORMACIÓN

Publicación científica #8:

"Evaluation of fungal- and photo-degradation as potential treatments for the removal of sunscreens BP3 and BP1"

por:

Pablo Gago-Ferrero, M. Silvia Díaz-Cruz, Damià Barceló

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Evaluation of fungal- and photo-degradation as potential treatments for the removal of sunscreens BP3 and BP1

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ABSTRACT

Photodecomposition might be regarded as one of the most important abiotic factors affecting the fate of UV absorbing compounds in the environment and photocatalysis has been suggested as an effective method to degrade organic pollutants. However, UV filters transformation appears to be a complex process, barely addressed to date. The white rot fungus *Trametes versicolor* is considered as a promising alternative to conventional aerobic bacterial degradation, as it is able to metabolise a wide range of xenobiotics. This study focused on both degradation processes of two widely used UV filters, benzophenone-3 (BP3) and benzophenone-1 (BP1). Fungal treatment resulted in the degradation of more than 99% for both sunscreens in less than 24 h, whereas photodegradation was very inefficient, especially for BP3, which remained unaltered upon 24 h of simulated sunlight irradiation. Analysis of metabolic compounds generated showed BP1 as a minor by-product of BP3 degradation by *T. versicolor* while the main intermediate metabolites were glycoconjugate derivatives. BP1 and BP3 showed a weak, but significant estrogenic activity (EC50 values of 0.058 mg/L and 12.5 mg/L, respectively) when tested by recombinant yeast assay (RYA), being BP1 200-folds more estrogenic than BP3. Estrogenic activity was eliminated during *T. versicolor* degradation of both compounds, showing that none of the resulting metabolites possessed significant estrogenic activity at the concentrations produced. These results demonstrate the suitability of this method to degrade both sunscreen agents and to eliminate estrogenic activity.

1. Introduction

UV filters are a diverse group of chemical additives used in everyday products with a continuously increasing rate of use. These substances are used as protection against solar UV radiation and can be found not only in sunscreen cosmetics but also in other personal care products, food packaging, pharmaceuticals, domestic and industrial commodities or vehicle maintenance products (Ash and Ash, 2004). Sunscreen agents enter the aquatic environment as a direct release or through sewage, where monitoring data of wastewater treatment plants (WWTPs) indicates that current techniques are not effective at removing UV filters since several of them were found in treated wastewater (Snyder et al., 2006; Li et al., 2007; Rodil et al., 2008; Negreira et al., 2009), and sewage sludge (Plagellat et al., 2006; Gago-Ferrero et al., 2011a). UV filters are considered environmental contaminants of increasing concern since most of the commonly used are known to cause endocrine disrupting effects (Schreurs et al., 2005) and bioaccumulate in both aquatic and terrestrial organisms (Balmer et al., 2005).

The organic UV filters most frequently found in water samples are 2,4-dihydroxybenzophenone (BP1), benzophenone-3 (BP3), 2-Hydroxy-4-methoxybenzophenone-5-sulphonic acid (BP4), 2-phenylbenzimidazole-5-sulfonic acid (PBSA), 4-methyl-benzylidene camphor (4-MBC), ethylhexyl methoxycinnamate (EHMC), isoamyl methoxycinnamate (IAMC), octocrylene (OC) and octyl dimethyl-p-aminobenzoate (OD-PABA) (Rodil et al., 2008; Kasprzyk-Hordern et al., 2008; Negreira et al., 2009). The widespread use of BP3 in personal care products was recently documented in an extensive survey (Calafat et al., 2008). Maximum values reported in raw wastewaters

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for this sunscreen agent are 462 ng/L in Spain (Negreira et al., 2009), 722 ng/L in China (Li et al., 2007) and 2930 ng/L in USA (Snyder et al., 2006). BP3 was also detected in sludge, soils, sediments and industrial drainage (Jeon et al., 2006; Gago-Ferrero et al., 2011b). Regarding its toxicity, BP3 is under study by the European Commission (EU, 2007) as substance with potential evidence of endocrine disrupting effects and it is regulated by the 2002/72/EC Directive, relating to compounds in contact with food.

Since biological conventional treatments in WWTP are not effective at degrading most UV filters, new approaches are being developed. Two of them are assessed in the present work, one physicochemical and one biological. Because of UV filters are substances designed to absorb solar radiation, their fate in natural waters might be driven by sun radiation mediated mechanisms. To date, very few studies have examined UV filters response under UV radiation when exposed in aqueous samples (Vanquerp et al., 1999; Rodil et al., 2009) and in none of them degradation products were identified. On the other hand, it has been widely proved that white rot fungi (WRF) have the ability to oxidise a large number of organic contaminants (Pointing, 2001; Blánquez et al., 2004; Auriol et al., 2008; Marco-Urrea et al., 2009) due to their powerful enzymatic system, characterised by a high unspecificity given by the oxidation mechanism, partly based on the generation of free radicals and the presence of extracellular enzymes such as laccases and peroxidases. The basidiomycete *Trametes versicolor* is one of the most studied WRF. Nevertheless, these degradation processes are only useful if they do not lead to the formation of new compounds with higher toxicity or bioaccumulation capacity. Therefore, it is necessary to identify and characterise the derivatives formed during the transformation processes and to assess the potential toxicity (or any potentially deleterious biological activity) not only of the parental compounds, but also of their degradation products, in order to draw a complete picture of the process.

The present work was aiming to study the photolysis of BP3 and one of its main derivatives (BP1, also used as UV filter) in aqueous samples using artificial UV radiation, and to determine the feasibility of the ligninolytic fungus *T. versicolor* to degrade them and the intermediate products generated during fungal degradation. Further identification and structural characterization of these metabolites was performed by HPLC-MS/MS. Finally, an assessment of the total estrogenic activity after and before both treatments was carried out using the recombinant yeast assay (RYA).

2. Materials and methods

2.1. Reagents and fungal and yeast strains

The UV filter BP3 was kindly given by Merck (Darmstadt, Germany). BP1 (also known as DHB), 4-hydroxybenzophenone (4HB) and 4,4′-dihydroxybenzophenone (4DHB) were purchased from Sigma-Aldrich (Munich, Germany), and 2-2′dihydroxy-4-methoxybenzophenone (DHMB) and 2,3,4-trihydroxybenzophenone (THB) were from Dr Ehrenstorfer (Augsburg, Germany). The isotopically labelled compound 2-hydroxy-4-methoxy-2′,3′,4′5′,6′-d_5 (BP3-d_5) used as internal standard in HPLC-MS/MS analyses, was obtained from CDN isotopes (Quebec, Canada). All other chemicals used were of analytical grade.

T. versicolor (ATCC#42530) was from the American Type Culture Collection and was maintained by subculturing on petri dishes in malt extract (2%) and agar (1.5%) medium at 25 °C.

For Estrogen Receptor Assay (ER-RYA), yeast strain BY4741 (MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0) from EUROSCARF (Frankfurt, Germany) was transformed with plasmids pH5HE0 (hER) and pVitBX2 (ERE-LacZ) as described in Noguerol et al., 2006.

2.2. Biodegradation by T. versicolor

2.2.1. Media and cultures for pellets production

Pellets production was done as previously described by Font et al., 2003. For the experiments, a defined medium was used (Blánquez et al., 2004). For the bioreactor experiments, few drops of antifoam (204, Sigma, USA) were also added to the media.

2.2.2. Experiments of UV filter degradation at bioreactor scale

A glass bioreactor (1.5 L) at experimental conditions described in Blánquez et al. (2004) was used for the degradation experiments in batch operational mode. Pellets were added in an amount equivalent to a final concentration of 2 g/L dry weight (dw) and UV filters at 250 μg/L from a concentrated stock standard solution in ethanol. Samples taken at scheduled times were filtered by vacuum with Withman GF/C filters and concentrated by solid phase extraction (SPE) with Oasis HLB cartridges (Waters, Mildford, USA) as described elsewhere (Radjenovic et al., 2007). The extracts were reconstituted with 0.5 ml 40:60 v/v ethanol:water 1% acetic acid. The percentage of recovery was 78.2 ± 7.5 for BP3 and 96.3 ± 1.3 for BP1. For ER-RYA assay, samples were evaporated and reconstituted with 0.5 ml of methanol (MeOH).

2.2.3. Experiments of UV filter degradation at Erlenmeyer scale

The experiments were performed on 125 mL amber serum bottles (Wheaton, Mealville, NJ) with cotton plugs in a total reaction volume of 25 mL. Pellets were added to a final concentration of 5 g/L dw and BP3 or BP1 at 10 mg/L. The experiments were carried out at 25 °C and 130 rpm of orbital agitation. Treatments were done by triplicate and fresh bottles were sacrificed at each sample time point. Apart from the experimental bottles (EB), uninoculated controls (UNI), without fungi but with UV filter to take into account potential natural photodegradation or other abiotic processes; heat killed fungal control (HK), with the same amount of fungi than the EB but killed by the autoclave (30 min at 121 °C) to quantify the amount of adsorbed contaminant; and blank controls (BC), with solely alive fungi to detect diminishing in the fungal growing due to toxicity of the compound or to compare laccase production activation by the contaminant, were also included. At each sample time, 1 mL of liquid medium was filtered with a Millex-GV (Millipore) 0.22 μm syringe filter in order to determine the glucose concentration and the laccase enzymatic activity. Then, ethanol at a final concentration of 40% v/v was added to each bottle to achieve the total solubilisation of the UV filters. Finally, the bottle content was filtered by vacuum, and then analyzed by either HPLC-UV or HPLC-MS/MS.

Degradation was determined by comparing BP3 or BP1 concentration in the UNI controls with that in the EB flasks. All the degradation values were corrected for the sorption concentration values determined in HK control flasks, whereas UV filter's removal were only referred to UNI concentration values.

2.3. Photodegradation experiments

Photolysis studies were carried out in 25 mL Duran glass UV reactor by exposing 20 mL of the aqueous solutions spiked with BP3 or BP1 at 250 μg/L in HPLC grade water under simulated UV radiation. Irradiation was performed using a SunTest apparatus from Heraeus (Hanan, Germany) equipped with a Xenon arc lamp providing a light intensity of 400 W/m^2. Aliquots of 1 mL at scheduled times were taken for analysis. In order to ensure that observed transformations were only due to photochemical processes, sunscreens stability in aqueous solutions was verified, by storing 500 mL spiked solutions in HPLC grade water under the same experimental conditions at initial concentrations, and a blank control for each sample in the dark and at room temperature (dark controls). For ER-RYA assay, irradiated unitary

samples were taken at the scheduled times, and further concentrated as described in Section 2.5.

2.4. Analytical methods

2.4.1. HPLC–UV analysis

The samples of biodegradation experiments were placed in amber HPLC vials to avoid natural photodegradation during the analysis. A Dionex 3000 Ultimate HPLC equipped with UV detector and autosampler Dionex were used. The chromatographic separation was achieved on a LiChrosphere RP-18 (125 mm × 4 mm, 5 μm) LC column from Merck. The method used was based on that of Salvador and Chisvert (2005). Flow rate and injection volume were set up to 0.5 mL/min and 20 μL, respectively. The mobile phase consisted of ethanol (A) and acetic acid 1% in MilliQ water (B). The eluent gradient started with 20% A, from 5 to 10 min, increased to 50% and from 10 to 15 until 70%. Then, from 15 to 20 it was set to 100% A and finally, returned to initial conditions within the next 10 min.

2.4.2. HPLC-MS/MS analysis

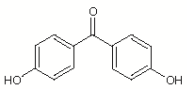
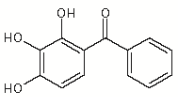
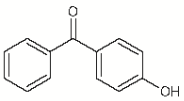
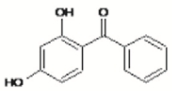
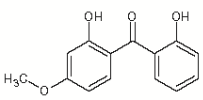
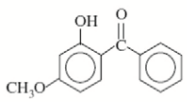
Target analysis of BP3 and its known metabolites, namely BP1, 4HB, 4DHB, DHMB and THB, in samples from biodegradation and photodegradation experiments were performed by HPLC using a hybrid triple quadrupole-linear ion trap mass spectrometer (HPLC–QqLIT-MS/MS) from Applied Biosystems-Sciex (Foster City, California, USA). The chromatographic separation was achieved on a Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 5 μm) LC-column from Merck. In the optimised method, the mobile phase consisted of a mixture of HPLC grade water and acetonitrile (ACN), both with 0.15% formic acid. Analyte elution was achieved by increasing the organic composition of the mobile phase from 5% to 25% in 7 min, and then to 100% in the following 3 min. Pure organic conditions were kept constant for 2 min and then initial conditions were reached in the next 2 min. The injection volume and the mobile phase flow-rate were set to 10 μL and 0.3 mL/min, respectively.

MS/MS detection was performed in positive (PI) electrospray ionization (ESI) mode operating under the selected reaction monitoring (SRM) mode. Two major characteristic fragments of the protonated molecular ion $[M+H]^+$ were monitored for improved sensitivity and selectivity (see Table 1). The most abundant transition was used for quantification, whereas the other was used for confirmation. Fragmentation voltage and collision energy were optimised for each transition. The experimental conditions were investigated by infusion experiments of single UV filter standard solutions at 500 ng/L in ACN. The optimised values were selected as a compromise using the optimum values for the majority of the analytes. This procedure was in compliance with the European Council Directive, 2002/657/EC, that although it was initially conceived for food residue analysis, it has been accepted by the scientific community for environmental analysis. Molecular structures of target analytes, their chromatographic retention times (t_R) and the optimum MS/MS acquisition parameters are shown in Table 1.

Non-target analyses for the identification of BP3 unknown metabolites produced during the fungal treatment were performed by high performance liquid chromatography coupled to quadrupole-time of flight-tandem mass spectrometry (HPLC-QqTOF-MS/MS) using a Waters Acquity UPLC™ system attached to a Waters/Micromass QqToF-Micro™ (Waters/Micromass, Manchester, UK). Chromatographic conditions were the same as those described above for the HPLC–QqLIT-MS/MS method.

For identification of biodegradation products full-scan analyses were carried out on selected samples in the PI and in the negative (NI) ESI ionization modes in the range *m/z* 50–700 at different cone voltages (15–35 V). PI mode allowed higher sensitivity as compared to NI mode, and as NI mode did not provide additional information further MS and MS/MS analyses were carried out in PI mode. Collision

Table 1
SRM experimental conditions used in the HPLC–MS/MS determination of UV filters and proposed product ions.

Target compound	Chemical structure	Retention time (min)	Transition[a]	Fragmentation voltage (V)	Collision energy (eV)	Proposed product ion
4,4′-Dihydroxybenzophenone (4DHB)		5.63	215 > 121 215 > 93	8	27 45	$[M-C_6H_5OH]^+$ $[C_6H_4OH]^+$
2,3,4-Trihydroxybenzophenone (THB)		6.68	231 > 153 231 > 105	10	31 31	$[M-C_6H_5OH]^+$ $[C_6H_5C{=}O]^+$
4-Hydroxybenzophenone (4HB)		7.07	199 > 121 199 > 105	8	25 27	$[M-C_6H_5]^+$ $[C_6H_5C{=}O]^+$
2,4-Dihydroxybenzophenone (BP1)		7.70	215 > 137 215 > 105	8	27 29	$[M-C_6H_5]^+$ $[C_6H_5C{=}O]^+$
2,2′-Dihydroxy-4-methoxybenzophenone (DHMB)		8.20	245 > 151 245 > 121	10	27 29	$[M-C_6H_5OH]^+$ $[C_6H_4(OH)C{=}O]^+$
Benzophenone 3 (BP3)		9.31	229 > 151 229 > 105	15	25 27	$[M-C_6H_5]^+$ $[C_6H_5C{=}O]^+$

[a] All compounds were determined in ESI(PI) mode. Parent ion correspond in all cases to $[M+H]^+$.

induced fragmentation (CID) of selected *m/z* ions was evaluated at different collision energies (10–40 eV), using argon as collision gas at 1.5 bars. Data were collected in the centroid mode, with a scan time of 0.3 s and an inter scan delay time of 0.1 s, with a full width at half maximum (FWHM) resolution of 5000. Other MS parameters were set as follows: 600 L/h for the desolvation gas at 350 °C, 50 L/h for the cone gas and 120 °C as source temperature. A valine–tyrosine–valine (Val-Tyr-Val) reference solution (*m/z* 380.2185 of $[M+H]^+$) was used to tune the instrument and also as lock mass to achieve mass accuracy. This solution was analyzed every 4 s by infusion through an independent reference probe (LockSpray™). Elemental compositions and accurate masses of the molecular ions and their fragments were determined using MassLynx V4.1 software.

2.4.3. Additional analyses

Glucose concentration was measured with a biochemical analyser YSI 2700 SELECT (Yellow Spring Instruments) in the concentration range 0–20 ± 0.04 g/L.

Laccase activity can be measured through the oxidation of 2,6-dimetoxyphenol (DMP) by the enzyme laccase. The analysis process is based on the measure of the absorbance variance at 468 nm and 30 °C during 2 min in a Varian Cary 3 UV/Vis spectrophotometer. The reaction was done with 600 µl of sample, 200 µL of sodium malonate 250 mM pH 4.5 and 50 µl of DMP 20 mM. Activity units per litre (U/L) are defined as the amount of DMP in micromoles per litre which are oxidised per minute (µmol DMP/L min). The molar extinction coefficient of DMP is 24.8 mM^{-1} cm^{-1} (Wariishi et al., 1992).

Biomass amount was determined as the constant weight at 100 °C.

2.5. Estrogenic Recombinant Yeast Assay (ER-RYA)

This yeast-based bioassay, which harbours the human estrogen receptor (hER), is able to monitor and quantify the interactions between the ER and the compounds present in the medium by activation of the *lacZ* gene. The protocol carried out is described elsewhere (Noguerol et al., 2006). The incubation period was 6 h before adding Y-PER®. Samples from photodegradation and biodegradation experiments for ER-RYA assays were concentrated by SPE using Oasis HLB cartridges as previously described (Radjenovic et al., 2007) and further evaporated to 0.5 mL MeOH, which corresponded to a concentration of 40-fold the original concentration (250 µg/L).

3. Results and discussion

3.1. Comparison of photodegradation and biodegradation of BP3 and BP1

As shown in Fig. 1A, BP3 was not photodegraded after 24 h of irradiation. This result is in accordance with previous studies (Rodil et al., 2009). In contrast its derivative BP1 was readily photodegraded, disappearing after 24 h of irradiation (Fig. 1B).

High biodegradation rates were observed by *T. versicolor* during 24 h batch operating. Initial levels of BP3 dropped to non-detectable levels in 8 h (Fig. 1C). In the case of BP1, about 95% of the initial concentration was removed after 2 h of treatment and completely eliminated at 24 h (Fig. 1D). During the biodegradation treatment, new peaks were observed in the chromatograms indicating that these contaminants were degraded to some extent by the fungus and that their elimination from the medium was not only driven by their adsorption onto the biomass. For BP3 the new peak was observed at $t_R = 13.4$ min (M1), which reached a 40% of the initial area of BP3 ($t_R = 18.5$ min) upon 24 h of treatment. Similarly, for BP1 the new peak ($t_R = 13.7$ min, N1) reached a maximum (50% of initial BP1 signal at $t_R = 15.5$ min after 8 h, to further decrease. Any of the detected compounds presented toxicity to the fungus since glucose consumption was in the usual range.

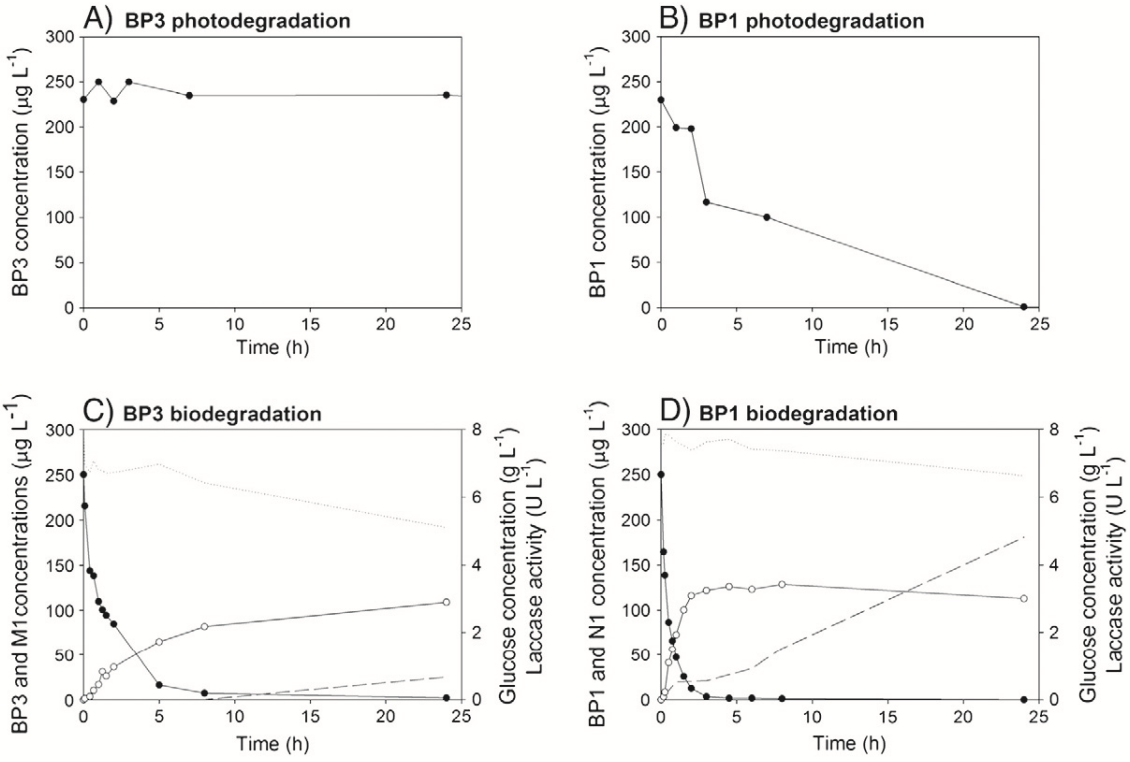


Fig. 1. Concentration profiles of A) BP3 and B) BP1 during UV irradiation and C) BP3 and D) BP1 during fungal degradation in the bioreactor at initial concentration of 250 µg/L (close circles). Glucose concentration and laccase activity in bioreactors are shown in dotted line and long dashes, respectively, and concentrations of main metabolites M1 and N1 in white circles.

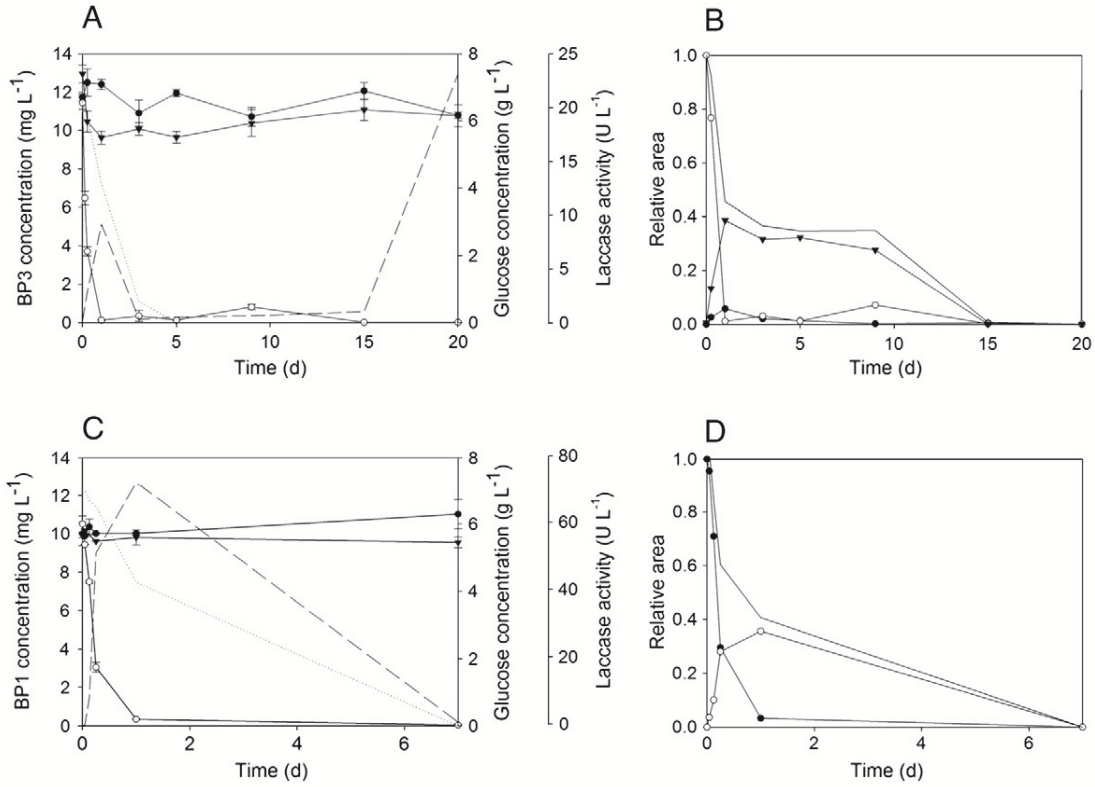


Fig. 2. A) BP3 concentration profile degradation experiment by *T. versicolor* at Erlenmeyer scale at 10 mg/L BP3 initial concentration and B) evolution of peak areas in HPLC chromatograms. Legend: (○) BP3 peak, at t_R 18.5 min; (▼) M1 peak, at t_R 13.4 min; (●) M2 peak, at t_R 12.7 min. C) BP1 concentration profile in degradation experiment by *T. versicolor* at Erlenmeyer scale and at 10 mg/L BP1 initial concentration and D) evolution of peak areas in HPLC chromatograms. Legend: (○) BP1 peak, at t_R 15.5 min; (●) N1 peak, at t_R 13.7 min. In A) and C): Treatments: (●) UNI, (○) EB and (▼) HK. Glucose concentration and laccase activity in EB are also plotted in a dotted line and long dashes, respectively. Values plotted are means ± standard error for triplicates. In B) and D): Sum of all areas of the main peaks is plotted in a solid line.

3.2. Target analysis of BP3 and BP1 metabolites

In order to characterise the biodegradation products formed, and to assess the capacity of the fungus to degrade them, experiments at Erlenmeyer scale were carried out. To facilitate metabolites identification, higher concentrations of UV filters (10 mg/L) and fungus (5 g/L dw) were used. The results obtained in long term experiments for BP3 are shown in Fig. 2A. Abiotic degradation processes were discarded, since no decrease of the BP3 concentration in the UNI controls was observed. Dead fungi controls were also performed in order to ensure that the sunscreen agent's elimination was not only produced by its adsorption onto the biomass. The little adsorption observed (between 0 and 19.6%) was in agreement with the relative low hydrophobicity of BP3 (log K_{OW} = 3.79, experimental value from database of physico-chemical properties. Syracuse Research Corporation: http://www.syrres.com/esc/physdemo.htm) and the step of the solubilisation applied.

In the experimental bottles BP3 exhibited a high degree of elimination, reaching >99% between 6 and 24 h. During 20 days (Fig. 2A), elimination of BP3 was maintained near 100% without abiotic elimination or high adsorption in HK controls. Therefore, taking into account that amount of BP3 adsorbed on the biomass, at least 80.4% of the initial concentration could be assigned to degradation processes at 24 h. During the biodegradation the M1 peak appeared again and another one at a t_R = 12.7 min (M2) not detected in bioreactor experiments (Fig. 2B). None appeared at HK controls. The maximum concentration of both was observed at 24 h of culture, M1 with a relative area of 40% compared to the initial concentration of BP3, while M2 reached only a relative area of 5%. Thus, the sum of all degradation products areas did not achieve the corresponding initial concentration of BP3. As between 10 and 15 days all peaks disappeared (Fig. 2B), it can be assumed that, finally, the aromatic ring was broken. It must be remarked that this biotransformation occurred approximately 10 days after glucose depletion in the media. Thus, fungus was still alive even at 20 days of culture, as it is corroborated by the production of laccase at that moment (Fig. 2A).

Similarly, 7 days-long fungal degradation experiments were performed for BP1. Results showed a similar but faster degradation profile to that of BP3 (see Fig. 2C and D).

The formation of derivatives during biodegradation of BP3 by the fungus *T. versicolor* was investigated. Firstly, target compounds, namely BP1, 4HB, 4DHB, DHMB and THB, which have been previously identified as metabolites in rats and humans, were considered (Felix et al., 1998; Díaz-Cruz et al., 2008; Jeon et al., 2008). These compounds were analysed in the samples by HPLC-MS/MS according to the methodology described in Section 2.4.2. The developed procedure exhibited excellent linearity (R^2 > 0.99) in a wide range of concentrations (0.1–300 μg/L). Good instrumental precision was obtained, with intraday RSD values between 0.5% and 2% and interday RSD values ranging from 1% to 4%. Method limits of detection (MLODs) and method quantification limits (MLOQs) were calculated in spiked samples as the concentration corresponding to a signal-to-noise ratio of 3 and 10, respectively. Estimated values were in the ranges 0.4–11.1 μg/L for MLOD and 1.3–37.0 μg/L for MLOQ.

BP1, 4DHB and 4HB were identified as metabolites produced during the degradation experiments of BP3 with the fungus. BP1 was detected up to 6 h of treatment at 3.6 μg/L maximum concentration, indicating a high fungal degradation rate. 4DHB and 4HB were determined after 3 days of treatment. 4DHB was detected at 3, 5 and 9 days at a concentration of 11.5, 50.7 and 31.3 μg/L, respectively, while 4HB

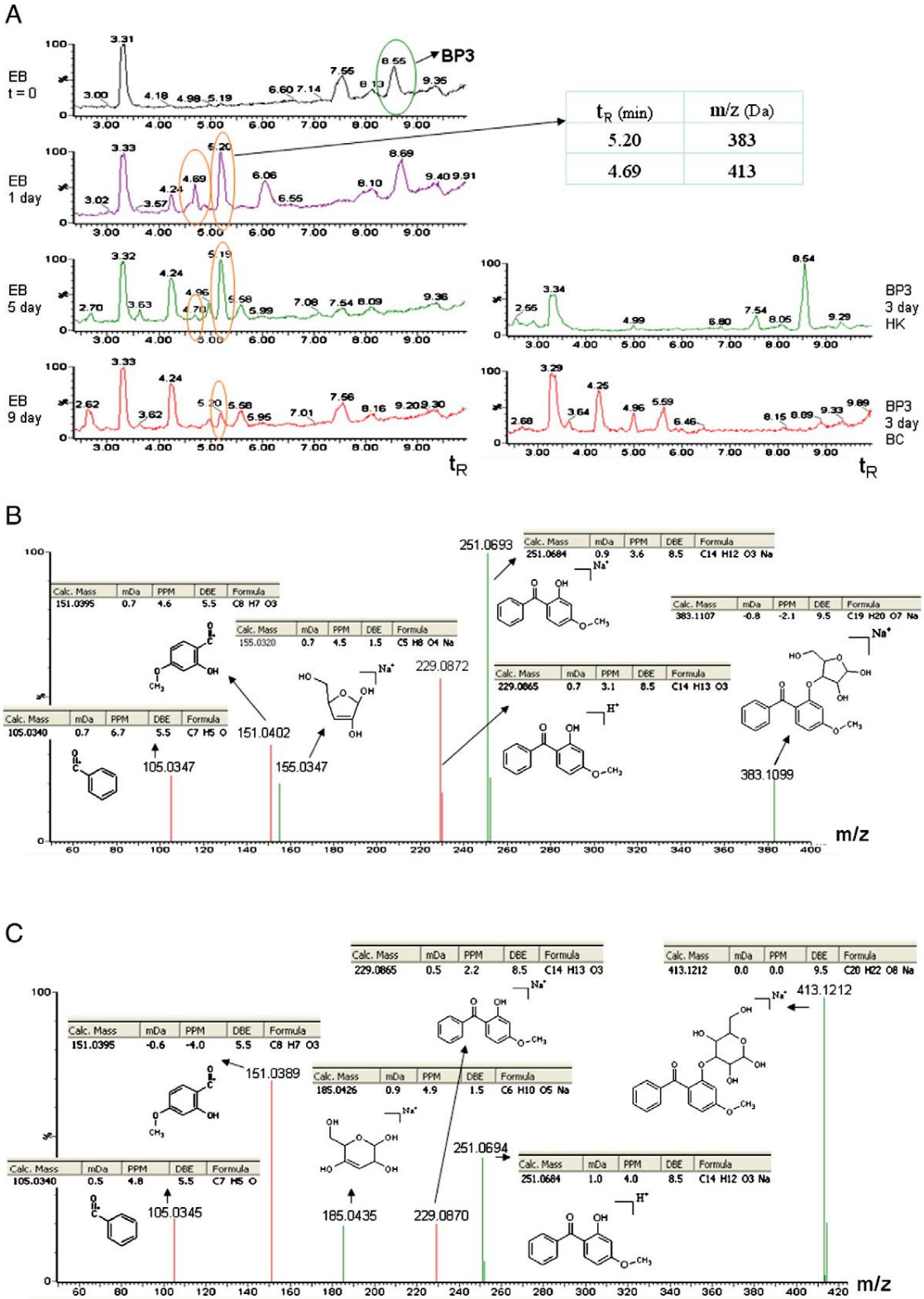


Fig. 3. A) Total ion current chromatograms (TIC) of samples collected at t = 0, 1, 5 and 9 days. HK and BC TIC are also shown. B) and C) ESI(PI)-MS/MS spectra of the molecular ions (as sodium adduct) of the metabolites at B) m/z: 383 and C) m/z 413 in t = 1 day samples (cone voltage = 20 V, collision energies = 10 eV (green) and 35 eV (red)). Additionally, the experimental and theoretical exact masses obtained for the molecular and fragment ions, expressed as m/z, the proposed elemental composition, together with recalculated mass errors (in mDa and mg/g) and double bond equivalents (DBEs) given by the software (mass measurements accuracy threshold of 5 mg/g) are provided.

was always below MLOQ. THB and DHMB were apparently not produced by the treatment with the fungus, since they were not detected in any sample.

Fungal degradation of BP1 resulted in the formation of 4HB and 4DHB. Their concentrations after 3 and 6 days of treatment were similar to those reported for BP3 degradation experiments. Likewise in

BP3 degradation tests, THB and DHMB were not detected in the analysed samples.

Since the relative concentrations of these metabolites were quite low suggesting that other major metabolites might be formed, further studies were carried out in order to identify them.

3.3. Identification and characterization of non-target metabolites of BP3 and BP1

As depicted in the chromatograms of Fig. 3A, solely BP3 ($t_R = 8.55$ min) was present before starting the biodegradation experiment but readily decreased to be undetectable upon 24 h of fungal treatment. At this time two new chromatographic peaks at t_R 4.69 and 5.20 min were observed in the chromatogram, corresponding to m/z ratios of 413 and 383 Daltons (Da), respectively. Maximum levels of these metabolites were determined after 1 day, to almost disappear between 9 and 15 days of treatment.

Fig. 3B and C summarises the MS/MS information obtained and the fragmentation patterns proposed for these two degradation products (with m/z 383 and 413). In both cases, higher sensitivity was obtained for the sodium adduct of the molecule ($[M+Na]^+$) as compared to that of the protonated molecule $[M+H]^+$, which was hardly detected. Thus, further MS/MS experiments were performed taking the sodium adduct as the precursor ion.

As shown in Fig. 3B, fragmentation of m/z 383 at high collision energy completely fragmented the molecular ion and provided the m/z 229.0865 as the major fragment ion, corresponding to the molecular formula $C_{14}H_{13}O_3$, which fits with the formula of the BP3 molecular ion $[M+H]^+$. Additionally, two other product ions were generated with m/z 151.0402 and m/z 105.0347, which gave the best-fit formula $C_8H_7O_3$ and C_7H_5O, respectively. The first fragment, is produced by the loss of the phenyl group of BP3 $[M\text{-}C_6H_5]^+$, whereas the other one corresponded to the benzoyl cation $[C_6H_5C{=}O]^+$. In the light of these results, it appears that BP3 is part of the molecular structure of the unknown metabolite with m/z 383. In the spectrum obtained at low collision energy the molecular ion at m/z 383 was still observed ($C_{19}H_{20}O_7Na$) together with two product ions at m/z = 251.0693 ($C_{14}H_{12}O_3Na$), which likely corresponds to the adduct $[BP3+Na]^+$, and to m/z 155.0327 ($C_5H_8O_4Na$), which would not correspond to any part of the BP3 molecule, unless aromatic ring cleavage occurred, which is not likely at low collision energy. Therefore, those data suggested that the metabolite at m/z 383 might be BP3 conjugated with a molecule of 132 Da. Several studies claim that the formation of conjugated metabolites with pentoses (mainly xylose and ribose) and hexoses (mainly glucose) is a predominant pathway in the degradation of chemicals by WRF, especially in presence of phenolic hydroxyl groups (Kondo et al., 1993; Gesell et al., 2004). In this case, the addition of a pentose via glycosidic bond to the BP3 with the consequent loss of one molecule of water corresponds to an increase in the molecular mass of 132 Da. Thus, the metabolite at m/z 383 may be produced by the addition of one pentose molecule to BP3.

CID spectra obtained for the metabolite at m/z 413 was similar to that of the previously discussed metabolite (m/z 383). At high collision energy the fragments with m/z 229.0870 ($C_{14}H_{13}O_3$), m/z 151 and m/z 105 were also detected. In the spectrum obtained at low collision energy, besides the sodium adduct of BP3 (m/z 251.0694, $C_{14}H_{12}O_3Na$ ($[BP3+Na]^+$), two other fragment ions were observed. One at m/z 413.1212, which gave the best fit formula $C_{20}H_{22}O_5Na$, corresponding to an increase of 162 Da of the BP3 molecular weight, and the other at m/z 185.0435 ($C_6H_{10}O_5Na$). Following the rationale discussed above, it appears that the metabolite at m/z 413 might be the result of the addition of one hexose molecule to BP3, likely glucose, via glycosidic bond and consequent loss of one molecule of water.

The results obtained in a similar study carried out with BP1 were pretty similar. After 24 h of treatment, BP1 disappeared and only one new chromatographic peak ($t_R = 5.09$ min), was observed. This compound reached the maximum concentration upon 24 h of treatment, and then, decreased until not detectable levels after 6 days of treatment. Its MS/MS spectra at m/z 347 were very similar to those obtained for BP3, however, in this case the most abundant molecular ion was found to be the protonated form $[M+H]^+$. Its CID fragmentation allowed to observe the ion $[BP1+H]^+$ and its two main fragments $[BP1\text{-}C_6H_5]^+$ (m/z 137.0233), and $[C_6H_5C{=}O]^+$ (m/z 105.03043). Additionally the molecular ion m/z 347.1131 ($C_{18}H_{19}O_7$) and the fragment m/z 133.0507 ($C_5H_9O_4$) were observed. As it was reported for BP3 a difference in 132 Da in the molecular mass suggests the addition of one pentose molecule to BP1 (m/z 347). In a previous study on fungal degradation of the sunscreen agent 4-MBC similar conjugated metabolites were identified (Badia-Fabregat et al., 2012).

Non-target identified metabolites constitute conjugated forms of BP3 and BP1, and thus, further enzymatic activity of the fungus might revert them to the parent compound. Nevertheless, the fact that all identified metabolites, together with BP3 and BP1, disappeared along the treatment confirms further fungal degradation with potential cleavage of the aromatic rings.

The increase in the polarity of the identified metabolites caused by the addition of sugars is in agreement with the lower t_R associated to them in comparison to those of the respective parental compounds. Taking into account that absorbance of conjugates is similar to that of the corresponding parent, the metabolite generated in a higher amount in BP3 degradation (M1) would be the pentose conjugate, while that less abundant (M2) would be the glucose conjugate.

These findings support the hypothesis that conjugation processes constitute one of the defensive mechanisms that fungi have against toxic hydroxylated compounds (Hundt et al., 2000). Thus, glycoconjugation appears to be the first step in the BP3 and BP1 metabolism. The active enzymes appear to be UDP-xylosiltransferase when conjugation occurs with a xylose (Kondo et al., 1993) or UDP-glucosyltransferase if the added molecule is a glucose, as it has been previously reported for other xenobiotics upon the action of *T. versicolor* (Hundt et al., 2000). Conjugation with ribose, also described for other fungi (Gesell et al., 2004), constitutes a possibility that cannot be ruled out since the molecular weight of this conjugate fits with the results obtained in the HPLC-MS/MS analyses. Sugar residues would bind to the molecule through an O-glycosidic bond to the unique free hydroxyl of BP3 and to one of the two free hydroxyl groups present in BP1. Both approaches must be considered since the fragmentation of the conjugate yield BP3 and BP1 molecules, but any other breakdown product.

Later, O-glycosidic bond would break down and other fungal enzymes would act. Likely, the monooxygenase cytochrome P450 would oxidise BP3 and BP1 by adding hydroxyl groups (Hammer et al., 2001) or eventually demethylation (Campoy et al., 2009) for BP3. This would lead to the formation of BP1, 4HB and 4DHB, following a metabolic pathway similar to the one reported for mammals (Jeon et al., 2008). In fact, similar experiments of cytochrome P450 inhibiton described at Marco-Urrea et al. (2009) pointed to the possible involvement of this intracellular enzymatic system in the first steps of BP3 degradation (data not shown). Laccase could oxidise BP3 as well, although only with the help of mediators (Garcia et al., 2010).

Summarizing, BP3 and BP1 are rapidly transformed in their glycoconjugated forms to decrease its toxicity and to increase its bioavailability. Then, conjugation would steadily revert and oxidation of compounds and further ring cleavage would occur.

3.4. Estrogenic activity of BP3 and BP1

Estrogenic activity of BP3 and BP1 was determined by ER-RYA obtaining EC50 values of 12,5 mg/L and 0.058 mg/L for each compound and a LOEC of 1,6 mg/L and 0.015 mg/L, respectively. BP1 was three orders of magnitude less estrogenic than 17β-estradiol and 200-fold

higher estrogenic than BP3. Similar values have been described in the literature (Kunz et al., 2006).

3.5. Endocrine disruption evaluation of the treatments

In order to evaluate the evolution of the endocrine disruption during the degradation processes either by photodegradation or by *T. versicolor* at bioreactor-scale, the estrogenic activity was monitored at the same scheduled times than chemical analyses. During BP3 treatments, estrogenic activity was below the detection limit in all checked points. This indicates that putative estrogenic metabolites formed by *T. versicolor* degradation, such as BP1 were readily metabolised and, therefore, they were not present at sufficiently high concentration to elicit biological response. This is in agreement with the low BP1 concentrations found during fungal degradation.

The estrogenic activity of BP1 was reduced by both degradation processes, although *T. versicolor* treatment was faster and more effective than photodegradation. Fig. 4 shows that *T. versicolor* eliminated almost completely the estrogenic activity of BP1 after 4 h of treatment, whereas photodegradation needed more than 7 h to show a significant decrease of the estrogenic activity, and did not fully remove the activity until 24 h of incubation. These results are in agreement with the chemical analyses of BP1 (see Fig. 1B and D). These outcomes suggest that neither bidegradation by *T. versicolor* nor photodegradation of BP3 and BP1 produced significant amounts of estrogenic metabolites.

4. Conclusions

Comparison between photodegradation and biodegradation studies of one of the most widely used sunscreen agents, BP3, and one of its degradation products, BP1, under both high and environmental-like concentrations evidenced that high degradation rates were achieved by both approaches for BP1 (>95% at 3 h in bioreactor and 100% after 24 h UV irradiation). Conversely, BP3 was only degraded by the fungus but not by UV irradiation. Therefore, we conclude that the biodegradation approach appears as the most effective treatment in degrading both BP1 and BP3. BP1 was formed during the BP3 degradation process by *T. versicolor*, but it was readily degraded and never reached significant concentrations. The developed UPLC-ESI(PI)-QqTOF-MS/MS method allowed the univocal identification of BP3 and BP1 fungal metabolites by providing their exact mass and molecular formula. Up to 6 derivatives were identified; BP1, 4DHB, 4HB, and the conjugates BP3-pentose (xylose or ribose) and BP3-hexose (glucose) for BP3, and the conjugate BP1-pentose for BP1. Finally, estrogenic disruption capacity profiles of the biodegradation and photodegradation treatments indicated that none of the evaluated treatments induced the formation of estrogenic metabolites at concentrations sufficiently high to exceed the activity of the parental compound.

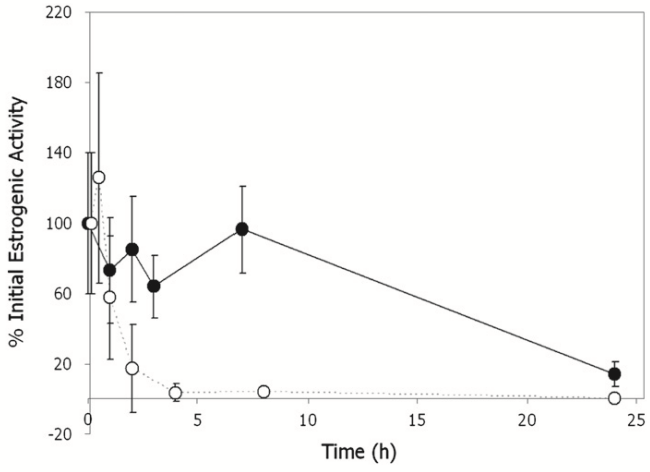


Fig. 4. Estrogenic activity profiles of BP1 degradation mixture by either fungal biodegradation (○) or photodegradation (●).

Acknowledgements

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3.3.2 IDENTIFICACIÓN DE LOS PRODUCTOS DE TRANSFORMACIÓN DE 4MBC EN LA DEGRADACIÓN DE ESTE COMPUESTO EN MEDIO LÍQUIDO

Degradación de 4MBC en medio líquido: Los experimentos de degradación de este compuesto en medio líquido se llevaron a cabo exactamente de la misma manera que los descritos para BP3 en el apartado anterior. La concentración inicial de 4MBC fue de 10 mgL^{-1}, notablemente superior de la que se encuentra en el medio ambiente, pero necesaria para poder determinar con éxito los productos de transformación, los cuales se encuentran normalmente en concentraciones mucho menores que sus precursores.

La Figura 3.2. presenta los resultados del experimento de degradación para este compuesto durante las primeras 24 horas (EB). También se hicieron experimentos control, con hongo muerto para asegurar que la disminución en la concentración del analito se debe a la biodegradación y no a procesos de absorción o adsorción. En estos controles su utilizó hongo muerto por tratamiento térmico (HK) y por tratamiento con azida sódica (SAK).

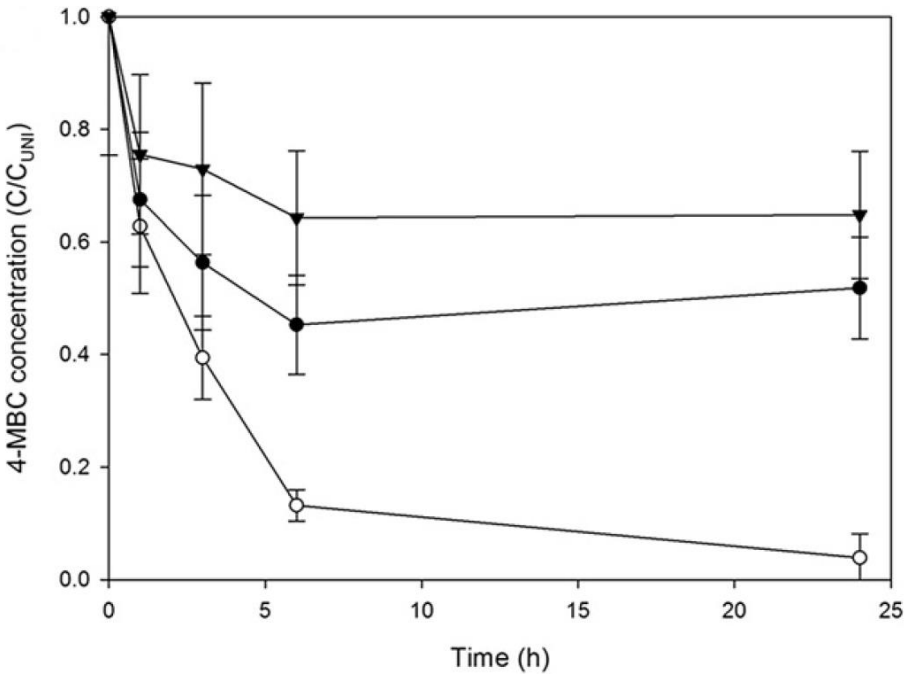


Figura 3.2. Perfil de concentración de 4MBC durante 24 horas de experimento. EB (○), HK (●), SAK (▼).

El tratamiento con el hongo mostró altas tasas de eliminación, superiores al 95% entre 6 y 24 horas de tratamiento. Teniendo en cuenta la cantidad que ha podido quedar adsorbida en la biomasa, en el peor escenario la eliminación que puede ser debida a la biodegradación sería al menos del 48%. Durante todo este proceso se observaron algunos picos cromatográficos, distintos a los obtenidos en los experimentos de control. Estos picos se corresponden con metabolitos del 4MBC. Aunque después de 24 horas de tratamiento el 4MBC es eliminado, la metabolización total del compuesto no ocurre hasta 21 días después, momento en que desaparecen todos los picos asociados a productos de transformación.

Identificación de los productos de degradación: Los productos de transformación del 4MBC en medio líquido se analizaron mediante HPLC-QqTOF-MS/MS de manera análoga a la descrita en el apartado anterior para la identificación de los productos de transformación de BP3 y BP1.

La Tabla 3.2 resume las masas exactas de los iones moleculares y los iones fragmento con los errores recalculados y los dobles enlaces equivalentes (DBEs) que proporciona el software (errores de precisión máximos de 5 mgL^{-1}). Estos datos se obtuvieron bajo condiciones optimizadas de voltage de cono y energía de colisión en los experimentos de MS/MS en el instrumento QqTOF.

Para descartar la formación de otros productos diferentes a los provenientes de la degradación del 4MBC por la acción del hongo, se realizaron diversos experimentos control con hongo muerto HK y blancos (BC).

Antes de empezar los experimentos de degradación (t=0) sólo se observa un pico, perteneciente a 4MBC (E) (t_R=10.27 min, $[M+H]^+$=255 Da). La intensidad de este pico, de acuerdo con lo comentado previamente, disminuye rápidamente hasta alcanzar el 5% de su área inicial al cabo de 24 h. Cabe destacar que ya durante las primeras horas de reacción se empieza a detectar un pico a t_R=10.73 min, con el mismo ratio masa/carga (*m/z*) e idéntico patrón de fraccionamiento (FP) que el 4MBC. Este hecho sugiere que el otro isómero del 4MBC, el 4MBC (Z), podría también estar presente. El 4MBC (Z) no es comercial, sino que es un producto de fotodegradación del producto comercialmente disponible 4MBC (E) [102]. Así mismo, la isomerización de 4MBC (E)

a 4MBC (Z) por acción de organismos vivos está también descrita [103]. Estos mismos trabajos también indican que el t_R del isómero Z es mayor que el del isómero E, como ocurre en nuestro estudio.

Tabla 3.2. Valores de masa exacta para los iones fragmento del 4-metilbenciliden canfor (4MBC) y sus productos de degradación Pr271, Pr287, Pr425 y Pr441 obtenidos mediante UPLC-QqTOF-MS/MS.

Compuesto	Precursor y ión producto	Fórmula elemental	Masa (m/z)		Error		DBE[a]	tr[b], min
			Experimental	Teórica	mDa	ppm		
4MBC	$[M+H]^+$	$C_{18}H_{23}O$	255.1755	255.1749	0.6	2.4	7.5	10.37; 10.73
	$[M+Na]^+$	$C_{18}H_{22}ONa$	277.1578	277.1568	1.0	3.6	7.5	
	$[M+H-H_2O]^+$	$C_{18}H_{21}$	237.1644	237.1643	0.1	0.4	8.5	
	$[M+H-C_3H_6]^+$	$C_{15}H_{17}O$	213.1289	213.1279	1.0	4.7	7.5	
	$[M+H-C_3H_7]^+$	$C_{15}H_{16}O$	212.1210	212.1201	0.9	4.2	8.0	
	$[M+H-C_5H_8O]^+$	$C_{13}H_{15}$	171.1178	171.1174	0.4	2.3	6.5	
	$[M+H-C_6H_{10}O]^+$	$C_{12}H_{13}$	157.1023	157.1017	0.6	3.8	6.5	
	$[MeC_6H_4CH_2]^+$	C_8H_9	105.0709	105.0704	0.5	4.8	4.5	
Pr271	$[M+H]^+$	$C_{18}H_{23}O_2$	271.1708	271.1698	1.0	3.7	7.5	7.96; 8.22
	$[M+Na]^+$	$C_{18}H_{22}O_2Na$	293.1530	293.1517	1.3	4.4	7.5	
	$[M+H-H_2O]^+$	$C_{18}H_{21}O$	253.1601	253.1592	0.9	3.6	8.5	
	$[M+H-C_3H_6]^+$	$C_{15}H_{17}O_2$	229.1235	229.1229	0.6	2.6	7.5	
	$[M+H-C_3H_7]^+$	$C_{15}H_{16}O_2$	228.1159	228.1150	0.9	3.9	8.0	
	$[M+H-C_5H_8O]^+$	$C_{13}H_{15}O$	187.1114	187.1123	-0.9	-4.8	6.5	
	$[M+H-C_6H_{10}O]^+$	$C_{12}H_{13}O$	173.0968	173.0966	0.2	1.2	6.5	
	$[MeC_6H_3(OH)CH_2]^+$	C_8H_9O	121.0659	121.0653	0.6	5.0	4.5	
Pr287	$[M+H]^+$	$C_{18}H_{23}O_3$	287.1657	287.1647	1.0	3.5	7.5	7.17; 7.65
	$[M+Na]^+$	$C_{18}H_{22}O_3Na$	309.1480	309.1467	1.3	4.2	7.5	
	$[M+H-H_2O]^+$	$C_{18}H_{21}O_2$	269.1553	269.1542	1.1	4.1	8.5	
	$[M+H-C_3H_6]^+$	$C_{15}H_{17}O_3$	245.1190	245.1178	1.2	4.9	7.5	
	203 $[M+H-C_5H_8O]^+$	$C_{13}H_{15}O_2$	203.1081	203.1072	0.9	4.4	6.5	
	$[M+H-C_6H_{10}O]^+$	$C_{12}H_{13}O_2$	189.0914	189.0916	-0.2	-1.1	6.5	
	$[MeC_6H_2(OH)_2CH_2]^+$	$C_8H_9O_2$	137.0610	137.0603	0.7	5.1	4.5	
Pr425	$[M+Na]^+$	$C_{23}H_{30}O_6Na$	425.1946	425.1940	0.6	1.4	8.5	6.31; 6.74
	$[Pr271+Na]^+$	$C_{18}H_{17}O_7Na$	293.1527	293.1517	1.0	3.4	7.5	
	$[C_5H_8O_4+Na]^+$	$C_5H_8O_4Na$	155.0327	155.0320	0.7	4.5	1.5	
	$[Pr271+H]^+$	$C_{18}H_{23}O_2$	271.1702	271.1698	0.4	1.5	7.5	
	$[Pr271+H-H_2O]^+$	$C_{18}H_{21}O$	253.1599	253.1592	0.7	2.8	8.5	
	$[Pr271+H-C_3H_6]^+$	$C_{15}H_{17}O_2$	229.1237	229.1229	0.8	3.5	7.5	
	$[Pr271+H-C_3H_7]^+$	$C_{15}H_{16}O_2$	228.1160	228.1150	1.0	4.4	8.0	
	$[Pr271+H-C_5H_8O]^+$	$C_{13}H_{15}O$	187.1125	187.1123	0.2	1.1	6.5	
	$[Pr271+H-C_6H_{10}O]^+$	$C_{12}H_{13}O$	173.0970	173.0966	0.4	2.3	6.5	
	$[MeC_6H_3(OH)CH_2]^+$	C_8H_9O	121.0655	121.0653	0.2	1.7	4.5	
Pr441	$[M+Na]^+$	$C_{23}H_{30}O_7Na$	441.1891	441.1889	0.2	0.5	8.5	5.01; 5.36
	$[Pr287+Na]^+$	$C_{18}H_{22}O_3Na$	309.1458	309.1467	-0.9	-2.9	7.5	
	$[C_5H_8O_4+Na]^+$	$C_5H_8O_4Na$	155.0315	155.0320	-0.5	-3.2	1.5	
	$[Pr287+H]^+$	$C_{18}H_{23}O_3$	287.1638	287.1647	-0.9	-3.1	7.5	
	$[Pr287+H-H_2O]^+$	$C_{18}H_{21}O_2$	269.1550	269.1542	0.8	3.0	8.5	
	$[Pr287+H-C_5H_8O]^+$	$C_{13}H_{15}O_2$	203.1079	203.1072	0.7	3.4	6.5	
	$[Pr287+H-C_6H_{10}O]^+$	$C_{12}H_{13}O_2$	189.0912	189.0916	-0.4	-2.1	6.5	
	$[MeC_6H_2(OH)_2CH_2]^+$	$C_8H_9O_2$	137.0609	137.0603	0.6	0.4	4.5	

Además del pico descrito, también desde las primeras horas observamos dos picos a t_R=7.96 y 8.22 min. Estos picos alcanzan su máxima intensidad a t=1 día, disminuyendo hasta no ser detectables a t=3 días. Los dos picos muestran una *m/z* de 271 Da y tienen FP idénticos entre sí, lo que sugiere que son isómeros de un mismo compuesto, que llamamos Pr271. La Figura 3.3. y 3.4.a muestran los espectros y estructuras propuestas para 4MBC y Pr271 respectivamente. Considerando que la masa de Pr271 pasó a ser 16 Da mayor que la del compuesto precursor y los fragmentos que obtenemos también tienen una *m/z* 16 Da superior en relación a los fragmentos del precursor, asumimos que

se produce la hidroxilación mediante radicales hidroxilo (•OH). La fragmentación de Pr271 da lugar a fragmentos con *m/z* 229 (desprendimiento del puente de la molécula), *m/z* 187 (pérdida de C_5H_8O), *m/z* 173 (rotura de la fracción canfor) y *m/z* 121. El hecho de que la masa de este último fragmento, que corresponde a la parte aromática del compuesto, sea 16 Da mayor que en el correspondiente fragmento del compuesto precursor (*m/z* 105), sugiere que el grupo hidroxilo, -OH, se ha insertado también en el anillo aromático o el grupo metil adyacente al anillo aromático. De todos modos, basándonos únicamente en el FP obtenido mediante MS/MS, no es posible elucidar inequívocamente la estructura molecular, por lo que en este caso la posición del grupo hidroxilo la consideraremos como deslocalizada.

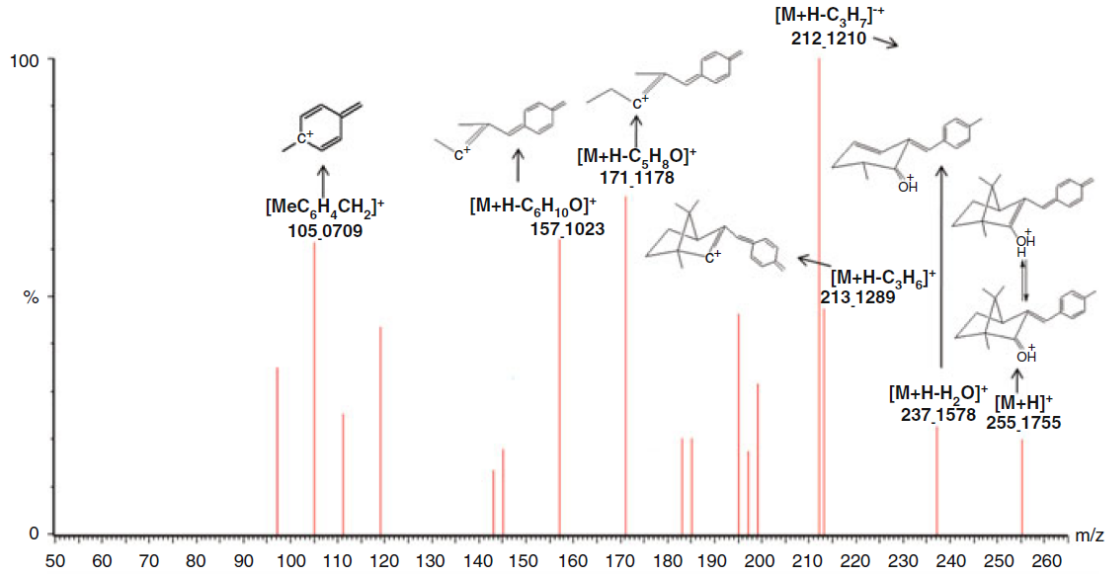


Figura 3.3. Espectros de masas (MS/MS) de los iones producto del 4MBC obtenidos mediante HPLC-QqTOF-MS/MS.

Un comportamiento prácticamente idéntico al descrito anteriormente para el Pr271 muestran los isómeros del compuesto con *m/z* 287 (Pr287) que encontramos a t_R =7.17 y t_R=7.65 min (misma *m/z* y FP). En la Figura 3.4.b Se puede observar que los iones fragmento corresponden a un compuesto 16 Da mayor con respecto a Pr271 y 32 Da al precursor. Estos hechos sugieren una doble hidroxilación del 4MBC y que los dos grupos hidroxilos se añaden, como en el caso anterior, en alguna posición del anillo aromático o a su grupo metil colindante.

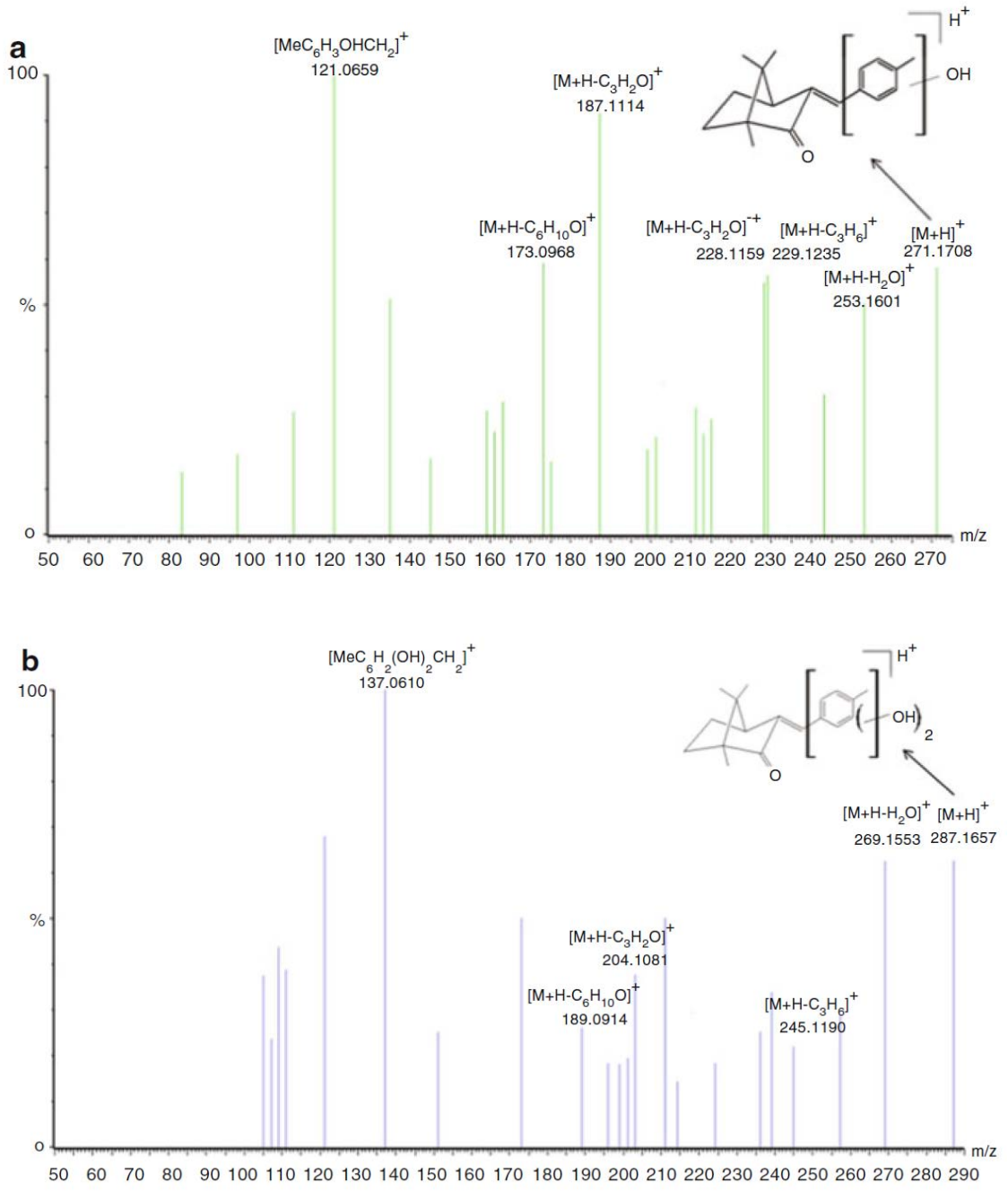


Figura 3.4. Espectros de masas (MS/MS) de los iones producto de los metabolitos de 4MBC. Experimentos realizados mediante HPLC-QqTOF-MS/MS. (a) Pr271, (b) Pr287.

Aunque son perfectamente detectables, la intensidad de los picos de Pr271 y Pr287 son muy bajas en comparación con la disminución de la intensidad para el 4MBC. Este hecho sugiere que éstos no son los productos de degradación mayoritarios. Los principales picos en cuanto a intensidad, los cuales probablemente corresponden a los metabolitos mayoritarios, los encontramos a t_R=6.31 y t_R=6.74 minutos (idéntica *m/z* y FP) con una *m/z* 425 (Pr425). La fórmula molecular exacta para este compuesto

corresponde a $C_{23}H_{30}O_6Na$. El aducto de este compuesto protonado se puede detectar pero la sensibilidad para este es mucho más baja y no permite obtener buenos espectros MS/MS. En este caso obtuvimos mejores resultados trabajando con el aducto de sodio.

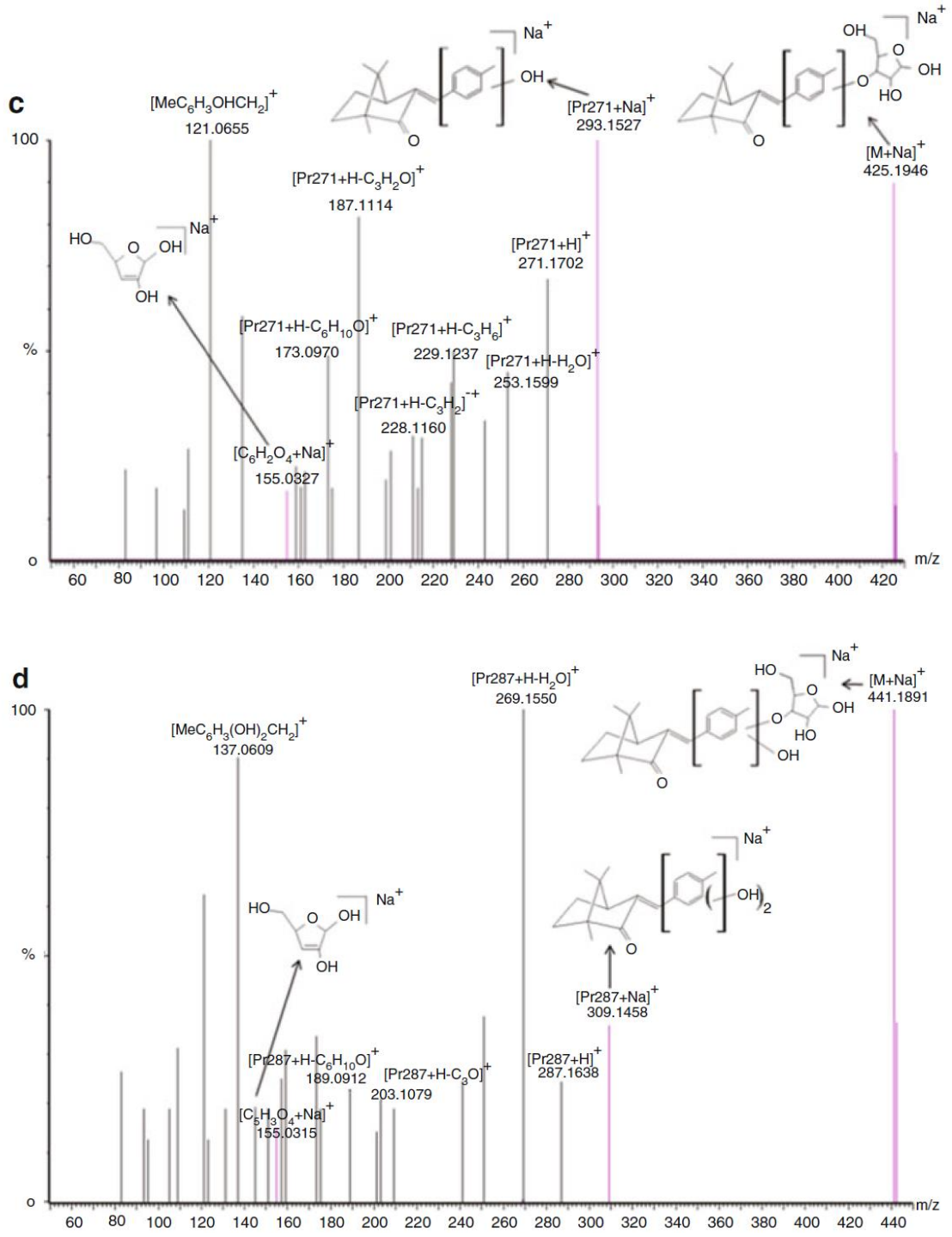


Figura 3.4. (continuación) Espectros de masas (MS/MS) de los iones producto de los metabolitos de 4MBC. Experimentos realizados mediante HPLC-QqTOF-MS/MS. (c) Pr425, (d) Pr441.

La Figura 3.4.c muestra la superposición de los espectros MS/MS para *m/z* 425 y *m/z* 271 a t_R=6.31 min (a t_R=6.74 son exactamente iguales). El espectro Pr425 tiene *m/z* 293 como principal ión fragmento, al que le corresponde una fórmula molecular de $C_{18}H_{22}O_2Na$. Esto se corresponde con el aducto de sodio del metabolito Pr271. El otro ión fragmento es *m/z* 155 con una fórmula molecular elemental correspondiente a $C_5H_8O_4Na$. Los espectros MS/MS para *m/z* 271, además de mostrar los picos a t_R=7.96 y t_R=8.22 min descritos anteriormente, nos muestra dos de mayor intensidad justo a t_R=6.31 y t_R=6.74 minutos. Este espectro es idéntico al que obtenemos para el 271 a t_R=7.96 y t_R=8.22. Estos datos sugieren que el metabolito Pr425 puede tratarse de Pr271 conjugado con una molécula de 132 Da. Pr425 podría tratarse de una molécula de pentosa conjugada a Pr271 mediante enlace glucosídico con la correspondiente pérdida de agua. Esta posibilidad se discute con más detalle posteriormente.

En los cromatogramas también se observaron dos picos de gran intensidad a t_R=5.01 y t_R=5.36 min (misma *m/z* y FP), con una *m/z* 441 (Pr441). La fórmula molecular exacta para este compuesto corresponde a $C_{23}H_{30}O_7Na$. En este caso, el aducto protonado no fue detectable. La Figura 3.4.d muestra la superposición de los espectros MS/MS para m/z 441 y m/z 287 a t_R=5.01 (el espectro a t_R=5.36 es exactamente igual). Se produce una analogía con el caso anterior entre Pr425 y Pr271. El espectro MS/MS para *m/z* 287 a t_R=5.01 min es exactamente igual al de *m/z* 287 a t_R=7.17 min. Por otra parte, los iones fragmento obtenidos del análisis MS/MS de *m/z* 441 corresponden a *m/z* 309, con fórmula molecular $C_{18}H_{22}O_3Na$, asociado al aducto de sodio de Pr287 y *m/z* 155, con fórmula molecular $C_5H_8O_4Na$ (la misma que la obtenida en el caso de Pr425). Estos hechos sugieren que Pr441 podría tratarse de una pentosa conjugada a Pr287 mediante enlace glucosídico.

Tanto Pr425 como Pr441 alcanzan su intensidad máxima a t=3 días, tras los que su intensidad va decreciendo hasta no ser detectables a t=12 días. A partir de ese momento no se detectó ningún metabolito.

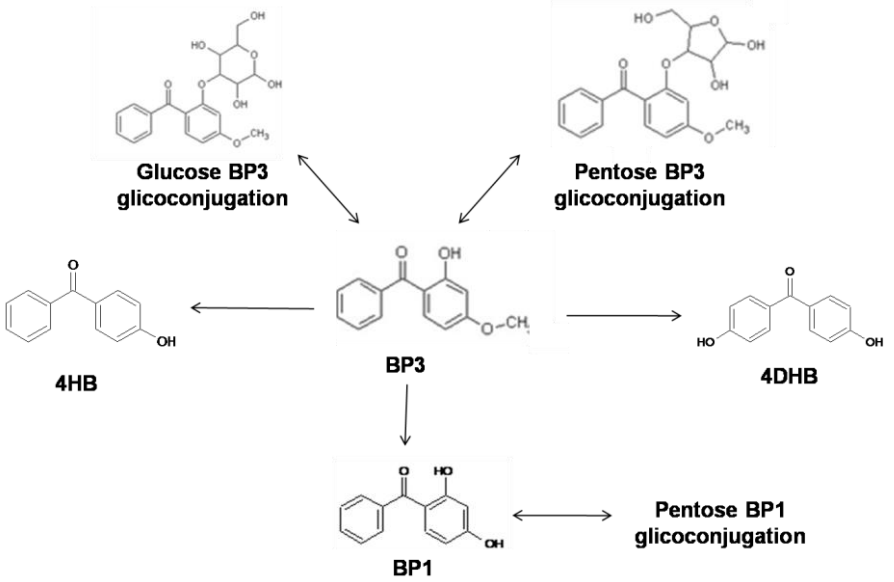
3.4. DISCUSIÓN SOBRE LOS MECANISMOS DE REACCIÓN EN LOS PROCESOS DE DEGRADACIÓN EN MEDIO ACUOSO PARA BP3, BP1 Y 4MBC

Para todos los UV-F para los que se evaluó su degradación en medio acuoso por la acción del hongo *T. versicolor* se identificaron metabolitos correspondientes a hidroxilaciones de los compuestos originales. Sin embargo, estos compuestos se detectaron a niveles de concentración muy bajos, no siendo los mayoritarios. Los principales metabolitos formados se correspondieron a conjugaciones de el compuesto original o hidroxilado con una pentosa o una hexosa. Hay muchas formas de metabolitos conjugados producidos mediante tratamiento con hongo descritos en la literatura. Los más comunes son el glucurónido, glutatión, el sulfato y las conjugaciones glucosídicas [104, 105]. Sin embargo, los metabolitos conjugados de 4MBC, BP3 y BP1 mediante el hongo *T. versicolor* solo se formaron mediante la adición de una molécula de pentosa al compuesto original (y también una molécula de glucosa en el caso de BP3). Las pentosas más comunes en las conjugaciones son la ribosa y la xilosa. Por lo tanto, las enzimas que llevan a cabo estos procesos serían la UDP-glucosiltransferasa, cuando la molécula añadida es la glucosa [106] y la UDP xylosiltransferasa, cuando la conjugación se produce con xilosa, tal y como se ha descrito anteriormente para otros xeniobioticos con *T. versicolor* [107]. La conjugación con ribosa también ha sido descrita con otros hongos [108], y también puede ocurrir con *T. versicolor* ya que el peso molecular del conjugado resultante sería el mismo que para el metabolito conjugado con xilosa. Estas moléculas pueden adherirse al compuesto a través de un enlace O-glicosídico con los grupos de hidroxilo presentes en el anillo aromático [107, 108]. En el caso de BP3, la conjugación tiene que producirse mediante el único hidroxilo libre de la molécula, mientras que BP1 presenta dos alternativas posibles. Estos metabolitos conjugados contienen el compuesto original, ya que las estructuras de BP3 y BP1 se encontraron sin alteraciones en los análisis de fragmentación HPLC-MS/MS. En el caso de 4MBC, la conjugación solo se produce después de la mono- o di-hidroxilación del anillo aromático o el grupo metilo adyacente, pero no es posible especificar la posición exacta en nuestros análisis.

Los principales metabolitos identificados para todos los UV-F estudiados fueron los que se encontraban en formas conjugadas. El producto predominante en la degradación de BP3 fue la conjugación con una molécula de pentosa, alcanzando su máximo de

concentración a las 24 h, con un área de pico equivalente al 40% de de la cantidad inicial de contaminante. La composición del medio puede modificar la forma de los metabolitos que se originan o, al menos, las proporciones de éstos. Por ejemplo, en el caso de BP3 la falta de glucosa en el medio genera una disminución de la forma conjugada de la glucosa.

a



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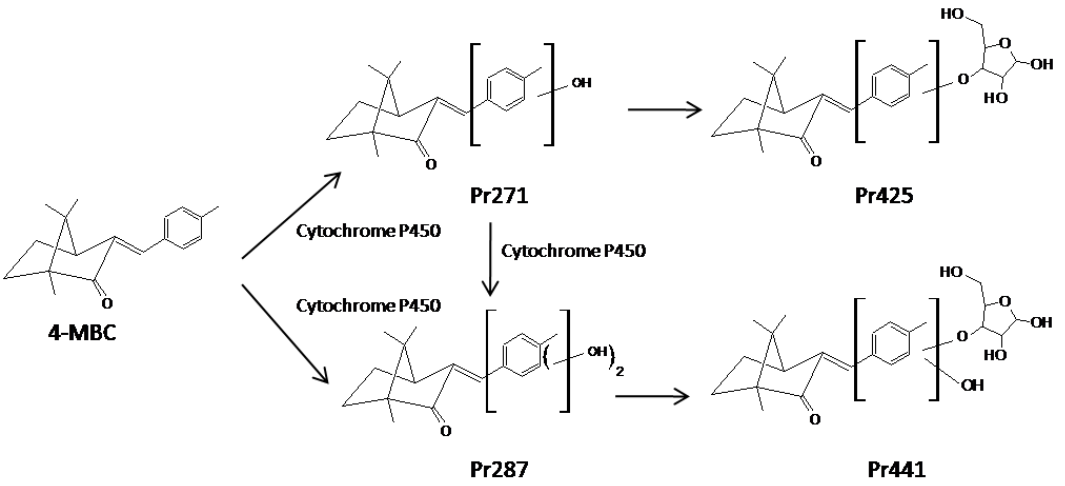

Figura 3.5. Mecanismos de biodegradación propuesto para el tratamiento con *T. versicolor* de (a) BP3 y (b) 4MBC.

Como se ha comentado antes, un tratamiento no tiene mayor interés si no se produce la metabolización tanto del metabolito conjugado como del compuesto original libre. No obstante, tal y como se describió anteriormente, en nuestros experimentos los metabolitos desaparecieron gradualmente del medio, lo que significa que el enlace O-glucosídico se va rompiendo y actuando a la vez otras enzimas del hongo. En los estudios con derivados de la benzofenona, el citocromo P450 monooxigenasa probablemente oxidaría BP3 y BP1 mediante la adición de grupos hidroxilo o mediante desmetilación (sólo en el caso de BP3). Esto conduciría a la formación de los metabolitos detectados BP1, 4HB y 4DHB. Sin embargo, el sistema enzimático de los hongos es amplio [109] y otras enzimas intracelulares o extracelulares podrían estar involucradas. Para el 4MBC también tuvo lugar la posterior degradación de los metabolitos glicoconjugados.

En conclusión, los procesos de conjugación constituyen uno de los mecanismos de defensa que tienen los hongos contra los compuestos tóxicos hidroxilados, disminuyendo su toxicidad y aumentando su biodisponibilidad [106]. Así, la glicoconjugación sería el primer paso en la degradación de BP3 y BP1. Para 4MBC, cualquier reacción de conjugación requiere un paso previo de hidroxilación (o dihidroxilación) por el citocromo P450. En todos los casos, la conjugación revertirá gradualmente y dará lugar a la oxidación de los compuestos y la subsiguiente rotura de los anillos, lo que conduce a la formación de fragmentos más pequeños y no identificados. La Figura 3.5. resume las etapas iniciales propuestas para los mecanismos de degradación de BP3 y de 4MBC, respectivamente.

El estudio de los mecanismos de degradación para PCPs en los tratamientos con hongo es relativamente reciente y no hay demasiados datos al respecto. En general, los mecanismos de degradación de xenobióticos con hongos más estudiados son aquellos relacionados con los PAHs [90]. Como los PCPs presentan también estructuras aromáticas, se entiende que las enzimas implicadas en su degradación no deberían diferir considerablemente de las implicadas en el metabolismo de los PAHs. Entre estas enzimas se incluyen las enzimas extracelulares, manganeso peroxidasa, las lacasas y la lignina peroxidasa, así como el sistema intracelular del citocromo P450.

Existen estudios relacionados con el metabolismo de los PCPs llevados a cabo en ratas y posteriormente en humanos, así como también se han realizado estudios

toxicogenéticos para evaluar sus riesgos asociados. Sin embargo, los UV-F son un ejemplo donde el metabolismo degradativo difiere entre mamíferos y hongos [110], a pesar de que las enzimas implicadas son de la misma familia (citocromo P450). No obstante, algunas etapas si que son equivalentes tanto en los estudios con ratas como con el hongo (que dan lugar a los mismos metabolitos, como BP1 a partir de BP3). Los estudios toxicogenéticos llevados a cabo en ratas han revelado que el mecanismo metabólico principal para BP3 es la desalquilación de la cadena lateral hidroxilada, lo que nos conduce al metabolito BP1. Entre los mecanismos secundarios encontramos las hidroxilaciones aromáticas llevadas a cabo mediante el sistema del citocromo P450 que lleva a la formación de los metabolitos hidroxilados DHMB y 2,3,4-Trihidroxibenzofenona (THB) [11]. No obstante, los metabolitos predominantes en muestras de orina humana y de semen durante las primeras veinticuatro horas tras la aplicación tópica son los glucurónidos conjugados de BP3 y BP1 [29]. Ninguno los compuestos comentados, con la excepción de BP1, se ha encontrado en los experimentos con hongo. En los ensayos con 4MBC, los metabolitos identificados en ratas y humanos fueron 3-(-4 carboxibenciliden)-6-hidroxicanfor (como metabolito mayoritario) y 3-(-4 carboxibenziliden) canfor y sus respectivos conjugados glucurónidos [111]. Como en los mamíferos, las enzimas de fase I y fase II parecen participar en la degradación de BP3, BP1 y 4MBC mediante el tratamiento con hongo *T. versicolor* [67, 112].

Los ensayos *in vitro* con lacasa concluyen que esta enzima no es capaz de transformar BP3 y 4MBC por sí sola, aunque BP3 puede degradarse si se añaden mediadores externos que incrementen el intervalo de oxidación de la enzima. Teniendo en cuenta que los hongos tienen sus propios mediadores, la oxidación de BP3 (la del 4MBC es más difícil aunque posible) por el sistema endógeno lacasa-mediador es en principio viable. Estos resultados coinciden con los publicados por García et al. [113]. En este estudio también se observó que las concentraciones bajas de BP3 (1 ngL^{-1}) requieren mayores ratios molares mediador/BP3 que las concentraciones altas de BP3 (1 mgL^{-1}) para mantener la misma tasa de degradación para una concentración dada de enzima (1UmL^{-1}).

Los hongos expresan otras encimas extracelulares oxidativas, como MnP o LiP. Sin embargo, no se han publicado estudios respecto a su participación en la degradación de los UV-F.

Otras enzimas importantes en la degradación de los xenobióticos son las monoxigenasas citocromo P450, que son una amplia familia de enzimas intracelulares presentes en todos los organismos eucariotas y también en algunos microorganismos procariotas. Estudios *in vivo*, consistentes en la adición de un inhibidor del citocromo P450 en el cultivo de hongos con los contaminantes, permiten saber si ese sistema enzimático participa o no en los primeros pasos de transformación de dichos contaminantes. La presencia del inhibidor 1-aminobenzotriazol (ABT) afecta a las tasas de degradación de BP3 y 4MBC, pero de diferente manera. La presencia de ABT inhibe completamente la degradación de 4MBC, lo que parece indicar que la primera etapa en su degradación, la hidroxilación del anillo aromático, se lleva a cabo mediante el citocromo P450. Por otro lado, ABT no inhibe completamente la degradación BP3 aunque sí que afecta negativamente. De diferentes formas, tanto BP3 como 4MBC presentan grupos hidroxilados que permiten la conjugación en ausencia de actividad del citocromo P450 hidroxilasa.

Los metabolitos producidos por las lacasas, P450 u otras enzimas están más oxidados que los compuestos precursores, por lo general mediante la hidroxilación de anillos aromáticos. En el caso de BP3, el citocromo P450 puede llevar a cabo también una desmetilación como la descrita previamente para 2,4,6-tricloroanisol (2,4,6-TCA) [114]. No obstante, los metabolitos conjugados también juegan un papel importante en la transformación de los xenobióticos. La conjugación aumenta la polaridad de los compuestos, su biodisponibilidad y su posterior degradación. Estos procesos generalmente derivan en una disminución de la toxicidad de los xenobióticos [115, 116], aunque en algunos casos la mayor disponibilidad del metabolito resultante también puede incrementar su toxicidad [117, 118]. Un problema de los metabolitos conjugados es que el proceso puede ser reversible fácilmente dando lugar a la formación del compuesto original. Por lo tanto, se debe confirmar que al final del tratamiento tanto el precursor como las formas conjugadas de este se degradan. En el caso de BP3 esto se produjo después de 9-15 días de tratamiento, mientras que los metabolitos conjugados 4MBC desaparecieron después de alcanzar el máximo a los 3 días.

3.5. DEGRADACIÓN DE FILTROS UV EN LODOS DE DEPURADORA CON EL HONGO *TRAMETES VERSICOLOR*

Problemática de los lodos y de su degradación

La eliminación y el reciclado de los residuos que se generan como consecuencia de la actividad humana es un problema de grandes dimensiones y difícil de acometer que requieren la adopción de medidas urgentes para la conservación del medio ambiente. Entre estas substancias encontramos los lodos de depuradora, que son un residuo semisólido que se genera en las EDARs durante el tratamiento primario (físico y/o químico), secundario (biológico) y terciario (normalmente de eliminación de nutrientes). En los últimos años y con el fin de cumplir las exigencias de las directivas europeas (91/271/EEC y 98/15/EEC) que establecen que todas las poblaciones con más de 2000 habitantes deben depurar sus aguas residuales, se ha construido una gran cantidad de EDARs. Este hecho ha conllevado un gran incremento en la generación de lodos de EDAR. Los datos recogidos en 2005 en el informe sobre la implementación de la legislación sobre residuos estiman en 9 millones de toneladas de materia seca la producción de lodos de depuradora en los estados miembros de la Unión Europea. De todos estos lodos generados, aproximadamente el 45% es reciclado, mayoritariamente para su uso en la agricultura como fertilizante, el 18% es transportado a los vertederos, el 17% es incinerado y el 20% restante no está especificado.

Debido a los procesos fisicoquímicos relacionados con el tratamiento en las EDARs, los lodos tienden a acumular metales pesados y compuestos orgánicos poco biodegradables, así como organismos potencialmente patogénicos como bacterias o virus que están presentes en las aguas residuales. Los lodos son también ricos en nutrientes como nitrógeno y fósforo y contienen gran cantidad de materia orgánica, que es muy útil como fertilizante en agricultura.

Los criterios de calidad sobre los lodos producidos en las EDARs para definir sus posibles usos, tales como la aplicación de estos a la agricultura, se centran fundamentalmente sobre su contenido en metales pesados (Real Decreto 824/2005 sobre productos fertilizantes. BOE 19/07/2005). En la Unión Europea, desde 1986, a los lodos les es aplicable la directiva 86/278/EEC, que recomienda la regulación de los usos en base a la calidad del lodo. Actualmente se está trabajando en el desarrollo de una nueva normativa (Working Document on Sludge, 3rd Draft, 2000) sobre la calidad de los

biosólidos y se prevé una mayor restricción y control de estos residuos para minimizar el riesgo de efectos nocivos en la salud y el medio ambiente. Este documento propone límites de concentración para algunos contaminantes prioritarios, pero se prevé que también será necesario incluir límites para contaminantes emergentes.

Teniendo en cuenta todo esto, es evidente la necesidad de llevar a cabo una correcta gestión de los lodos de depuradora, ya que un uso no controlado en agricultura puede conllevar problemas medioambientales como un exceso y/o desequilibrio en el aporte de nutrientes, dispersión de patógenos humanos, animales y vegetales o la introducción de contaminantes inorgánicos, tales como metales pesados y contaminantes orgánicos. Estos contaminantes introducidos pueden llegar a aguas superficiales, aguas residuales e incluso introducirse en la cadena alimentaria.

Debido a su lipofilicidad y su baja biodegradabilidad, los UV-F, que son el objeto de nuestro estudio, tienden a acumularse en los lodos de depuradora. Estos compuestos se encuentran presentes en altas concentraciones, del orden de los $\mu g g^{-1}$ como ya se ha descrito en el capítulo anterior.

Estudio de su degradación con el hongo *T. versicolor*

Toma de muestra: Para este estudio de degradación de UV-F en lodos de EDAR mediante la acción del hongo, se han utilizado lodos reales provenientes de la depuradora de El Prat de Llobregat. Esta depuradora está incluida en el estudio descrito con anterioridad en el artículo científico #4. La depuradora seleccionada es la mayor de Cataluña tanto en número de habitantes equivalentes como de toneladas de materia seca producidas anualmente. En esta depuradora el tratamiento que se aplica a los lodos es la digestión anaerobia, que es el tratamiento más común en las EDARs europeas. El estudio que llevamos a cabo anteriormente descrito en el artículo científico #4, mostró que esta EDAR es la que contiene una mayor carga de UV-F en sus lodos, por lo que constituye un punto de muestreo muy interesante para evaluar la acción del hongo. Los lodos utilizados para llevar a cabo los experimentos y cuantificar la degradación obtenida son los llamados lodos térmicos. La Figura 3.1. muestra el diagrama de procesos de la EDAR de El Prat de Llobregat.

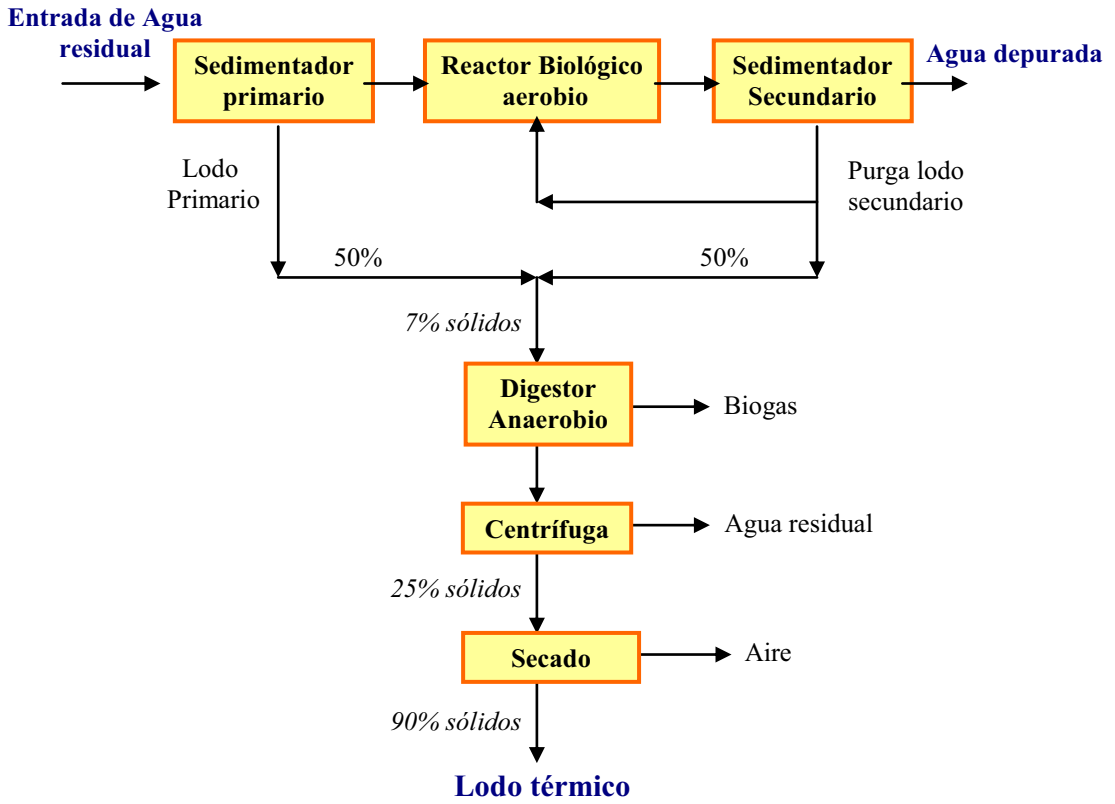

Figura 3.1. Diagrama de flujo para la obtención de los lodos en la EDAR de El Prat de Llobregat.

Metodologías: Los experimentos se llevaron a cabo siguiendo el proceso descrito por Rodríguez-Rodríguez et al. [99]. Brevemente, los sistemas (en fase sólida) contenían lodo esterilizado con inóculos de *T. versicolor* al 38% en peso, el cual previamente había sido incubado con pellets de paja de trigo. Estos sistemas se incubaron por un periodo de 42 días a 25 °C, durante los cuales fueron homogeneizados y hidratados periódicamente. El proceso de esterilización consistió en autoclavar las muestras a 121 °C durante 30 minutos.

La concentración de UV-F en los lodos, tanto antes de tratar como después del tratamiento se determinó siguiendo el método descrito incluido en el Capítulo II, artículo científico #4.

Los datos de toxicidad se obtuvieron mediante ensayos basados en levaduras recombinantes (Recombinant Yeast assay, RYA), siguiendo los protocolos descritos por Noguerol et al. [100]. Así, se estudiaron la posible estrogenicidad y el efecto tipo

dioxina (dioxina-like activity). Los resultados se expresaron en equivalentes de estradiol (E2 eq., ER_RYA) o equivalentes de β-naptoflavona (BNF Eq., AhR-RYA), para la estrogenicidad y la actividad dioxina, respectivamente.

Caracterización del lodo y eliminación de UV-F: La Tabla 3.1. muestra las concentraciones de UV-F en el lodo antes y después del tratamiento con el hongo. El compuesto más abundante fue el OC, con una concentración de 8.0 μgg^{-1}, seguido del 4MBC y EHMC. Para BP3 y su metabolito 4DHB se determinaron menores concentraciones. Los compuestos BP1, 4HB y OD-PABA no se detectaron en estas muestras. OC, 4MBC y EHMC están entre los compuestos más lipofílicos (log K_{ow} 4.95 – 6.88), por lo que el hecho de que estén en concentraciones mayores era esperable, debido a la relación entre log K_{ow} y su potencial eliminación en la EDAR por adsorción [41]. Estas concentraciones están en concordancia con los resultados previamente obtenidos en el estudio de evaluación de estos compuestos en lodos de diferentes EDARs (artículo científico #4 de esta Tesis). Se encontró que los UV-F están en concentraciones mucho más altas que otras familias de contaminantes emergentes, como por ejemplo, los fármacos, que también se analizaron en estos mismos lodos (< 100 ngg^{-1}) [99].

Tabla 3.1. Eliminación de UV-F por biodegradación por el hongo *Trametes Versicolor.*

UV-F	Lodo de EDAR antes de tratar (μgg^{-1} dw) ± RSD (%)	Lodo de EDAR tratado con el hongo (μgg^{-1} dw) ± RSD (%)	% Eliminación
BP1	n.d	n.d	**-**
4DHB	0.07 ± 6	n.d	**100**
4HB	n.d	n.d	**-**
BP3	0.06 ± 11	n.d	**100**
4-MBC	3.10 ± 9	0.40 ± 11	**87**
OC	8.00 ± 11	0.92 ± 10	**89**
OD-PABA	n.d	n.d	**-**
EHMC	2.20 ± 5	0.16 ± 5	**93**
Oestrogenicidad (μgL^{-1} E2 Eq.)	0.67 ± 0.12	n.d.	
Dioxin-like (μgL^{-1} BNF Eq.)	603 ± 114	1430 ± 200	

n.d: no detectado

Observando Tabla 3.1. queda claro que el hongo T. *versicolor* proporcionó una reducción muy significativa de las concentraciones de UV-F. BP3 y 4DHB se eliminaron totalmente y los otros compuestos que están presentes en el lodo, después del tratamiento sufrieron eliminaciones entre el 87-93%. El hecho de una eliminación un poco más baja para los compuestos OC, 4MBC y EHMC se puede explicar parcialmente por sus valores más altos de $\log K_{ow}$, que pueden traducirse en una reducción de la biodisponibilidad para el hongo. Hay que tener en cuenta que los valores que se dan en la Tabla 3.1 se han corregido previamente teniendo en cuenta la cantidad de hongo añadida, por lo que el efecto dilución está considerado.

El estudio de degradación de estos contaminantes xenobióticos se ha complementado con ensayos toxicológicos. El tratamiento del lodo ya había mostrado una disminución significativa de la toxicidad global en ensayos previos con *Dafnia magna*, *Vibrio fischeri* y tests de germinación [99]. En este trabajo se llevaron a cabo análisis para determinar el efecto disruptor endocrino y la actividad tipo dioxina. Los lodos mostraron una actividad estrogénica moderada que fue totalmente eliminada tras el tratamiento, hecho que está de acuerdo con resultados previos de otros estudios [101]. Por el contrario, la actividad tipo dioxina se incrementó en el lodo después del tratamiento, llegando a valores de mgL^{-1} equivalentes de BNF. Sin embargo, en el lodo hay muchos otros compuestos a parte de los UV-F, y estos datos pueden ser interpretados como indicadores de bioactivación de algunos de los otros compuestos presentes en el lodo mediante el tratamiento con el hongo.

3.6. POTENCIAL Y APLICABILIDAD DE LAS TECNOLOGÍAS INVESTIGADAS

Este estudio ha sido el primero en evaluar la eliminación de UV-F presentes en lodos de EDAR mediante un tratamiento con hongos. Estos compuestos se encuentran en concentraciones medioambientales muy relevantes, del orden de $\mu g g^{-1}$ en muchos casos. En la cuantificación de UV-F y sus metabolitos en el lodo, se han detectado en mayor concentración los que tienen mayor Log K_{ow}. Sin embargo, se han obtenido porcentajes de eliminación cercanos al 100% para todos los compuestos después del tratamiento. Aunque el proceso que se describe requiere de una profunda optimización para ser aplicado a mayor escala, los prometedores resultados que se extraen muestran que el hongo *T. versicolor* es una opción con mucho potencial para la bioremediación de UV-F y, en general, de compuestos xenobióticos en lodos EDAR. El paso que debería seguir a estos estudios sería llevar a cabo los ensayos en condiciones no estériles, para determinar la capacidad del hongo de degradar estos contaminantes compitiendo con otros microorganismos presentes en el lodo. Finalmente, se debería comprobar la viabilidad de los experimentos a mayor escala, donde habría que mediar con otras complicaciones de tipo técnico, como por ejemplo encontrar soluciones para gestionar grandes volúmenes de lodo y hongo de una manera que el proceso funcione eficazmente. El estudio del "up-scale" indicaría finalmente la viabilidad de la implantación de estos sistemas en EDARs reales.

Dado que la mayor parte de los lodos, incluido el que se estudia en esta sección, son empleados en suelos agrícolas, queda demostrado que grandes cantidades de contaminantes pasan al medio ambiente a través de su aplicación. Estos estudios pueden dar lugar a una etapa extra en el proceso de depuración de los lodos, de relativamente fácil implantación y de bajo coste energético y de mantenimiento. Estos costes quedarían compensados si se consiguiese una reducción sustancial de los contaminantes así como de la toxicidad de los lodos que se producen. Por lo que, en mi opinión, vale la pena investigar tecnologías innovadoras y respetuosas con el medio ambiente como puede ser el tratamiento con hongos.

3.7. CONCLUSIONES

En estos estudios, que ofrecen los primeros datos sobre la biodegradación de UV-F con hongos ligninolíticos, se puede observar que el hongo *T. versicolor* es capaz de degradar estos compuestos, tanto en medio acuoso como en lodos de depuradora mediante los diferentes procesos descritos. Por otra parte, hay que resaltar los productos de transformación generados durante estos tratamientos. Es imprescindible conocer cuales son así como determinar si son también biodegradables por el hongo o, por el contrario, permanecen en el medio.

A la vista de los resultados obtenidos podemos concluir que:

- El tratamiento en fase sólida de lodos de EDAR con el hongo es efectivo para los UV-F presentes, con porcentajes de eliminación entre el 87 y 100%.

- La estrogenicidad de los lodos después del tratamiento decrece considerablemente mientras la actividad tipo dioxina aumenta. Estos cambios en la toxicidad del lodo no se pueden atribuir a las transformaciones que puedan sufrir los UV-F, ya que en el lodo hay miles de sustancias, muchas de ellas más tóxicas, pero si evidencia la necesidad de controlar un espectro de toxicidades lo más amplio posible con el fin de poder hacer una mejor evaluación del proceso.

- El tratamiento con hongo en fase acuosa ha resultado efectivo para la completa degradación de BP3 y BP1 en un tiempo inferior a 24 h. Mediante análisis con espectrometría de masas de alta resolución se comprobó que BP1, así como 4DHB y 4HB son productos de transformación de BP3 durante este proceso, pero no los mayoritarios y solo se han podido detectar a bajas concentraciones. Los principales metabolitos identificados fueron, en el caso de BP3, la conjugación de una pentosa a la estructura de BP3 (una ribosa o una xilosa) y la conjugación de una hexosa (probablemente glucosa). En el caso de la BP1 el principal metabolito fue la conjugación de una pentosa a su estructura.

- Los experimentos de fotodegradación muestran que BP1 se degrada completamente mientras BP3 es susceptible de eliminación mediante fotólisis.

- En el caso del 4MBC, también se obtuvo una degradación completa del compuesto en el tratamiento con hongo en menos de 24 horas. Los principales

metabolitos que identificamos fueron hidroxilaciones del compuesto así como la conjugación de una pentosa a la molécula de 4MBC (ribosa o xilosa).

- Todos los productos de transformación detectados en los experimentos con BP3, BP1 y 4MBC se degradaron y no se detectaron tras 12 días de tratamiento. A partir de este momento no se apreciaron picos cromatográficos diferentes a los presentes al mismo tiempo en los experimentos control, lo que sugiere una completa degradación de los compuestos.

- Estos resultados muestran la importancia de otras reacciones, además de las oxidaciones en los mecanismos de degradación de contaminantes mediante el hongo *T. versicolor*. Estás reacciones, como las conjugaciones mediante enlace glicosídico, aumentan la biodisponibilidad de los contaminantes, facilitando posteriores procesos de transformación.

CAPÍTULO IV

PROCESOS DE OXIDACIÓN AVANZADA: OZONIZACIÓN Y PEROXONIZACIÓN APLICADOS A LA DEGRADACIÓN DE BP3

Procesos de oxidación avanzada: Ozonización y peroxonización aplicados a la degradación de BP3

Los procesos de oxidación avanzada (advanced oxidation processes, AOPs) han emergido como una potente tecnología capaz de eliminar una gran variedad de contaminantes orgánicos en matrices acuosas. Estos procesos se basan en la generación de radicales hidroxilo (•OH) y otras especies radicalarias que atacan las moléculas orgánicas mineralizándolas u oxidándolas a productos intermedios de menor complejidad.

Estos procesos suelen ser muy efectivos, pero tienen la desventaja de un elevado coste energético. Se pueden combinar con otras tecnologías, como los procesos biológicos convencionales, aumentando así considerablemente la efectividad de éstos debido al aumento de la biodegradabilidad de las aguas. Estas técnicas, que tienen un gran potencial, están siendo ampliamente estudiadas para la degradación de contaminantes. Pese a ello, su aplicación para la eliminación de residuos de PCPs es muy escasa.

La ozonización y su combinación con peróxido de hidrógeno (H_2O_2) son potentes técnicas para la eliminación de microcontaminates, con mucho potencial de aplicación en la potabilización de agua y en el tratamiento de aguas residuales.

Objetivos

El objetivo de este estudio fue evaluar el proceso de ozonización y su combinación con la adición de H_2O_2 para la degradación de BP3 en medio acuoso. Primero fue necesario comprobar que este proceso era efectivo para degradar este compuesto, ya que como se comentará posteriormente los datos existentes hasta la fecha no eran concluyentes. Posteriormente, se estudió como afectan al proceso diferentes variables operacionales mediante un estudio paramétrico en el que se tiene en cuenta el flujo y la concentración de ozono (O_3) inyectado, la temperatura, el pH, la adición de H_2O_2 y la adición del *t*-butanol. Estos datos también nos permitieron obtener información sobre los mecanismos de reacción que tienen lugar durante el proceso oxidativo.

Otro de los principales objetivos de este capítulo fue identificar y cuantificar los productos de transformación generados en este proceso, evaluando si es posible llegar a

su completa mineralización o, por el contrario, se generan productos de transformación recalcitrantes. Ello es sumamente importante, ya que los productos de transformación generados mediante estos procesos oxidativos, pueden ser incluso más tóxicos que sus precursores.

Estructura

Este capítulo consta de una pequeña introducción sobre los AOPs, sobre el ozono y las reacciones directas e indirectas involucradas en los procesos de ozonización y sobre la combinación O_3/H_2O_2. Posteriormente se muestran los resultados de la evaluación de la degradación de BP3 mediante ozonización y su combinación con H_2O_2, así como la identificación de los productos de transformación generados en el proceso. Los resultados de este estudio se explican detalladamente en la siguiente publicación:

Publicación ciéntífica #9: Ozonation and peroxone oxidation of benzophenone-3 in water: Effect of operational parameters and identification of intermediate products (Science of the Total Environment (2013) 443:209-217).

Posteriormente también se discute y se compara con el anterior trabajo el proceso de ozonización para BP1, el principal producto de transformación de BP3. Finalmente, se lleva a cabo una comparación entre el proceso de degradación para BP3 con el hongo *T. versicolor* y mediante ozonización.

- *Estos estudios se realizaron en el contexto de una estancia científica predoctoral en la Facultad de Ingeniería de la Universidad de Gante (Universiteit Gent), Bélgica, concretamente en el grupo "Environmental Organic Chemistry and Technology (EnVoc)" bajo la dirección del profesor Kristof Demeestere.*

4.1. PROCESOS DE OXIDACIÓN AVANZADA (AOPS)

Los AOPs son ampliamente utilizados en el tratamiento de agua de bebida y pueden suponer una alternativa o complemento en el tratamiento de aguas residuales para la degradación de todo tipo de compuestos orgánicos, incluyendo familias de contaminantes emergentes como fármacos o disruptores endocrinos [119-128]. Los distintos AOPs tienen lugar mediante diferentes reacciones pero pueden definirse como procesos que implican la formación de radicales hidroxilo. Estos radicales son capaces de oxidar compuestos orgánicos principalmente por abstracción de hidrógeno o por adición electrofílica a dobles enlaces, generando radicales orgánicos libres, que pueden reaccionar a su vez con moléculas de oxígeno y que dan lugar a una serie de reacciones de oxidación que pueden mineralizar los contaminantes o bien oxidarlos a productos menos complejos y más fácilmente degradables. En el trabajo de Ikehata et al. [129] se presenta una visión general sobre varios AOPs y se puede consultar su efectividad en la degradación de diversos tipos de microcontaminantes.

En la literatura se han descrito diferentes tipos de AOPs: ozonización, electrólisis, fotocatálisis homogénea y heterogénea basada en UV, UV-cercana o UV-visible, reactivos de Fenton y otros procesos menos convencionales como radiación ionizante, microondas o plasma por impulsos. Muy frecuentemente, estas técnicas se aplican combinadas con otros procesos fisicoquímicos y biológicos. Algunas de las combinaciones más utilizadas son: UV combinada con H_2O_2, UV combinada con O_3, O_3 en combinación con H_2O_2, UV combinada con O_3 y H_2O_2, foto oxidación heterogénea mediante dióxido de titanio (TiO_2/UV), fotocatálisis homogénea (foto-Fenton), ultrasonidos combinados con reactivos de Fenton o electrólisis combinada con los reactivos de Fenton.

En principio las técnicas de AOPs pueden mineralizar totalmente los contaminantes orgánicos. Sin embargo, en muchos casos ese no es el objetivo. Por ejemplo, la mineralización de fármacos mediante AOPs ha demostrado no ser económicamente rentable [130], produciéndose además un considerable aumento en el consumo de energía y en las emisiones de CO_2 [131]. La degradación parcial, que da lugar a la formación de especies más fácilmente biodegradables y menos tóxicas es una opción muy interesante que nos proporcionan los AOPs. Como ya se ha comentado, en todo proceso de degradación se pueden formar productos de transformación, que pueden ser

más tóxicos que los compuestos precusores. En consecuencia, se debe llevar a cabo un riguroso estudio de los productos de transformación para asegurar un tratamiento seguro de las aguas, ya sean residuales o de otro tipo, y prevenir así posibles efectos adversos tanto en la salud humana como en el medio ambiente. De los productos de transformación de los AOPs se debe caracterizar su estructura química, su toxicidad así como su estrogenicidad y otros efectos biológicos.

Se sabe que la presencia de otras sustancias presentes en las aguas a tratar, como materia orgánica, sólidos suspendidos y disueltos como también el pH y la temperatura del agua influyen en los AOPs [129]. Por ejemplo, los sólidos en suspensión y partículas con color pueden obstaculizar reacciones fotoquímicas, debido a la dispersión de la luz y a la absorción. Los iones carbonato, bicarbonato y cloruro, así como otros compuestos orgánicos, actúan como inhibidores de los radicales. Estos compuestos compiten con los microcontaminantes que queremos degradar mediante los radicales hidroxilo generados en el medio, por lo que su presencia incrementa la demanda de oxidantes y disminuye la eficacia del tratamiento. Además, el coste del equipo y materiales, así como los requerimientos energéticos deben ser tenidos en cuenta para evaluar el rendimiento global de los AOPs.

4.2. OZONO

El ozono es un gas a temperatura y presión ambiente, pero es inestable. Así, para utilizarlo como agente oxidante se debe generar *in-situ*. Al igual que el oxígeno, su solubilidad en agua depende de la temperatura y de la presión parcial de ozono en la fase gaseosa. Su solubilidad también es altamente dependiente del pH. El ozono se genera por oxidación electrolítica del oxígeno proveniente de aire seco o bien de oxígeno puro. Teóricamente, se pueden producir 1058 g de ozono por kilovatio-hora (kW·h) de energía eléctrica pero, en la práctica, se puede esperar una producción de alrededor de 150 $gkW^{-1}h^{-1}$.

El ozono es un poderoso agente oxidante, con un potencial redox estándar de 2.07 V y una polaridad débil (0.53 D). El ozono es altamente reactivo, hecho que se puede atribuir a la configuración electrónica de la molécula. Debido a la ausencia de electrones en una parte de la molécula y el exceso en la otra, el ozono tiene carácter electrofílico y

nuecleofílico al mismo tiempo [132]. El principal oxidante formado a partir del ozono es el radical hidroxilo, el cual es más oxidante y menos selectivo que el ozono [133].

Reacciones de ozonización directas e indirectas

Reacciones directas: Las reacciones directas con ozono se pueden dividir en cuatro grupos: reacciones de oxidación-reducción, reacciones de cicloadición y reacciones de substitución nucleofílica y electrofílica. Las reacciones de oxidación-reducción son importantes principalmente para especies inorgánicas mientras que las reacciones de substitución nucleofílica solo han sido confirmadas en sistemas no acuosos [132], por lo que quedarían fuera de nuestro objeto de estudio: la ozonización de contaminantes orgánicos en sistemas acuosos.

Las reacciones de cicloadición tienen lugar cuando el ozono reacciona con una olefina mediante una adición electrofílica. Estas reacciones siguen el mecanismo de Criegee, donde el ozónido primario (1,2,3-trioxolano) formado se descompone para dar un compuesto carbonílico y un óxido de carbonilo. En las reacciones de substitución electrofílica el ozono ataca una posición nucleofílica de la molécula orgánica, resultando en una substitución de una parte de la molécula. Esta reacción es muy común en compuestos aromáticos, donde los grupos sustituyentes pueden incrementar o disminuir la estabilidad del carbocatión dependiendo de la capacidad para liberar o aceptar electrones. Los grupos activantes, entre los que se incluyen –OH, -O-, $-NH_2$, -NHR, $-NR_2$, -OR, $-C_6H_5$ o –Alquilo, incrementan la estabilidad del carbocatión y facilitan la sustitución de hidrógeno en posiciones orto y para. Por el contrario, los grupos desactivantes entre los que se incluyen $-NO_2$, $-NR_3^+$, -CN, -CHO, -COOH, -F, -Cl, -Br o –I, desestabilizan el carbocatión y favorecen la sustitución en posición meta.

Reacciones indirectas: El ozono es inestable en sistemas acuosos, tanto en condiciones ácidas como básicas. El ozono se descompone mediante un complejo mecanismo radicalario en cadena en el cual el radical hidroxilo se considera la especie oxidante principal en las reacciones indirectas [132]. En solución alcalina el ozono se descompone dando lugar a radicales hidroxilo mediante las siguientes etapas:

$O_3 + OH^- \rightarrow O_2^{-}\bullet + O_2$

$O_3 + H_2O \rightarrow 2OH\bullet + O_2$

Esta es la razón por la que la velocidad de oxidación en medio básico es varios órdenes de magnitud superior que en medio ácido, como pudimos constatar en nuestro estudio de degradación de BP3 y BP1 que se incluye a continuación.

En aguas naturales (subterráneas, superficiales o residuales), a menudo hay una disminución en la tasa de oxidación mediante reacciones indirectas debido al efecto de inhibición que producen los iones carbonato y bicarbonato. HCO_3^- y CO_3^{2-} tienen constantes de reacción con radicales hidroxilo de $8.5 \cdot 10^6$ y $4.2 \cdot 10^8$ $M^{-1}s^{-1}$ [132], respectivamente. Esto produce que en medios acuosos muy alcalinos la velocidad de la oxidación pueda decrecer a medida que el pH aumenta debido a la formación de iones carbonato, que son más efectivos como inhibidores de •OH que los iones bicarbonato.

4.3. OZONO/PERÓXIDO DE HIDRÓGENO

Durante la descomposición del O_3 se pueden formar cantidades significativas de H_2O_2, de acuerdo con Vandersmissen et al. [134]. La concentración del H_2O_2 formado no depende de la concentración inicial de O_3 o del pH, sino que depende de la temperatura, cuanto más elevada es ésta más H_2O_2 se forma. Esta formación de H_2O_2 proviene directamente de la descomposición del O_3 a través de la reacción $O_3 + \bullet OH \rightarrow O_2 + HO_2^-$ o bien de la hidrólisis de productos orgánicos de ozonización. El H_2O_2 formado de esta manera parece que intensifica la tasa de descomposición del O_3. La oxidación indirecta se intensifica cuanto más elevadas son las concentraciones de radicales para una misma concentración de O_3.

Una posibilidad para aumentar la concentración de radicales con respecto al O_3 es el proceso de peroxonización, donde se añade H_2O_2 a disoluciones que contienen O_3. El H_2O_2 inicia el proceso de descomposición del O_3 y lo acelera considerablemente. Este tipo de AOP se utiliza en tratamientos de purificación para aguas subterráneas y superficiales contaminadas con compuestos difíciles de degradar.

Tanto en la ozonización como en la peroxonización, el pH y la alcalinidad del bicarbonato juegan un papel importante en la efectividad de estos procesos, básicamente por las reacciones competitivas de los iones carbonato y bicarbonato con los radicales libres, ya comentadas anteriormente.

Mediante la adición de H_2O_2 al agua, puede verse mejorada la transferencia de O_3 de la fase gaseosa a la fase liquida, debido al incremento de la tasa de reacción del O_3. Añadiendo pequeñas concentraciones de H_2O_2 ($< 10^{-5}$ M) a las soluciones acuosas se pueden llevar a cabo AOPs a pH ácido, neutro y básico. La tasa de descomposición del O_3 en sistemas de agua pura puede verse mejorada hasta en un orden de magnitud mediante la adición de una pequeña cantidad de H_2O_2 ($\sim 6 \cdot 10^{-6}$ M). Si trabajamos a pH alcalino, se necesitan concentraciones de H_2O_2 más elevadas para observar el efecto, debido a la mayor tasa de descomposición del O_3 a esos pH [134].

4.4. EVALUACIÓN DE LA DEGRADACIÓN DE BP3 MEDIANTE OZONIZACIÓN Y SU COMBINACIÓN CON PERÓXIDO DE HIDRÓGENO: ESTUDIO PARAMÉTRICO E IDENTIFICACIÓN DE PRODUCTOS DE TRANSFORMACIÓN

Publicación científica #9

"Ozonation and peroxone oxidation of benzophenone-3 in water: Effect of operational parameters and identification of intermediate products"

por:

Pablo Gago-Ferrero, Kristof, Demesteere, M. Silvia Díaz-Cruz, Damià Barceló

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Ozonation and peroxone oxidation of benzophenone-3 in water: Effect of operational parameters and identification of intermediate products

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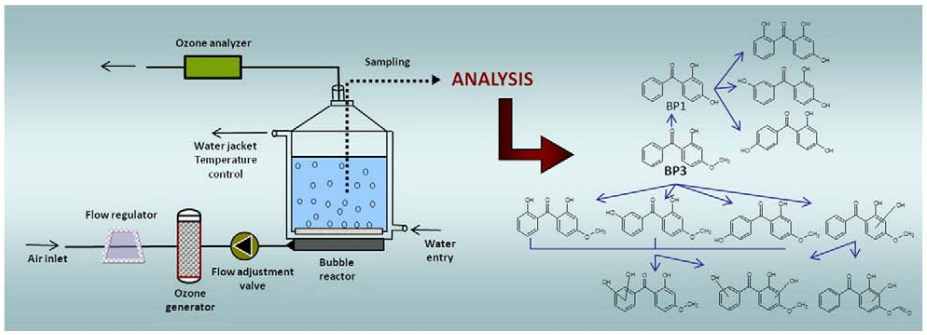
HIGHLIGHTS

- Ozonation shows to be a promising technology for the elimination of BP3.
- New data are obtained on the effect of process parameters on BP3 removal.
- Conditions favoring hydroxyl radical formation accelerate the degradation process.
- The reactivity is higher through radicalar pathways compared to direct ozonation.
- Seven major transformation products of BP3, including BP1 and DHMB, are identified.

GRAPHICAL ABSTRACT



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ABSTRACT

The goal of this study was to bring forward new data and insights with respect to the effect of operational variables and reaction pathways during ozonation and peroxone oxidation of the UV filter compound benzophenone-3 (BP3) in water. A systematic parameter study, investigating the effect of the ozone inlet concentration, temperature, pH, H_2O_2 and t-butanol addition in a lab-scale bubble reactor, showed the promising potential of ozonation towards BP3 degradation. pH showed to be a major process parameter, with half-life times (5.1–15.0 min) being more than two times shorter at pH 10 compared to neutral and acid conditions. This indicates the important role of hydroxyl radicals as supported by the addition of H_2O_2 and t-butanol as HO• promoter and scavenger, respectively. Ozonation intermediate products were identified by liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry (HPLC–QqTOF-MS/MS). Demethylation and non-selective HO• attack proved to be the major reaction mechanisms. Where available, identified intermediates were confirmed using analytical standards, and concentration profiles along the ozonation process were determined through selective targeted MS/MS analysis. Benzophenone-1 (BP1), also being a UV-filter compound, and 2,2′-dihydroxy-4-methoxybenzophenone (DHMB) revealed to be the major BP3 degradation products, showing a maximum concentration at about the half-life time of BP3.

1. Introduction

Benzophenone-3((2-hydroxy-4-methoxyphenyl)-phenylmethadone, BP3) absorbs and dissipates UV irradiation, and constitutes one of the most commonly used UV filters. This compound is used in personal care products such as cosmetics, beauty creams, lotions and shampoos, or as

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an additive in polymeric materials that have to be protected from sunlight-initiated disruption (FDA, 1999; Council Directive, 1976).

Recent studies (Fent et al., 2010a; Gago-Ferrero et al., 2012a) indicate that sunscreen agents, including BP3, are persistent and bio-accumulative compounds (Log K_{OW} = 3.79, http://www.syrres.com/esc/physdemo.htm). The increasing use of UV filters constitutes a potential risk for the environment. BP3 was detected in several environmental matrices such as in surface waters (Balmer et al., 2005), sediments (Gago-Ferrero et al., 2011a) and fish (Fent et al., 2010a), as well as in human milk (Hany and Nagel, 1995; Schlumpf et al., 2010). BP3 shows estrogen-like activity in in vitro and in vivo assays (Schlumpf et al., 2004; Calafat et al., 2008, Blüthgen et al., 2012). Dermal and oral administration of BP3 to rats and mice has shown alterations in liver, kidney, and reproductive organs (Calafat et al., 2008). A recent study of Kunisue et al. (2012) indicates that exposure to elevated levels of benzophenone type UV filter compounds may be associated with estrogen-dependent diseases such as endometriosis.

Current wastewater treatment techniques are not effective in removing UV filters (Li et al., 2007; Negreira et al., 2009). These compounds are preferably retained in sewage sludge (Gago-Ferrero et al., 2011b; Negreira et al., 2011), which might be further used as a fertilizer. BP3 was measured at relatively high values (37 to 3810 ng L^{-1}) in raw wastewater and primary effluent (Snyder et al., 2006). The latter value is not far from the predicted no observed effect concentration (PNEC) for BP3 (6000 ng L^{-1}, chronic effects), as estimated in a recent tentative environmental risk assessment for fish and *Daphnia magna* (Fent et al., 2010b). In this context, there is an urgent need of new approaches for wastewater treatment for the removal of UV-absorbing compounds.

Advanced oxidation processes (AOPs), which generate hydroxyl radicals (HO•), are a promising tool for the removal of persistent organic pollutants (POPs) at an acceptable cost (0.05–0.20 euros per m^3 for ozonation) (Joss et al., 2008). There is abundant information about removal of pharmaceuticals and pesticides via innovative physical–chemical processes, but for personal care products the data available are limited. The feasibility of BP3 removal from sewage or treated gray water by ozone has been demonstrated in a few studies (Table 1), but a detailed insight in the mechanisms and the parameters affecting ozonation and peroxone oxidation processes are lacking so far. There are some studies reporting the conversion of BP3 into other benzophenone-type degradation products like BP1 during fungal biodegradation (Gago-Ferrero et al., 2012b) or human metabolism (Okereke et al., 1993, 1995; Kunisue et al., 2012), but its behavior during advanced oxidation processes is largely unknown. Moreover, apart from mechanistic considerations, also the data reported on removal efficiency are somewhat ambiguous. For example, whereas Rosal et al. (2010) did not detect any BP3 elimination by ozone, other studies report removal efficiencies above 80%. Hernández-Leal et al. (2011) recently studied BP3 degradation in Milli-Q water along with 17 other micropollutants and their results evidenced that BP3 was removed up to 94% (from 673 to 40 ng L^{-1}).

Given the lack of data and knowledge in this field, the aim of this research was to systematically investigate the removal of BP3 in water when treated with ozone and/or hydrogen peroxide (H_2O_2). The scope is twofold. First, the focus is put on the effect of operational variables on BP3 removal and on the ozone consumption. To the author's best knowledge, this study constitutes the first one investigating the effect of important process parameters like pH, H_2O_2 addition, temperature, and inlet ozone concentration on the ozonation of UV filters. Second, by use of advanced analytical techniques based on liquid chromatography coupled to quadrupole-time-of-flight tandem mass spectrometry (HPLC–QqTOF-MS/MS), BP3 main ozonation and peroxone byproducts are identified and structurally characterized to gain insight in the advanced oxidation pathway of BP3 in water.

2. Materials and methods

2.1. Standards and reagents

BP3 (CAS N. 131-57-7) and BP1 (CAS N. 131-56-6) were purchased from Sigma-Aldrich (Germany). 2,2′-dihydroxy-4-methoxybenzophenone (DHMB, CAS N. 131-53-3) and 2,3,4-trihydroxybenzophenone (THB, CAS N. 1143-72-2) were supplied by Dr. Ehrenstorfer (Germany). The isotopically labeled compound 2-hydroxy-4-methoxy-2′,3′,4′,5′,6′-d5 (BP3-d5), used as an internal standard, was obtained from CDN isotopes (Canada). The organic solvents (>99.8% purity) methanol, acetone, acetonitrile and HPLC grade water were provided by Biosolve (The Netherlands). H_2O_2 (35 wt.%), KH_2PO_4, K_2HPO_4 and $NaB_4O_7 \cdot 10(H_2O)$ were supplied by Acros Organics (Belgium). H_3PO_4 (≥85%, 15 M) was obtained from Merck (Belgium). Clean dry air ($[H_2O]$<3.0 ppm_v; $[CO_2]$<1.0 ppm_v; $[C_xH_y]$<0.5 ppm_v) was provided by L'Air Liquide (Belgium). Solid phase extraction (SPE) was carried out with Oasis HLB cartridges (60 mg sorbent, Waters, Spain).

2.2. Experimental setup

The ozonation experiments were performed in a temperature controlled bubble column with a height of 41.8 cm and an inner and outer diameter of 10.3 and 14.1 cm, respectively. Ozone was generated in dry air by a LAB2B ozone generator (Ozonia, Switzerland) and after flow adjustment dosed through a sintered glass plate at the bottom of the reactor. The reaction solution was prepared by adding BP3 powder (0.219 mmol L^{-1} (50.0 mg L^{-1})) to deionized water at controlled temperature conditions. After stirring this oversaturated solution for 24 h, the undissolved fraction was removed by filtration over a 0.45 μm filter, and the reactor was filled with 2.4 L of the BP3 saturated aqueous solution (dissolved concentration, 22.3 μmol L^{-1} (5.1 mg L^{-1}); RSD = 5%, n = 10). At the standard conditions, i.e. those applied during the initial experimental setup, the ozone inlet concentration was 85.7 μmol L_{gas}^{-1}, the gas flow rate 120 mL min^{-1}, and the reactor temperature 25 °C. The ozone mass transfer coefficient (k_La) in the bubble column was determined to be 5.5 h^{-1} (De Witte et al., 2010). The gas holdup and specific gas–liquid contact area are calculated (Tizaoui et al., 2009; Heynderickx et al., 2011) to be 0.014 (dimensionless) and 15 m^2m^{-3}, respectively. The water was buffered by a 10.12 mM phosphate buffer (pH 3 and pH 7) or a 2.5 mM borax buffer (pH 10). For the peroxone experiments, H_2O_2 was added in concentrations of 10–600 μM. Ozonated liquid samples were taken by a tap at 6 cm water height. Since the liquid phase in

Table 1
Overview of reported studies dealing with the ozonation of BP3 in sewage water.

Type of water	Scale	Mode	Time (min)	AOD[a] (mg L^{-1})	C_o[b] (ng L^{-1})	Removal (%)	Reference
STP effluent	Lab-scale	Semi-batch	15	16.3	123	NR[c]	Rosal et al. (2010)
Tertiary effluent	Pilot-scale	Continuous	24	7.3	6	>83	Snyder et al. (2006)
Tertiary effluent	Full-scale	Continuous	180	5–6	311	20	Li et al. (2007)
Aerobically treated gray water	Lab-scale	Batch	45	1.22	285	>94	Hernández-Leal et al. (2011)

[a] AOD: applied ozone dose.
[b] C_o: initial BP3 concentration.
[c] NR: not removed.

the bubble column reactor can be considered to be well mixed under the action of the bubbles (Heynderickx et al., 2011; Beltran, 2004), this sample is representative for the liquid phase in the whole reactor. Immediately after sampling, the samples were flushed with nitrogen for 2 min at 15 mL min^{-1} in order to remove residual ozone. Blank experiments, performed at the same conditions as applied during BP3 degradation but without addition of BP3, were carried out to obtain ozone consumption profiles also in the absence of BP3. In order to discard the effect of photodegradation during the experiments, preliminary experiments were carried out with 22.3 μmol L^{-1} BP3 but without ozone addition, showing no significant variations in the BP3 concentration.

2.3. Analytical methods

The ozone concentration in the gas flow was measured by an ozone analyzer through UV-light absorption at 254 nm. Aqueous BP3 concentrations were measured by HPLC attached to a photodiode array detector (Surveyor, Thermo Finnigan) without preconcentration. For details, see the Supplementary material. Aqueous hydrogen peroxide concentrations were determined by spectrophotometry based on 2,9-dimethyl-1,10-phenanthroline (DMP) and Cu(II) (Kosaka et al., 1998). pH measurements were done using a Jenway 3310 electrode.

Identification of BP3 degradation products formed during the ozonation process was performed by means of HPLC–QqTOF-MS/MS using a Waters Acquity UPLC™ system attached to a QqTOF-Micro™ (Waters/Micromass, UK). Chromatographic separation and detection conditions are given in the Supplementary material. Full-scan analyses were carried out on selected samples in both positive (PI) and negative (NI) electrospray (ESI) ionization modes. Further MS/MS analyses were carried out on identified molecular ions for their structural characterization. Exact masses were calculated and the elemental composition of the molecular ions and their fragments were determined using the MassLynx V4.1 software.

The ozonated water samples selected for byproduct identification by HPLC–QqTOF-MS/MS were preconcentrated by SPE using Oasis HLB cartidges. The applied SPE method was based on that of Rodil et al. (2008). Briefly, the cartridges were preconditioned sequentially with 2.5 mL of methanol and 2.5 mL of deionized water at pH 4.5. Twenty mL of the sample at pH 4.5 was loaded. After drying the cartridge under vacuum, elution was performed with twice 2.5 mL of methanol, followed by concentration to dryness under a gentle nitrogen stream. Finally, the extracts were reconstituted with 0.5 mL 25:75 (v/v) acetonitrile–water solution containing the internal standard BP3-d_5.

For the identified intermediates having commercially available analytical standards, selective target analysis was performed to determine their concentrations in non-concentrated aqueous samples collected as a function of ozonation time. For the most sensitive analysis, a liquid chromatography hybrid triple quadrupole-linear ion trap mass spectrometry (HPLC–QqLIT-MS/MS) method operating in the selected reaction monitoring (SRM) mode (Gago-Ferrero et al., 2012b) was used.

3. Results and discussion

3.1. BP3 degradation at standard conditions

At the standard conditions described in Section 2.2 and at pH 7, BP3 showed a half-life time ($t_{1/2}$) of 12.6 min (n = 5) and a 95% removal efficiency after 40–50 min, indicating a good BP3 degradability by ozonation. The BP3 degradation and ozone consumption profiles, with the latter representing the ozone inlet minus the ozone outlet concentration as a function of time, are shown in Fig. 1. Considering the data points up to 5% of the initial BP3 concentration, pseudo-first order reaction kinetics relative to the BP3 concentration were observed:

$$\ln\frac{[BP3]_t}{[BP3]_0} = -k_1 t \quad (1)$$

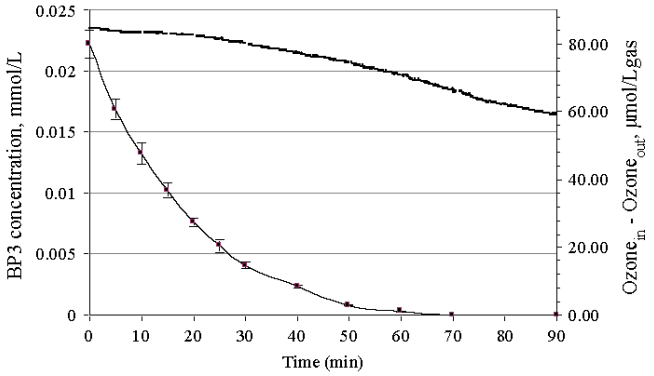

Fig. 1. Ozonation of BP3 at standard conditions (85.7 μmol L_{gas}^{-1} O_3, 25 °C, pH 7) (full line) and ozone consumption profile versus time (dotted line). Error bars represent standard deviations (n = 5).

in which $[BP3]_0$ and $[BP3]_t$ represent the BP3 concentration at time 0 and at reaction time t, respectively. The pseudo-first order rate constants ($k_{1,BP3}$) obtained at all investigated conditions are summarized in Table 2. These rate constants, which are function of the concentration of ozone, hydroxyl radicals and other oxidative species like $O_2^{\bullet -}$, $O_3^{\bullet -}$, $HO_2^{\bullet}$ and $HO_4^{\bullet}$, cannot be seen as fundamental reaction kinetics but they allow a quantitative comparison between the different experiments. At pH 7 and standard conditions, a $k_{1,BP3}$ value of 0.0556 min^{-1} (RSD = 6%, n = 5) was calculated. After 60 min of ozonation, 0.571 mmol (RSD = 5.4%, n = 5) of ozone was consumed, compared to 0.278 mmol for the blank experiment. The latter may be the result of ozone instability in aqueous systems. It may decompose and lead to complex radical mechanisms in which the hydroxyl radical is considered as the main responsible species for indirect reactions (Beltran, 2004).

3.2. Effect of the ozone inlet concentration

The effect of the inlet ozone gas concentration on the degradation of aqueous BP3 was investigated by dosing ozone at concentrations ranging from 32.6 to 151 μmol L_{gas}^{-1}, corresponding to an ozone load of 1.63 to 7.55 μmol min^{-1} L_{water}^{-1}. Other operational parameters, including the initial BP3 concentration, pH, and temperature were kept constant at 22.3 μmol L^{-1}, pH 7 and 25 °C, respectively. The experimental results (Table 2) revealed a faster BP3 removal at higher inlet ozone gas concentrations, with $k_{1,BP3}$ values increasing from 0.0229 to 0.1173 min^{-1}. This can be explained by an increased ozone concentration in the aqueous phase. Since BP3 is a non-volatile compound (vapor pressure = $7 \cdot 10^{-4}$ Pa at 25 °C), reactions in the gas phase are negligible. After the mass transfer of ozone from gas to liquid phase, however, it may either directly react with BP3 or decompose to produce other reactive species which in turn attack BP3. A linear relationship was found when the pseudo-first order constants $k_{1,BP3}$ were plotted against the gaseous ozone inlet concentrations ($y = 8.0 \cdot 10^{-4}x - 0.007$ ($R^2 = 0.992$, n = 5)), with the intercept not significantly different from zero ($\alpha > 0.05$).

The ozone consumption after 60 min of ozonation increased from 0.240 to 0.900 mmol. Plotting the ozone consumption versus the ozone inlet concentration also yielded a linear relationship ($y = 5.7 \cdot 10^{-3}x + 0.066$ ($R^2 = 0.994$, n = 5)), which means that, within the concentration interval studied, the ozone consumption is first order in the ozone inlet concentration. The total increase in ozone consumption might be explained not only by a faster BP3 degradation, but also by the formed reactive species and BP3 degradation byproducts.

3.3. Temperature effect

The effect of reaction temperature on the BP3 ozonation was investigated between 25 °C and 65 °C (Table 2). Within this range,

Table 2
Pseudo-first order BP3 rate constants and ozone consumption during 60 min of ozonation for experiments at 22.3 μmol L^{-1} initial BP3 concentration (RSD: relative standard deviation; n: number of repetitions).

Inlet O_3 concentration (μmol L_{gas}^{-1})[a]	Temperature (°C)	pH	H_2O_2 (μmol L^{-1})	t-butanol (mM)	$k_{1,BP3}$ (min^{-1}); RSD (%)	O_3 consumption during 60 min (mmol)
32.6	25	7	–	–	0.0229	0.240
61.2	25	7	–	–	0.0421	0.408
85.7	25	7	–	–	0.0556; 6 (n=5)	0.571
126.5	25	7	–	–	0.0973	0.822
151.0	25	7	–	–	0.1173	0.900
85.7	25	7	–	–	0.0556; 6 (n=5)	0.571
85.7	45	7	–	–	0.0624	0.598
85.7	55	7	–	–	0.0788	0.571
85.7	65	7	–	–	0.0912	0.608
85.7	25	3	–	–	0.0463; 2 (n=3)	0.436
85.7	25	7	–	–	0.0556; 6 (n=5)	0.571
85.7	25	10	–	–	0.1348; 4 (n=3)	0.517
85.7	25	3	–	30.45	0.0417; 7 (n=3)	0.369
85.7	25	7	–	30.45	0.0475; 5 (n=3)	0.477
85.7	25	10	–	30.45	0.0890; 5 (n=3)	0.514
85.7	25	7	10	–	0.0796	0.560
85.7	25	7	50	–	0.0815; 0.5 (n=3)	0.550
85.7	25	7	100	–	0.0911; 0.3 (n=3)	0.514
85.7	25	7	300	–	0.0778	0.536
85.7	25	7	600	–	0.0805	0.552
85.7	25	3	50	–	0.0679	0.408
85.7	25	7	50	–	0.0815; 0.5 (n=3)	0.550
85.7	25	10	50	–	0.1126	0.528

[a] Corresponding to an ozone load of 1.63 to 7.55 μmol min^{-1} L_{water}^{-1}.

the BP3 rate constants increased from 0.0556 to 0.0912 min^{-1} at higher temperature, whereas no significant effect in the consumption of ozone was observed.

The reaction temperature may influence ozonation processes in two aspects. First, the Henry's law coefficient of ozone increases by more than a factor of 2 at higher temperature (25–65 °C) (Phattaranawik et al., 2005), limiting the mass transfer from gas to liquid phase and thus negatively affecting the BP3 degradation efficiency (see Section 3.2). Second, higher temperature may increase both the instability of ozone itself, as well as the activation of the reactive species leading to the enhancement of the BP3 degradation rate (Zhao et al., 2009). At the conditions applied in this study, it appears that the second temperature effect dominates the first one. Since the amount of ozone dissolved in the water phase is expected to be smaller at higher temperature, and given that the consumed amount of ozone is almost independent of temperature, it seems that a higher fraction of the available ozone is converted at higher temperature, which indicates a more efficient use of the aqueous ozone for direct or radicalar BP3 degradation.

3.4. pH effect

Since pH may affect both the ozonation rates and mechanistic pathways of organic micropollutants (Chelme-Ayala et al., 2011; Garoma and Matsumoto, 2009), the ozonation of BP3 was investigated at acid, neutral and alkaline conditions. Table 2 shows a clear increase of the BP3 removal rate at higher pH, particularly between pH 7 and pH 10. This can be explained by the higher rate of ozone decomposition at higher pH as the hydroxyl ions catalyze the decay of ozone to form hydroxyl radicals serving as reactive species (Hoigné and Bader, 1983). At pH 3, when no hydroxyl radical formation is expected and molecular ozone is presumed to be the most important reactive species, the decomposition of BP3 is slower than at neutral and basic conditions. This is also in agreement with Blüthgen et al. (2012) who noticed that benzophenone-derivatives (including BP3) are stabilized under acidic conditions. At pH 10, the BP3 decomposition rate is more than 2-fold higher than at acid pH, showing the importance of the formed hydroxyl radicals. The reactivity of BP3 with HO• is significantly higher than with aqueous ozone, as is the case with most organic compounds (Garoma and Matsumoto, 2009). Moreover, since BP3 has a pKa of 8.06, it is mainly dissociated at higher pH, which might result into an enhancement of the reaction rate since ozone is an electrophilic reagent.

Blank experiments showed an increase of ozone consumption with increasing pH, which can be explained by the enhanced mass transfer due to the chemical conversion of ozone into hydroxyl radicals keeping the driving force high. The quantity of ozone consumed during the first 60 min of ozone dosing in the blank experiments amounted 0.204, 0.278 and 0.513 mmol at pH 3, 7 and 10, respectively. When subtracting these amounts from those consumed during the BP3 degradation experiments, values of 0.232 mmol (pH 3), 0.292 mmol (pH 7) and 0.005 mmol (pH 10) are obtained. The fact that at pH 10 almost no extra consumption of ozone is observed in the presence of BP3 supports the dominating effect of the indirect BP3 degradation by formed radicals compared to the direct ozonation pathway. By dividing ozone net consumption by the amount of BP3 degraded in the same time interval, values of 4.32 (pH 3), 5.46 (pH 7) and 0.08 (pH 10) mmol of ozone consumed per mmol BP3 removed, are obtained. However, it should be considered that part of the ozone consumed is due to the formed intermediate products.

In order to better understand the role of hydroxyl radicals during BP3 ozonation, t-butanol (TBU) was added in excess (30.45 mM) at each investigated pH. TBU is a strong hydroxyl radical scavenger having reaction rates of 6×10^{8} M^{-1} s^{-1} with HO• (Buxton et al., 1988) and 3×10^{-3} M^{-1} s^{-1} with ozone (Hoigné and Bader, 1983). Fig. 2 illustrates the effect of TBU on the BP3 degradation at pH 3, 7 and 10. Although TBU addition increased the BP3 half-life time at each pH (10% at pH 3 and 16% at pH 7), the effect is most pronounced at pH 10 (51%). This is in agreement with the expected higher hydroxyl radical concentration at alkaline conditions. Moreover, given the small ozone consumption (0.005 mmol) attributed to BP3 degradation at pH 10 as noticed in the absence of TBU, the effect of TBU could have been

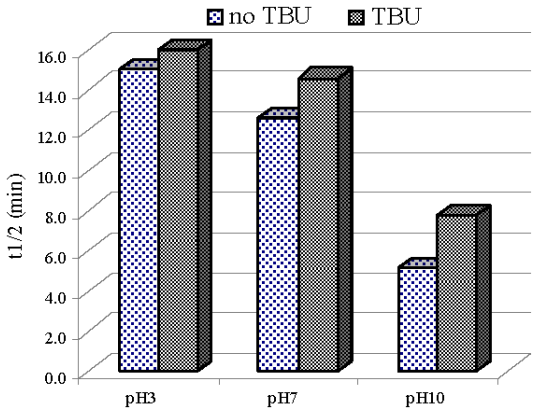

Fig. 2. Effect of t-butanol (TBU) on the BP3 half-life time during ozonation at an initial concentration of 22.3 μmol L^{-1}, 85.7 μmol L_{gas}^{-1} ozone inlet concentration, T = 25 °C, and at different values of pH.

expected to be even more pronounced. However, mass transfer aspects should also be taken into consideration. TBU addition is reported to yield smaller gas bubbles and thus higher interfacial area with the liquid phase, resulting into an increased k_La (Lopez-Lopez et al., 2007). Next to that, also the role of other reactive radicals, different from HO• might not be excluded.

3.5. O_3/H_2O_2 process

Given the relevance of hydroxyl radicals as shown in Section 3.4, peroxone experiments were performed at pH 7 by adding various H_2O_2 dosages (10 to 600 μmol L^{-1}) in the aqueous phase as a source for HO• generation in the O_3/H_2O_2 process. The degradation of BP3 by using O_3/H_2O_2 still followed the pseudo-first-order decay, and rate constants are shown in Table 2. Although not statistically significant, the H_2O_2 dosage influences the oxidation process rate. Differences are relatively low (<7% deviation from the mean value) in the 10–600 μmol L^{-1} range, but clearly higher than those observed when repeating three times the experiment at $[H_2O_2] = 100$ μmol L^{-1} (RSD = 0.3%). As a result of a promoted HO• radical formation (Gogate and Pandit, 2004), an increment of the BP3 degradation rate is observed as the H_2O_2 concentration increases, to reach a maximum ($k_{1,BP3} = 0.0911$ min^{-1}) at 100 μmol L^{-1} H_2O_2, being 64% higher than without H_2O_2. At higher H_2O_2 dosages, however, the BP3 degradation rate decreased, showing similar values at 10 μmol L^{-1} H_2O_2 and 600 μmol L^{-1} H_2O_2. This inhibiting effect on the oxidation of BP3 may be explained by the scavenging behavior of H_2O_2 towards hydroxyl radicals (Chu and Lau, 2007; Yasunaga et al., 2006; De Witte et al., 2009). The ozone consumption as a function of H_2O_2 concentration followed an opposite trend to that of the BP3 degradation rate. The lowest ozone consumption was measured at 100 μmol L^{-1} H_2O_2 (Table 2), i.e. at the maximum BP3 removal rate. This fact may be attributed to the higher concentration of radicals present in the aqueous phase, reducing the ozone consumption due to direct reaction with BP3.

The effect of pH (3, 7, 10) was also studied in the peroxone process (Table 2). Adding 50 μmol L^{-1} H_2O_2 in the aqueous solution did increase the BP3 removal rate by 47% at pH 3, which is completely in line with the results obtained at neutral pH. In contrast, the BP3 degradation at pH 10 was almost 20% slower when H_2O_2 was added. Since in this case high concentrations of hydroxyl radicals and H_2O_2 are present simultaneously, the observed rate retardation most probably results from the consumption/scavenging of hydroxyl radicals by H_2O_2, yielding less reactive radicals (such as HO_2•) in the solution (Sun and Pignatello, 1992; Poulopoulos et al., 2006).

3.6. Ozonation byproduct identification

Table 3, summarizes for all major peaks detected during HPLC–QTOF-MS/MS analysis the retention time, the exact mass and elemental formula of both the molecular and fragment ions. It also lists the calculated mass errors and double bond equivalents (DBEs) of the proposed reaction products formed during BP3 ozonation. These data were obtained under optimized conditions of collision energy and cone voltage in both ESI(+)- and ESI(−)-MS/MS experiments. Since almost all fragment ions had an even number of electrons (as exemplified by half-integer DBE values), most of the neutral losses had likewise even electron configurations. Fig. S1 (Supplementary information) shows the HPLC–ESI-MS/MS chromatograms of BP3 and its formed ozonation products.

Fig. 3a–e shows the ESI(+)-MS/MS product ion spectra of the detected compounds. The CID fragmentation of BP3 (molecular ion $[M+H]^+$ 229), which elutes at 8.51 min, shows the split of the molecule to leave the positive charge in the keto group that is resonantly stabilized by the adjacent aromatic ring. The fragment at m/z 151 is produced by the loss of the phenyl group of BP3 $[M-C_6H_5]^+$, whereas the fragment at m/z 105 corresponds to the benzoyl cation $[C_6H_5C{=}O]^+$. Apart from BP3, 5 other chromatographic peaks were observed during ESI(+) full scan analysis of samples collected during the first 25 min of BP3 ozonation at standard conditions. Four peaks with a molecular ion $[M+H]^+$ 245 were observed at 7.88, 6.93 and 6.82 min (ozonation byproduct m/z 244a (Pr244a)) and 6.50 min (ozonation byproduct m/z 244b (Pr244b)). Considering that the mass of these compounds is shifted 16 Da upwards relative to the parent compound, hydroxylation by HO• attack is the most plausible explanation. The peaks at 7.88, 6.93 and 6.82 min show an identical fragmentation pathway in the ESI(+)-MS/MS experiments (Fig. 3b), with clear similarities with the MS/MS spectra obtained for BP3. Indeed, the same fragment at m/z 151 is formed, and instead of the fragment at m/z 105 (BP3), a fragment at m/z 121 (+16 Da) is detected for Pr244a. In light of these results, it appears that BP3 has been hydroxylated in the unsubstituted aromatic ring of the molecule as a consequence of a non-specific HO• attack. The three peaks represent the ortho-, meta- and para-hydroxylated forms. The peak at 7.88 min (ortho position) corresponds to DHMB, as confirmed by the analysis of a commercial analytical standard solution. The peak at 6.50 min (Pr 244b) also having the molecular ion at $[M+H]^+$ 245 (Fig. 3c) results from the hydroxylation of BP3 at the other moiety of the molecule. This is supported by the fragment at m/z 167 instead of m/z 151 and by the presence of the fragment at m/z 105. The fifth peak (ozonation byproduct m/z 214, (Pr214)) detected already after 5 min of BP3 ozonation, elutes at 6.78 min and corresponds to the molecular ion $[M+H]^+$ 215. ESI(+)-MS/MS spectra show two fragments (Fig. 3d), one at m/z 105 (the same as that observed for BP3, $[C_6H_5{=}O]^+$) and another one at m/z 137 $[M-C_6H_5]^+$. The difference in mass (14 Da) with the $[M-C_6H_5]^+$ ion of BP3 strongly suggests demethylation of BP3. The resulting molecule corresponds to BP1, which is also a commonly used UV filter compound and whose identity was confirmed by the analysis of a commercial analytical standard. After 15 min of BP3 ozonation, a sixth peak occurred in the ESI(+) full scan analysis, eluting at 5.13 min (ozonation byproduct m/z 258 (Pr258)). From the ESI(+)-MS/MS spectra and the structures proposed for the fragments (Fig. 3e), it is highly probable that Pr258, having a molecular ion 14 Da higher in mass than Pr244b, is formed through the oxidation of the methyl group in Pr244b converting it to an aldehyde functionality.

At the same conditions, ozonated samples collected as a function of time were also analyzed by ESI(−)-MS/MS full scan analysis. For the products Pr244a, Pr244b and Pr214 (BP1), corresponding peaks were detected at the same retention time as obtained in ESI(+) analysis (Table 3). In the case of BP1, CID fragments in ESI(−) were obtained at m/z 135, 109 and 91 (Fig. 3f). Similar results were obtained for DHMB in the negative mode (results not shown), being in agreement

Table 3
Retention time, accurate mass measurement and elemental formula of the molecular and fragment ions of both benzophenone-3 (BP3) and its ozonation degradation products, as elucidated by HPLC-QqTOF analysis in both ESI(+) and ESI(−) ionization modes.

t_R[a] (min)	Compound	Precursor and production	Elemental formula	Mass (m/z)		Error		DBE[b,c]
				Experimental	Theoretical	mDa	ppm	
8.51	BP3	$[M+H]^+$	$C_{14}H_{13}O_3$	229.0870	229.0865	0.5	2.2	8.5
		$[M-C_6H_5]^+$	$C_8H_7O_3$	151.0399	151.0395	0.4	2.6	5.5
		$[C_6H_5{=}O]^+$	C_7H_5O	105.0346	105.0340	0.6	5.7	5.5
7.88, 6.93, 6.82	Pr244a	$[M+H]^+$	$C_{14}H_{13}O_4$	245.0812	245.0814	−0.2	−0.8	8.5
		$[M-C_6H_5OH]^+$	$C_8H_7O_3$	151.0400	151.0395	0.5	3.3	5.5
		$[C_6H_4(OH){=}O]^+$	$C_7H_5O_2$	121.0294	121.0290	0.4	3.3	5.5
		$[M-H]^-$	$C_{14}H_{11}O_4$	243.0650	243.0657	−0.7	−2.9	9.5
		$[M-CH_3]^-$	$C_{13}H_8O_4$	228.0412	228.0423	−1-1	−4.8	10
		$[C_6H_4(OMe)O]^-$	$C_7H_7O_2$	123.0452	123.0446	0.6	4.9	4.5
		$[C_6H_5O]^-$	C_6H_5O	93.0335	93.0340	−0.5	−5.4	4.5
6.50	Pr244b	$[M+H]^+$	$C_{14}H_{13}O_4$	245.0812	245.0814	−0.2	−0.8	8.5
		$[M-C_6H_5]^+$	$C_8H_7O_4$	167.0349	167.0344	0.5	3.0	5.5
		$[C_6H_5{=}O]^+$	C_7H_5O	105.0338	105.0340	−0.2	−1.9	5.5
		$[M-H]^-$	$C_{14}H_{11}O_4$	243.0648	243.0657	−0.9	−3.7	9.5
		$[M-CH_3]^-$	$C_{13}H_8O_4$	228.0424	228.0423	0.1	0.4	10
		$[C_6H_4(OMe)(OH)O]^-$	$C_7H_7O_3$	139.0389	139.0395	−0.6	−4.3	4.5
6.78	Pr214	$[M+H]^+$	$C_{13}H_{11}O_3$	215.0710	215.0708	0.2	0.9	8.5
		$[M-C_6H_5]^+$	$C_7H_5O_3$	137.0241	137.0239	0.2	1.5	5.5
		$[C_6H_5{=}O]^+$	C_7H_5O	105.0345	105.0340	0.5	4.8	5.5
		$[M-H]^-$	$C_{11}H_9O_3$	213.0549	213.0552	−0.3	−1.4	9.5
		$[C_6H_3(O)_2C{=}O]^{\bullet\bullet-}$	$C_7H_3O_3$	135.0079	135.0082	−0.3	−2.2	6.5
		$[C_6H_5(OH)O]^-$	$C_6H_5O_2$	109.0290	109.0290	0	0	4.5
		$[C_6H_3O]^{\bullet\bullet-}$	C_6H_3O	91.0180	91.0184	−0.4	−4.4	5.5
5.13	Pr258	$[M+H]^+$	$C_{14}H_{11}O_5$	259.0597	259.0606	−0.9	−3.5	9.5
		$[M-C_6H_5]^+$	$C_8H_5O_5$	181.0136	181.0137	−0.1	−0.6	6.5
		$[M-C_6H_5-C{=}O]^+$	$C_7H_5O_4$	153.0184	153.0188	−0.4	−2.6	5.5
		$[C_6H_5{=}O]^+$	C_7H_5O	105.0345	105.0340	0.5	4.8	5.5
6.34*	Pr260a	$[M-H]^-$	$C_{14}H_{11}O_5$	259.0601	259.0606	−0.5	−1.9	9.5
		$[C_6H_4(OMe)O]^-$	$C_7H_7O_2$	123.0452	123.0446	0.6	4.9	4.5
		$[C_6H_5(OH)O]^-$	$C_6H_5O_2$	109.0286	109.0290	−0.4	−3.7	4.5
		$[M-CH_3]^-$	$C_{13}H_8O_5$	244.0365	244.0372	−0.7	−2.9	10
5.12*	Pr260b	$[M-H]^-$	$C_{14}H_{11}O_5$	259.0612	259.0606	0.6	2.3	9.5
		$[M-CH_3]^-$	$C_{13}H_8O_5$	244.0362	244.0372	−1.0	−4.1	10
		$[C_6H_4(OMe)(OH)O]^-$	$C_7H_7O_3$	139.0390	139.0395	−0.5	−3.6	4.5
		$[C_6H_5O]^-$	C_6H_5O	93.0344	93.0340	0.4	4.3	4.5
6.13, 5.61, 5.31	Pr230	$[M-H]^-$	$C_{13}H_9O_4$	229.0496	229.0501	−0.5	−2.2	9.5
		$[C_6H_3(O)_2C{=}O]^{\bullet\bullet-}$	$C_7H_3O_3$	135.0077	135.0082	−0.5	−3.7	6.5
		$[C_6H_5(OH)O]^-$	$C_6H_5O_2$	109.0285	109.0290	−0.5	−4.6	4.5
		$[C_6H_5O]^-$	C_6H_5O	93.0337	93.0340	−0.3	−3.2	4.5
		$[C_6H_3O]^{\bullet\bullet-}$	C_6H_3O	91.0184	91.0184	0	0	5.5

[a] t_R: HPLC retention time.
[b] DBE: double bond equivalent.
[c] Several peaks around this time.

with Negreira et al. (2009). Next to that, the ESI(−)-MS/MS full scan analysis showed one group of peaks around 6.34 min (ozonation byproduct m/z 260a (Pr260a)) and another one around 5.12 min (ozonation byproduct m/z 260b (Pr260b)), both with the molecular ion $[M-H]^-$ 259. All the peaks of the first and second group show the same fragmentation pattern, given in Fig. 3g and h, respectively. The mass of the molecular ion is 16 Da upwards relative to Pr244a and Pr244b, so that the most probable explanation is another non-specific HO• oxidation of these compounds. After 20 min of ozonation, three additional peaks were observed in ESI(−), eluting at 6.13, 5.61 and 5.31 min (ozonation byproduct m/z 230 (Pr230)). The mass of the molecular ion is 16 Da upwards relative to Pr214, and the same fragmentation pathway is observed for the three peaks (Fig. 3i). Given the similarity with the ESI(−)-MS/MS spectra of BP1, it is suggested that Pr230 represents the ortho-, meta- and para-hydroxylation products of BP1 resulting from a HO• attack in the non-hydroxylated moiety of the molecule. The peak at 6.13 min (ortho position) corresponds to THB, as confirmed by the analysis of a commercial analytical standard solution.

Among the identified intermediate products, the commercially available BP1, DHMB and THB compounds have also been identified as BP3 degradation products or metabolites in other studies dealing with rats, human urine or fungal degradation (Gago-Ferrero et al., 2012b; Felix et al., 1998; Jeon et al., 2008). In order to obtain a quantitative profile of their formation/elimination during BP3 ozonation (Fig. 4), these compounds were measured in non-preconcentrated aqueous samples via HPLC–MS/MS SRM analysis (Section 2.3). BP1 and DHMB concentrations reach their maxima (0.055 and 0.047 μmol L^{-1} respectively) at about 15 min, to drop below the detection limit after 40 min of BP3 ozonation. Based on the integrated areas below the curves presented in Fig. 4, it can be estimated that of the total amount of BP1 degraded during the first 40 min of ozonation, about 0.5% was detected as BP1 and DHMB. THB was not detected in the non-preconcentrated samples. A supporting experiment, investigating BP1 ozonation at pH 7 in the same reactor and at the standard conditions (Section 2.2), revealed that BP1 (23.3 μmol L^{-1}; 5 mg L^{-1}) ozonation is slower than that of BP3, as exemplified by a half life time of 16.1 min (n = 3), compared to 12.6 min for BP3. This slower BP1 degradation supports its temporary accumulation during BP3 ozonation. Although the detected concentrations of BP1 are relatively low compared to the initial BP3 concentration, its formation should be taken into account when considering the application of ozonation for BP3 removal from wastewater. Since yeast based bioassay (ER-RYA) analysis showed that BP1 is about 200 times more estrogenic than its parental compound BP3, and three orders of magnitude less estrogenic than 17β-estradiol (Gago-Ferrero et al., 2012b), the ozonation time should

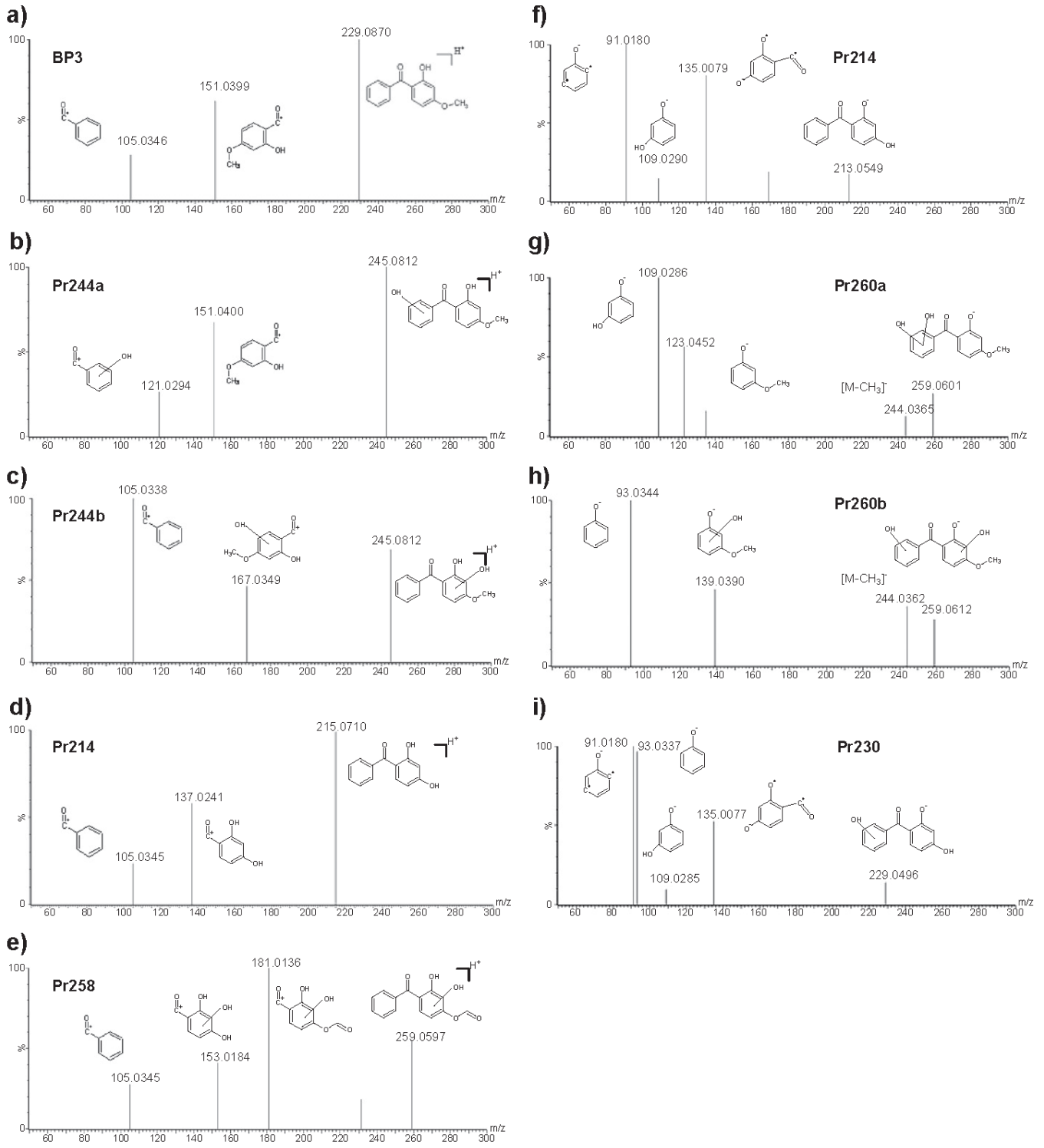

Fig. 3. Spectra obtained from full-scan ESI-QqTOF-MS/MS analysis of BP3 and its detected ozonation products. ESI(+)-MS/MS: (a) BP3, (b) Pr244a, (c) Pr244b, (d) Pr214 (BP1), and (e) Pr258. ESI(−)-MS/MS: (f) Pr214 (BP1), (g) Pr260a, (h) Pr260b, and (i) Pr230.

be long enough in order to remove both BP3 and BP1 from the reaction medium.

The fact that both BP1 and DHMB show a similar concentration profile, rising from the first minutes of ozonation, strongly indicates that both compounds are formed directly out of BP3, independently from each other. This is shown in Fig. 5, proposing the first steps in the BP3 ozonation pathways. The involvement of hydroxyl radicals in these pathways is further exemplified by byproduct identification analysis performed at the other pH values. During ozonation at pH 10 and during the peroxone experiments, the same hydroxylated degradation products were detected. DHMB concentrations were, however, up to 100% higher than at pH 7 and reached their maximum at already 6 min while the BP1 maximum kept similar to pH 7 experiments. By contrast, at pH 3, when direct ozonation is expected to be

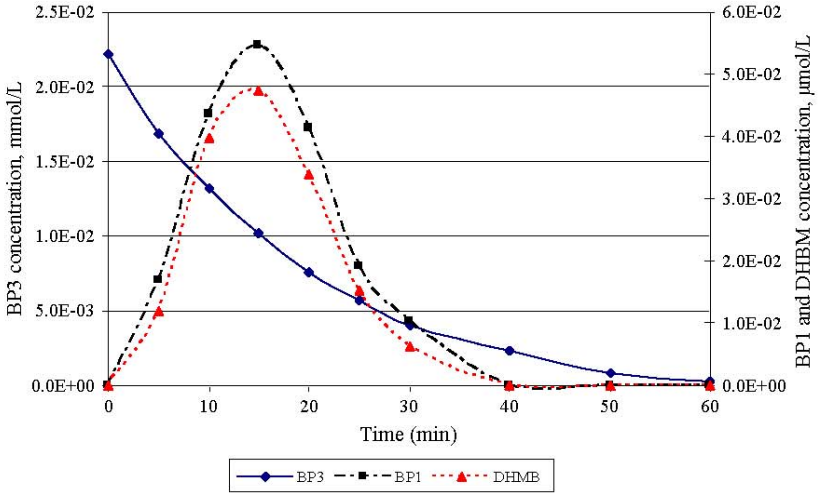

Fig. 4. BP3, DHMB and BP1 concentration profiles measured during BP3 ozonation at standard conditions (85.7 µmol L_{gas}^{-1} O_3, 25 °C, pH 7).

one of the major reaction pathways, maximum concentrations achieved for DHMB and BP1 are respectively 80% and 20% lower than those obtained at pH 10, and maximum concentrations were reached at 20 min for both compounds.

4. Conclusions

This study brings forward new data and insights with respect to both the effect of operational variables (ozone inlet gas concentration, temperature, pH, and H_2O_2 addition) on and reaction pathways of benzophenone-3 ozonation in water. A major conclusion is that process conditions that favor hydroxyl radical formation have a pronounced positive effect on BP3 degradation, which shows the higher reactivity through radicalar pathways compared to direct ozonation. Through advanced LC-MS/MS analysis, up to 7 BP3 ozonation intermediate products could be identified and structurally characterized. As far as we know, this is the first study providing data on the identification of BP3 ozonation byproducts. Apart from benzophenone-1 (BP1), which is a result of

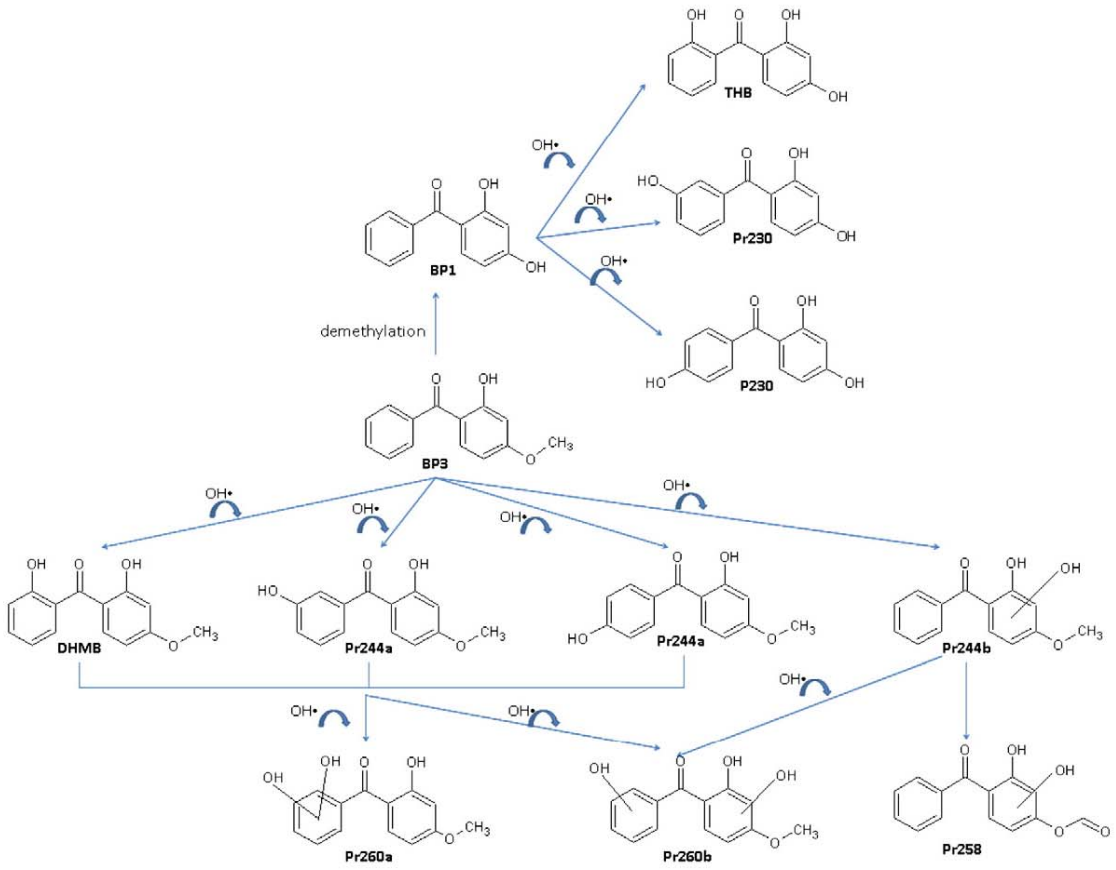

Fig. 5. Proposed initial degradation pathways of BP3 during ozonation in aqueous solution at standard conditions (85.7 µmol L_{gas}^{-1} O_3, 25 °C, pH 7).

BP3 demethylation, all other identified degradation products were hydroxylated derivatives, formed by a non-specific attack of HO• radicals at different moieties of the molecule.

Since this work – as being one of the first on this topic – has been performed in deionized water at relatively high concentrations, the results should be interpreted within its context and goal of this work, and might not be quantitatively transferable to real conditions. However, the acquired knowledge is of the utmost use to better understand the process and to further investigate the application potential of ozonation for UV-filter removal in real environmental or wastewaters. In order to avoid the introduction of estrogenic activity in the aquatic environment, the focus should not only be put on the removal of the parent compound but also the further oxidation of harmful reaction products like BP1 is certainly a topic for a more in-depth study.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2012.10.006.

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Información suplementaria para:

Ozonation and peroxone oxidation of benzophenone-3 in water: effect of operational parameters and identification of intermediate products

Pablo Gago-Ferrero, Kristof Demeestere, M. Silvia Díaz-Cruz, Damià Barceló

Analytical Methods – Supplementary information related to Section 2.3

Quantification of BP3 during ozonation and peroxone oxidation experiments was done by HPLC with a photodiode array detector (Surveyor, Thermo Finnigan), without preceding SPE. A Luna C18(2) column (150 mm x 3.0 mm, 3 µm, Phenomenex) was used as the stationary phase and the column temperature was kept at 35 ºC. The optimized chromatographic conditions were as follows: solvent A consisted of HPLC grade water (0.1% formic acid) and solvent B acetonitrile. The gradient elution (0.3 $Lmin^{-1}$) started with 100% solvent A. Solvent B increased to 85% in 3 min, raised to 100% in the next 8 min to keep it constant for 2 min. Then, conditions returned to the initial values in 2 min, and were kept constant for 4 additional minutes to allow the equilibration of the column. The sample volume injected was 10 µL and the wavelength of quantification was set at 324 nm.

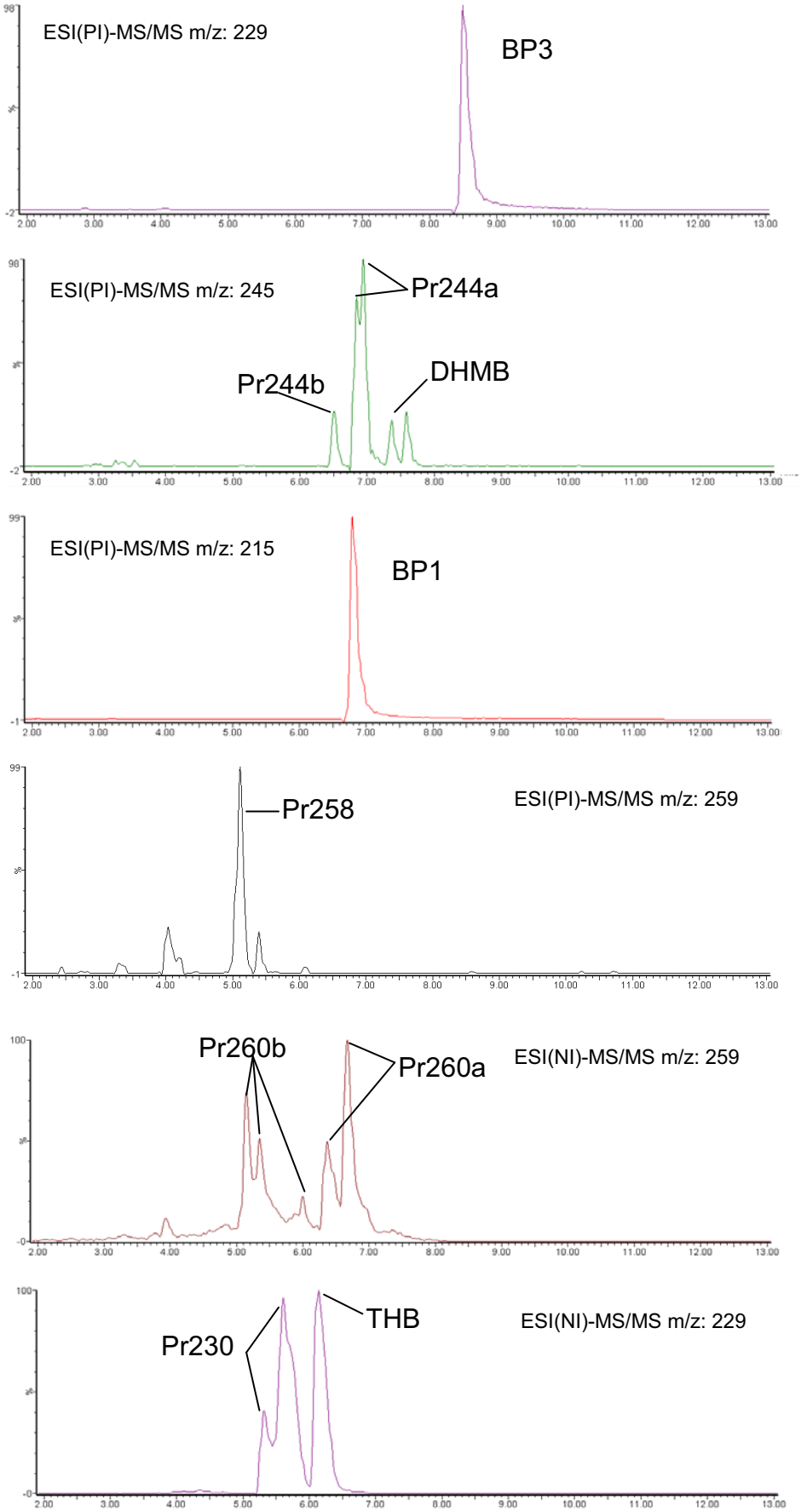
Linearity was assessed by constructing a seven point calibration curve in triplicate at concentration levels ranging from 0.3 mgL^{-1} to 10 mgL^{-1}. Least-square linear regression analysis was performed by plotting the peak area as a function of the analyte concentration and the correlation coefficient (R^2) was higher than 0.999. The limit of quantification (LOQ), calculated at a signal-to noise (S/N) ratio of 10, was lower than 0.3 mgL^{-1} for BP3. Precision, expressed as interday repeatability, was calculated by repeated analyses of the same sample sets, providing RSD values lower than 4%. Samples were injected in simple injection mode and quantification was performed by external standard calibration.

Identification of BP3 degradation products formed during the ozonation process was performed by means of HPLC-QqTOF-MS/MS using a waters Acquity UPLC™ system attached to a QqToF-Micro™ (Waters/Micromass, Manchester, UK). Chromatographic separation was achieved on a Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm,

5 µm, from Merck), thermostated at 40 °C. In the optimized method, when working with positive ionization mode (PI), the mobile phase consisted of a mixture of HPLC grade water and acetonitrile, both with 0.15% formic acid. Analyte elution was achieved by increasing the organic fraction of the mobile phase from 5% (initial conditions) to 25% in 7 min, and then to 100% in the following 3 min. Pure organic conditions were kept constant for 2 min, and finally the initial conditions were reached in the next 2 min. The injection volume was set to 10 µL and the mobile phase flow-rate to 0.3 $mLmin^{-1}$. Working with negative ionization mode (NI), the chromatographic conditions were the same than for the PI but without using formic acid in any of the phases. Full-scan analyses were carried out on selected samples in both PI and NI electrospray (ESI) ionization modes. Acquisition in full scan mode was performed in the range *m/z* 50-700 at different cone voltages (15 V, 25 V and 35 V) and with a capillary voltage of 3000 V (PI) or 2800 V (NI). Further MS/MS analyses were carried out on identified molecular ions for their structural characterization. Collision induced fragmentation (CID) of selected *m/z* ions was evaluated at different collision energies between 10 and 40 eV, using argon as the collision gas at a pressure of 22 psi. Data were collected in the centroid mode, with a scan time of 0.3 s and an inter scan delay time of 0.1 s, and with a full width at half maximum (FWHM) resolution of 5000. Other MS/MS parameters were set as follows: 600 Lh^{-1} for the desolvation gas at a temperature of 350 ºC, 50 Lh^{-1} for the cone gas and 120 ºC as source temperature. A valine-tyrosine-valine (Val-Tyr-Val) reference solution (*m/z* of $[M+H]^+$=380.2185 (PI) and $[M-H]^-$=378.2029 (NI)) was used to tune the instrument and also as lock mass to ensure mass accuracy. The reference was analyzed by infusion in the MS analyzer by means of an independent reference probe (LockSpray™).

For the identification of the BP3 degradation byproducts, samples were selected at pH3, at pH 7 (both at the defined standard conditions and in addition of 100 $\mu molL^{-1}$ H_2O_2), and at pH 10. Also samples taken during BP1 degradation experiments were analysed for byproduct identification. Samples were taken at initial time, 5, 10, 15, 20, 30 and 50 min.

Figure S1. HPLC-ESI-MS/MS chromatograms of BP3 and its ozonation byproducts obtained from an experiment performed at standard conditions.



4.5. COMPARACIÓN ENTRE LOS EXPERIMENTOS DE OZONIZACIÓN DE BP3 Y BP1:

El compuesto BP1, como ya se ha comentado en otras secciones, además de ser el principal producto de transformación de BP3 en los estudios de ozonización, es el principal metabolito de BP3 observado en animales y humanos [11, 29]. Este compuesto también se forma y es detectado en la degradación de BP3 mediante el hongo *T. versicolor* [67]. BP1 también se utiliza como UV-F para prevenir el daño debido a la radiación UV en pigmentos y aromas en PCPs tales como perfumes y jabones. Así mismo, esta sustancia se añade a plásticos de envasado para protegerlos de la degradación debido a la radiación UV. La actividad estrogénica de BP1 se determinó mediante ER-RYA, y se observó que este compuesto es tres órdenes de magnitud menos estrogénico que el 17-β-estradiol, pero 200 veces más estrogénico que su precursor BP3 [67]. Kunz et al. determinaron otros efectos biológicos de este compuesto en peces, que afectan a la reproducción y al desarrollo [8-10, 135] y también se le asocia con enfermedades en humanos relacionadas con los estrógenos como la endometriosis [12]. Esta dolencia se considera una enfermedad del sistema endocrino ya que se sabe que los estrógenos fomentan el desarrollo de la enfermedad.

Tener información sobre los productos de transformación que se forman durante la degradación de BP3 mediante ozonización es de suma importancia, ya que de nada serviría conseguir una degradación óptima de BP3 en el medio acuoso si luego su principal producto de transformación, el cual es mucho más tóxico que el propio precursor, no se elimina y permanece en el medio.

La Tabla 4.1. resume los resultados obtenidos para la degradación de BP1 durante los experimentos de ozonización llevados a cabo en condiciones estándar (definidas en la publicación). BP1 mostró un tiempo de vida media de 16.1 min (n=3), alcanzando un 95% de degradación entre 30 y 40 min. El hecho de que la velocidad de degradación alcanzada sea más baja que la correspondiente a BP3 permite detectar BP1 en concentraciones apreciables en los experimentos de degradación de BP3. No obstante, la degradación para este compuesto es también eficaz, por lo que su concentración es siempre indetectable al final de los experimentos. La degradación de BP1 y el perfil de consumo de ozono se muestra en la Figura 4.1. Considerando todos los puntos por encima del 5% de la concentración inicial de BP1, se observó que la reacción sigue una

cinética de pseudo-orden cero con una pseudo constante $K_{0,\ BP1}$ de 0.1753 (RSD= 1%, n=3) $mgL^{-1}min^{-1}$en condiciones estándar.

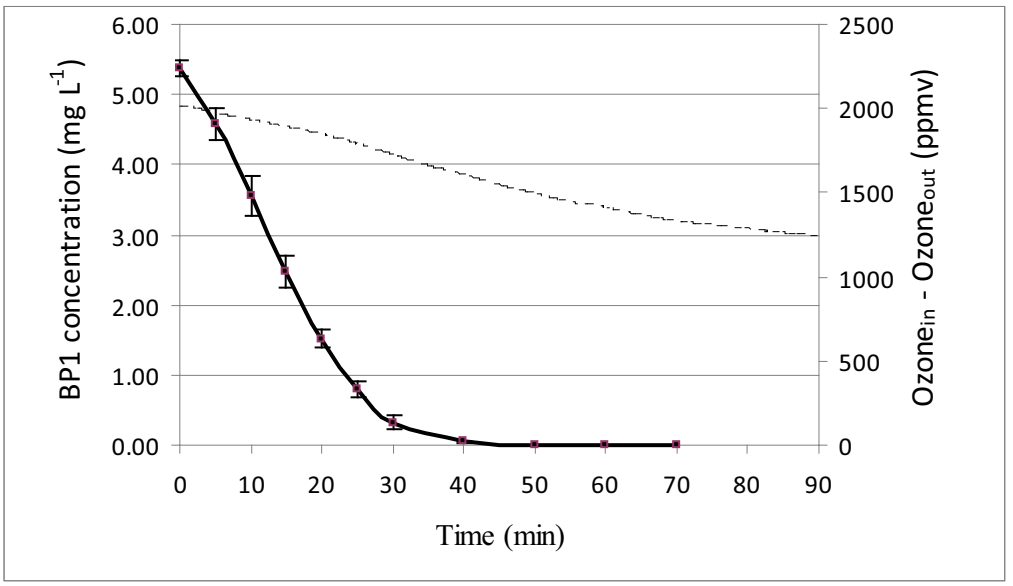
$$[BP1]_t = [BP1]_0 - k_{0,BP1}\int_0^t dt = [BP1]_0 - k_{0,BP1}t$$

En la que $[BP1]_0$ indica la concentración de BP1 a tiempo 0 y $[BP1]_t$ la concentración de BP1 a un tiempo determinado.

Tabla 4.1. Constantes de reacción, tiempo de vida medios y consumo de ozono durante 60 min de ozonización para BP1 y BP3, ambos a concentración inicial de 5 mgL^{-1}, 2100 ppmv de concentración de ozono inyectada, T = 25 °C y pH 7.

	Orden de reacción	Constante de reacción	$t_{1/2}$ (min)	Consumo de ozono durante 60 min (mmol)
BP1	0	0.0553 min^{-1}	16.1	0.571
BP3	1	0.1753 $mgL^{-1}min^{-1}$	12.5	0.525

Figura 4.1. Ozonización de BP1 en condiciones estándar (línea continua) y perfil de consumo de ozono con respecto al tiempo (línea punteada). Las barras de error representan la desviación estándar (n=5).



4.6. COMPARACIÓN ENTRE EL TRATAMIENTO MEDIANTE EL HONGO TRAMETES VERSICOLOR Y LA OZONIZACIÓN

Tanto el proceso de degradación mediante el hongo como la ozonización demostraron ser capaces de degradar completamente la BP3, y lo que es más importante, también degradaron hasta niveles no detectables los productos de transformación identificados durante el transcurso de los experimentos. La velocidad de degradación es mucho más alta en los experimentos de ozonización pero hacer una comparación en este sentido es sumamente difícil, ya que esta depende directamente de la concentración y el flujo de O_3 inyectado, lo cual está relacionado con el gasto energético.

Por una parte la ozonización ofrece ventajas ambientales como por ejemplo no transferir contaminantes entre fases, ya sea mediante procesos de adsorción, precipitación química o producción de biosólidos. Pero por otra parte, los costes de material, implementación y sobre todo energéticos son muy altos, limitando así su aplicación.

El tratamiento mediante el hongo es muy ventajoso económicamente porque no requiere fuentes externas de energía, solamente nutrientes externos, lo cual es relativamente barato. Otra ventaja es que el hongo *T. versicolor* es muy común y de muy fácil obtención, en términos de producción y costes.

Es necesario tener en cuenta que los experimentos no se han llevado a cabo en aguas reales y estos resultados podrían variar considerablemente, especialmente trabajando con aguas residuales. En el caso del hongo *T. versicolor*, el proceso se podría ver seriamente afectado por la adsorción o por posibles inhibiciones debido a la toxicidad del medio o a procesos competitivos con bacterias presentes en el sistema. En el caso de la ozonización es de esperar procesos competitivos con materia orgánica presente y otros contaminantes que pueden disminuir considerablemente la eficacia del proceso. Es sin duda necesario estudiar estas tecnologías en aguas reales siendo uno de los objetivos futuros en los cuales ya se está trabajando.

Aun en el caso de que estos procesos no consigan mineralizar los contaminantes orgánicos en los que estamos interesados, lo cual es frecuente con la mayoría de AOPs (especialmente en aguas residuales), los contaminantes son oxidados en general a productos menos complejos, lo que afecta positivamente a la biodegradabilidad de este agua, siendo un buen complemento a los tratamientos biológicos convencionales.

En mi opinión, la elección de una tecnología se tiene que hacer en base a cada caso particular. La evaluación económica tanto de la implantación como del mantenimiento de la tecnología a escoger es un factor muy importante. No obstante, es necesario disponer de conocimiento científico previo, tanto en agua pura como en aguas reales y en diferentes escalas para poder tomar una decisión correcta. Teniendo en cuenta el alto coste de procesos como la ozonización, esta técnica se debería emplear preferiblemente en aplicaciones concretas, donde haya una gran carga de contaminantes específicos y su no eliminación suponga un riesgo para el medioambiente. Un ejemplo serían las aguas provenientes de hospitales, en el caso que el objetivo sea eliminar fármacos, o las aguas de desecho de determinadas industrias para otros tipos de compuestos. Como se comentaba anteriormente, la combinación de AOPs y tratamientos biológicos puede ser una buena estrategia. Un ejemplo, sería aplicar la ozonización para oxidar total o parcialmente los microcontaminantes de algunos de los flujos residuales más problemáticos de una industria y aplicar posteriormente un tratamiento biológico para la mezcla de todos sus efluentes, el cual será mucho más efectivo debido a la mayor biodegradabilidad del efluente final. El uso de estas tecnologías, no obstante, debe aplicarse cuidadosamente para que los beneficios en los tratamientos de agua obtenidos sean claramente superiores al gasto energético que comporta.

4.7. CONCLUSIONES

Este estudio revela nuevos datos acerca de la cinética y los mecanismos de reacción de la eliminación de BP3 mediante el proceso de ozonización en agua. Hasta la fecha, aunque existe bastante información sobre la eliminación de contaminantes emergentes como fármacos o pesticidas mediante el proceso de ozonización, la información sobre PCPs y especialmente UV-F es muy limitada y poco concluyente. Por ejemplo, Rosal et al. [136] no observó degradación alguna de BP3 en experimentos con ozonización, mientras que otros estudios indicaron eficiencias de degradación superiores al 80% [82, 137]. Este capítulo aporta información clara y consistente sobre el proceso de ozonización de BP3 en agua, que se resume a continuación.

Respecto a la cinética y el estudio de los diferentes parámetros experimentales podemos concluir:

- La ozonización parece ser una alternativa prometedora a las tecnologías convencionales de tratamiento de agua para la eliminación de BP3, como lo ejemplifican los tiempos de vida media en el intervalo 6 – 30 min.

- En las condiciones experimentales en las que se llevaron a cabo los experimentos, la ozonización de BP3 siguió una cinética de pseudo-primer orden, mientras que para BP1 fue de pseudo orden cero.

- La pseudo constante de reacción muestra una correlación lineal con respecto a la dosis de ozono, y el efecto de la temperatura en la reacción está dominado por el decrecimiento en la transferencia de masa de O_3 de la fase gaseosa a la líquida a mayor temperatura.

- Las condiciones que favorecen la formación de radicales hidroxilo tienen un pronunciado efecto positivo en la degradación de BP3, que muestra más reactividad mediante mecanismos radicalarios en comparación con la reacción directa con ozono.

- Siguiendo el argumento anterior, la degradación es mucho más rápida en condiciones alcalinas de pH, como también tras la adición de pequeñas cantidades de H_2O_2 (10 – 100 $\mu molL^{-1}$). Sin embargo, la adición de cantidades

mayores de H_2O_2 y la adición de t-butanol hacen disminuir la velocidad de degradación debido a su efecto inhibidor sobre los radicales hidroxilos.

Respecto a los productos de identificación:

- Mediante el análisis con LC-MS/MS de alta resolución, se identificaron y se caracterizaron estructuralmente siete productos intermedios de transformación, siendo el primer estudio que aporta datos en la identificación de productos de transformación de BP3 en el proceso de ozonización.

- Además de BP1, que es resultado de la desmetilación de BP3, los otros productos de transformación son derivados hidroxilados, formados mediante un ataque no específico de radicales hidroxilo en diferentes posiciones de la molécula. Esta ruta de degradación mediante hidroxilaciones es común en otros procesos bióticos y abióticos.

- El análisis cuantitativo reveló que BP1 y DHMB siguieron un perfil de formación/degradación similar en función del tiempo de ozonización de BP3. El hecho de que la degradación de BP1 sea ligeramente más lenta que la de BP3 en las mismas condiciones explica su acumulación temporal en el medio de reacción.

CONCLUSIONES GENERALES

CONCLUSIONES GENERALES

Uno de los principales objetivos de este trabajo ha sido el desarrollo de metodologías analíticas sensibles que permitan la detección de UV-F en muestras ambientales. Los métodos analíticos desarrollados y validados en esta Tesis Doctoral han demostrado ser herramientas útiles que han permitido ampliar la información disponible sobre el impacto ambiental de estos compuestos. Mediante los métodos propuestos, basados en LC-MS/MS, se han podido determinar residuos de UV-F a nivel traza en aguas naturales, aguas residuales, sedimentos, lodos de EDAR y biota. Cabe destacar el desarrollo de la primera metodología completamente automatizada para el análisis de estos compuestos en aguas, que permite obtener una gran sensibilidad y precisión reduciendo muy significativamente tanto el volumen de muestra como el tiempo de trabajo en el laboratorio. Las metodologías para matrices sólidas se han basado en una extracción mediante PLE. La utilización de adsorbentes como la Alumina o el Florisil ha permitido llevar a cabo una purificación en la misma celda y prescindir, en muchos casos, de una etapa extra de purificación. En el análisis de UV-F en muestras de pez esto no fue posible y se añadió una etapa de purificación mediante SPE para conseguir la sensibilidad necesaria para analizar los UV-F en este tipo de muestras, alcanzando los limites de detección más bajos descritos hasta el momento para la mayoría de UV-F analizados.

Mediante la aplicación de estas metodologías a muestras reales se ha determinado la presencia de estos compuestos en todas las matrices estudiadas. Por primera vez se aportan datos sobre la presencia de UV-F en aguas subterráneas. Se determinaron concentraciones relevantes para aguas superficiales y, especialmente, para aguas residuales, que parecen ser el principal mecanismo de entrada de estos compuestos en el ecosistema acuático. Los compuestos más lipofílicos se determinaron en concentraciones muy elevadas en lodos de EDAR, lo que evidencia una pobre degradación de los UV-F mediante los tratamientos biológicos convencionales. En este trabajo se determinaron por primera vez algunos productos de transforamación de BP3. Estos lodos son utilizados ampliamente en la agricultura por lo que los contaminantes que quedan presentes se vuelven a reintroducir en el medio ambiente, pudiendo llegar incluso a la cadena alimentaria.

La bioacumulación de UV-F en organismos acuáticos queda demostrada en los estudios llevados a cabo en peces y delfines. Cabe señalar que en esta Tesis Doctoral se han presentado los primeros datos de presencia de UV-F en peces de ríos Ibéricos. Así mismo, el estudio en delfines es el primero que demuestra la presencia de residuos de estos compuestos en mamíferos marinos. Estos estudios parecen indicar cierto grado de biomagnificación, ya que las concentraciones resultaron ser más elevadas en especies depredadoras de niveles superiores de la cadena trófica. No obstante, harían falta más estudios para obtener datos más concluyentes.

En esta Tesis Doctoral también se han evaluado tecnologías innovadoras, sostenibles y respetuosas con el medio ambiente para la degradación de UV-F en agua y lodos de EDAR. La biodegradación con el hongo ligninolítico *T. versicolor* ha demostrado ser muy efectiva para degradar UV-F en lodos de EDAR, demostrando el potencial de este tipo de tratamiento para complementar los procesos de tratamientos actuales que no consiguen biodegradar muchos microcontaminantes orgánicos. También resultó efectivo este tratamiento para la biodegradación en medio acuoso de los UV-F estudiados (BP3, BP1 y 4MBC). En estos casos, se identificaron los principales productos de transformación del proceso, que resultaron ser mayoritariamente hidroxilaciones y conjugaciones con pentosas y hexosas. No obstante, todos los productos de transformación detectados también fueron degradados en un periodo inferior a 12 días.

También se ha llevado a cabo el estudio del proceso de ozonización y la combinación de éste con H_2O_2 para degradar uno de los UV-F más utilizados, BP3. En este estudio se investigó el efecto de diferentes variables operacionales como la temperatura, el pH o la adición de H_2O_2, así como sobre los mecanismos de reacción en la degradación de de este compuesto. La ozonización demostró ser una tecnología muy eficaz para degradar BP3. Las condiciones que favorecieron la formación de radicales hidroxilo favorecieron muy considerablemente la degradación de BP3, que mostró más reactividad mediante mecanismos radicalarios en comparación con la reacción directa con ozono. Un pH alcalino o la adición de pequeñas cantidades de H_2O_2 aumentaron notablemente la velocidad de degradación. En este estudio se identificaron los productos de transformación generados durante el proceso de ozonización, entre los que se incluyen BP1 y DHMB. Todos estos productos de transformación fueron eliminados en un tiempo inferior a 60 min.

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ANEXOS

ANEXO I. LISTADO DE ACRÓNIMOS

3BC: 3-benziliden canfor.

4DHB: 4,4'-dihidroxibenzofenona.

4HB: 4-hidroxibenzofenona.

4MBC: 4-metilbenciliden canfor

4MBC-d_4: 4-metilbenciliden canfor deuterado.

ABT: 1-aminobenzotriazol.

AOPs: Procesos de oxidación avanzada.

APCI: Ionización química a presión atmosférica.

APPI: Fotoionización a presión atmosférica.

BM-BDM: Butil metoxidibenzoilmetano.

BNF: β-naptoflavona.

BOD: Demanda biológica de oxígeno.

BOE: Boletín Oficial del Estado.

BP1: Benzofenona-1.

BP2: Benzofenona-2.

BP3: Benzofenona-3.

BP3-d_5: Benzofenona-3 deuterada.

BP4: Benzofenona-4.

BP-d_5: Benzofenona deuterada.

CE: Electroforesis capilar.

CO_3^{2-}: Ión carbonato.

DBE: Dobles enlaces equivalentes.

DBT: Dietilhexil butamido triazona.

DHMB: 2,2'-dihidroxi-4-metoxibenzofenona.

d.w.: Peso seco.

EB: Experimento de degradación.

EC: Comisión Europea.

EC_{50}: Concentración efectiva para el 50% de la población.

EDAR: Estación depuradora de aguas residuales.

EHMC: Etilhexil metoxicinamato.

EHS: 2-Etilhexil salicilato.

EHT: Etilhexil triazona.

ER-RYA: Ensayo basado en levaduras recombinantes para la detección de disruptores endocrinos.

ESI: Ionización por electrospray.

Et-PABA: Etil-PABA.

EU: Unión Europea.

FP: Patrón de fraccionamiento.

GC: Cromatografia de gases.

H_2O_2: Peróxido de hidrógeno.

HCO_3^- : Ión bicarbonato.

HF-LPME: Microextracción en fase líquida con fibra hueca.

HK: Control muerto por tratamiento térmico.

HMS: Homosalato.

HPLC: Cromatografía de líquidos de alta resolución.

IAMC: Isoamil metoxicinamato.

INCI: Nomenclatura internacional para ingredientes cosméticos.

IR: Infrarojo.

K_{oc}: Coeficiente de partición entre el carbono orgánico y el agua.

K_{ow}: Coeficiente de partición octanol-agua.

LC: Cromatografia de líquidos.

LC-MS/MS: Cromatografia de líquidos acoplada a espectrometría de masas en tándem.

LOD: Límite de detección.

LOQ: Limite de cuantificación.

MEPS: Microextracción mediante sorbentes empaquetados.

MM: Melanoma maligno.

MS: Espectrometría de masas.

MS/MS: Espectrometría de masas en tándem.

m/z: Relación masa/carga.

O_3: Ozono.

OC: Octocrileno.

OD-PABA: Octil dimetil PABA.

PABA: Ácido p-aminobenzoico.

PAHs: Hidrocarburos aromáticos policíclicos.

PBSA: Ácido fenilbencimidazol sulfónico.

PCBs: Policlorobifenilos.

PCP: Producto para el cuidado personal.

pKa: logaritmo negativo de la constante de disociación de un ácido.

PLE: Extracción con líquidos presurizados.

QqLIT: Analizador de masas en tándem híbrido tipo cuadrupolo-trampa lineal de iones.

QqQ: Analizador de masas en tándem tipo triple cuadrupolo.

QqTOF: Analizador de masas en tándem híbrido cuadrupolo-tiempo de vuelo.

SAK: Control muerto con azida sódica.

SBSE: Microextracción con barras agitadoras.

SDME: Microextracción con gota suspendida.

S/N: Relación señal-ruido.

SPE: Extracción en fase sólida.

SPF: Factor de protección solar.

SPME: Microextracción en fase sólida.

SRM: Monitorización de reacciones seleccionadas (selected reaction monitoring).

THB: Trihidroxibenzofenona.

TiO_2: Dióxido de titanio.

t_R: Tiempo de retención cromatográfico.

T. versicolor: *Trametes versicolor.*

UPLC: Cromatografia de líquidos de ultra-alta resolución.

USE: Extracción por ultrasonidos.

UV: Ultravioleta.

UV-F: Filtros solares.

UVA: Ultravioleta de onda larga (320-400 nm).

UVB: Ultravioleta de onda media (290-320 nm).

UVC: Ultravioleta de onda corta (100-290 nm).

ANEXO II. AMPLIACIÓN DEL ESTUDIO SOBRE PRESENCIA DE FILTROS UV EN PECES DE LAS CUENCAS HIDROGRÁFICAS DE LOS RÍOS LLOBREGAT, EBRO, JÚCAR Y GUADALQUIVIR.

Tabla A.1. Concentraciones de UV-F en peces (ngg^{-1}).

LLOBREGAT							
Punto de muestreo	Nombre común	Nombre científico	% lípidos	BP3	EHMC	4MBC	OC
LLO3 (n=3)	Barbo del Ebro (Juvenil)	Luciobarbus Graellsii	14	n.d.	n.d.	n.d.	n.d.
LLO4 (n=3)			14	n.d.	n.d.	n.d.	n.d.
LLO6 (n=3)			16	n.d.	n.d.	n.d.	n.d.
LLO3 (n=3)	Barbo del Ebro (Adulto)	Luciobarbus Graellsii	20	n.d.	n.d.	n.d.	n.d.
LLO4 (n=2)			27	n.d.	n.d.	n.d.	n.d.
LLO6 (n=3)			21	n.d.	n.d.	n.d.	n.d.
LLO3 (n=3)	Carpa común	Cyprinus Carpio	27	n.d.	n.d.	n.d.	n.d.
LLO4 (n=1)			n.a.	n.d.	n.d.	n.d.	n.d.
LLO5 (n=3)			21	n.d.	n.d.	n.d.	n.d.
LLO6 (n=3)			25	n.d.	n.d.	n.d.	n.d.
LLO7 (n=3)			23	n.d.	n.d.	n.d.	<LOQ

EBRO							
Punto de muestreo	Nombre común	Nombre científico	% lípidos	BP3	EHMC	4MBC	OC
OCAn (n=4)	Barbo del Ebro (Juvenil)	Barbus Graellsii	12	n.d.	n.d.	n.d.	n.d.
EBR2 (n=3)			12	n.d.	n.d.	n.d.	n.d.
EBR3 (n=3)			13	n.d.	n.d.	n.d.	n.d.
EBR4 (n=3)			13	n.d.	n.d.	n.d.	n.d.
EBR5 (n=3)			15	n.d.	n.d.	n.d.	n.d.
OCA (n=3)	Barbo del Ebro (Adulto)	Barbus Graellsii	17	n.d.	n.d.	n.d.	n.d.
EBR2 (n=3)			24	n.d.	n.d.	n.d.	n.d.
EBR3 (n=3)			n.a.	2.2	n.d.	2.7	n.d.
EBR4 (n=3)			n.a.	n.d.	n.d.	n.d.	n.d.
EBR5 (n=2)			n.a.	n.d.	n.d.	n.d.	n.d.
EBR2 (n=1)	Carpa común	Cyprinus Carpio	11	n.d.	n.d.	n.d.	n.d.
EBR3 (n=3)			9	n.d.	n.d.	n.d.	n.d.
EBR4 (n=3)			8	n.d.	n.d.	n.d.	n.d.
EBR5 (n=3)			13	n.d.	n.d.	n.d.	<LOQ
EBR4 (n=2)	Sirulo	Silurus Glanis	25	n.d.	12.2	n.d.	<LOQ
EBR5 (n=2)			24	<LOQ	30.4	<LOQ	25.7

GUADALQUIVIR							
Punto de muestreo	Nombre común	Nombre científico	% lípidos	BP3	EHMC	4MBC	OC
GUA1 (n=1)	Barbo de Andalucía (Adulto)	Luciobarbus sclateri	27	n.d.	n.d.	n.d.	n.d.
GUA3 (n=9)			29	n.d.	19.0	n.d.	<LOQ
GUA4 (n=9)			41	24.3	241.7	n.d.	30.4
GUA5 (n=9)			34	16.5	63.0	n.d.	n.d.
GUA3 (n=9)	Carpa común	Cyprinus Carpio	9	11.2	<LOQ	n.d.	n.d.

JÚCAR							
Punto de muestreo	Nombre común	Nombre científico	% lípidos	BP3	EHMC	4MBC	OC
JUC1 (n=3)	Trucha común (Adulto)	Salmon Trutta	48	4.6	n.d.	n.d.	n.d.
JUC2 (n=2)	Boga del Tajo	Pseudochondrostoma polylepis	19	n.d.	n.d.	n.d.	n.d.
JUC2 (n=13)	Gobio ibérico (Juvenil)	Gobio Lozanoi	n.a.	n.d.	n.d.	n.d.	n.d.
JUC4 (n=10)			n.a.	n.d.	n.d.	n.d.	n.d.
JUC4 (n=4)	Gobio ibérico (Adulto)	Gobio Lozanoi	n.a.	n.d.	n.d.	n.d.	n.d.
JUC6 (n=4)			n.a.	n.d.	n.d.	<LOQ	n.d.
JUC4 (n=6)	Perca americana	Mycropterus salmpoides	13	n.d.	n.d.	n.d.	n.d.
JUC5 (n=5)			n.a.	n.d.	n.d.	n.d.	<LOQ
JUC6 (n=2)			19	n.d.	n.d.	n.d.	n.d.
JUC5 (n=6)	Alburno	Alburnus alburnus	n.a.	n.d.	n.d.	n.d.	n.d.
JUC6 (n=16)			n.a.	n.d.	n.d.	n.d.	n.d.
JUC5 (n=3)	Anguila	Anguila anguila	11	n.d.	n.d.	n.d.	n.d.
JUC6 (n=3)			45	n.d.	<LOQ	n.d.	30.0
JUC6 (n=1)	Perca sol	Leponis Gibbosus	n.a.	n.d.	n.d.	n.d.	n.d.
JUC6 (n=2)	Barbo del Mediterráneo (Juvenil)	Barbus guiraonis	12	n.d.	n.d.	n.d.	n.d.
JUC6 (n=1)	Barbo del Mediterráneo (Adulto)	Barbus guiraonis	15	n.d.	n.d.	n.d.	n.d.
JUC6 (n=1)	Lucio	Esox Lucius	8	n.d.	n.d.	n.d.	n.d.

n.d.: no detectado; n.a.: no disponible

ANEXO III. LISTA DE TABLAS Y FIGURAS

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Figura 3.2. Perfil de concentración de 4MBC durante 24 horas de experimento. EB (○), HK (●), SAK (▼).

Figura 3.3. Espectros de masas (MS/MS) de los iones productos del 4MBC de los obtenidos mediante HPLC-QqTOF-MS/MS.

Figura 3.4. Espectros de masas (MS/MS) de los iones producto de los metabolitos de 4MBC. Experimentos realizados mediante HPLC-QqTOF-MS/MS. (a) Pr271, (b) Pr287, (c) Pr425 y (d) Pr441.

Figura 3.5. Mecanismos de biodegradación propuestos para el tratamiento con *T. versicolor* de (a) BP3 y (b) 4MBC.

Figura 4.1. Ozonización de BP1 en condiciones estándar (línea continua) y perfil de consumo de ozono con respecto al tiempo (línea punteada). Las barras de error representan la desviación estándar (n=5).