NOVEL ANALYTICAL STRATEGIES FOR THE ANALYSIS OF ORGANIC MICROPOLLUTANTS IN ENVIRONMENTAL SAMPLES

NUEVAS ESTRATEGIAS ANALÍTICAS PARA EL ANÁLISIS DE CONTAMINANTES ORGÁNICOS EN MUESTRAS AMBIENTALES

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Memoria para obtener el título de Doctor con mención europea por la Universidad Autónoma de Madrid

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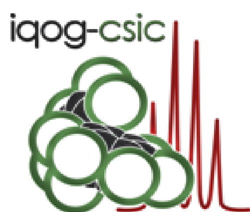

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CERTIFICA:

Que el trabajo aquí presentado bajo el título: **"Novel analytical strategies for the analysis of organic micropollutants in environmental samples"**, que constituye la memoria que presenta Dª. Miren Pena Abaurrea para optar al grado de Doctor, ha sido realizado bajo su dirección en el Departamento de Análisis Instrumental y Química Ambiental del Instituto de Química Orgánica General (IQOG-CSIC). Así mismo, manifiesta que el trabajo descrito en la presente memoria reúne, en su opinión, todos los requisitos para su defensa y aprobación, por lo que autoriza su presentación para que sea defendido como **Tesis Doctoral con mención de Doctorado Europeo.**

Y para que conste, firma el presente certificado en Madrid, a 26 de Marzo de 2012.

Fdo: Dra. Lourdes Ramos Rivero

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LIST OF ACRONYMS/LISTA DE ACRÓNIMOS

COP/POP Persistent organic pollutant – Contaminante orgánico persistente

^{1}D First dimension – Primera dimensión

^{2}D Second dimension – Segunda dimensión

DPX Disposable pippete extraction – Extracción con puntas de pipetas desechables

EC European Commission – Comisión Europea

ECD Electron capture detector – Detector de captura electrónica

EI Electronic impact – Impacto electrónico

GC Gas chromatography – Cromatografía de gases

GC × GC Comprehensive two-dimensional gas chromatography – Cromatografía de gases completa en dos dimensiones

HNPs Halogenated natural products – Compuestos naturales halogenados

ITD Ion trap detector – Detector de trampa de iones

LOD Limit of detection – Límite de detección

LOQ Limit of quantification – Límite de cuantificación

MeO-PBDE PBDE methoxy derivative – Derivado metoxilado de PBDE

MRL Maximum residual level – Límite máximo residual permitido

MS Mass Spectrometry – Espectroscopía de masas

MS/MS Tandem mass spectrometry – Espectroscopía de masas en modo tándem

MSPD Matrix solid-phase dispersion – Dispersión de la matriz en fase sólida

NCI Negative chemical ionization – Ionización química negativa

OB Organobromine – Compuesto organobromado

PAH Polycyclic aromatic hydrocarbon – Hidrocarburo aromático policíclico

PBDE Polybrominated diphenyl ether – Polibromodifenil éter

PBHD Polybrominated hexahydroxanthene derivative – Derivado de polibromohidroxanteno

PCB Polychlorinated biphenyl – Bifenilo policlorado

PLE Pressurised liquid extraction – Extracción con líquidos a presión

SIM	Selected ion monitoring – Monitorización de iones seleccionados
SPE	Solid-phase extraction – Extracción en fase sólida
TEF	Toxic equivalent factor – Factor de equivalencia tóxica
TEQ	Toxic equivalent of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin – Equivalente tóxico de 2,3,7,8-tetraclorodibenzo-*p*-dioxina
TIC	Total ion chromatogram – Cromatograma de iones totales
ToF	Time of flight – Analizador de tiempo de vuelo
t_R	Retention time – Tiempo de retención
^1t_R	Retention time in the first dimension – Tiempo de retención en la primera dimensión
^2t_R	Retention time in the second dimension – Tiempo de retención en la segunda dimensión
UAE	Ultrasonic assisted extraction – Extracción asistida con ultrasonidos

The present PhD work focuses in the development of new analytical procedures for the analysis of trace organic pollutants in relevant environmental samples. Studies have focused in two aspects: the development and validation of novel miniaturised, simplified/generic, and green sample preparation procedures, and the evaluation of the feasibility of comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC–ToF MS) to solve specially difficult coelution problems that can hardly be solved using monodimensional GC-based techniques, and to develop multiresidual and non-target analysis in complex extracts.

The first section of this PhD book is an Introduction chapter (**Section 1**). The first part of this chapter summarizes the physicochemical properties, origin, environmental fate, toxicity and, when available, current legislative situation of the several analytes considered in the study. Then, an overview of both conventional and miniaturised sample preparation methods in use for the determination of the test analytes in complex (solid and semi-solid) biotic and abiotic matrices is presented. The main practical advantages and limitations of both types of analytical approaches as well as their relative merits for routine analysis are deeply discussed. At the end of this section, a review on miniaturization in analytical chemistry, corresponding to a chapter included in the book entitle "Challenges in Green Analytical Chemistry", is included. The main instrumental requirements demanded for this type of determination have been summarized in the last part of this section, and a book chapter reviewing relevant environmental application studies involving GC×GC is also presented.

The main and specific objectives of the present work are summarised in the second section of the book (**Section 2**). Objectives have been set on the base of present state of knowledge in the fields of sample preparation and of GC×GC–ToF MS for the analysis of organohalogenated compounds and microcontaminants in complex (semi-)solid environmental samples.

The most relevant results concerning the development of miniaturised, simplified and generic sample preparation methods for the determination of the investigated trace compounds are presented in the third part of the book. **Section 3.1.1** reports on the use of hot Soxhlet for the determination of different organobromines (including PBDEs, MeO-PBDEs, TBA, MHC-1 and PBHDs) in tuna muscles from the

Mediterranean sea. In the next two sections (**Sections 3.1.2 and 3.1.3**) the combined use of matrix solid-phase dispersion (MSPD) and pressurised liquid extraction (PLE) with in-cell packing of the clean-up sorbents is proposed as an efficient analytical strategy for exhaustive extraction and simultaneous purification of the studied microcontaminants. The feasibility of the approach has been demonstrated for the simultaneous analysis of PCBs and PBDEs in feedstuffs (**Section 3.1.2**) and of PCBs in sediments (**Section 3.1.3**). Finally, in **Section 3.1.4**, the use of ultrasonic-probe assisted extraction (UAE) in combination with solid phase extraction in disposable pipette tip is proposed for the fast and cost-effective extraction and purification of PCBs in small-size (semi-)solid samples (i.e., 50 mg of sample).

The second section of the third chapter of this book (**Section 3.2**) evaluates the feasibility of GC×GC–ToF MS for the chromatographic separation of specific pairs/groups of analytes that are difficult to determine accurately by monodimensional GC-based approaches, the simultaneous analysis of different families of pollutants, and the screening and tentative identification of analytes from which previous information is not available (non-target analysis). Thereby, in **Section 3.2.1** the potential of GC×GC–ToF MS for the simultaneous determination of several families of organobromines, including PBDEs, MeO-PBDEs, PBHDs, diMeO-PBDEs and other emerging analytes, in tuna muscles has been investigated. Moreover, considering both the typical structured chromatograms obtained with GC×GC and the MS structural information provided by ToF MS, a tentative identification of additional non-target organobromines was also achieved. Interestingly, some novel PBHD isomers, not previously described in the literature could be also identified. **Section 3.2.2** describes a GC×GC–ToF MS-based methodology for the analysis of the 15+1 EU PAHs in sediments from a protected area in the South of Spain, The Cadiz Bay. The enhanced identification power provided by the use of GC×GC–ToF MS allowed the complete separation of critical co-eluting PAH pairs/groups which had not been previously resolved when using monodimensional GC-based techniques.

Finally, the main conclusions of the most relevant results achieved in the present study are summarised in **Section 4**.

El presente trabajo de doctorado se ha centrado en el desarrollo de nuevos protocolos analíticos para el análisis de contaminantes orgánicos traza en muestras de interés ambiental. El estudio se ha orientado tanto al desarrollo y validación de procedimientos de preparación de muestra miniaturizados, simplificados, genéricos y medioambientalmente sostenibles, como a la evaluación del potencial de la cromatografía de gas en dos dimensiones acoplada a espectrometría de masas de tiempo de vuelo (GC×GC–ToF MS) para la resolución de problemas de coelución imposibles de resolver empleando técnicas cromatográficas monodimensionales, para el análisis multicomponente en extractos complejos y para el denominado *non-target analysis*.

La primera parte de esta memoria es una Introducción general que recoge una revisión detallada de las principales características estructurales, físico-químicas y toxicológicas de los diversos contaminantes orgánicos objeto de estudio en este trabajo (**Sección 1**). Para la mayoría de ellos, sus niveles en muestras ambientales, especialmente en aquellas destinadas al consumo animal y humano, han sido regulados por diferentes Directivas Europeas debido a sus potenciales efectos tóxicos. En el segundo apartado de la Introducción se describen y discuten las técnicas de extracción y purificación convencionales y modernas empleadas en la actualidad para el análisis de contaminantes orgánicos traza en matrices ambientales complejas. Al final de este apartado se presenta un capítulo del libro "Challenges in Green Analytical Chemistry" que hace una introducción a la miniaturización en la Química Analítica actual. El tercero y último apartado de la Introducción revisa las técnicas instrumentales más comunes empleadas para la separación y detección instrumental de este tipo de microcontaminantes. Se ha prestado especial atención al caso de la GC×GC, incluyéndose un capítulo de libro que recoge una revisión bibliográfica de las aplicaciones de esta técnica en el campo medioambiental.

Los objetivos generales y concretos del presente trabajo se presentan en la segunda sección de la memoria (**Sección 2**). Estos objetivos se establecieron en base al estado actual de los conocimientos descrito en la primera sección de la memoria y, abordan tanto la preparación de muestra como la aplicación de la GC×GC–ToF MS para el análisis de contaminantes y compuestos organohalogenados en matrices ambientales (semi-)sólidas complejas.

Los resultados más relevantes obtenidos en relación con el desarrollo de métodos miniaturizados, simplificados y genéricos de preparación de muestra se presentan en la tercera parte de la memoria en forma de publicaciones científicas (**Sección 3**). La primera de ellas (**Sección 3.1.1**) propone el empleo de hot Soxhlet para el análisis de una variedad de compuestos organobromados (PBDEs, MeO-PBDEs, TBA, MHC-1 y PBHDs) en músculo de atunes del Mediterráneo. Las dos secciones siguientes (**Secciones 3.1.2 y 3.1.3**) proponen el empleo combinado de la dispersión de la matriz en fase sólida (MSPD) con la extracción con líquidos a presión (PLE) y el empaquetado de los sorbentes requeridos para la purificación en línea en la misma celda de extracción. Con ello se consiguió un método basado en PLE miniaturizada y selectiva para el análisis simultáneo de PCBs y PBDEs en piensos (**Sección 3.1.2**) y de PCBs de sedimentos (**Sección 3.1.3**). La última sección (**Sección 3.1.4**) propone el empleo de la extracción asistida por sonda focalizada de ultrasonidos (UAE) para la extracción de PCBs de muestras (semi-)sólidas de tamaño muy limitado (unos pocos mg) y el empleo de la técnica de extracción en fase sólida dispersiva en pipetas desechables para la purificación rápida de los extractos obtenidos con mínimo consumo de reactivos.

La segunda sección del tercer capítulo de la memoria (**Sección 3.2**) evalúa el potencial de la GC×GC–ToF MS para la resolución de ciertos pares/grupos de microcontaminantes que no pueden ser determinados de manera adecuada con técnicas monodimensionales para la evaluación simultánea y en un único análisis cromatográfico de la presencia de diferentes familias de contaminantes, y para el screening e identificación preliminar de analitos de los que no se dispone de información previa (*non-target analysis*). Así, en la **Sección 3.2.1** se evaluaron las posibilidades de análisis simultáneo de distintas familias de compuestos bromados, incluyendo PBDEs, MeO-PBDEs, PBHDs, diMeO-PBDEs y otros compuestos emergentes bromados, en muestras de atunes. En este estudio se aprovechó la capacidad de la GC×GC para generar cromatogramas estructurados y del ToF MS para obtener información estructural para la identificación tentativa de diferentes contaminantes organobromados de los que no se disponía de patrones. También se identificaron ciertos isómeros de PBHDs que no habían sido descritos con anterioridad. Por su parte, la **Sección 3.2.2** propone una metodología basada en el

uso de GC×GC–ToF MS que, una vez optimizada, permitía la determinación inequívoca de los 15+1 EU PAHs en muestras complejas, como los sedimentos. Este método se aplicó posteriormente al análisis de los contaminantes investigados en sedimentos recogidos en la bahía de Cádiz, en los que se observó que la elevada capacidad de resolución ofrecida por la técnica permitía la separación adicional de algunos otros pares de isoméros cuya resolución mediante GC monodimensional es problemática.

El último apartado de la memoria (**Sección 4**) expone de manera resumida las conclusiones más relevantes del trabajo.

Chapter – Capítulo 1

GENERAL INTRODUCTION

INTRODUCCIÓN GENERAL

El fenómeno de "contaminación ambiental" surgió a lo largo del último siglo, como consecuencia colateral de los avances tecnológicos que han permitido el desarrollo industrial de sectores estratégicos como el químico, el agrícola o el sanitario. Este fenómeno, fruto de la utilización de ciertos compuestos que mejoraban los rendimientos y/o calidad de los productos obtenidos o de los procesos industriales, ha propiciado la incorporación al medio de nuevos productos de síntesis y subproductos de estos que, una vez liberados, quedan distribuidos en la totalidad de los compartimentos ambientales. Sin embargo, la mayor importancia reside en aquellos compuestos que, si bien pueden liberarse a la naturaleza de forma accidental o no intencionada, tienen efectos perjudiciales sobre el medioambiente y los organismos que en él residen.

Entre los xenobióticos vertidos al medio, merecen especial atención aquellos cuyas características de persistencia, lipofilia y toxicidad favorecen que puedan permanecer en él durante periodos de tiempo relativamente largos. Esto posibilita su incorporación y distribución en las redes tróficas en las que experimentan procesos de bioacumulación y biomagnificación piramidal, lo que contribuye a aumentar sus potenciales efectos nocivos.

Bajo la denominación de Contaminantes Orgánicos Persistentes (COPs), se engloban una serie de xenobióticos cuya producción, uso y comercialización se reguló por primera vez con el Convenio de Estocolmo en 2001 (*1*). Entre los primeros compuestos incorporados a este tratado cabe citar el pesticida organoclorado diclorodifeniltricloroetano (DDT), otros pesticidas organohalogenados (endrín, mirex, dieldrín, clordano, aldrín, heptacloro y los más de 700 congéneres de la familia de los toxafenos), algunas sustancias químicas utilizadas en la industria como los bifenilos policlorados (PCBs) y el hexaclorobenceno (HCB), y otros productos generados en reacciones secundarias inintencionadas como las policloro dibenzo-*p*-dioxinas (PCDDs) o los policloro dibenzofuranos (PCDFs). Dicho tratado ha sufrido modificaciones posteriores que responden a la necesitad de incluir en dicha lista nuevos contaminantes orgánicos que, de la misma manera que los anteriores, requerían de un control exhaustivo de su presencia en el medio debido a sus potenciales efectos tóxicos (*2*). Este es el caso de las mezclas de menor grado de bromación de los polibromo difeniléteres (PBDEs), algunos derivados clorados de los

hexaciclohexanos (HCHs), las sales sulfónicas perfluoradas y pesticidas como el lindano y la clordecona, que fueron adheridos al convenio en 2009. Hace apenas unos meses, abril de 2011, dicho convenio volvió a ser revisado, adhiriéndose al Anexo A el pesticida endosulfán (*3*). Sobre estos nuevos COPs y los doce inicialmente calificados como contaminantes peligrosos para la salud humana, han sido aplicadas diversas estrategias que limitan, cuando no prohíben, su producción y uso. Como ya se ha mencionado, esta lista no es estanca y un comité regulador supervisa periódicamente aquellos xenobióticos de los cuales se sospeche que puedan alterar la salud humana y/o medioambiental.

Son numerosos los episodios de contaminación relacionados con los COPs que fortalecieron la idea de creación de un comité único que unificase estrategias legales para regular las emisiones de dichos compuestos (España fue uno de los primeros en ratificarlo, de los más de 100 países de todo el mundo que integran el convenio.) Por recordar algunos de los episodios que ocasionaron alarmas alimentarias en Europa durante la pasada década, podemos citar el ocurrido a finales de 2008 en Irlanda, donde se retiraron del mercado toneladas de carne de cerdo y productos porcinos al detectarse cantidades de PCDD/Fs 200 veces superiores al límite máximo permitido. También en 2008 saltó a la luz pública en Italia el caso de la mozzarella de búfala contaminada con PCDD/Fs. En julio de 2007, la Comisión Europea envió a los Estados Miembros una advertencia sanitaria relacionada con la presencia de altas concentraciones de PCDD/Fs en un aditivo alimentario, la goma guar, utilizado en pequeñas cantidades como espesante en carnes, productos lácteos, postres y platos precocinados. El origen de la contaminación fue una goma guar procedente de la India y contaminada con pentaclorofenol, un plaguicida cuyo uso está prohibido en numerosos países. En 2006, en los Países Bajos, se encontraron niveles altos de PCDD/Fs en piensos, cuyo origen estaba en una grasa contaminada utilizada como ingrediente base. También en este país, en 2004, se retiró del mercado leche en la que se habían detectado concentraciones elevadas de PCDD/Fs y cuyo origen estaba en una arcilla utilizada en la producción de piensos. Otros casos que quedan ya más lejanos son los incidentes en Bélgica en 1999, con la contaminación de piensos destinados a la alimentación de aves de corral; en 1998, en Alemania, el episodio de contaminación de leche con una pulpa de cítrico proveniente de Brasil; y el primer

incidente de gran magnitud, ocurrido en Italia en 1976, cuando se incendió una fábrica de productos químicos. Por último, y como ejemplo más mediático, recordar el episodio de envenenamiento del Presidente de Ucrania, Viktor Yushchenko, cuyo rostro quedó desfigurado por el cloroacné provocado por la ingesta de un herbicida defoliante, el agente naranja, que llevaba en su composición altas cantidades de 2,3,7,8-tetraclorodibenzo-*p*-dioxina.

La alarma social causada por estos episodios de contaminación y las continuas evidencias de toxicidad en un número cada vez mayor de compuestos orgánicos han llevado a reconsiderar, de manera más frecuente y exhaustiva, los niveles máximos permitidos (MRLs) para estos compuestos en los alimentos destinados al consumo humano y animal, así como la frecuencia y amplitud de los controles oficiales para determinar la presencia en los mismos (*4*). Las evidencias patológicas obtenidas en los distintos estudios toxicológicos que están profundizando en el metabolismo y en los mecanismos de toxicidad de estos compuestos y otros emergentes (en buena medida, metabolitos derivados de los anteriores (*5*)), ratifican la necesidad de establecer una legislación más estricta que se revise regularmente para garantizar la salubridad de los alimentos y otras matrices medioambientales con las que el ser humano entra en contacto directa o indirectamente.

En la actualidad, los niveles a los que los COPs deben ser monitorizados en muestras biológicas y ambientales son, salvo caso de contaminación aguda, residuales, del orden de las partes por billón (ppb, ng/mL) y partes por trillón (ppt, ng/μL). Este hecho, unido a la complejidad de muchas de las matrices a estudiar, han contribuido en gran medida a que buena parte de las metodologías convencionales de preparación de muestra empleadas para la determinación de estos compuestos sean procedimientos laboriosos y tediosos, que involucran un elevado consumo de reactivos y una manipulación constante de los extractos, con el consiguiente riesgo de contaminación y pérdida de los analitos. La creciente demanda, antes apuntada, tanto en la frecuencia de los controles como en el número y tipo de matrices sobre los que estos deben efectuarse, requieren el desarrollo de nuevos métodos de tratamiento de muestras que, cumpliendo los requisitos de calidad exigidos para estas determinaciones, sean más rápidos, automáticos y respetuosos con el medio ambiente. Este último aspecto, conocido como "química verde", centra sus esfuerzos en la

reducción del impacto ambiental que pueden generar este tipo de análisis convencionales, y aplica nuevas metodologías que minimicen tanto la cantidad de reactivos empleados para llevar a cabo el análisis como la cantidad de desechos químicos generados y vertidos al medioambiente.

Las propuestas analíticas adoptadas en la actualidad para abordar estos objetivos siguen dos estrategias: (i) el uso de nuevas técnicas aceleradas de extracción, como, por ejemplo, la extracción con líquidos a altas presiones (PLE) o con agua subcrítica (SWE); y (ii) la miniaturización en las técnicas instrumentales; tendiendo en ambos, y en la medida de lo posible, al acoplamiento entre las distintas etapas del proceso analítico, a fin de lograr una mayor automatización del proceso y solventar problemas asociados con la degradación y pérdida de los analitos o los largos tiempos de análisis.

En el presente trabajo de tesis doctoral se evaluarán distintas técnicas y estrategias de preparación de muestra para el análisis de COPs y otros compuestos orgánicos emergentes en diferentes matrices medioambientales. El uso combinado de estas técnicas con los sistemas de separación cromatográfica y detección seleccionados, en general, cromatografía de gases mono- y bi-dimensional (GC y GC×GC, respectivamente) con detectores selectivos, como el de captura electrónica (micro-ECD) o la espectrometría de masas (MS), permitirá la propuesta de distintos protocolos analíticos modernos y sostenibles para la determinación de los compuestos orgánicos organohalogenados en matrices ambientales complejas, como suelos, sedimentos y muestras bióticas con alto contenido lipídico.

En los siguientes apartados, se hará una revisión detallada de las tendencias analíticas actuales para el análisis de compuestos orgánicos en matrices sólidas y (semi-)sólidas complejas, prestando especial atención a aquellas técnicas orientadas a la miniaturización en la preparación de muestra, y la separación y detección simultánea de mezclas complejas de contaminantes mediante GC×GC.

1.1. CONTAMINANTES ORGÁNICOS DE INTERÉS AMBIENTAL

1.1.1. Contaminantes orgánicos persistentes

Bajo la denominación de COPs se engloban una variedad de familias de compuestos con estructuras químicas afines y, en general, basadas en la combinación de heterociclos cuya base principal es el átomo de carbono. Sus particulares propiedades físicas y químicas, derivadas de su conformación estructural, favorecen que, una vez liberados al medio, permanezcan inalterados en él durante largos periodos de tiempo y se distribuyan en los distintos compartimentos ambientales como resultado de procesos naturales (evaporaciones, filtraciones, deposiciones). Este hecho, unido a su carácter apolar, favorece su acumulación en los tejidos adiposos de los organismos vivos, en los que experimentan procesos de bioconcentración y biomagnificación. Además, suelen resultar tóxicos tanto para los seres humanos como para otros organismos animales.

En general, los COPs son compuestos semi-volátiles halogenados, y más frecuentemente clorados. Exhiben una gran resistencia a las descomposiciones químicas, biológicas y fotoquímicas, siendo éstas tanto mayores cuanto mayor sea el grado de halogenación del compuesto. La gran estabilidad de los enlaces carbono-átomo halógeno les proporcionan, además, una gran estabilidad frente a la hidrólisis. Como ya se ha mencionado, experimentan procesos de bioacumulación en tejidos grasos, siendo su logaritmo de coeficiente de reparto octanol-agua (log K_{OW}) superior a 5 y la relación entre el factor de bioacumulación (BAF) y el factor de bioconcentración (BCF), BAF/BCF, mayor de 5000.

Los efectos tóxicos asociados a los COPs suelen ser específicos para cada familia de compuestos y dentro de éstas congénere-dependiente. Sin embargo, a grandes rasgos, incluyen efectos tales como hipersensibilidades, alergias, daños en los sistemas nervioso central y periférico, alteraciones reproductivas, fallos en los sistemas endocrinos y reproductivos así como afecciones carcinogénicas y del desarrollo fetal.

Ante la necesidad de regular la producción y uso de estos compuestos, a la vista de las evidencias de toxicidad, persistencia y ubicuidad, el Consejo de Administración del Programa de las Naciones Unidas para el Medio Ambiente (United Nations Environment Programme, UNEP) publicó a través del Convenio de Estocolmo un

tratado en 2001 para promover la reducción, cuando no su completa eliminación, de las emisiones no intencionadas de los COPs (*1*). Inicialmente, doce compuestos fueron reconocidos como potencialmente adversos para los humanos y los ecosistemas y se clasificaron en tres categorías en función de su origen:

- **Pesticidas:** aldrín, clordano, DDT, dieldrín, endrín, heptacloro, mírex y toxafenos
- **Sustancias químicas industriales:** hexaclorobenceno (HCB) y bifenilos policlorados (PCBs)
- **Productos secundarios no intencionados:** policlorodibenzo-*p*-dioxinas (PCDDs) y policlorodibenzofuranos (PCDFs)

No obstante, el comité regulador del Convenio de Estocolmo, encargado del cumplimiento y revisión del tratado según el Artículo 8 de dicho convenio, prosigue en su labor de gestión con propuestas de evaluación de nuevos compuestos químicos. Así, en la cuarta conferencia, celebrada en Génova en 2009, se presentó una enmienda a los Anexos A, B y C del Convenio con el fin de añadir nueve nuevos compuestos a la lista de COPs: clordecona, α-hexaclorociclohexano, β-hexaclorociclohexano, lindano, pentaclorobenceno, hexabromobifenilo, las familias tetra-, penta-, hexa- y hepta-bromodifenil éter y los perfluorooctanos en su formas ácida, fluorada y las sales (*2*). El pasado abril de 2011 se celebró en Génova la quinta conferencia de las partes en la que, como punto destacado, se ha acordado la inclusión del pesticida endosulfán en el Anexo A del Convenio (*3*). Además, se continúan evaluando nuevas propuestas y recomendaciones para incluir otros contaminantes emergentes en dicha lista, entre los que se encuentran las parafinas cloradas de cadena corta, los hexabromociclododecanos, el hexaclorobutadieno y el pentaclorofenol.

En las siguientes secciones se resumirán algunas de las características más relevantes de las distintas familias de COPs, prestando especial atención a los compuestos considerados en el presente estudio.

Bifenilos policlorados

Los PCBs son una familia de compuestos organoclorados formados por dos anillos aromáticos unidos entre sí mediante un enlace C-C y con cloro-sustituciones sobre sus posiciones libres (Fig. 1.1).

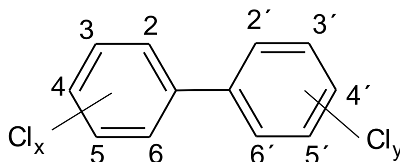

Fig. 1.1. Estructura general de los PCBs.

Su fórmula empírica es $C_{12}H_{10-n}Cl_n$ siendo $n=x+y$, y con n comprendido entre 1 y 10. De este modo, según el grado de cloro-sustituciones sobre la molécula, los PCBs se clasifican en diez grupos de homólogos, caracterizados por un mismo peso molecular pero con diferente disposición espacial. En total, 209 posibles congéneres de PCBs (Tabla 1.1).

La nomenclatura adoptada para los distintos congéneres ha sufrido diversas modificaciones a lo largo del tiempo. Fueron Ballschmister y Zell en 1980 quienes propusieron denominar a los 209 congéneres por orden numérico ascendente. Esta nomenclatura es la más utilizada en la actualidad, está aceptada por la IUPAC y se empleará en este trabajo (*6*).

Si bien los PCBs fueron sintetizados por primera vez a finales del siglo XIX, su producción y uso comercial no se inició hasta el año 1929, siendo los años 60 cuando se alcanzó su máxima producción. Su síntesis se basaba en la cloración del bifenilo en presencia de catalizadores, lo que daba lugar a mezclas con distinto grado de cloración en función del tiempo de mezclado de los reactivos. Dichas mezclas complejas de isómeros se comercializaron con distintos nombres (Aroclor®, Kaneclor®, Clophen® o Pyroclor®), e iban acompañadas de cuatro dígitos que describían el número de carbonos de la estructura y el grado de cloración del compuesto (por ejemplo, Aroclor® 1254 indicaba que el producto comercial contenía 12 carbonos y un 54% en peso de cloro).

Tabla 1.1. Características generales para los distintos congéneres de PCBs agrupados según grado de sustitución.

Grupo de homólogos	Fórmula molecular	Peso molecular	Nº de isómeros	Nº Nomencl. IUPAC
Monoclorados	$C_{12}H_9Cl$	188,0	3	1–3
Diclorados	$C_{12}H_8Cl_2$	222,0	12	4–15
Triclorados	$C_{12}H_7Cl_3$	256,0	24	16–39
Tetraclorados	$C_{12}H_6Cl_4$	289,9	42	40–81
Pentaclorados	$C_{12}H_5Cl_5$	323,9	46	82–127
Hexaclorados	$C_{12}H_4Cl_6$	357,8	42	128–169
Heptaclorados	$C_{12}H_3Cl_7$	391,8	24	170–193
Octaclorados	$C_{12}H_2Cl_8$	425,8	12	194–205
Nonaclorados	$C_{12}H_1Cl_9$	459,7	3	206–208
Decaclorado	$C_{12}Cl_{10}$	493,7	1	209

La alta estabilidad física y química, baja inflamabilidad y la capacidad de aislamiento eléctrico y térmico que ofrecen los PCBs propiciaron su aplicación industrial como fluidos dieléctricos en capacitores y transformadores, fluidos hidráulicos, lubricantes y como aditivo en pinturas adhesivos, plásticos, retardantes de llama e implantes quirúrgicos. Se estima que entre 1 y 2 millones de toneladas de PCBs fueron manufacturadas a escala mundial durante los años en los que tuvo lugar su producción y comercialización (*7-8*). No obstante cabe mencionar que los PCBs también pueden llegar indirectamente al medio, como productos secundarios en la síntesis de compuestos clorados, como clorobenceno o alcanos (*9*).

De los 209 posibles congéneres, entre 140 y 150 se encuentran en las mezclas comerciales y son, por tanto, los que pueden llegar a encontrarse en el medio. Sin embargo, entre ellos, los más relevantes son aquellos caracterizados por su elevada persistencia y/o toxicidad. Los organismos internacionales de seguridad ambiental han propuesto diversas listas de congéneres prioritarios. La más aceptada es la del BCR (Community Bureau of Reference), que engloba a 7 congéneres prioritarios, también denominados "indicadores": PCBs 28, 52, 101, 118, 138, 153 y 180 (*10*). Los congéneres más tóxicos son los llamados PCBs coplanares o *dioxin-like* (PCBs 81, 77, 126, 169, 105, 114, 118, 123, 156, 157, 167 y 189) (*11*). Estas dos series de congéneres son las habitualmente determinadas en muestras ambientales bióticas y de alimentos.

Propiedades físico-químicas

Los PCBs, que son sólidos a temperatura ambiente, exhiben una baja tensión de vapor, baja solubilidad en agua, alta estabilidad térmica y son sensibles a la exposición solar y ultravioleta. Son resistentes a ácidos, bases y otros agentes corrosivos, siendo muy complicada su descomposición por métodos que no sean muy enérgicos o involucren catalizadores. Esta gran estabilidad física y química es la responsable de su elevada persistencia en el medio una vez que se ha producido una contaminación accidental. Asimismo, son muy apolares, lo que favorece su bioacumulación en los tejidos adiposos de los organismos vivos, en los que experimentan procesos de bioconcentración y biomagnificación una vez ingresan en las cadenas tróficas. Todo ello justifica su amplia diseminación en el medio, que sean considerados contaminantes ubicuos y que, incluso hoy en día, aún se detecten concentraciones importantes en los distintos substratos ambientales (*12-13*).

Toxicidad. Vías de exposición

Como ya se ha mencionado, los efectos tóxicos asociados a los PCBs son congénere-dependiente. Es decir, no todos los congéneres presentan el mismo grado de toxicidad, estando íntimamente relacionada con su estructura química. La sustitución sobre las posiciones libres de los anillos bencílicos marca la disposición espacial que pueda adoptar la molécula. Son los derivados no-*orto* y mono-*orto* los que permiten alcanzar una disposición semiplana a la molécula, lo que la hace capaz de desarrollar un mecanismo de acción semejante a la de los PCDD/Fs. Éstos congéneres coplanares o *dioxin-like* (ver lista más arriba) son los que presentan una mayor toxicidad y, por tanto, los de mayor interés desde un punto de vista toxicológico, ambiental y químico.

Su toxicidad deriva de la capacidad para interaccionar con el receptor celular arilhidrocarbonasa (AhR) formando un dímero receptor-PCB capaz de penetrar en los núcleos celulares, donde se disocia en dos subunidades para posteriormente unirse a otra proteína (*14*) y, en último término, interaccionar con el ADN tras la activación en la síntesis de ciertos citocromos (P-4501A1) y de sus actividades monooxigenasas dependientes (*15*). La Figura 1.2 muestra un ejemplo de la interacción de dos PCBs coplanares, 77 y 169, con los receptores celulares de una proteína y un anticuerpo.

La toxicidad relativa de cada uno de los congéneres coplanares viene definida por un factor de equivalencia tóxica (TEF), que es una medida de la capacidad de un determinado congénere de PCB para la activación, *in vivo* o *in vitro*, del receptor AhR. Es una medida de activación relativa puesto que es evaluada respecto al congénere de dioxina de mayor toxicidad, la 2,3,7,8-TCDD, a la que se le asigna un valor arbitrario de 1. La Tabla 1.2 resume los valores de TEF asignados a cada congénere de PCB por la organización mundial de la salud (OMS) en 1998 (*18*) para distintas especies y los actualizados para mamíferos en 2005 (*11*).

El concepto de TEF asume que la carga tóxica asociada a los diferentes congéneres de cada una de estas familias de contaminantes (PCBs, PCDDs y PCDFs) es aditiva, de manera que el total de equivalentes tóxicos (TEQs) de 2,3,7,8-tetraCCD en una muestra puede calcularse según la ecuación:

$$TEQ = \sum([PCDD_i] \times TEF_i) + \sum([PCDF_j] \times TEF_j) + \sum([PCB_k] \times TEF_k)$$

El valor total así obtenido se considera como un valor de referencia para la cuantificación de la carga tóxica total debida a estas familias de xenobióticos en los distintos substratos ambientales, de tal forma que, a partir de estos datos, es posible

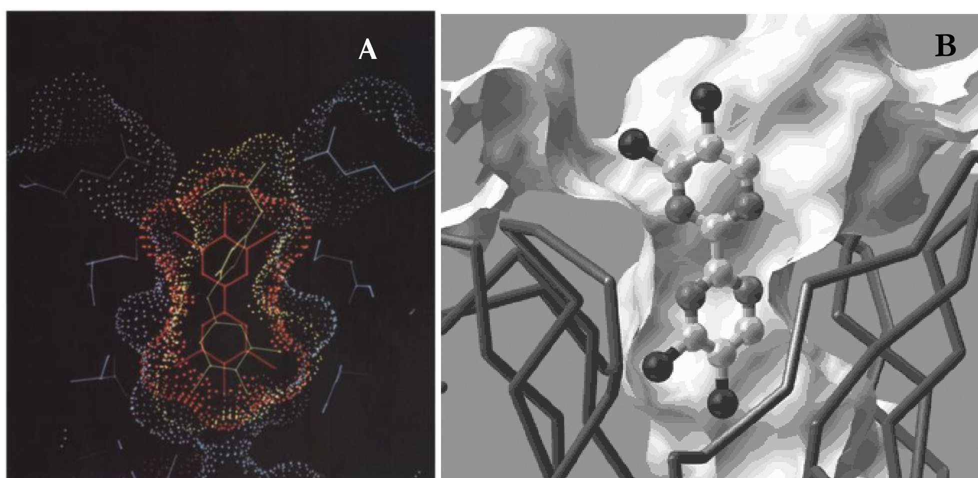

Fig. 1.2. (A) Representación tridimensional del PCB 169 y el complejo tirosina obtenido por rayos X con dominio de unión con la proteina humana prealbúmina (uniones azules) (*16*). (B) Modelo estructural de la unión entre el anticuerpo S2B1 (los tubos grises oscuros representan el esqueleto carbonado y la superficie blanca corresponde a los sitios activos de unión) y el PCB 77 (átomos de cloro en negros). La unión antígeno-anticuerpo se ve favorecida en este caso por los átomos de carbono en posición *orto* libres (en gris oscuro) (*17*).

Tabla 1.2. TEFs asignados a los distintos congéneres tóxicos de PCBs en diferentes especies animales por la OMS en 1998 (*18*) y los actualizados para mamíferos en 2005 (subrayado) (*11*).

		TEF_{98}			**TEF_{05}**
Congénere	**Estructura química**	**Aves**	**Peces**	**Mamíferos**	**Mamíferos**
2,3,7,8–TCDD	2,3,7,8	1	1	1	1
PCB 81	3,4,4´,5	0,1	0,0005	0,0001	0,0003
PCB 77	3,3´,4,4´	0,05	0,0001	0,0001	0,0001
PCB 126	3,3´,4,4´,5	0,1	0,005	0,1	0,1
PCB 169	3,3´,4,4´,5,5´	0,001	0,00005	0,01	0,03
PCB 105	2,3,3´,4,4´	0,0001	<0,000005	0,0001	0,0003
PCB 114	2,3,4,4´,5	0,0001	<0,000005	0,0005	0,0003
PCB 118	2,3´,4,4´,5	0,00001	<0,000005	0,0001	0,0003
PCB 123	2´,3,4,4´,5	0,00001	<0,000005	0,0001	0,0003
PCB 156	2,3,3´,4,4´,5	0,0001	<0,000005	0,0005	0,0003
PCB 157	2,3,3´,4,4´,5´	0,0001	<0,000005	0,0005	0,0003
PCB 167	2,3´,4,4´,5,5´	0,00001	<0,000005	0,00001	0,0003
PCB 189	2,3,3´,4,4´,5,5´	0,00001	<0,000005	0,0001	0,0003

establecer MRLs en las distintas matrices que permitan controlar la exposición a dichos contaminantes en el ser humano y los distintos substratos ambientales.

Para aquellos congéneres cuya conformación espacial no es plana, las alteraciones no afectan a nivel transcripcional del ADN, sino que se asocian, en general, a desórdenes en los sistemas endocrino, reproductor y respiratorio de los seres vivos.

Además, la capacidad teratogénica, mutagénica y oncogénica de los PCBs varía en función de la especie animal. La Tabla 1.3 resume alguna de las patologías asociadas a la acción tóxica de los PCBs no coplanares en diferentes especies animales (*19*), si bien la lista no es cerrada y nuevos estudios basados en el uso de biomarcadores y técnicas matemáticas avanzadas contribuyen a completarla día a día (*20-22*).

En general, la principal vía de exposición a PCBs para los seres vivos es la alimentación (alrededor del 90%) (*23*). Otras vías, como la inhalación o la exposición cutánea, tienen una incidencia menor, salvo en el caso de exposiciones ocupacionales y accidentales, como las que pueden suceder durante reparaciones y mantenimientos de equipos que contengan PCBs, derrames involuntarios o incendios.

Tabla 1.3. Patologías descritas para los congéneres no coplanares de PCBs en diferentes seres vivos (*19*).

Especies / Patologías	Rata	Ratón	Cerdo	Mono	Pollo	Humanos
Pérdida de peso	×	×	×	×	×	×
Acne/Alopecia					×	×
Edema		×		×	×	×
Atrofia linfática	×	×	×	×	×	
Hepatomegalia	×	×	×	×	×	×
Necrosis	×	×	×	×	×	×
Hipertrofia vesícula urinaria			×			
Inducción tumoral	×	×				

Legislación

A partir de los años 70 se iniciaron medidas legales para controlar y reducir paulatinamente las emisiones de PCBs y PCDD/Fs al medioambiente. Entre ellas, cabe destacar las relativas a la incineración de residuos (Directiva 2000/76/EC (*24*)), la adoptada para la prevención de efectos co-laterales en accidentes industriales fortuitos (Directiva 96/82/CE (*25*)), las directivas marco sobre aguas (Directiva 2000/60/EC (*26*)) y aquellas relativas a las restricciones de comercialización y eliminación de residuos químicos que contengan PCBs (Directivas 85/467/EEC y 96/59/EC respectivamente (*27-28*)).

El establecimiento de los TEQs de los PCBs coplanares llevó a la elaboración de diversas regulaciones que fijaban los contenidos máximos permitidos de PCBs en matrices destinadas, preferentemente y de manera directa o indirecta, al consumo humano. La primera directiva europea (Reglamento 466/2001 (*29*)) sobre el contenido máximo de dioxinas y compuestos *dioxin-like* y la pertinente recomendación sobre la frecuencia de muestreo y análisis (Directiva 2004/705/EC (*30*)) fueron sustituidas en 2006 por las vigentes hasta finales de 2011 (Directiva 2006/794/EC (*31*) y 1881/2006 (*32*)), que fijan los controles y niveles máximos permitidos de PCBs en varios alimentos grasos (carnes, pescado, leche y huevos, entre otros). Esta legislación ha sido recientemente modificada, adoptándose nuevos valores máximos para dioxinas, furanos y PCBs en alimentos destinada al consumo animal y humano que entrará en vigor a partir de enero de 2012 (*33*).

El número mínimo de muestreos anuales requerido varía en función del país, al estar directamente asociado con la producción industrial del mismo. Con un mínimo de 150 muestras analizadas/año, España es uno de los países con mayor número de controles. A grandes rasgos, los niveles máximos permitidos para la suma de PCBs y PCDD/Fs varían entre 1,5 y 12 pg/g de TEQs en peso graso del producto alimenticio considerado.

Otras directivas hacen referencia al contenido máximo de dichos contaminantes en alimentación destinada al consumo animal (Directiva 2006/13/EC (*34*)) y a los métodos de muestreo y análisis a aplicar en estos estudios de monitorización (*35*).

Por último citar que, de acuerdo con el Anexo A -parte II- del Convenio de Estocolmo, los países miembros acordaron eliminar antes del año 2025 todos los equipos y aceites en uso que contengan PCBs, y gestionar el tratamiento de los desechos generados antes de 2028. Para ello, se ha impulsado la creación de un grupo de seguimiento que facilite la información en relación a dichas obligaciones y gestiones (*36*).

Polibromodifenil éteres

Los PBDEs son una familia de compuestos bromados de fórmula empírica $C_{12}H_{10-n}Br_nO$ ($n=x+y$; y $1<n<10$), cuya estructura base consta de dos anillos aromáticos sustituidos por átomos de bromo pero, a diferencia de los PCBs, la unión entre dichos anillos se produce mediante un enlace éter C-O-C (Fig. 1.3).

Al igual que los PCBs, existen 209 posibles congéneres de PBDEs, que se subdividen en diez grupos de homólogos (de mono- a deca-PBDEs). El número de posibles isómeros de posición o congéneres de cada grupo de homólogos y su nomenclatura son similares a las adoptadas para los PCBs (Tabla 1). Los pesos moleculares, sin embargo, oscilan entre 482 y 950 u.m.a.

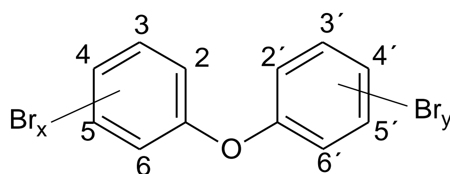

Fig. 1.3. Estructura general de los PBDEs.

A medida que los PCBs fueron retirados progresivamente de la actividad industrial, empezaron a comercializarse nuevas formulaciones de compuestos, entre las que se encontraban los PBDEs. Fue a finales de los años 70 cuando se comenzaron a introducir los PBDEs, aunque su auge comercial no llegó hasta finales de los años 90, con la expansión del sector electrónico, en el que se empleaban como aditivos retardantes de llama (*37*).

Los PBDEs se sintetizan a escala industrial por bromación directa del bifeniléter en presencia de tribromuro de aluminio o hierro como catalizadores (*38*). Según el foro BSEF (Bromine Science and Environmental Forum), la demanda de mercado para el año 2001 se estimó en más de 65.000 toneladas (*39*), siendo Asia y América los continentes con mayor consumo. Como consecuencia de esta evolución comercial, la concentración de PBDEs en muestras ambientales ha sufrido un aumento considerable y progresivo en los últimos años, llegando hoy día a alcanzar niveles detectables en todos los compartimentos ambientales (suelos, peces, mamíferos, aves y humanos) (*40*).

Por su alta estabilidad química y térmica, los PBDEs se han empleado sobre todo como aditivos retardantes de llama para prevenir la ignición o ralentizar las fases iniciales de los procesos de combustión. En este sentido, presentan una excepcional eficiencia en la captura de los radicales libres que se originan durante el proceso de ignición y que son imprescindibles en la propagación del fuego. En general, todos los halógenos son efectivos en la eliminación de los radicales libres, si bien los compuestos bromados y clorados son los que poseen las mejores propiedades. Entre ellos, son los retardantes de llama bromados los que por su alta eficiencia y resistencia térmica (la temperatura de ebullición está comprendida entre 301 y 425 ºC) se han empleado en mayor medida en la industria, donde llegan a representar más del 40% del total de los retardantes empleados. Sin embargo, al contrario que los PCBs, no todos los congéneres de PBDEs han sido sintetizados a nivel industrial, siendo las denominadas mezclas penta-, octa- y deca-bromadas las más comunes. Así, sólo 69 congéneres de los 209 posibles han sido identificados en las mezclas comerciales (Tabla 1.4). De estos, los congéneres más frecuentemente detectados en muestras ambientales son los PBDEs 28, 47, 99, 100, 153, 154, 183 y 209 (*41*).

Tabla 1.4. Composición porcentual (%) de las mezclas comerciales de PBDEs (*47*).

Mezcla comercial	Tetra-BDE	Penta-BDE	Hexa-BDE	Hepta-BDE	Octa-BDE	Nona-BDE	Deca-BDE
Penta-BDE	24-38	50-60	4-8				
Octa-BDE			10-12	43-44	31-35	9-11	<1
Deca-BDE						0.3-3	97-98

Propiedades físico-químicas

Los PBDEs son, al igual que otros COPs, sustancias con altos coeficientes de partición K_{ow} (entre 4 y 9) aumentando este valor con el grado de bromación de la molécula (*42*). Asimismo, los PBDEs presentan una baja presión de vapor, elevada estabilidad térmica y sensibilidad a la exposición ultravioleta. Presentan una resistencia elevada a ácidos, bases y otros agentes corrosivos, lo que justifica su elevada persistencia una vez que son vertidos al medio. Por su elevado carácter apolar, al igual que los PCBs, tienden a acumularse y concentrarse en los tejidos adiposos de los seres vivos y, por sus características físicas y químicas, una vez que ingresan en las cadenas tróficas, también experimentan procesos de biomagnificación.

Toxicidad. Vías de exposición

Como sucede en otras familias de COPs, no todos los congéneres de PBDEs exhiben el mismo grado de toxicidad. Diferentes estudios han concluído que la exposición de cultivos primarios a mezclas de PBDEs inducen apoptosis y necrosis (*43*) y que, de las tres mezclas comerciales, la mezcla penta- parece ser la de mayor toxicidad (*44-45*).

Los principales efectos de los PBDEs observados en animales se refieren a su actividad como disruptores endocrinos. Todas las mezclas técnicas han mostrado actividad disruptora del tiroides por unión a sus receptores hormonales (*39*). Además, llegan a alterar las funciones hepáticas debido a desequilibrios en la producción de hormonas tiroideas y de vitamina A, siendo la eliminación de la hormona tiroidea T4 la que causa mayores disfunciones en el organismo (*46*).

La mezcla penta-BDE también parece influir en el desarrollo del aparato reproductor provocando disformismos sexuales que ocasionan retraso en la llegada de la pubertad y la disminución de la formación de folículos (*48*). También se ha descrito

la unión de esta mezcla de PBDEs al receptor andrógeno, lo que resulta en una interferencia en el desarrollo de los tejidos reproductivos masculinos, con disminución en el tamaño de los mismos (*49*).

La exposición perinatal a PBDEs provoca efectos similares a los observados para los PCBs, con alteraciones en el desarrollo intelectual y, además, disfunciones neurológicas (*50*). Además, se han detectado comportamientos especiales para el PBDE 209 cuyos efectos nocivos en neonatos se agravan con el avance del estado gestacional (*51*).

Aunque se han observado efectos carcinogénicos en animales para la mezcla deca-BDE, los datos referentes a humanos son aún escasos (*52*). Hasta la fecha, y a pesar de la analogía estructural de los PBDEs con las PCDD/Fs, no existe acuerdo entre los autores sobre la posible interacción de estos xenobióticos con el receptor AhR. Sin embargo, sí parece claro que, de existir, sería al menos seis veces inferior a la descrita para la 2,3,7,8-TCDD (*53*). Por este motivo, hasta el momento no se les ha asignado un valor de TEF (*54*). En cualquier caso, se estima que el nivel más bajo para el cual estos contaminantes presentan efectos adversos (Lowest Observed Adverse Effect Level, LOAEL) es de 0,8 mg/Kg de peso corporal/día (*55*).

Las principales vías de exposición a PBDEs en humanos son la dieta, la exposición en espacios cerrados y el polvo doméstico (*56*). Entre los productos alimenticios, los aceites, pescados, carnes y huevos son los alimentos con mayores niveles de PBDEs y, por tanto, de mayor relevancia para la evaluación de su incidencia en humanos (*57*).

Legislación

La presencia de PBDEs en los diferentes substratos ambientales y en el hombre (en 2005 se publicó un artículo científico en el que se mencionaba la mayor concentración de PBDEs detectada en tejido adiposos humano hasta la fecha, 9,63 ng/g (*58*)), unido a su demostrada persistencia y toxicidad, impulsaron a que distintas organizaciones gubernamentales de protección ambiental emprendieran acciones legales para regularizar su uso y producción.

Ya en 2001 la Comisión para la protección del medio marino del Noreste Atlántico (OSPAR) tomó las primeras medidas de acción para la no propagación de

los PBDEs en el medio marino (*59*). A ellas se sumó una directiva europea cuyo objetivo era crear un marco comunitario de actuación en el ámbito de la política de aguas y que establecía una lista de sustancias prioritarias, entre las que se encontraban los penta-BDEs (*60*). En enero de 2003, la directiva 2002/95/EC (*61*) fijó el 1 de Julio de 2006 como fecha límite para el cese en la utilización de las mezclas penta- y octa-bromada de PBDEs en equipos electrónicos y eléctricos. Poco después, la directiva 2003/11/EC prohibió, mediante una enmienda paralela, el uso general de las mezclas penta- y octa-bromadas a partir de agosto de 2004. No obstante, ante la imposibilidad de cumplir con los plazos inicialmente previstos, en 2005 la directiva 2002/95/EC fue sustituida por la decisión 2005/717/EC que modificaba el anexo anterior y restringía el uso del congénere deca-bromado en aplicaciones poliméricas (*62*). En 2006, el Parlamento Europeo junto con las comisiones de supervisión presentaron una demanda en la Corte Europea de Justicia para que revisasen las directivas anteriores respecto a la utilización de la mezcla decabromada. Esta demanda fue aceptada y, así, la directiva 2008/C 116/02 menciona específicamente que, hoy día, existen otras formulaciones químicas que podrían sustituir a dicha mezcla en sus aplicaciones industriales (*63*). La Agencia para el control de sustancias tóxicas y enfermedades relacionadas (Agency for Toxic Substances and Disease Registry, ATSDR), fijó en 2004 como MRL para la exposición humana a los PBDEs una dosis diaria máxima de 7 μg/Kg de peso seco/día.

Por último, cabe mencionar que las mezclas tetra-, penta-, hexa- y hepta-bromadas fueron incluidas en el anexo A del Convenio de Estocolmo (referente a sustancias cuyo uso y producción debe ser eliminados de los procesos industriales) durante su cuarta reunión, que tuvo lugar en 2009 en Génova.

Otros COPs de relevancia medioambiental

Policlorodibenzo-*p*-dioxinas y policlorodibenzofuranos

Las PCDDs y PCDFs son dos familias de éteres aromáticos cuya estructura básica consta de dos anillos de benceno unidos entre sí por un heterociclo que contiene un atómo de oxígeno, en el caso de los PCDFs, y dos átomos de oxígeno, en el caso de las PCDDs (Fig. 1.4). Las posiciones libres sobre los anillos pueden ser ocupadas por átomos de cloro, con un total de 75 posibles isómeros de PCDDs y 135 para el caso de los PCDFs.

La rigidez que aporta el heterociclo a la molécula impide la rotación de los anillos de benceno y confiere a todos los congéneres de ambas familias una estructura espacial plana, lo que justifica que tengan propiedades físico-químicas muy parecidas a las de los PCBs coplanares y, en menor medida, a las de los PBDEs.

En general, las PCDD/Fs son sólidos cristalinos de color blanco con puntos de fusión y ebullición elevados. Presentan una gran estabilidad física, química y biológica, si bien su degradación térmica se ve favorecida en presencian de otros compuestos clorados. Al igual que todos los COPs, son apolares y se acumulan en el tejido adiposo de los organismos vivos, en el que tienden a bioconcentrarse.

Las PCDD/Fs se generan en pequeñas concentraciones, y de manera no intencionada, como subproductos de procesos industriales y naturales (principalmente combustiones), no habiéndose sintetizado nunca con fines industriales y/o comerciales, a excepciones de los patrones analíticos empleados en investigación.

Las fuentes principales de emisión natural de PCDD/Fs son los incendios forestales, las erupciones volcánicas y algunas reacciones enzimáticas y fotolíticas. Sus principales fuentes antropogénicas son los procesos de combustión (incineradoras, motores), ciertos procesos químicos e industriales (producción de compuestos

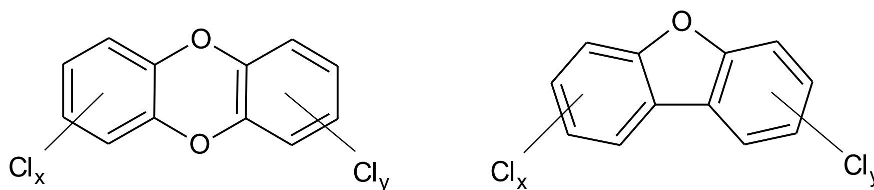

Fig. 1.4. Estructura general de las PCDDs y PCDFs.

organoclorados y otros retardantes de llama, blanqueo de la pasta de papel con cloro, la industria eléctrica y textil y la fabricación de pinturas y metales). Pero también pueden formarse de manera accidental y verterse junto con los productos de desecho generados en vertederos, depuradoras y potabilizadoras (*64*).

A pesar de que todos los congéneres de PCDDs y PCDFs se consideran planos, los derivados 2,3,7,8-sustituidos son los que presentan una mayor toxicidad y, por tanto, un mayor interés analítico. Los congéneres 2,3,7,8-sustituidos son además los únicos presentes en muestras biológicas. Como ya se ha mencionado antes, su toxicidad deriva de su capacidad para interaccionar con el receptor celular AhR. La 2,3,7,8-TCDD está clasificada por la agencia internacional de investigación sobre cáncer (International Agency for Research on Cancer, IARC) como un agente cancerígeno en humanos del grupo 1. El resto de las PCDDs y PCDFs están clasificadas dentro del grupo 3; es decir, como no carcinogénicos en humanos. Otros efectos de las PCDD/Fs en humanos incluyen alteraciones en los sistemas nervioso y endocrino, manifestaciones cutáneas severas (enrojecimiento de la piel y cloroacné entre otros) y trastornos psíquicos, si bien muchos de estos efectos pueden desaparecer al finalizar la exposición.

Tanto la OMS (*23*) como las directivas europeas antes expuestas para los PCBs, y que asignan valores máximos de ingesta en muestras biológicas para el consumo humano y animal, incluyen valores máximos permitidos para estas dos familias de COPs (*32, 34*).

Diclorodifeniltricloroetano y sus metabolitos

El *p,p´*-DDT, o simplemente DDT, es un insecticida organoclorado que fue profusamente empleado durante la Segunda Guerra Mundial para proteger a las tropas y a los civiles contra la malaria, el tifus y otras enfermedades transmitidas por insectos. En años posteriores, también fue empleado de forma masiva en multitud de cultivos agrícolas con el fin de controlar las plagas y ciertas enfermedades de expansión vectorial.

El DDT es insoluble en agua y semivolátil y presenta gran facilidad para liberarse a la atmósfera y transportarse en ella. Se considera un contaminante ubicuo habiendo sido detectado incluso en el Ártico (*65*). Al igual que otros COPs, tiende a acumularse

en los tejidos grasos de los seres vivos, donde experimenta fenómenos de bioacumulación. A pesar de su estabilidad (la vida media del DDT oscila entre 8 y 10 años), el DDT puede sufrir degradaciones transformándose en sus metabolitos principales: el 2,2-bis(4-clorofenil)-1,1-dicloroeteno, *p,p´*-DDE, y 2,2-bis(4-clorofenil)-1,1-dicloroetano, *p-p´*-TDE, (Fig. 1.5), que también son ubicuos y exhiben, en el caso del DDE, una elevada persistencia en el medio y una toxicidad incluso superior a la del DDT.

Los efectos asociados a la exposición al DDT se relacionan con supresiones del sistema inmune, posiblemente por una depresión humoral de la respuesta inmune. Tanto el DDT como el DDE han mostrado actividad disruptora endocrina, al mimetizar la acción de las hormonas endógenas y bloquear los sitios activos de unión con los receptores correspondientes.

También se han observado alteraciones de la membrana nerviosa provocando movimientos involuntarios, calambres y convulsiones. No obstante, sus efectos más negativos se producen por exposición perinatal, cuando provoca alteraciones en los niveles de estrógenos que inducen alteraciones del desarrollo reproductivo, y se asocia con un aumento en el riesgo de padecer cáncer de pecho.

El DDT está considerado como posible carcinógeno humano (grupo 2B) por la IARC y se encuentra incluido desde 2001 en el anexo A del Convenio de Estocolmo (sustancias químicas consideradas para su eliminación).

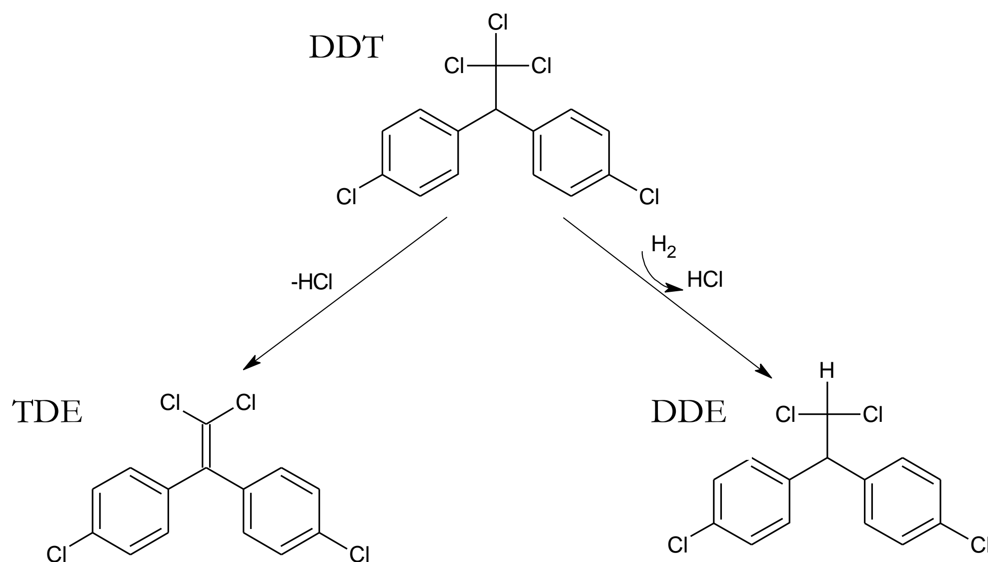

Fig. 1.5. Estructuras generales del DDT y sus principales metabolitos, TDE y DDE.

Las primeras prohibiciones de uso de DDT comenzaron en Estados Unidos en 1972, tras las primeras evidencias de toxicidad y la publicación del libro de Rachel Carson "*Silent Spring*". En Europa, las restricciones a su comercialización y uso comenzaron en 1979, con el convenio sobre la contaminación atmosférica transfronteriza asociada a ciertos contaminantes orgánicos, ratificado en el año 2004 (*66*). No obstante, directivas previas ya habían abordado el control de sus niveles en aguas y su uso como pesticidas (Directivas 76/464/EC y 79/117/EC, respectivamente (*67-68*)).

Otros pesticidas organoclorados

Bajo la denominación de pesticidas organoclorados se engloban ciertos compuestos como el aldrín, clordano, dieldrín, endrín y mírex que han sido ampliamente empleados como insecticidas para el control de plagas producidas por insectos como las termitas, saltamontes o gorgojos, y de otras especies como gusanos y ratas de campo en cosechas agrícolas de vegetales, cereales, maíz, semillas oleaginosas, patatas, caña de azúcar, frutas, algodón, frutos secos y yute. Como se observa en la Figura 1.6, estos compuestos presentan una estructura química bastante similar entre sí, con una estructura base bicíclica sustituidas por átomos de cloro en sus posiciones libres.

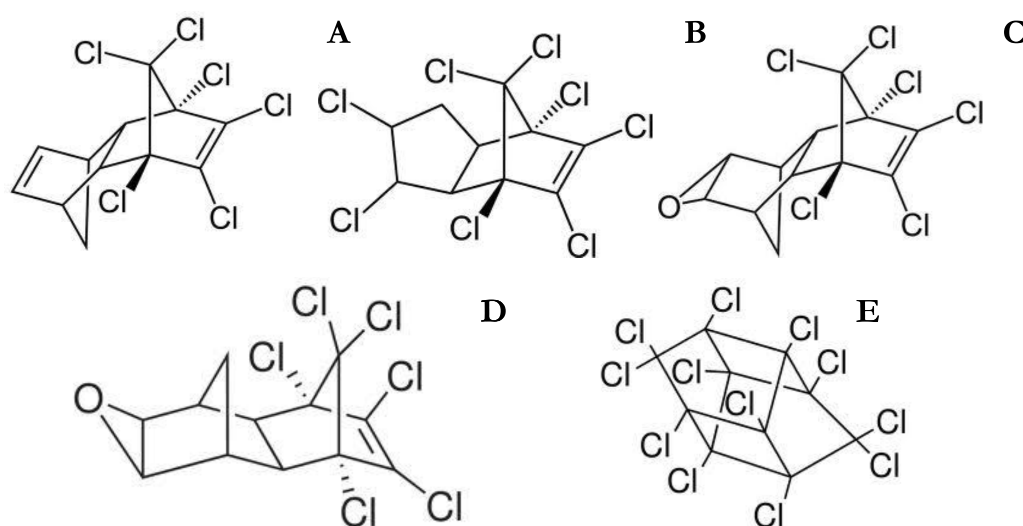

Fig. 1.6. Estructuras generales de distintos pesticidas organoclorados: (A) aldrín, (B) clordano, (C) dieldrín, (D) endrín y (E) mírex.

A pesar de la alta estabilidad de estos compuestos, algunos de ellos pueden biotransformarse en otros compuestos químicamente similares por vías metabólicas sencillas. Este es el caso del aldrín, que se biotransforma en dieldrín de manera rápida en el hígado, siendo los mecanismos de toxicidad aguda y crónica de ambos compuestos muy similares (*69*). En general, todos estos pesticidas organoclorados son insolubles en agua, ubicuos y tienden a acumularse sobre los tejidos adiposos de los organismos vivos.

Otros insecticidas de acción no sistémica que se incluyen dentro de esta familia y, que han sido catalogados por la IARC como posibles carcinógenos en humanos (grupo 2B), son los toxafenos y el heptacloro, también recogidos dentro del Convenio de Estocolmo (Fig. 1.7). Las mezclas comerciales del toxafeno pueden llegar a contener más de 700 compuestos en función del grado de cloro-sustitución del biciclo (entre 6 y 10 átomos de cloro).

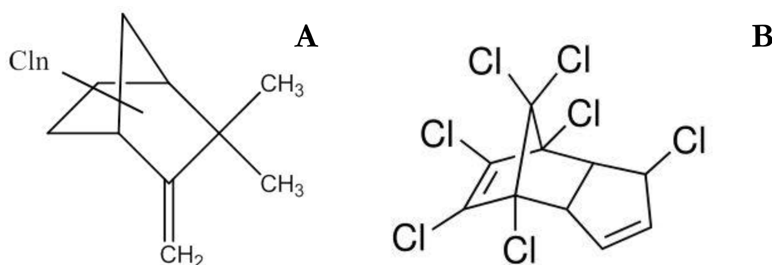

Fig. 1.7. Estructura general de (A) un bornano policlorado (toxafeno) y (B) heptacloro.

Como sucede con otros COPs, la principal vía de exposición a estos plaguicidas en humanos es la ingesta de alimentos que hayan estado expuestos a estos contaminantes y/o exposición a zonas contaminadas con los mismos.

1.1.2. Otros compuestos orgánicos de interés ambiental

Hidrocarburos aromáticos policíclicos

Los hidrocarburos aromáticos policíclicos (PAHs) son un grupo de contaminantes químicos que se originan durante la combustión incompleta de compuestos orgánicos como carbón, aceites, grasas, madera, gas, tabaco o en procesos de pirólisis a altas temperaturas (cercanos a los 700 ºC). Existen más de 100 PAHs, aunque en general se encuentran en la naturaleza como mezclas cuya composición varía según su origen.

La estructura química de los PAHs está formada por condensación de varios anillos aromáticos que pueden existir en distintas disposiciones isoméricas. Los PAHs de menor peso molecular detectados en la naturaleza están formados por condensación de 2 anillos, aunque se han llegado a detectar isómeros de hasta 8 anillos (Tabla 1.5).

Los PAHs son en su mayoría sólidos cristalinos con diferentes formas (laminadas, agujas, prismas), con ausencia o presencia de color (en general, suelen ser blancos o amarillo-verde pálido) y tienen puntos de ebullición altos y baja solubilidad en agua. Aunque normalmente son compuestos generados de manera involuntaria en reacciones naturales, algunos de ellos (como el antraceno, fluoreno, fenantreno y fluorantreno), aparte de para uso científico, son comercializados para aplicaciones en medicina, asfaltos, carbón y como aditivo en tintes, plásticos y pesticidas.

Los PAHs ingresan principalmente en el medioambiente por liberación al aire en erupciones volcánicas, incendios forestales, fugas naturales accidentales de petróleo, quema doméstica de madera; pero también por la actividad de las industrias de acero y metales y el tráfico. Los suelos también pueden verse afectados a través de vertidos de plantas industriales y de plantas de tratamiento de aguas.

Al igual que todos los COPs previamente revisados, los PAHs son poco solubles en agua y suelen encontrarse sobre superficies sólidas como pequeñas partículas aisladas. Pueden viajar a través de la atmósfera, lo que favorece su ubicuidad en el medio. Han sido detectados en los distintos compartimentos medioambientales,

Tabla 1.5. Nombre y estructura de los PAHs más frecuentemente monitorizados en muestras ambientales según el Comité Científico Europeo para el control de los alimentos (Scientific Committee for Food, SCF), la Agencia de Protección Medioambiental (Environmental Protection Agency, EPA) y la Unión Europea (UE).

Lista	***Nombre común***	***Estructura química***	***Lista***	***Nombre común***	***Estructura química***
EPA	Naftaleno		EPA	Acenafteno	
EPA	Fluoreno		EPA	Acenaftileno	
EPA	Antraceno		EPA	Fenantreno	
EPA	Fluoranteno		UE	Benzo[*c*] fluoreno	
EPA SCF UE	Benzo[*a*] antraceno		EPA SCF UE	Criseno	
EPA	Pireno		SCF UE	5-Metilcriseno	H_3C
EPA SCF UE	Benzo[*b*] fluoranteno		SCF UE	Benzo[*j*] fluoranteno	
EPA SCF UE	Benzo[*k*] fluoranteno		SCF UE	Ciclopenta [*cd*]pireno	
EPA SCF UE	Benzo[*a*] Pireno		EPA SCF UE	Dibenzo[*a,h*] antraceno	
EPA SCF UE	Indeno[1,2,3 -*cd*]pireno		EPA SCF UE	Benzo[*ghi*] perileno	
SCF UE	Dibenzo[*a,e*] pireno		SCF UE	Dibenzo[*a,h*] pireno	
SCF UE	Dibenzo[*a,i*] pireno		SCF UE	Dibenzo[*a,l*] pireno	

aunque las concentraciones observadas en suelos y organismos vivos presentan varios órdenes de magnitud superior a las encontradas en agua o aire (*70-72*).

Estudios toxicológicos con mezclas de contaminantes que incluían algunos PAHs (benzo[*a*]pireno, benzo[*a*]atraceno, benzo[*b*]fluoranteno y dibenzo[*a,h*]antraceno y criseno entre otros) han asociado el incremento de la mortalidad por cáncer de pulmón en humanos con la exposición a emisiones de coque y de cubiertas de alquitrán, y al humo de los cigarrillos. Sin embargo, no se pudo definir la carga tóxica asociada a los PAHs debido a la variedad de compuestos con propiedades cancerígenas de la mezcla investigada (*73*). No obstante, diversos estudios han proporcionado evidencias del potencial carcinogénico de los PAHs individuales antes mencionados (*74-76*). Así, la IARC ha clasificado a varios PAHs como carcinógenos humanos, desde constatados (grupo 1) a probables (grupo 2A), posibles (grupo 2B) y no verificados (grupo 3) (*77*). Algunos PAHs son también capaces de actuar como disruptores endocrinos e inducir alteraciones en el sistema inmune y reproductivo de los seres vivos, siendo sus efectos menos pronunciados en presencia de luz ultravioleta (*78*).

A pesar de que los PAHs no están incluidos en el Convenio de Estocolmo (no están considerados como COPs porque sufren procesos de metabolización una vez que ingresan en los organismos), fueron clasificados como contaminantes atmosféricos transfronterizos de largo alcance por la Comisión Económica Europea de las Naciones Unidas (UNECE) en 1979 (*66*), y distintas directivas europeas han regulado su producción, emisión y control en alimentos desde el año 2000. La Directiva 2004/107/EC fue la primera que limitó las emisiones de PAHs al aire y consideraba al benzo[*a*]pireno uno de los congéneres con mayores propiedades mutagénicas y teratogénicas (*79*). Directivas previas ya habían limitado la cantidad máxima de dos isómeros de PAHs (benzo[*a*]pireno y benzo[*a*]antraceno) en humos de tabaco (*80*), aunque no fue hasta 2006 cuando el SCF publicó la primera lista recomendando los isómeros cuyos niveles debían ser controlados regularmente en productos alimenticios (*81*). También la EPA y la UE han publicado sus propias listas de PAHs prioritarios, denominadas las listas de los 16 PAHs de la EPA y de los 15+1 de la UE, respectivamente, para el control de los niveles ambientales y en alimentos de esta familia de xenobióticos. En la UE, se publicó en 2006 una directiva común

para ciertos contaminantes químicos que fijaba niveles máximos de ingesta por alimentos, entre ellos el benzo[*a*]pireno, con niveles para alimentos grasos en el intervalo 1,0-10 μg/Kg de peso seco (*32*). Recientemente una nueva directiva europea publicó una enmienda a esta anterior legislación fijando nuevos niveles máximos para cuatro PAHs en alimentos, el benzo[*a*]pireno, benzo[*a*]antraceno, benzo[*b*]fluoranteno y el criseno. El cumplimiento de esta nueva directiva entrará en vigor en unos meses, septiembre de 2012, junto con la nueva regulación que establece los métodos de muestreo para análisis de benzo[*a*]pireno en alimentos (*82-83*).

Derivados metoxilados de polibromodifenil éteres

Los metoxi-polibromodifenil éteres (MeO-PBDEs) son metabolitos metoxilados de PBDEs que se forman por sustitución de un átomo de hidrógeno por un grupo metoxi. En general, estos compuestos de origen natural (halogenated naturally-products; HNPs) son sintetizados por esponjas (*Dysidea herbacea*) (*84*), algas verdes (*Cladaphora fasciculatis*) (*85*) y algas rojas como la *Ceramium tenuicorne* (*86*), aunque también se ha sugerido su síntesis por metilación de los PBDEs o de sus derivados hidroxilados por microflora intestinal y/o por metilación microbiana en sedimentos (*87*). La nomenclatura aplicada a estos compuestos es análoga a la utilizada para los PBDEs con la especificación de la posición del grupo metoxi en la molécula. De todos los congéneres de MeO-PBDEs existentes son dos, el 2´-MeO-BDE-68 y el 6-MeO-BDE-47 (Fig. 1.8), los de mayor incidencia en las muestras biológica, en las que llegan a representar hasta el 98% del total de los metabolitos metoxilados (*84*). Una mayor contribución del congénere 2´-68 apuntaría a las esponjas como fuente principal de dichos compuestos, mientras que la presencia mayoritaria del derivado 6-47 indicaría una predominancia de la síntesis por algas (*88*).

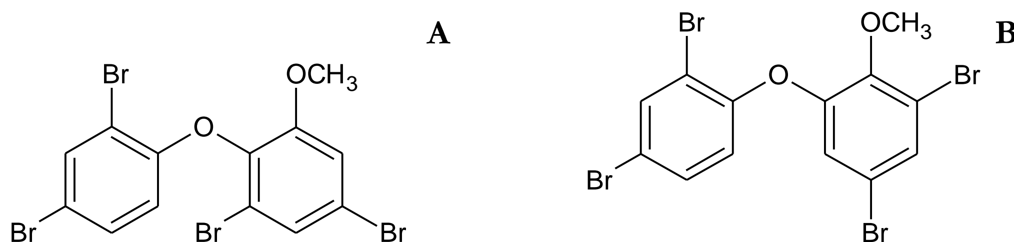

Fig. 1.8. Estructuras de los congéneres mayoritarios de MeO-PBDEs: 6-MeO-BDE-47 y (B) 2´-MeO-BDE-68.

Aunque todavía no hay demasiados ensayos toxicológicos que hayan evaluado la carga tóxica asociada a estos compuestos, algunos artículos científicos ya han mostrado ciertas alteraciones en las actividades aromatasas y en las expresiones de genes esteroidogénicos (*89*). No obstante, cabe mencionar que los derivados hidroxilados de los PBDEs (OH-PBDEs) tienen una mayor capacidad de activación a nivel genético que los correspondientes metoxilados.

Polibromohidroxantenos

En los últimos años se ha detectado en algunas especies acuáticas ciertos compuestos halogenados que, tras diferentes estudios de elucidación estructural, fueron identificados como derivados bromados de xantenos. Dentro de esta familia de polibromohexahidroxantenos (PBHDs), los congéneres más abundantes en las muestras hasta ahora analizadas han sido dos derivados tri y tetra-BHD, el 2,7-dibromo-4a-bromometil-1,1-dimetil-2,3,4,4a,9,9a-hexahidro-1H-xanteno y el 2,5,7-tribromo-4a-bromometil-1,1-dimetil-2,3,4,4a,9,9a-hexahidro-1H-xanteno (Fig. 1.9).

Al igual que los MeO-PBDEs, los PBHDs son compuestos de origen natural. Fueron detectados por primera vez como subproductos de la esponja *Cacospongia genus*, recolectada en Australia (*90*) y, más recientemente, en una esponja típica de la zona mediterránea, *Scalarispongia scalaris* (*91*). Los niveles detectados de estos compuestos en especies acuáticas son mayores que los del resto de compuestos organobromados habitualmente detectados en muestras ambientales, con concentraciones que, de manera puntual, han alcanzado las 17 ppm en peces de aguas profundas (*92*). Sin embargo, al ser compuestos de reciente descubrimiento, no hay todavía demasiada información respecto a su incidencia, su presencia en productos alimentarios o sus posibles efectos tóxicos.

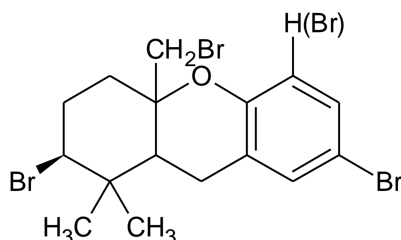

Fig. 1.9. Estructura general de los derivados PBHDs.

Tribromoanisol y monoterpernos halogenados

Dentro de este último grupo de HNPs se encuentran el 2,4,6-tribromoanisol (TBA) y un monoterpeno organohalogenado mixto denominado MHC-1. La elucidación de la estructura de este último tuvo lugar en 2008, tras ser aislado del alga marina *Plocamium cartilagineum* (*93*) (Fig. 1.10).

Los estudios toxicológicos para estos compuestos están aún en fases iniciales, si bien en 2005 se publicó un estudio en el que se analizaron dos compuestos halogenados emergentes, entre los que estaba el TBA, detectándose una leve pero medible actividad biológica para este último (*94*). Además, apuntar que estos compuestos están siendo detectados con frecuencia y en niveles similares a los de otros COPs (PCBs y PBDEs) en especies marinas, algunas de ellas destinadas al consumo alimentario (*91, 93*).

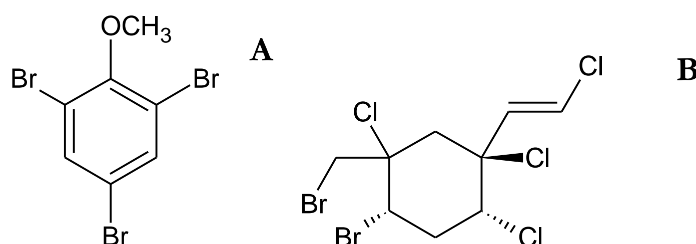

Fig. 1.10. Estructura química de los compuestos emergentes TBA y (B) MHC-1.

Finalmente, resaltar la reciente publicación de un revisión que resume las características físicas, químicas y metodológicas para más de diez nuevos compuestos organobromados que han sido utilizados en los últimos años como retardantes de llama y que se están detectando en distintos substratos ambientales (*95*).

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1.2. PREPARACIÓN DE MUESTRAS (SEMI-)SÓLIDAS PARA ANÁLISIS DE RESIDUOS DE CONTAMINANTES ORGÁNICOS

El gran cuello de botella, hoy día, en el análisis de contaminantes orgánicos a niveles traza en matrices ambientales y de alimentos, reside en aquellos procesos involucrados en el tratamiento de la muestra. Bajo la denominación de tratamiento o preparación de muestra se engloban una serie de etapas que incluyen el muestreo, el tratamiento y acondicionamiento preliminar de la muestra, la extracción de los analitos de la matriz en la que se encuentran, su aislamiento de otros compuestos presentes en el extracto y co-extraídos en la etapa anterior y su concentración previa al análisis instrumental. La complejidad de ciertas matrices ambientales y los bajos niveles a los que los compuestos orgánicos objeto de estudio en este trabajo suelen detectarse en muestras reales (en general, en concentraciones iguales o inferiores a las partes por billón, ppbs), han contribuido a que las metodologías convencionales de preparación de muestra empleadas en este tipo de estudios sean protocolos laboriosos y complejos que involucran un elevado número de etapas independientes, frecuentemente desarrolladas de forma manual, y que conllevan un elevado consumo de reactivos y disolventes orgánicos de elevada calidad. Se trata, por tanto, de procedimientos analíticos caros, tanto en términos de tiempo como de fungibles, y que suelen requerir de personal altamente cualificado. Además la continua manipulación de las muestras y extractos potencia el riesgo de contaminación y/o de pérdida de los analitos y, por tanto, contribuye a aumentar la probabilidad de error en la determinación del analito en cuestión.

En este apartado, se revisarán algunos de los aspectos más relevantes relacionados con la preparación de muestras para la determinación de residuos de COPs y otros contaminantes orgánicos de interés en matrices ambientales complejas. Se prestará especial atención a muestras biológicas (semi-)sólidas, como los tejidos animales y los alimentos grasos; y matrices abióticas (suelos y sedimentos) por ser las investigadas en este estudio.

1.2.1. Pretratamiento de muestra

Al margen de la necesidad de una primera etapa de muestreo que garantice la representatividad de la muestra objeto de estudio (tema que a pesar de su interés escapa al objetivo de esta memoria) existen una serie de operaciones asociadas al tratamiento de la muestra cuyos objetivos son garantizar la homogeneidad de la muestra, su integridad hasta el momento del tratamiento y favorecer la eficacia de la primera etapa de extracción. Entre estas operaciones previas se incluyen, por ejemplo, el triturado, disgregado y, cuando es necesario, el tamizado de las muestras y otros tratamientos como la eliminación de agua (necesario en muchos casos para garantizar la eficacia de la etapa posterior de extracción). En el caso de muestras abióticas, este secado se suele realizar a temperatura ambiente o por calentamiento en estufa a temperaturas inferiores a 100 ºC; por el contrario, la liofilización suele ser la técnica más empleada para muestras bióticas.

En el caso de los estudios llevados a cabo en esta tesis, las muestras homogeneizadas y estabilizadas se almacenaron hasta su análisis en frascos de vidrio, previamente lavados con diferentes disolventes orgánicos, y se mantuvieron a - 4 ºC, en el caso de las muestras bióticas, y a temperatura ambiente, en el de las abióticas, y protegidas de la luz hasta su análisis.

Otro de los pasos previos al tratamiento de muestra, con un profundo efecto en el resultado final, es la suplementación de la muestra con un patrón interno adecuado. El objetivo de este paso es evaluar la recuperación y bondad del proceso analítico y, si fuera necesario, poder corregir las posibles desviaciones detectadas en el mismo con el fin de garantizar la validez del resultado. Las sustancias químicas empleadas con estos fines deben tener un comportamiento químico similar al de los analitos estudiados durante todo el proceso analítico, y no encontrarse a niveles detectables en la matriz investigada. Por ello, las mejores opciones consisten en el uso como patrón interno de (i) un compuesto perteneciente a la misma familia de los analitos que se quieren analizar pero que no exista o se detecte en las muestras, (ii) un compuesto de estructura química afín y comportamiento químico análogo o (iii) el mismo compuesto analizado marcado isotópicamente. Esta última sería la solución idónea al problema y, de hecho, su utilización es obligatoria en algunos tipos de análisis de contaminantes traza, como para las PCDD/Fs y los PCBs coplanares *(1)*. Presenta,

sin embargo, el inconveniente de su elevado precio, por lo que en los últimos años se han intentado sintetizar otros compuestos de similares características físico-químicas pero de menor coste, como es el caso de los análogos fluorados *(2-4)*. Es importante mencionar que, desde un punto de vista práctico, en análisis multirresiduo es conveniente el empleo de más de un patrón interno, de manera que estos cubran la totalidad del análisis cromatográfico a fin de que cada compuesto pueda ser referido y corregido con el patrón interno que eluya en la región cromatográfica más cercana al analito considerado.

La suplementación con el patrón interno debe realizarse en un disolvente adecuado que permita su correcta incorporación a la matriz y garantice un comportamiento químico similar al del compuesto endógeno. Así, la suplementación suele llevarse a cabo mediante disolución del patrón interno en un volumen de disolvente orgánico suficiente para cubrir la totalidad de la muestra. Tras agitar la mezcla durante un tiempo suficiente para una distribución homogénea (10-30 min), el disolvente se deja evaporar a temperatura ambiente en la oscuridad, para que los compuestos interaccionen con la matriz y se incorporen a la misma *(5)*.

1.2.2. Etapa de extracción

La primera etapa dentro del proceso de preparación de muestra en un análisis orgánico de trazas es la extracción. En ésta, el principal objetivo es la extracción exhaustiva de los analitos contenidos en la matriz. La necesidad de que el proceso de extracción sea exhaustivo viene determinada por los bajos niveles a los que suele ser necesario detectar los COPs y otros analitos relacionados en muestras reales. Esta etapa no suele ser demasiado selectiva y frecuentemente conlleva la co-extracción de otros componentes de la matriz que podrían interferir en la determinación final de los analitos de interés. Por ello, suele ser necesaria la incorporación de una (o varias) etapas de purificación previas al análisis instrumental con el fin de aislar los interferentes analíticos potenciales y obtener un extracto final, más limpio y concentrado, apto para su determinación analítica final.

A continuación se revisarán algunas de las principales técnicas de extracción más empleadas para determinación de contaminantes orgánicos en muestras ambientales (semi-)sólidas *(6)*.

Técnicas convencionales

Extracción sólido-líquido

La extracción sólido-líquido (SLE) es una de las técnicas más convencionales y sencillas utilizadas en este campo de investigación. Consiste en la puesta en contacto de la muestra (semi-)sólida a analizar con un disolvente, o mezcla de disolventes, que presenten afinidad por los analitos de interés. El proceso de reparto se acelera mediante agitación manual o mecánica, al favorecer el contacto entre las dos fases. Es un proceso sencillo y poco costoso pero no muy selectivo, siendo necesarios largos tiempos de análisis y que, además, puede dar lugar a la formación de emulsiones.

Extracción con Soxhlet

La extracción con Soxhlet es uno de las técnicas estándar de extracción de muestras (semi-)sólidas y es ampliamente utilizada en los laboratorios analíticos desde su creación, hace ya más de un siglo. Es además, uno de los métodos de referencia más frecuentes para análisis de COPs en muestras ambientales y de alimentos y cuenta con una amplia aceptación en los procedimientos oficiales de organizaciones como la EPA y la Food and Drug Administration (FDA).

En este caso, la muestra finamente pulverizada se coloca en un cartucho de material poroso (de celulosa o vidrio) que se sitúa en la cámara del extractor Soxhlet. El disolvente extractante elegido se calienta en un matraz situado en la parte inferior del extractor, en la que está instalado algún equipo de calentamiento (mantas calefactoras o placas eléctricas), y se hace pasar a través de la muestra por condensación de sus vapores sobre un serpentín refrigerado. Cuando el nivel del disolvente condensado en la cámara de extracción alcanza el sifón lateral, la fracción extraída sifona y retorna al matraz. Este proceso se repite el número de veces necesario para completar la extracción de los analitos. A continuación, y a fin de concentrar el extracto graso obtenido, el/la mezcla de disolvente/s se evapora.

Las ventajas principales de esta técnica son: (i) el continuo contacto entre la muestra y el disolvente, (ii) que la solubilización de los analitos en el disolvente se ve favorecida por la aplicación de temperatura, (iii) que no es necesaria la filtración del extracto después de la extracción, (iv) que proporciona excelentes recuperaciones, (v)

que no depende de la naturaleza de la matriz y (vi) que es muy simple, poco costosa, universal y que permite realizar varias extracciones en paralelo.

Entre las desventajas se pueden destacar: (i) los largos tiempos de extracción (entre 6 y 24 horas), (ii) el gran consumo de disolventes (50-300 mL), (iii) problemas de descomposición en el caso de compuestos termolábiles, (iv) la necesidad de incorporar una etapa final de evaporación del extracto obtenido y (v) la limitada automatización del sistema, si bien en los últimos años se han introducido nuevos equipos basado en esta técnica pero con mayor grado de automatización *(7)*.

Dispersión de la matriz en fase sólida

La dispersión de la matriz en fase sólida (MSPD) ha crecido en popularidad desde su introducción y hoy día es frecuentemente utilizada en estudios ambientales y en análisis de alimentos. De manera general, la muestra se pone en contacto con un adsorbente adecuado, como C18, sílice, Alúmina o Florisil® (entre otros), o con un material inerte, como las tierras diatomeas, hidromatrix, sulfato sódico o arena, con el que se mezcla profusamente hasta conseguir su dispersión homogénea en la superficie del adsorbente. El polvo seco así obtenido se empaqueta en una columna o cartucho de extracción en fase sólida (SPE) para su tratamiento como si de un sistema de SPE se tratara. Es decir, la mezcla muestra-adsorbente puede ser lavada, secada y/o extraída con el disolvente extractante elegido. Una de sus principales ventajas es que permite una pre-purificación de la muestra ya que, en función de las propiedades del sorbente elegido para la dispersión, algunos de los interferentes potenciales presentes en la muestra pueden quedar irreversiblemente retenidos en la superficie del adsorbente, o ser selectivamente eluídos de la columna, antes de proceder a la extracción de los analitos de interés.

Técnicas de extracción aceleradas

Extracción con líquidos presurizados

La extracción con líquidos presurizados (PLE) consiste en la extracción con disolventes de polaridad variable a altas presiones (hasta 3500 psi) y temperaturas (40-200 ºC). Esta técnica es también conocida como extracción acelerada con disolventes (ASE), extracción con fluidos presurizados (PFE), extracción a altas presiones (HPSE), extracción a altas presiones y altas temperaturas (HPHTSE), extracción a temperaturas y disolventes calientes (PHSE) o extracción con disolventes en estado subcríticos (SSE).

La PLE aprovecha la disminución de la viscosidad de los disolventes conseguida al emplear altas temperaturas para favorecer la solubilización de los analitos de interés. Las altas temperaturas favorecen la rotura en las interacciones muestra-analito facilitando la difusión de este último fuera de la matriz en la que está atrapado. Bajo estas condiciones, los disolventes tienen una mayor capacidad de solvatación y, por tanto, se incrementan las velocidades de extracción.

Por último, mencionar que la PLE se puede llevar a cabo en modo estático o dinámico, y que se pueden añadir a la celda de extracción otros sorbentes, bien mezclados en la muestra como en el caso de la MSPD, bien en forma de capas sucesivas, de manera que la selectividad del proceso de extracción pueda ser modulada e incrementada.

Extracción con fluidos supercríticos

El uso de fluidos supercríticos como disolventes extractante permite, en principio, una mayor selectividad en el proceso de extracción, con cinéticas de extracción más rápidas que las proporcionadas por otros disolventes en condiciones atmosféricas. Los principales parámetros que controlan este aspecto son la temperatura, la presión aplicada y la adición de modificadores como, por ejemplo, metanol (aunque esto último suele implicar la presencia de otras sustancias co-extraídas en el extracto final y, por tanto, una menor selectividad del proceso).

Los fluidos utilizados en la extracción con fluidos supercríticos (SFE) suelen ser gases inertes y económicos, siendo el dióxido de carbono el más usado. Como en

otras técnicas, para el análisis de contaminantes traza, se requieren elevados grados de pureza de los gases y reactivos empleados.

La SFE es una técnica automática especialmente adecuada para el análisis de muestras particuladas, como suelos y material seco, aunque se ha empleado también para el análisis de matrices con alto contenido lipídico, como alimentos y piensos. En todos los casos, la recogida del extracto resultante se realiza por despresurización del fluido y atrapamiento cuantitativo de los analitos de interés en trampas líquidas o sólidas que permiten la eliminación simultánea del extractante. El elevado coste de la instrumentación y la dependencia la complejidad matricial en la eficacia del proceso, serían sus principales limitaciones y el motivo por el que, en los últimos años, está siendo desplazada por otras técnicas como la PLE en campos como el ambiental.

Extracción asistida por microondas

La extracción asistida por microondas (MAE) consiste en el calentamiento mediante energía de microondas del disolvente extractante (un disolvente orgánico en el caso de análisis de compuestos orgánicos) que está en contacto con la muestra. Este tipo de energía, a diferencia de otros procedimientos, calienta directamente y de forma simultánea la muestra y el disolvente sin transferir energía al recipiente, lo que contribuye a reducir los tiempos de extracción *(8)*. En general, en la MAE, los tiempos de extracción y los volúmenes de disolventes utilizados son relativamente pequeños. Sin embargo, esta técnica no es recomendable para el análisis de compuestos termolábiles y en aquellas extracciones en las que se requiera el uso de disolventes apolares, al no absorber éstos la energía microondas.

Extracción con ultrasonidos

Al igual que la MAE, la extracción con ultrasonidos (UAE) mejora la eficacia de extracción respecto a la SLE al aplicar directamente la energía de ultrasonidos para favorecer la disgregación de la muestra y aumentar la difusión de los analitos desde la matriz al disolvente de extracción. La energía ultrasonidos provoca, mediante cavitación acústica, un aumento de la temperatura y presión del proceso de extracción, acortando los tiempos de análisis y aumentando la eficacia de la extracción, que puede ser estática o dinámica. La instrumentación es sencilla y

relativamente económica. Entre las principales desventajas de la técnica cabe destacar la baja automatización del proceso, la necesidad de etapas posteriores de filtrado y concentración de los extractos obtenidos y, por último, su limitada selectividad.

1.2.3. Etapa de purificación

Dada la limitada selectividad de buena parte de las técnicas empleadas en la etapa de extracción, en la mayoría de las ocasiones es necesario incorporar al proceso de tratamiento de muestra una etapa o etapas posteriores para la purificación del extracto obtenido. Su objetivo es la eliminación de los interferentes analíticos co-extraídos de la matriz y, dependiendo de la técnica instrumental elegida para la determinación final, el fraccionamiento final de las distintas familias de compuestos de características físico-químicas similares a los compuestos de interés que pudieran permanecer en el extracto purificado.

En el caso de las matrices bióticas, en general, la primera etapa de limpieza suele perseguir la eliminación de la materia grasa en la que se encuentran ab/ad-sorbidos todos aquellos xenobióticos con carácter lipofílico y que ha sido co-extraída con los compuestos que se pretende determinar. Para ello, pueden elegirse técnicas destructivas o no destructivas.

Entre las técnicas destructivas, se pueden citar como más habituales la oxidación con ácido sulfúrico concentrado y la saponificación, si bien esta última no es aconsejable para análisis de PCBs y PBDEs por la posibilidad de degradación de aquellos congéneres menos estables (los menos clorados y los más bromados, respectivamente). La oxidación puede llevase a cabo por adición directa del agente oxidante sobre el extracto graso *(9)* o utilizando adsorbentes impregnados con ácidos, en general sílices modificadas con diferentes porcentajes de ácido sulfúrico *(7)*. Este último procedimiento permite eliminar los problemas asociados a la formación de emulsiones, típicos de purificaciones mediante extracción líquido-líquido y, además, el empaquetamiento de los adsorbentes necesarios en las columnas y celdas de extracción, lo que facilita el acoplamiento entre las etapas de extracción y purificación (se minimiza la manipulación de los extractos y reduce el tiempo total de análisis) *(10)*. Otros adsorbentes, como la Alúmina o el Florisil®, no necesitan modificaciones

químicas ya que poseen cierta capacidad para reterner la grasa, si bien su uso sólo es aconsejable para el tratamiento de muestras con bajo contenido en lípidos *(11)*.

En el caso de las muestras abióticas, la saponificación (o tratamiento con sílices modificadas con NaOH o KOH alcohólicas) suele ser imprescindible para la eliminación de la materia orgánica. Este tratamiento suele además combinarse con el de sílices modificadas y/o neutras para completar el proceso de purificación y neutralizar la basicidad del eluato. El cobre es, asimismo, otro adsorbente muy utilizado en purificaciones de muestras abióticas, como suelos y sedimentos, para la eliminación de compuestos con azufre que interferirían en la determinación instrumental de los COPs.

Como alternativa a estas técnicas destructivas, existen otras técnicas de purificación no destructivas que conservan la naturaleza de los diferentes componentes del extracto. Entre ellas cabe destacar por su mayor frecuencia de uso la diálisis, la cromatografía de exclusión molecular (SEC) y las técnicas de purificación basadas en el uso de adsorbentes no modificados que incluyen, entre otras, la extracción en fase sólida (SPE) *(12)*, la microextracción en fase sólida (SPME) *(13)* y la extracción mediante adsorción sobre barra de agitación (SBSE) *(14)*, estas tres últimas muy empleadas en el análisis de COPs en muestras acuosas.

La diálisis es muy eficaz para la eliminación de grandes cantidades de materia grasa, aunque es un proceso que involucra largos tiempos de análisis (aprox. 40 h) y elevados consumos de disolventes orgánicos de elevada pureza. Por su parte, la SEC es una técnica relativamente selectiva pero que suele requerir de purificaciones adicionales del extracto, al obtenerse una fracción final en la que no siempre es posible un aislamiento completo de los analitos de interés de otros compuestos co-extraídos de la muestra.

Una vez obtenido un extracto limpio procedente de los tratamientos anteriores, en ciertas ocasiones y dependiendo de la selectividad de la técnica instrumental elegida para la determinación final y/o si el procedimiento analítico oficial lo exige, es necesario llevar a cabo un fraccionamiento adicional entre el analito o familia de analitos de interés y otros compuestos (o familias de compuestos) que no han sido eliminados en las etapas anteriores y que podrían interferir durante su determinación instrumental (bien por las marcadas diferencias en sus concentraciones, bien por

representar coeluciones cromatográficas que no puedan ser resueltas aumentando la selectividad del detector). Este fraccionamiento final suele basarse en la separación de los analitos en función de sus distintas propiedades estructurales, principalmente por planaridad, y no de sus propiedades químico-físicas como sucedería en los tratamientos anteriores. El adsorbente más utilizado en esta etapa es el carbón, ya sea en columnas abiertas o en formato de cartuchos de SPE *(15-16)*, aunque también se utilizan otros adsorbentes como el Florisil® *(17)*, Alúmina y ciertas fases de cromatografía líquida de alta eficacia (HPLC) como las de base pirenil *(18)*.

La Tabla 1 resume algunas aplicaciones representativas de las técnicas de extracción y purificación descritas en esta sección para el análisis de muestras (semi-) sólidas en el campo medioambiental.

Tabla 1. Comparativa de distintos tratamientos de preparación de muestra para el análisis de contaminantes orgánicos traza en matrices (semi-)sólidas abióticas y bióticas.

Muestra (g)	Analito	Pretratam.	Extrac.	t (min)	Disolvente (mL)	Purif.	Análisis	Recup. (%)	RSD[a] (%)	LOD[b] (ng/g)	Ref.
Suelo (1-2)	PCBs prioritarios + 105, 128, 149, 156, 170	radiación γ + mezclado	Soxhlet Soxtec USE SFE MAE ASE	1080 120 60 35 20 5+5	n-C_6[c]:ACE[d] (1:1) /tolueno, /CO_2 (variable)	Columnas multisílice y sulfato sódico	GC–(^{63}Ni) μECD	V.R. [e] 98-115 108-119 98-115 108-117 96-132	0-8 0-6 0-8 0-4 0-3 0-11	50-100 HPC[f]/g	*(7)*
Suelo (10)	PCBs (Aroclor 1242+1260)	secado y tamizado	SSLE[g]	27	n-C_6:ACE (1:1) (75)	Cu + Hg + H_2SO_4	GC–(^{63}Ni) μECD	56-88	6-13	NE	*(19)*
Sedimento (1,2)	PCBs	MSPD (Cu + Na_2SO_4)	SFE	30	CO_2	NA[h]	GC–MS	NE	3-12	NE	*(20)*
Sedimento (0,5)	PAHs	NR	FUSE[i]	2	n-C_6 (5)	Florisil	GC–MS	42-74	7-25	NE	*(11)*
Sedimento (5)	PAHs	secado+Cu	SFE	60	CO_2	NA	GC–MS	88-96	2-19	NE	*(21)*
Suelo y vegetales (30)	PAHs PCBs OCPs	lavado, homogen., tamizado	Soxhlet	1440	n-C_6:ACE (1:1) (300)	SPE	GC–MS	84-115 92-124 80-112	3-12 4-19 3-14	NE	*(22)*
Leche materna (1)	PBDEs	secado	ASE	10	n-C_6 + n-C_6:DCM[j] (6,6)	Alúmina	GC–NCI-MS	14-131	1-36	0,01-0,05	*(12)*
Leche en polvo (15-20)	PCBs copla. PCDD/Fs	MSPD (sílice)	SLE Soxhlet	NE[k] 960	n-C_6:ACE (1:1) (400:200)	SiO_2-HSO_4 Florisil	HRGC–ECD + GC–HR(MS)	95-101 80-94	1-7 2-11	NE	*(23)*
Pescado (1-5)	PBDEs	homogen	Soxhlet	720	DCM:ACE (1:1) (80)	Columnas multisílice y Na_2SO_4	GC–MS + GC–MS/MS	78-103	<10	0,01 (BDE# 209, 1)	*(24)*

Muestra (g)	Analito	Pretratam.	Extrac.	t (min)	Disolvente (mL)	Purif.	Análisis	Recup. (%)	RSD[a] (%)	LOD[b] (ng/g)	Ref.
Pescado (0,6-0,8)	PAHs	NR[l]	MSPD	NE	ACN[m] (10)	Florisil + C_{18}	HPLC-fluorescencia	88-106	2-8	0,04-0,32	*(25)*
Alimentos grasos (2-73)	PCBs copl PCDD/Fs	secado	ASE	10	*n*- C_7 (60)	Carbón	GC–HRMS	84-97	5-33	NE	*(15)*
Piensos (1,5)	PBDEs PBBs	NR	MSPD	NE	*n*-C_6 (30)	SiO_2-HSO_4 + Florisil	GC–MS/MS	70-96	1-9	0,02-0,9	*(26)*
Hígado de pollo (1)	PCBs	MSPD	USE	15	*n*-C_6:ACE :DCM (3:1:1)(10)	H_2SO_4	GC–MS	63-94	<10	0,06-0,63	*(13)*
Hígado de peces (0,6)	PBDEs HNPs PCBs	triturado	Soxhlet automát.	180	*n*-C_6:ACE (3:1)	SiO_2-HSO_4	GC–MS	70-100	<10	0,07-0,7	*(27)*
Grasa de ballena (2-5)	PCBs	NR	MAE	25-30	*n*-C_6 (20)	Columnas multicapa o SPD[n]	GC–HRMS GC–μECD	78-103	1-12	14	*(28)*
Equipos eléctricos (0,3)	PBDEs PBBs	desintegr. y tamizado	MAE	120	*n*-C_6 (10) + H_2O (4)	H_2SO_4 + SPE (LC-Si)	GC–MS	72-108	1-8	0,2-68	*(9)*

[a]RSD, desviación estándar relativa; [b]LOD, límite de detección; [c]*n*-C_6, *n*-hexano; [d]ACE, acetona; [e]V.R., valor referente; [f]HPC, número de platos heterotrópicos (heterothropic plate counts); [g]SSLE, extracción sólido-líquido con sonicación; [h]NA, no aplicable; [i]FUSE, extracción asistida por ultrasonidos focalizada; [j]DCM, diclorometano; [k]NE, no especificado; [l]NR, no requerido; [m]ACN, acetonitrilo; [n]SPD, sample preparation device (instrumento que combina en línea distintas columnas típicas para purificación de dioxinas y PCBs sobre a las que, además, se aplica temperatura)

1.2.4. Miniaturización en la química analítica

En apartados anteriores, se ha puesto de manifiesto algunas de las limitaciones más acuciantes de buena parte de las técnicas convencionales empleadas hoy día para el análisis de contaminantes traza en muestras complejas ambientales y de alimentos. Si a su laboriosidad se une la reducción constante experimentada en los últimos años en los MRLs permitidos para muchos xenobióticos, la necesidad de controlar de manera rutinaria sus niveles en un número creciente de matrices cada vez más variadas, y la inclusión de un número cada vez mayor de compuestos en los programas de monitorización, resulta evidente insistir en la necesidad de proponer alternativas analíticas que superen algunas de estas limitaciones, pero siempre manteniendo los estándares de calidad exigidos para este tipo de análisis. Una de las estrategias analíticas investigadas con este fin ha sido la miniaturización de las técnicas y/o metodologías empleadas en el tratamiento de muestra. Hoy día se pueden encontrar descritos en la bibliografía numerosos estudios que demuestran la validez de este tipo de aproximación como alternativa más rápida y de menor coste a los protocolos de preparación de muestra hasta ahora empleados para muestras líquidas. Los progresos en relación con el tratamiento de muestras (semi-)sólidas son mucho más limitados debido, fundamentalmente, a la falta de instrumentación analítica adecuada (es decir, miniaturizada) para abordar la etapa de extracción. Sin embargo, las ventajas inherentes a este tipo de estrategia justifican los esfuerzos llevados a cabo en los últimos años por algunos grupos de investigación en este campo. En este sentido, cualquier tipo de integración entre los distintos tratamientos a llevar a cabo, la simplificación o eliminación de etapas que supongan una reducción de la manipulación de la muestra por parte del analista, debe considerarse un avance respecto a las metodologías convencionales ya que simplificaría el proceso, reduciría sus costes tanto en términos de tiempo como de fungible, contribuiría al desarrollo de una química más verde, reduciría el riesgo de contaminación o pérdida de los analitos, y la exposición del operario a productos químicos tóxicos. La miniaturización de los procesos analíticos supone un factor clave en este sentido y suele ser, además, el paso previo imprescindible al posterior desarrollo de sistemas (semi-)automatizados y/o robotizados que puedan funcionar de manera (semi-)autónoma. La miniaturización representa también una valiosa alternativa en aquellos casos en los que el estudio de

un fenómeno concreto requiere tiempos de análisis inferiores a los involucrados por los procedimientos convencionales, pero, sobre todo, suele ser la mejor opción (e incluso a veces la única posible) cuando existe una limitación importante en el tamaño de la muestra disponible para el análisis.

A continuación se presenta una revisión bibliográfica en la que se discuten todos estos aspectos y se revisan los avances más relevantes conseguidos en los últimos años en relación con la miniaturización en química analítica para el análisis de trazas de xenobióticos en muestras líquidas, (semi-)sólidas y sólidas.

1.2.4.1. MINIATURIZACIÓN DE MÉTODOS ANALÍTICOS[1]

Abstract

This chapter highlights miniaturisation in sample preparation as a valuable alternative for green analytical chemistry. Current state-of-the-art will be discussed on the basis of representative examples selected from representative application areas, including biomedical, environmental and food analysis, and involving conventional instrumental techniques for final determination of the target compounds. Emphasis will be on those techniques and approaches that have already demonstrated their practicality by the analysis of real-life samples, and in particular to those dealing with the determination of minor components. The potential of lastest developments in this field for sample treatment simplification and complete hyphenation of analytical process will be discussed and the most pressing remaining limitations evaluated.

[1] M. Pena-Abaurrea, L. Ramos, Chapter 5. Miniaturisation of analytical methods, in "Challenges in Green Analytical Chemistry ". Edit.: M. de la Guardia and S. Garrigues. RSC Publishing. Cambridge (UK) (2011). ISBN: 978-1-84973-132-4

1. Miniaturisation as an alternative for green analytical chemistry: strengths and current limitations

Today, miniaturisation is an evident trend in many analytical application fields. Miniaturisation is the first required achievement when trying to develop coupled (or integrated) analytical procedures, which in their turn can be considered as the first necessary step for developing completely hyphenated, (semi-)automatic and/or unattended analytical systems. The several benefices associated to these types of analytical arrangements are evident and do not need any further explanation. These advantages partially explain the many efforts carried out during the last two decades to miniaturise the analytical processes and treatments, and the several attempts carried out to develop new analytical alternatives and techniques that contribute to achieve the hyphenation goal. But miniaturisation is both stimulated by and stimulating what can be considered another clear trend of modern analytical chemistry: the development of greener analytical methodologies and procedures that efficiently contribute to reduce the use of toxic solvents and reagents as well as the amount of generated wastes. Attempts carried out in this direction have promoted the development of new analytical techniques for both sample preparation and instrumental analysis and the use of alternative and original analytical approaches involving sometimes the use of already existing technologies under new and different perspectives. Some of these advances have been covered in previous chapters and many more will be presented in the following ones. The present chapter focuses in those achieved in the field of sample preparation for the analysis of organic components, and area in which progress has up to now been somehow more limited than in other application areas. This is especially true for the analysis of trace organic compounds, such as e.g. organic micro-contaminants, in environmental and biological samples. In these fields, the variety of matrices to be analysed, the complexity of most of them and the low levels at which the analytes should accurately be determined, has made that, despite the extremely powerful and sophisticated separation-plus-detection instrumental techniques used for final determination of the analytes, quiet often specific sample preparation protocols are still used for each particular analyte-matrix combination. Furthermore, the more complex the sample and the lower the analyte

concentration, the higher the number of treatments involved in the rather conventional, although often robust and well established, sample preparation procedures in use in this research field. Most of these analytical protocols start with an exhaustive extraction of the target compound(s) from the matrix using a conventional (but widely accepted) large-scale and time-consuming technique, such as liquid-liquid extraction (LLE) or Soxhlet extraction. This step should also effect the required trace enrichment of the analytes. However, because of the essentially non-selective nature of most of the extraction techniques, a laborious multi-step procedure is often needed to remove co-extracted material and isolate the analyte(s) of interest (unless, of course, separation-plus-detection is highly selective). The several analytical steps involved in such procedures are usually carried out off-line, which make them tedious and time-consuming, prone to loss of analytes, and to contamination, because of the continual manual manipulation of the extracts. In recent years, much effort has been devoted to eliminate these drawbacks, and faster and more powerful and/or more versatile extraction techniques are now available. However, the level of integration among the several treatment steps (i.e., extraction, purification and concentration), and among these and the final instrumental determination is still rather variable and certainly highly dependent on the matrix nature.

Today, on-line coupling (with or without automation) is a recognized feature in many areas of application which deal with gases or volatile analytes, and with a wide variety of analytes of divergent polarity in liquid samples (e.g., water, urine and plasma, soft drinks, and spirits). Initial problems regarding the compatibility of the various steps in terms of, e.g., sample size, chemicals required, time taken, and liquid or gas flows, have been solved satisfactorily. One main benefit of using on-line, i.e. integrated, systems is that instead of an aliquot of a sample extract of, often, 1% or even less, the entire sample is now subjected to the final separation-plus-detection. This enables considerable (and in some cases almost proportional) reduction of initial sample size required for the analysis. In its turn, such miniaturisation has helped to solve problems regarding the analysis of labile analytes, small (i.e., size limited) samples *(29-30)*, the study of processes that take place in times shorter than those involved by traditional methodologies, and/or the use of powerful separation techniques with limited sensitivity due to reduced loading capacity, e.g. narrow-bore

chromatographic or electrophoretic separation systems *(31-32)*. In some cases, developments have even led to the preparation procedure being minimised, e.g. by using highly efficient and selective preconcentration sorbents based on immuno-affinity recongnisition; or even discarded completely, e.g. substitution of the concentration step by large volume injection (LVI; typically 1 mL for liquid chromatography, LC, and 0.1 mL for gas chromatography, GC) and direct thermal desorption *(33-35)*.

For obvious reasons, the development of procedures similar to those mentioned above has been more limited for semi-solid and solid samples. For these, rather complex and large-scale (off-line) approaches are still the rule rather than the exception; in other words, improvement has been rather limited (*(36-38)* and references cited therein). In this research area, clearly, even miniaturisation of the basic processes and partial integration are, in most instances, highly demanding aspects, if not unachieved objectives.

Following the implications of the previous considerations, it is possible to conclude that the generic goal of miniaturisation in analytical chemistry is contributing to the development of integrated (i.e., hyphenated) systems for potential subsequent automation of the analytical process in the different research areas. In the analytical chemistry field, miniaturisation has also some specific goals including the development of greener and cheaper analytical processes (by reducing the required amount of reagents, solvents and wastes, the time and energy required per determination, the production costs…), speed up the analytical process (shorter analytical times, higher throughput), and the feasibility of setting up more integrated (simpler, smaller, portable….) and close (reducing the risk of decomposition, contamination or lost of the analytes, the exposure of the analyst to toxic chemicals, the amount of generated wastes…) systems. Other relevant goals, such as the (potential) suitability of the miniaturised analytical system for in-situ and continuous monitoring, the analysis of micro- and nano-samples, and the study, for the first time, of micro-environments (e.g., the analysis of the pore-water in soils and sediments) can be inferred from the previous considerations.

The tremendous analytical potential of these miniaturised approaches explain the many efforts conducted in this research field in recent years. However, as previously

mentioned, nowadays miniaturisation is a trend observed in many scientific and technical areas. Therefore, it is important to set the scale range of the analytical approached that will be reviewed in the present chapter. Table 2 summarises a simplified classification of the different scale ranges defined in analytical chemistry for the miniaturisation process.

For those readers who are not completely familiar with the terminology, Figure 1 shows a graphic description of the several possible degrees of integration in the analytical process.

- An analytical system or process is defined as on-line (integrated or hyphenated) when the extract (or effluent) from the sample preparation procedure is directly transferred, without intervention of the analyst, to the separation-plus-detection system selected for the determination of the target compound(s).
- When the final part of the sample treatment is carried out in the instrument used for final instrumental analysis, the system is so-called in-line.
- When the extract (or effluent) from the sample preparation procedure is transferred to the instrument selected for final determination via a mechanical or robotic system, the two systems are considered to be at-line.
- Finally, if the extract is transferred to the separation-plus-detection instrument by the analyst, the processes (or systems) involved in the analytical procedure are not coupled (or hyphenated) and the systems are defined as off-line.

Table 2. Characteristic size range of magnitudes, volumes and sample level associated to the different degrees of miniaturisation achieved in analytical chemistry.

Prefix	Typical size range	Typical volume	Sample size
Mini-	> 1 mm	µL	Cellular tissue
Micro-	1 mm – 1 µm	> 10 nL	Macromolecule
Nano-	< 1 µm	< 10 nL (fL)	Molecule; Ion

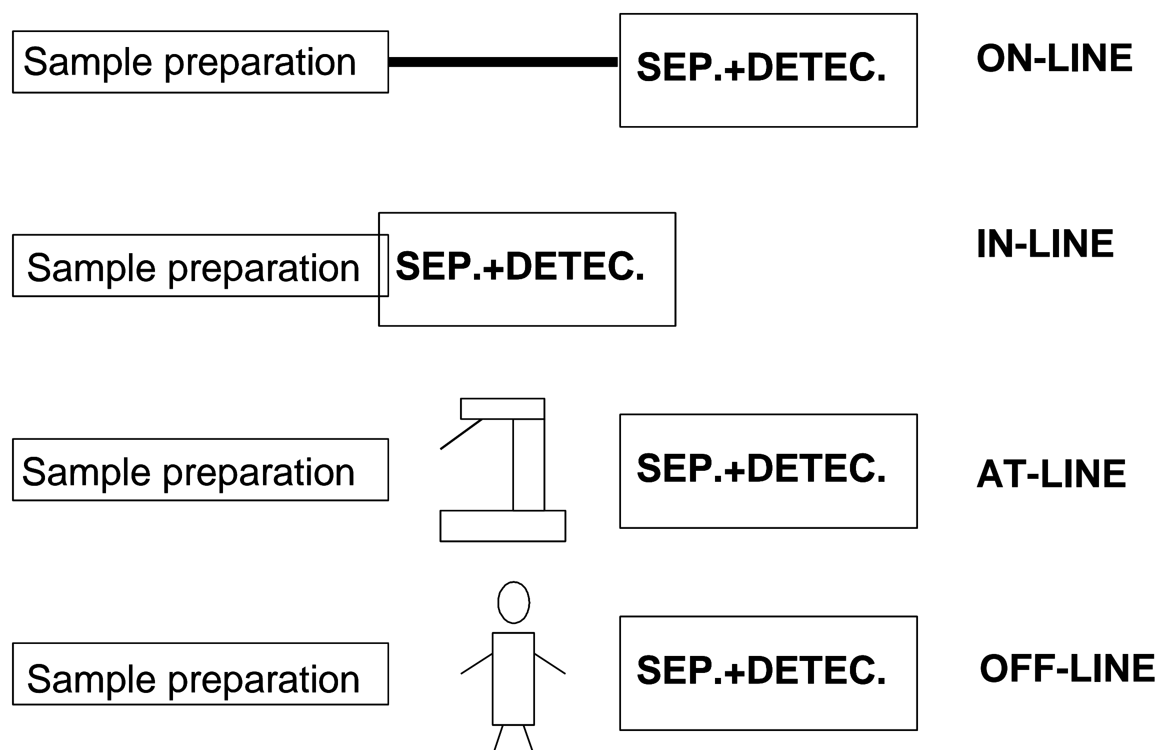

Fig. 1. Different levels of integration among analytical systems.

2. Miniaturised analytical techniques for treatment of liquid samples

<u>Solvent-based miniaturised extraction techniques</u>

Miniaturisation of a procedure can be achieved simply by reducing the dimensions of the systems used in earlier approaches or by developing completely new set-ups or techniques. As we will show in the next section, both strategies have been explored for LLE.

In-vial liquid-liquid extraction

LLE consists on the sequential extraction of an aqueous sample with an immiscible organic solvent in which the target compounds show a (much) higher solubility than in water. LLE is one of the simplest techniques for the extraction of liquid samples, does not require any especial equipment and allows several extractions

to be performed in parallel. These reasons could probably explain its wide acceptation in past and why is still in use in many laboratories. However, in a typical LLE experiment, relatively large volumes of sample (typically larger than 100 mL) are successively extracted with similar volumes of the selected organic solvent until complete extraction of the target compound from the original aqueous phase. This process obviously demands constant sample manipulation and the subsequent concentration of the organic extractant before instrumental determination to ensure proper detection. Apart from the high solvent consumption, the LLE process is prone to formation of emulsions, which lead to long analytical times and, in many instances, to analyte losses.

The goal of miniaturised LLE is to reduce the organic solvent/aqueous ratio as much as is possible to enhance analyte enrichment, and (virtually) eliminate the need for subsequent concentration of the separated organic layer. As in conventional LLE, stirring and salting-out of the aqueous phase are strategies often employed to improve transfer of the analytes from the sample to the extractant. Also derivatization of the target compounds either in the aqueous phase or in the organic layer have been used with satisfactory results.

When the volumes of the aqueous phase and the extractant are small enough (i.e., ca. 1-2 mL and 500µL, respectively), LLE can be developed in a chromatographic vial and the analytical approach is called in-vial LLE. In this case, a single extraction is carried out and, for obvious reasons, the selection of the extraction solvent and the optimisation of the different experimental parameters affecting the partition process become more critical. In any case, the analytical procedure is still very simple and samples can be treated in parallel, which contributes to increase the analytical throughput. However, probably the most interesting feature concerning this technique is that it allows a highly significant reduction of the organic solvent consumption, so contributing to green the analytical process. It also contributes to reduce enormously the time required per analysis as at this scale emulsions are (virtually) inexistent. This latter fact, together with the more favourable phases´ ratio, result in higher extraction efficiencies. Due to the higher enrichment factors achieved, concentration of the organic extract becomes in many instances unnecessary. Thereby, when the extractant is clean enough to avoid compromising the final

instrumental determination, this organic phase can directly be sampled by conventional autosamplers and transferred without any further treatment to the separation-plus-detection system. In other words, at-line coupling between the sample preparation and the instrumental steps are possible and, due to the simplicity of the several operations preformed, some degree of semi-integration (e.g., via a robotic arm or some of the sophisticated autosamplers nowadays commercialised) is possible.

In-vial LLE has been used, although with a variable success, to enrich polychlorinated biphenyls (PCBs) from spiked aqueous samples; for the semi-automatic miniaturised LLE of anilines and pesticides (in this case using membranes to avoid coextration of other matrix components); and for the analysis of tap, river and residual waters. In all cases, ready-to-analysis extracts of less than 1 mL were obtained, and, non-unexpectedly, the less polar the target analyte, the better the result obtained.

Unfortunately, somehow more disappointing results have been obtained when this approach has been applied to the analysis of non-aqueous samples, such foodstuffs. The higher complexity of these matrices lead to much lower extraction efficiencies and, due to the limited selectivity of the process, the introduction of additional clean-up step(s) became mandatory.

Solvent micro-extraction techniques

Several new micro-LLE-based techniques have been developed during the last fifteen years and grouped, under the name of solvent micro-extraction (SME) techniques, have recently been revised by Kokosa et al. *(39)*.

The various SME techniques and working modes described in the literature can be classified according to different criteria. In this text we will follow the terminology and classification proposed in *(39)*, which is essentially based on the number of phases involved in the extraction process and the two basic working modes: direct immersion sampling and headspace (HS) sampling (Table 3).

Table 3. Summarise of most commonly used SME techniques and working modes (adapted from *(39)*).

SDE technique	**Definition**
Direct immersion mode	
SDME	Single-drop microextraction
HF(2)ME	Hollow fiber-protected two-phases solvent microextraction
HF(3)ME	Hollow fiber-protected three-phases solvent microextraction
DLLME	Dispersive liquid-liquid microextraction
Headspace mode	
HS-SDME	Headspace single-drop microextraction
HS-HF(2)ME	Headspace hollow fiber-protected two-phases microextraction
Dynamic modes	
In-syringe	Repeated withdrawal of sample into syringe
In needle	Repeated withdrawal of sample into needle

Single-drop microextraction (SDME)

This miniaturised technique was firstly introduced by Jeannot y Cantwell *(40)* and by He and Lee *(41)* in 1997 when they simultaneously realised that a standard GC syringe could be used for SME. In SDME, the extractant phase is a single microdrop of a water-insoluble solvent (typically 1-8 µL) suspended at the tip of a syringe and immersed in an aqueous sample (ca. 1-10 mL) contained in a vial (Table 4).

After a pre-selected extraction time, the drop is withdrawn into the syringe and the enriched organic solvent directly transferred to the separation-plus-detection instrument. The experimental parameters affecting the efficiency of this SDME format are similar to those considered in LLE. Stirring of the sample, salting-out, application of temperature and analyte derivatization (to reduce its polarity or increase its volatility) are common practices that, in general, contribute to increase the extraction efficiency and reduce the analysis time.

However, to prevent drop dislodgement relatively slow stirring rates of up to 600 rpm should be used. The simplicity of the analytical procedure, which can be performed manually, or even better with a computer-controller autosampler, the extremely low solvent consumption and the relatively high enrichment factors achieved (typically in the range 10-100) can be considered key factors contributing to the fast development

of this miniaturised extraction technique and its application in different research fields.

Direct-immersion SDME has been demonstrated to be useful for the extraction of relatively non-polar and semivolatile analytes, including non-polar microcontaminants and drugs, from water samples that contain little or no particulate or dissolved matter (Table 4). For these types of samples, experimental results suggest that the nature of the sample has little effect on the enrichment process. However, when analysing more complex matrices such as urine *(42)*, previous filtration of the sample is recommended.

A recent modification of this method has been introduced by Wu et al. *(43)* and proved to be especially useful with size-limited biological samples. The technique is named *drop-to-drop extraction* (DDME) and involves very small volumes of both sample (ca. 10 μL) and organic solvent (ca. 0.5-1.0 μL). The sample is placed in a conical-bottomed microvial, in which the drop of organic solvent suspended at the tip of a microsyringe needle is immersed. Due to its format, the mass transfer rate of analytes into the extractant is high. Its main limitation is the relatively high LODs. However, it has been proved to be useful for the fast and simple extraction of drugs from blood, serum and urine.

A three-phase SDME-based alternative was introduced in 1999 by Ma and Cantwell *(60)* with the name *liquid-liquid-liquid microextraction* (LLLME). This technique enabled the simultaneous enrichment and purification of polar analytes from aqueous samples and consisted on the extraction of the deionised polar analytes from the aqueous sample into a few microlitres of organic phase, which acted as an organic liquid membrane contained in a PTFE ring. The preconcentrated analytes were then back-extracted into a micro-drop of aqueous receiving phase suspended in the organic phase. Next, the aqueous micro-drop was withdrawn into the syringed and directly subjected to liquid chromatography (LC) or capillary electrophoresis (CE).

Compared with regular SDME, this approach enables the use of higher stirring rates due to the improved stability of the drop-organic membrane. In addition, the small volume of receiving solution, enables high enrichment factors (in the range 200-500) to be obtained in rather short time (ca. 15 min), and the complete removal of the organic phase after each extraction prevents from cross-contamination. Several

examples have illustrated the feasibility of the approach for the determination of a variety of polar compounds from model buffered solution (Table 4) but, to the best of our knowledge, no application involving real-life samples has been reported up to now.

SDME can also be accomplished by direct exposure of the drop to the headspace of the samples. In this case, the technique is referred as headspace single-drop microextraction (HS-SDME). HS-SDME can be applied to aqueous, non-volatile liquids, solid and gas samples. Much higher stirring rates, without splashing, can be used, but for the rest all previous considerations apply also in this case. The technique performs very efficiently for the preconcentration of volatile non-polar analytes, and has the advantage over direct-immersion SDME of providing cleaner extracts in shorter analytical times.

Irrespective of the SDME mode used, a main factor with a profound effect on both the extraction efficiency and the extraction time is the diffusion of the extracted analytes from the drop surface to its inner part. In the previously described static SDME-based techniques, this diffusion rate can be increased by using less viscous solvents, stirring the sample or increasing the extraction temperature *(39)*. However, a probably more efficient alternative is the constant renovation of the solvent surface by using a dynamic approach. Two type of dynamic SDME are possible (Table 4). In the *in-syringe* dynamic method *(61)*, the aqueous sample or headspace is withdraw into the syringe needle or lumen and ejected repeatedly to perform the desired solvent enrichment. In the *in-needle* dynamic approach *(62-63)*, around 90% of the extraction drop is withdrawn into the syringe needle and then pushed out again repeatedly for sample exposure. The former dynamic approach is more effective when dealing with relatively pristine samples (i.e., without high contents of salts or major matrix components). The latter may be more useful for the analysis of relatively "dirty" samples (i.e., samples containing relatively high concentration of matrix components that could affect the subsequent instrumental analysis). It is important to highlight that in both types of dynamic approaches the use of a computer-controller autosampler for accurate and reproducible control of the syringe plunger movements is mandatory *(39)*.

Table 4. Selected applications of solvent micro-extraction techniques.

Matrix (mL)	Analytes	Extrac. solvent (µL)	Extract. mode	Ext. tim (min)	Enrich. factor	Ref.
SDME						
Spiked aqueous solution (1)	4-Methylacetophenone	*n*-C_8 (8)	D[a]	5	30	*(44)*
Spiked aqueous solution (1)	4-Methylacetophenone + 4-Nitrotoluene +progesterone +malathion	*n*-C_8 (1)	D	1	380	*(40)*
Spiked aqueous solution (4)	1,2,3-Trichlorobencene	Toluene (1)	E[b]	15	12	*(41)*
Spiked river water (5)	11 OCPs	*n*-C_6 (2)	D	5	21	*(45)*
Urine (2)	Cocaine and its metabolites	Chloroform (2)	D	6	7–17	*(42)*
LPME						
Spiked aqueous solution (0.06)	1,2,3-Trichlorobencene	Toluene (1)	D	3	27	*(41)*
River water (0.8)	PAHs	Chloroform (5) Toluene (3)	D E	20 20	>280 60–180	*(46)*
Wastewater (0.09)	10 chlorobenzenes	Isooctane (1)	D	2–3	130	*(47)*
Urine (1)	Basic drugs	Di-*n*-hexyl ether (20) +0.01M HCl (25)	D	45	60–140	*(48)*
Plasma (0.5)	Antidepressant drugs	Di-*n*-hexyl ether (25) + 0.01 M HCl (2)	D	45	20	*(49)*
Slurry sediment :water (4:100) (0.16 g)	Chlorobenzenes + OCPs	Toluene (3)	D	2	30–490	*(50)*
LLLME						
Spiked aqueous buffer, pH =13 (2)	7 aromatic amines	EtOAc [c] (150) +Na_2SO_4 buffer, pH =2.1 (2)	D	15.4	220–380	*(51)*

LLLME						
Cow milk + HCl 0.5M (8)	5 phenoxiacids	*n*-C_8 + NaOH 0.1M (7)	E	60	260–950	*(51)*
SLM						
Industrial wastewater (120)	7 aniline derivatives	Water, pH=3.3 (200)	4 [d]	30	12–30	*(52)*
Human plasma (0.5)	Anaesthetics	*n*-C_6 (360)	0.018 [g]	25	1.5	*(53)*
Assisted membrane LLE						
Spiked aqueous solution (15)	Triazines + apolar pollutants (OCPs, PAHs)	*n*-C_6 (500)	E	30	3	*(54)*
Tap water (60)	5 sulfonylurea herbicides	Chloroform (960)	3 [d]	20	55–60	*(55)*
DLLME						
River and lake water (100)	DDT and its metabolites	Chloroform (50)	E	2	200	*(56)*
Tap, spring and sea water [e] (12)	PAHs	Toluene (14)	D	0.5	857	*(57)*
CFLME [f]						
Spiked aqueous solution (80)	Bisphenol A	0.1 M Na_2SO_4 buffer, pH=12 (400)	0.8 [d]	40	200	*(58)*
Tap, sea and mineral water (20)	Sulfonylurea herbicides	0.2 M sodium carbonate buffer, pH=10 (50)	0.8 [d]	10	100	*(59)*

[a] Dynamic extraction; [b] Static extraction; [c] Ethyl acetate; [d] Flow rate, mL/min; [e] Ultrasonic-assisted DLLME; [f] Continuous flow liquid membrane extraction, combination of continuous flow LLE plus SLM.

Hollow fiber-protected two/three-phase solvent microextraction (HF(2/3)ME)

Hollow fiber-protected two-phase solvent microextraction, HF(2)ME, was firstly introduced by He and Lee in 1997 *(41)* with the name of *liquid-phase microextraction*. In its simplest version, the technique involves a small-diameter microporous polypropylene tube (the hollow fiber), usually sealed at one end, to contain the organic extracting solvent. The open end of the hollow fiber is attached to a syringe needle containing the selected extraction solvent and uses to fill the fiber with the organic solvent. The fiber is then immersed in the vial containing the studied aqueous sample. Enrichment is achieved by migration of the analytes through the fiber. Once the extraction time is completed, the solvent is withdrawal with the syringe and transferred to the instrument selected for final determination of the analytes. In practice, HFME can be considered as a liquid-liquid membrane extraction in which the porous polymer effectively protects the extraction solvent from contamination with particulate matter and soluble polymeric material, such as humic acids and proteins *(39)*. Consequently, HF(2)ME is more appropriate than SDME for the analysis of "dirty" aqueous samples. Another advantages of HF(2)ME over SDME are the use of larger extractant volumes (in the 4-20 µl range), which leads to higher extraction efficiencies, and the fact that the solvent cannot be dislodged, which allows the use of higher stirring rates. On the other hand, HF(2)ME usually involves longer extraction times than SDME (20-60 min *vs.* 5-15 min with SDME), and, at least LVI was used, only a fraction of the organic extractant is transferred to the instrument selected for final determination. When used in combination with LC, it also requires a previous solvent exchange. Although this technique can be adapted for use with an autosampler *(64)*, probably its main limitation is that each individual hollow fiber should carefully sized and prepared before use *(39)*.

The three phases involved in HF(3)ME are the aqueous sample investigated, the water-immiscible organic solvent that fills the pores of the hollow fiber polymer before this is attached to the syringe needle, and an aqueous acceptor phase that is placed in the lumen of the fiber with the help of the syringe *(65)*. HF(3)ME is operated in a way similar to HF(2)ME but, since the final acceptor solution is aqueous, the technique is used to extract water-soluble analytes from aqueous matrices. For obvious reasons, in HF(3)ME the pH of both the aqueous sample and

the acceptor phase are key parameters controlling the efficiency of the extraction process.

HF(3)ME shares with HF(2)ME its most pressing shortcomings, namely relatively long extraction times, difficulty of complete automation and intensive manual preparation of the fiber before use. However, despite these limitations, as HF(2)ME, HF(3)ME has been proved to be useful for the analysis of a variety of compounds in aqueous samples of limited size, for which allows high enrichment factors in relatively short times, specially when the technique is modified to favour the analytes migration through the porous fiber by, for instance, application of a difference of potential between the two phases *(66)*. This later modification is referred as *electromembrane extraction* (EME).

Interestingly, the fiber, filled with solvent can also be sealed at both ends. In this case, it can directly be placed into the stirred solution for extraction and, after a preselected extraction, retrieved. The enriched solvent is then removed by polymer puncture with a chromatographic syringe. This approach is also called *solvent bar microextraction (67)* and can be use as a two- or three-phase system.

HF(2/3)ME can be used in the previously described static versions or using dynamic (i.e., in syringe or in-needle) approaches similar to those described in the SDME section. Compared to previously described static approaches, regarding method development, their corresponding dynamic alternatives are more demanding due to the need of careful optimisation of a larger number of experimental parameters affecting the efficiency of the extraction process, including, apart from those described in the static approach, the speed of withdraw and ejection of the sample, the duration of the static extraction step in between these two actions, and the amount of sample aspirated. However, higher enrichment factors in shorter extraction times have been reported in all instances for these dynamic approaches.

In Table 4, the experimental conditions used and the typical enrichment factors achieved with the most common SME techniques are summarised and compared on the base of selected examples.

Dispersive liquid-liquid microextraction (DLLME)

The dispersive liquid-liquid microextraction (DLLME) was introduced in 2006 by Assadi´s group *(68)* and can be considered a modification of the miniaturised LLE. The technique consists on the fast injection into the studied aqueous sample (up to 10 mL) of a relatively small amount of a water-immiscible extraction solvent (typically 10-50 µL) dissolved in 0.5-2 mL of a water-soluble solvent with the help of a syringe. The fast injection of the mixture of organic solvents into the water makes the water-immiscible solvent to be efficiently dispersed in the aqueous mass as small micro-drops in which the target analytes are rapidly extracted. The enriched organic phase is then separated from the aqueous one by centrifugation or frozen (depending on its density) and directly subjected to instrumental analysis, typically by GC.

The feasibility of direct DLLME has been demonstrated for the accurate determination of non-polar compounds, including trace non-polar micro-contaminant families such as PAHs, chlorobenzenes and trihalomethanes for which enrichment factors in the 200-900 range have been reported (Table 4). The analysis of polar analytes demands previous pH adjustment and/or in-situ derivatization of the polar analytes to improve the extraction efficiency. The derivatization agent can be directly added to the sample or dispersed together with the extraction solvent.

The several manual manipulations involved in DLLME made the technique difficult to automate and the use of internal standards and surrogates even more necessary than for previously revised SME-based techniques.

Sorption-based miniaturised extraction techniques

Many pre-treatment techniques currently used for clinical or environmental analysis of gaseous, fluid, or liquid samples are based on trapping the target compounds on, or in, a suitable sorbent. The amount of sorbent that has to be used, i.e. the capacity, is determined by the amount of analyte(s), the level of matrix interferences, and the nature of the interactions between analyte(s) and interferences and the sorbent. Depending on the characteristics of the sorbent, analyte retention is governed by adsorption, i.e. by real chemical interaction between sorbent and analytes, by absorption, i.e. by partition of the analytes between sorbent and sample, by ionic interactions, and/or by a mixed retention mechanism. Frequently used

sorbents include alkyl bonded silicas, extremely hydrophobic styrene–divinylbenzene copolymers and polydimethylsiloxane (PDMS) for sorption and enrichment purposes, Tenax, carbon for the (more or less) selective trapping of highly polar or planar compounds, ion exchangers, mixed-mode materials, e.g. cation and anion exchangers mixed with C18-bonded silica for the simultaneous enrichment of both highly polar and non-polar compounds, and the class-selective immuno-sorbents (ISPEs) and molecular imprinted polymers (MIPs). For detailed overviews of the relevant characteristics of these and other types of sorbent, and the most commonly used formats, the reader is referred to reviews of a more specialist nature such as *(69-70).*

After preconcentration, analyte desorption can be accomplished by elution with a small volume of an appropriate solvent, either in a vial or in an appropriate interface, which is then partly or completely transferred to the instrument selected for final determination; or by thermal desorption, typically in the injection port of the instrument. Of course, thermal desorption offers the advantage of no dilution but it is obviously limited to (semi-)volatile thermally stable analytes.

This section revised the most relevant miniaturised sorption-based techniques in use on the base of selected applications dealing with the analysis of fluid and liquid real-life samples.

Solid-phase extraction

Today, solid-phase extraction (SPE) is the most widely used technique for the clean-up and enrichment of analytes from bio-fluids and environmental aqueous samples. During the last decades, a large variety of applications involving sorbents with increasingly improved selectivity and loading capacity, and even tailored-designed ISPEs and MIPs, have been described. Many of these sorptive phases are nowadays commercially available contributing to the extension of the technique. Although research in sorbent development continues, contributing to simplify the sample preparation procedures and to green the analytical process, probably the main achievement in this context was the introduction of on-line SPE-based procedures, with SPE–LC and SPE–GC as its most representative and relevant examples. The key factor which made possible these hyphenated systems was the miniaturisation of the SPE process. Reducing the size of the conventional 1-6 mL SPE syringe barrels to the

10 mm × 1-2 mm i.d. of the so-called Prospeckt-type cartridges used in the hyphenated systems led to a reduction of sample volumes from 0.5–1.0 L to less than 50–100 mL. Actually, quite often, even 5– 10 mL turns out to be sufficient to obtain similar LOD of 0.01–0.1 μg/L with SPE–LC and 1000-fold lower with SPE–GC that previously required 100-fold larger volumes. The relatively small particle size of the sorbent packed in the miniaturised SPE cartridge (ca. 40 μm) results in high retention efficiencies and adequate breakthrough volumes despite the small amount of sorbent used. More importantly, quantitative elution of the analytes can be achieved with 50-100 μL of the appropriate solvent, i.e. with a volume small enough to allow complete transfer to the instrument selected for final determination.

Prospeckt-type cartridges are typically mounted in holders similar to those used for the LC precolumns and integrated in systems containing 4-, 6- or 10-ports valves, depending on the complexity of the operations to be performed. LC pumps are used to pump the sample and solvent(s) through the system. Automation and computer control of the several operations can easily be achieved using programmable valves *(71)*.

As in any other SPE approach, in principle, two different working modes are possible: the sorbent can be used to retain either the interfering matrix components or the target compounds. The former working mode allows purification of the analytes but without concentration. The latter would ideally allow performing both operations, i.e. preconcentration plus clean-up of the studied analytes, in a single step. In practice, the SPE process is not as selective (or extracts as clean) as desirable and complete removal of potentially interfering matrix components is only accomplished after an extra purification step. In the on-line systems, this and other operations, such drying of the sorbent, can be performed by simply changing the valves position.

For those readers who are not completely familiar with the valve-based systems used for miniaturised SPE, Figure 2 shows the basic set-up required for on-line SPE with two cartridges.

Figure 2.A shows the valves position during sample loading. In this case, the sample, previously loaded in the injection loop, is pumped through both SPE cartridges. Analytes will be selectively retained in any of the cartridges according to their affinity for the respective sorbents; or alternatively eliminated via the waste port.

Then, changing the position of valves 1 and 3 and using a micro-LC pump, the analytes previously retained in cartridge 1 can be desorbed and directly transfer to the instrument selected to perform the analytical determination. When this step is completed, a simple change of the position of valves 2 and 3 will allow to proceed with the desorption of the analytes retained in cartridge 2, which can then be quantitatively transferred to the analytical system. The number of valves and configuration of the system would obviously depend on the goal of the analysis. As an example, a similar valve-based approach was used to study the so-called fast

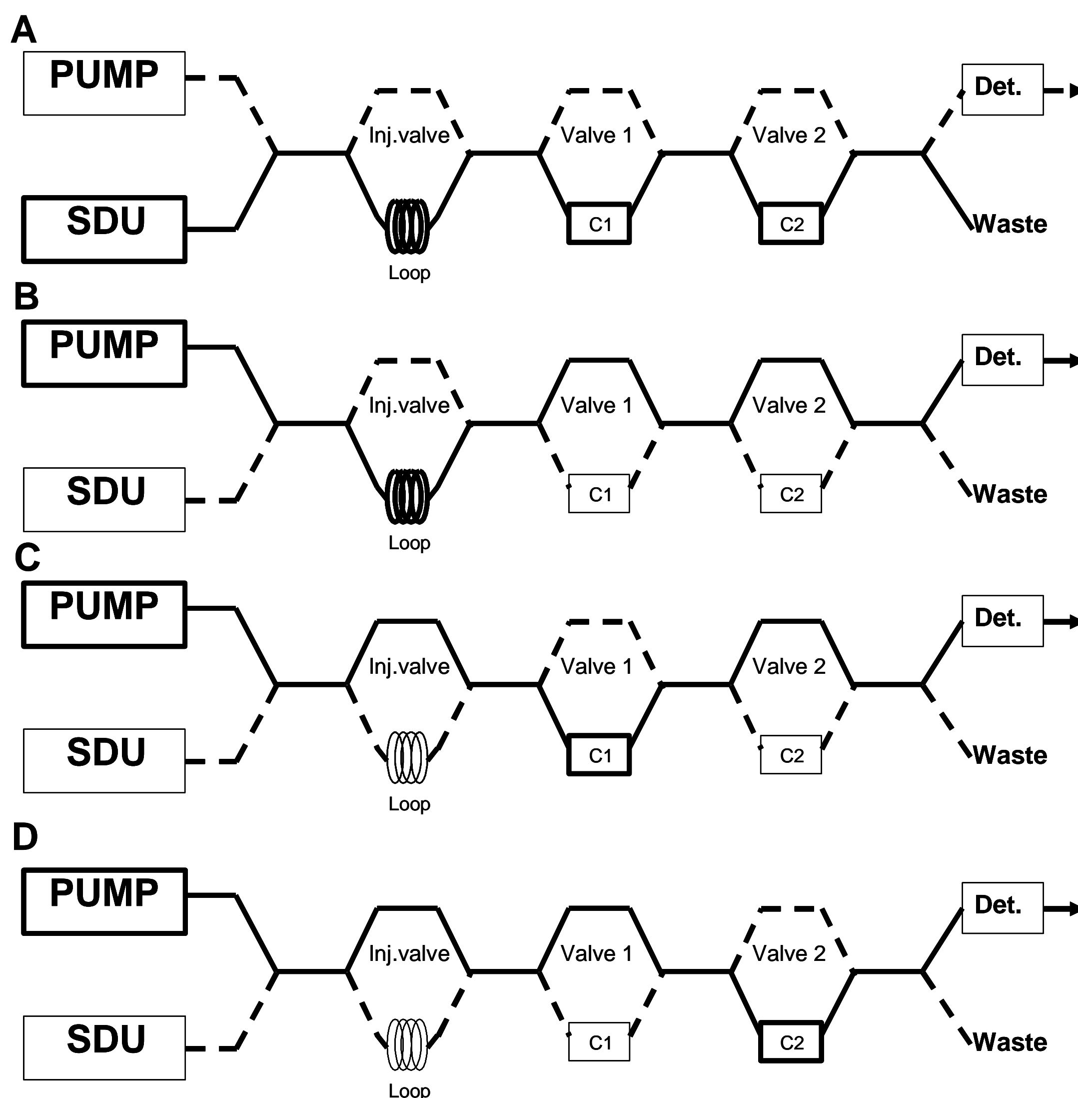

Fig. 2. Schematic of the basic valve set-up required for miniaturised on-line SPE with two cartridges (C1 and C2). (A) Configuration for sample loading, (B) test of the tube and injection loop, (C) cartridge 1 elution, and (D) cartridge 2 elution. SDU, solvent delivery unit; Det., detector.

adsorption of pesticides in soils and sediments *(72)*. In this set-up, the injection valve was slightly modified to allow direct injection of the contaminated soil/sediment slurry and a filter was incorporated for retention of the solid particles, while pesticides remaining in solution were concentrated in an SPE cartridge. Both pesticide fractions were separately collected for independent instrumental analysis.

That is, a single injection of the slurry in the system provided simultaneous information on the pesticides concentration in both phases in shorter times than the conventional batch approaches used at that moment so allowing a more accurate evaluation of the adsorption isotherms at early steps of the contamination process *(73)*. As a further illustration of the potential of this type of on-line SPE systems, Table 5 summarises the several steps involved in an SPE–LC analysis. The small volumes of solvents required for sorbent activation and equilibration, combined with the relatively high flow rates used (typically, 5 mL/min), allow to complete these steps in much shorter times (ca. 1 min) than those required for conventional SPE. Sample loading, in this case 500 µL of serum, should be carried out at slower flow rates to allow proper interaction of the analytes with the sorbent. For this step, a micro-LC pump must be preferred to ensure adequate flow control (typically in the 10-200 µL/min range). The subsequent washing of less retained matrix components out of the cartridge can be performed at intermediate flow rates of ca. 1 mL/min. Despite the relatively slow flow rates used in the loading and washing steps, both processes are completed in a rather short time (ca. 3 min) due to the small volume of sample and cleaning solvent pumped. Elution of the analytes from the cartridge is typically

Table 5. Different steps in an SPE–LC analysis; typical solvent volumes, flow rates and times involved in each case when using a 10 mm x 2 mm SPE cartridge (C18, 40 µm) are indicated.

Step	**Solvent (mL)**	**Flow (mL/min)**	**Time (s)**
Activation	Methanol (2)	5	24
Equilibration	Water (2)	5	24
Equilibration	Buffer (2)	5	24
Sample loading + washing (2.5 mL buffer)	Serum (0,5)	1	180
Elution	Mobile phase	0.5	45
Complete process			**297**

carried out at 100-500 μL/min to ensure quantitative desorption of the studied compounds in a minimum solvent volume and its transfer to the analytical system as a narrow band for optimal LC separation. In most instances, 50-75 μL suffices for the quantitative desorption of analytes from miniaturise SPE cartridge. Thereby, this step could be completed in 30-50 s, depending on the application. According to these considerations, it is possible to conclude that the average time required for complete sample preparation is ca. 5 min, which is significantly shorter that the time required for conventional (i.e., regular size and off-line) SPE.

The small volumes and short analytical times involved in on-line miniaturised SPE also made that method development and optimisation with theses systems were, in principle, faster than those needed in conventional approaches. The experimental parameters to consider during method development in miniaturised SPE are the same as for conventional SPE, namely the nature and amount of sorbent, the nature of the solvents used in the different SPE steps and, in particular, their flow rates. Also, the main reasons for low analyte recoveries are: reduced sorbent capacity or too strong retention, slow kinetic of the sorption process (or, to say differently, too high sample and/or solvent flow rates), and a possible adsorption of the analytes in the tube used to connect the different parts of the system. On the other hand, in these close systems the risk of analyte degradation and oxidation is greatly reduced as compared to the (open) conventional approaches.

Assuming that automation of on-line SPE-based systems can be considered an achieved goal, at present, development in SPE–LC is mainly orientated to the use of sorbents with (i) higher loading capacities that contribute to improve the present LODs; (ii) new functionalities to extend this type of routine analysis to more polar analytes, and (iii) more selective sorbents, such as ISPEs and MIPs, which contribute to reduce the risk of interference when less selective detectors are used as alternative to mass spectrometers (MS). It should be added that next to the on-line set-up, replacing the SPE–LC part for a single short column, SSC (1-2 cm length), and using MS *(74)* and especially MS–MS *(75)* detection has efficiently contributed to further reduction of both the analytical times and the LODs. As an example, it can be mentioned that this type of approach has facilitated the real-time study of analyte degradation at the trace level *(75-76)*, with LC run times of, frequently, only some 3

min. Finally, this progressive reduction of the sample size required to perform the analysis has made possible, in some cases, the direct injection of the aqueous sample *(77)* or of the aqueous extract obtained from fruits and vegetables *(78)*. In the former, 4 mL sufficed to achieve LODs of 0.01-0.1 μg/L. In the latter, the injection of the 3 mL extract provided LODs in the 0.5-2 μg/kg range for some particular pesticides, which can be appropriate for their fast screening. However, the use of highly selective and sensitive detectors, such MS-MS, becomes newly mandatory.

In principle, the on-line combination of SPE and GC could be considered more difficult than that of SPE-LC due to incompatibility of the solvents and flow rates used in both techniques. However, in practice, SPE-GC experienced a development parallel and similar to that SPE-LC. Experience has shown that with SPE cartridges as small as 10 mm·x 1 mm i.d., water samples of up to 10 mL suffice to reach LODs of 20-50 ng/L in the full-scan MS acquisition mode for a wide variety of (semi-)volatile micro-contaminants *(79)*. The better performance of the GC compared with the LC-based approach is mainly because of the superior detection/identification performance of GC–MS. Actually, if GC–MS/MS instead of GC–MS was used, LODs for several pesticides in surface water were 0.01-4 ng/L for 10-mL samples *(80)* or, alternatively, 0.2 μg/ L for 0.1-mL samples *(81)*. These low LODs and the progressive reduction of the sample volume required have promoted an important development in the field of LVI in GC. Some of the novel LVI interfaces *(33-34)* allow direct water injection in GC, and the introduction of up to 10 mL of solvent using slow injection or multiple fast injections in a packed linner. Nevertheless, the application field of these modifications depends strongly on the type and concentration of the interferences present in the sample.

For a detailed discussion on the feasibility of SPE–GC combined with different detectors for the accurate determination of trace compounds in aqueous samples, and on the possibility of on-line coupling of SPE with other modern separation techniques, including microbore LC and CE, the author is referred to a more specialised review *(82)*.

As regards other SPE formats, the so-called Empore disks (2–3 mm diameter,·0.5 mm thick disks cut from the larger original disks) have shown to be attractive alternatives to conventional cartridges, both for on-line SPE–LC *(83)* and SPE–GC

(84). Resin disks (0.7 mm diameter) have also been mounted inside the removable needle chamber of a 50 µL Hamilton gas-tight syringe and proved to be a valuable miniaturised automated alternative that enabled the efficient preconcentration of substituted benzenes from a volume of water as small as 2.5 mL. This method provided recoveries higher than 90% at the 10 ng/mL level with GC–FID and required only 5 µL of acetonitrile for desorption *(85)*. A recently introduced modification to this approach proposes the use of a sorbent chamber (or cartridge) placed at the top of the syringe needle to yield the so-called microextration in a packed syringe (MEPS) technique. This miniaturised technique, through successive withdrawn and ejection of the aqueous solvent, allows the preconcentration of the analytes in the aqueous sample on the selected sorbent. As in other SPE approach, a washing step (typically with 50 µL of water) can easily be incorporated to remove any undesirable matrix component. Then, analytes are eluted with an appropriate amount of solvent (ca. 20-50 µL) and transferred to the GC/LC port. MEPS applications includes, for instance, the determination of PAHs in water *(86)* and of drugs in blood *(87)*.

Finally, the 96-well plates, very popular in clinical research and for bio-applications, have up to now hardly been used in environmental studies, despite the high throughput and low LODs that can be achieved when they are combined with an appropriate separation-plus-detection system, as demonstrated for the trace-level determination of alachlor in water and vegetables, with an LOD of 0.4 µg/L by GC–MS *(88)*.

Solid-phase micro-extraction

Solid-phase micro-extraction (SPME) was introduced in 1990 by Pawliszyn´s group *(89)* as a solvent-free preconcentration technique in which the analyte(s) is(are) adsorbed onto a fused-silica fiber coated with an appropriate sorbent layer by simple exposure of the fiber for a pre-selected time to the gas or liquid sample. The preconcentrated analytes are then desorbed into a suitable instrument for separation-plus-detection. The device for SPME is extremely simple (Fig. 3). It consists of a syringe-like metallic body equipped with a needle that houses the SPME fiber and a

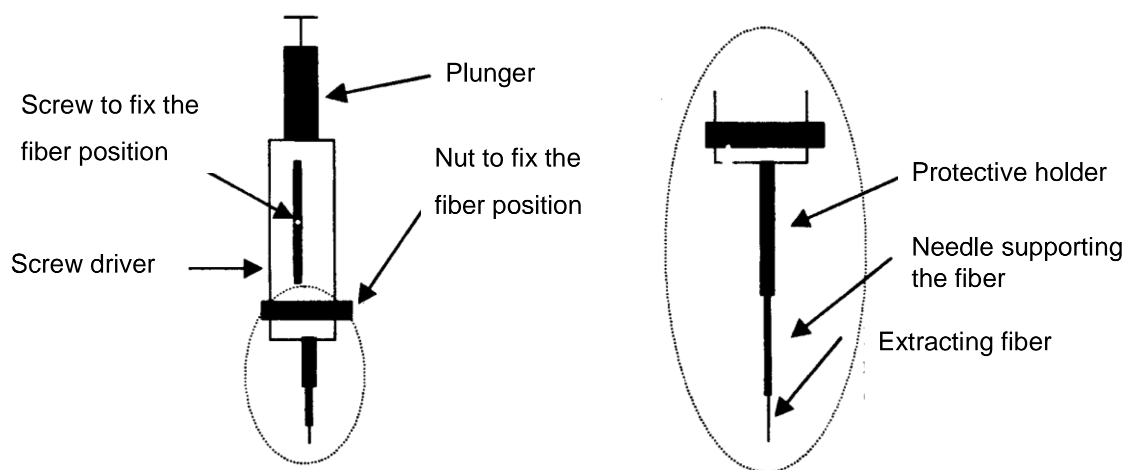

Fig. 3. SPME device.

plunger that allows to retract or to pull the fiber out of its protective holder (i.e., the syringe needle) for analyte sorption and desorption. The fiber position is adjusted with a screw placed in the syringe body. Other SPME-formats, such in-tube SPME *(90-91)* have achieved only a rather limited success.

In general, analyte preconcentration is achieved by exposure of the fiber to the HS of a 1-10 mL vial containing the gaseous, liquid or solid sample; or by direct immersion of the fiber in the liquid sample (or extract) in which the analytes are dissolved. Figure 4 shows an overview of the most common sorption and desorption approaches for SPME as well as some common analytical strategies adopted to increase either the selectivity or the application range of this miniaturised technique. In most instances, SPME is used for preconcentration but other applications, such as purification, phase exchange and field sampling are also possible. This chapter focuses in the first of these aims since all the rest can be considered particular cases of that one.

SPME is an equilibrium technique. In the HS approach, the volatile analytes are displaced from their original sample surface-headspace equilibrium to the fiber surface in which they are progressively preconcentrated. This working mode has been applied for the extraction at ambient temperature of compounds with Henry´s constants above 90 atm cm^3/mol from liquid (ca. 5 mL) or solid (a few mg) samples

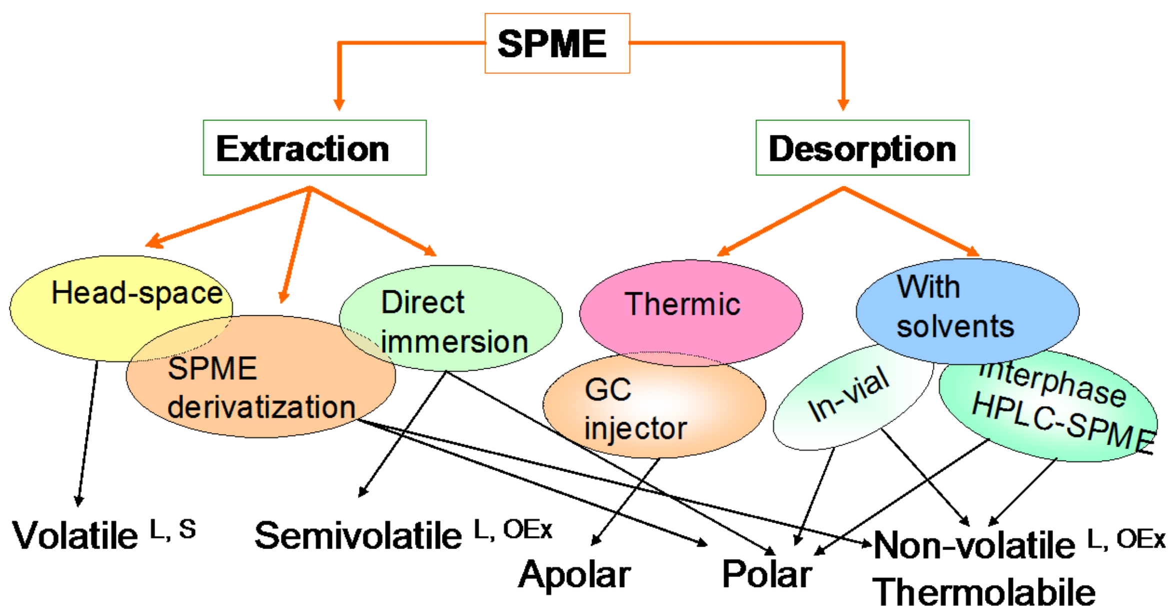

Fig. 4. Different working modes for SPME and their corresponding application fields. L: Liquid; S: Solid; OEx: Organic extracts of solid samples.

contained in a closed vial (or recipient). Extraction times as short as 1 min suffices for quantitative SPME of BTEX from water *(92)*. For less volatiles compounds, the extraction time can efficiently be shortened by reduction of the headspace volume, heating and salting-out of the sample, and by agitation of the liquid sample and the headspace. However, one should be aware that these treatments can also promote desorption of other less volatile matrix components from the sample, which will then compete with the target compounds for sorption in the fiber. Careful optimisation of the several experimental parameters affecting the SPME process is consequently mandatory. In any case, the selectivity of HS-SPME process results in rather clean (i.e., simple) chromatograms and has made this approach to be sometimes preferred for indirect determination of non-volatile analytes which are previously transformed onto more volatile derivatives in the extraction vial.

In principle, direct immersion of the fiber in a liquid sample should force the analyte partition between these two phases. In practice, the fiber is similarly exposed to all matrix components which, depending of their affinity for the selected sorbent, will compete with the target analytes for the active points on the fiber. Therefore,

direct fiber immersion allows to extend the application field of SPME to less-volatile compounds. However, as compared to HS-SPME, selectivity is somehow sacrificed and method optimisation is usually more demanding because of the higher possibility of matrix effect.

As in any other extraction technique based on analyte sorption on a sorbent, one of the main parameters affecting the efficiency of the SPME process are the nature and amount of sorbent. Several fibre coatings are now commercially available, including the non-polar PDMS, semi-polar polydimethyl siloxane–divinylbenzene (PDMS–DVB), and polar polyacrylate (PA), Carbowax–divinylbenzene (CW–DVB) liquid-like phases, the coated porous particle phase polydimethyl siloxane–Carboxen (PDMS– Carboxen), poly(3-methylthiophene), Nafion and, less frequently, carbon nanotubes *(93)*, MIPs *(94)* or simply anodized metals *(95)* and references cited therein, which contributes to expansion of the range of analyte classes that can be successfully analysed (Table 6). This variety of sorbents results in diverse retention mechanisms depending on the nature of the extracted analytes, which can be adsorbed, absorbed or react with the fiber coating.

In most applications, the analyte partitions into the stationary phase until plateau conditions are reached, which typically takes 2-60 min (with higher values for higher-molecular weight analytes). As previously mentioned, the process can be aided by salting-out, sample agitation, pH adjustment and/or heating, and matrix effects can be avoided by using the standard addition procedure for quantitation or, less frequently, protective membranes to prevent the adsorption of matrix components on the fibre *(96)*. At equilibrium, the amount of analyte sorbed in the fiber coating is directly related to its concentration in the sample according to the following equation:

$$n = \frac{K_{fs} V_f V_s C_o}{(K_{fs} V_f + V_s)}$$

where, n is the number of moles of compound retained by in the stationary phase, K_{fs} is the partition coefficient of the compound between the station at phase and the sample, V_f is the volume of the stationary phase, V_s is the volume of the sample, and C_o is the initial concentration of the compound in the sample. The relationship

between the amount of analyte preconcentrated in the fiber and that on the original sample is consequently linear and SPME can provide quantitative data. Moreover, when the volume of the sample is very large as compared to that of the fiber, the previous equation is simplified to

$$n\ V_s = K_{fs}\ V_f\ C_0$$

and that justifies why SPME can be used in field sampling. SPME allows to achive LODs at low ng/L levels for both volatile *(97)* and semi-volatile *(98-99)* analytes when selective detection is used, e.g. SIM-MS or AED *(100)*. Increasing fibre thickness, typically in the 7–100 μm range, helps to increase the sensitivity because of the improved partitioning ratio but it also increases equilibrium times and, sometimes, results in problems achieving complete desorption. Strategies involving derivatization of the analytes in the aqueous phase, combined with SPME, have extended the range of application to very polar *(101)* or ionic substances *(102-103)*.

Applications involving SPME with on-fibre derivatization require conversion of the analytes after extraction by applying the reagent as a gas and are, as far as we know, still scarce in the literature. The relatively high RSD values reported up to now when using this approach (e.g. 10–35% for chemical warfare agents at 1–20 μg/mL levels, n=6 *(108)*) can be regarded as an indicator of conditions which are difficult to control. Generally speaking, although the analysis of aqueous samples can be accomplished without (or with little) pre-treatment (Table 5), SPME of target compounds from more complex (solid) matrices typically requires a previous separation of the analytes from the main matrix components *(109)*, uses to involve longer extraction times and is frequently less exhaustive than for liquid samples because of the less favourable extraction conditions.

Table 6. SPME analytical approaches and typical applications.

Sample (mL/mg)	Analyte	SPME fibre (thickness, µm)	Fiber description	Sample pretreatm.	t (min)	Ref.
Head-space						
Tap and swimming-pool (10)	Haloacetic acids	PDMS–Carboxen (75)	Partially crosslinked	Derivatization + ion strength adjustment	35	*(104)*
Industrial harbour water (10)	PCBs	PDMS (100)	Non-bonded	–	30	*(98)*
Human urine (2)	BTEX	PDMS (100)	Non-bonded	–	30	*(97)*
Slurry plant:water (1:3) (5000)	Organophosphorous pesticides	PDMS (100)	Non-bonded	Homogenization	90	*(105)*
Waste oil (0.5)	PCBs	PDMS–DVB (65)	Partially crosslinked	Acid digestion +LLE+ water dilut	10	*(100)*
Direct immersion						
Surface water (3)	Herbicides	CW–DVB (65)	Partially crosslinked	–	30	*(99)*
Industrial wastewater (5.3)	Industrial organic pollutants	PA (85)	Partially crosslinked	pH adjustment	30	*(106)*
Fruit and fruit juice (3)	Organophosphorous pesticides	PDMS (100)	Non-bonded	Slurry soil:water (1:100)	20	*(107)*

Analyte desorption from the SPME fiber in which they have been preconcentrated is most frequently accomplished by direct thermal desorption in the GC inyection port for subsequent GC separation and detection (Fig. 3). In this approach, injection conditions should ensure complete analyte volatilization from the fiber and introduction in the GC column. The only practical limitation is the working temperature, which is determined by the nature of the fiber stationary phase (typically in the 260-300 ºC range, depending on the coating). Nowadays, a number of commercial GC autosamplers allows complete automation of the SPME process.

More polar or thermolabile analytes can be manually extracted by immersion of the fiber in a small amount of solvent contained in a vial. In this case, after a preselected extraction time, a fraction of the enriched solvent is typically injected in an LC or CE system for separation and analyte detection. This approach requires the careful optimisation of the several parameters affecting the solid-liquid extraction process, such as the nature and volume of the extraction solvent, extraction time and, when required, solvent agitation. One should also be aware that this approach is not applicable to fibres with non-bonded phases because they trend to swallow and dissolve in contact with organic solvents. Several interfaces have also been developed to allow automation of this liquid extraction process and direct transfer of the complete liquid phase to the LC system *(110)*, and references wherein]. However, the practical application of this configuration looks to be somehow more limited than that achieved by the GC-based approaches.

It is evident that the small size of the fiber is the main responsible of both the advantages and main shortcomings of the technique. Most of them have been discussed in detail in this section, but there is a particular type of analysis for which the small size of the needle is the key feature making SPME the only applicable technique. The approach is so-called non-depletive SPME (nd-SPME) and has been used, for instances, to the determine pollutants dissolved in the porewater with high precision *(111-112)*. The miniaturised fiber design was also the key aspect in the determination of both the free and total internal amounts of chlorfenvinphos in laboratory- and field-exposed small insects (*Trybliograpa rapae*, up to 3 mm long) extracted with only 200 μL of the selected solvent *(29)*. LODs ten times lower than

reported for solvent-based extraction procedures, i.e. below 0.5 ng, were obtained after 45 min SPME, thermal desorption, and GC–ECD analysis.

Stir-bar-sorptive extraction

One of the quoted limitation of SPME, the relatively small volume of bound stationary phase, prompted the development of a new miniaturised extraction technique, stir-bar-sorptive extraction (SBSE), introduced in 1999 by Sandra´s group *(113)* and marketed commercially as the Twister. In a typical SBSE experiment, a magnetic stir bar coated with 55 or 219 µL PDMS (corresponding to magnets 10 and 40 mm long, respectively) is rotated into an aqueous sample (or extract) for a selected, but often fairly long, extraction time. SBSE of the headspace of a gas, liquid or solid sample contained in a sealed vial is also possible, although less frequently used. The magnetic stir-bar can also be inserted into a short length of PDMS or silicon tubing. In any case, because the surface area of the stir bar is greater than that of the SPME fibre and the volume of the adsorbent is at least a factor of 100 larger, there is a higher phase ratio than in SPME and, hence, a higher extraction efficiency which results in lower LODs. After the extraction, the stir bar is removed, often manually, and transferred to the injection port of a GC for thermal desorption *(114)*, or into a solvent for LC analysis *(115-116)*. A novel desorption unit enables fully automated analysis of 98 or 196 PDMS-coated stir bars *(117)*.

The similarities between SPME and SBSE could easily make conclude that all working modes described for SPME (Fig. 3) are also possible for SBSE. However, it is important to note that the still rather limited variety of coating of materials available for SBSE limits the practicability of the technique. Anyway, SBSE has demonstrated to be a valuable simple, green and miniaturised analytical alternative for many applications and, in some of them, has proved to be superior to SPME. When combined with a selective GC detector such as MS and using sample volumes of typically 10 mL the technique is feasible for analysis of compounds ranging from non-polar PAHs *(115)* to some organotin compounds *(118)* in water, dicarboximide-type fungicides in wine *(119)*, and additives in beverage and sauce samples *(120)* at the µg/L level. However, and similarly to SPME, application to more complex samples such as biological fluids *(121-122)* or solid samples *(117)* can only be accomplished

after a pretreatment step which effects appropriate isolation of the target compounds from the matrix.

Compounds preconcentrated on the stir bar can also be extracted with a small volume of solvent, e.g. 500 μL, in a vial *(116, 123)*. However, the clear drawback is more manipulation and dilution of the analytes, i.e. loss of analyte detectability, because only a fraction of the extract is injected typically into the LC system.

3. MINIATURISED ANALYTICAL TECHNIQUES FOR TREATMENT OF SOLID SAMPLES

Most of the previously revised techniques can not directly applied to (semi-)solid environmental and biological matrices, i.e. to samples containing high amount of lipids, proteins or organic matter. In general, the analysis of these types of samples requires the initial extraction of the target compound(s) from the complex matrix in which they are entrapped. The non-selective character of most of the exhaustive extraction procedures used in this step made subsequent purification and/or fractionation of the studied analytes from the coextrated material mandatory to ensure the accurate instrumental determination of the target compounds. For these subsequent clean-up steps, techniques and analytical procedures similar to those described for liquid samples in previous sections are typically used.

This section reviews modern techniques that have already demonstrated to provide extraction efficiencies similar to those of other conventional (i.e., large scale) and widely accepted extraction techniques but that involve a much smaller sample size (i.e., < 0.5 g). Because one of the main goals of green sample treatment is the effective reduction of reagents consumption, techniques and analytical strategies allowing an enhanced selectivity, so contributing to the simplification (or even elimination) of subsequent clean-up treatment(s) of the generated extracts before instrumental analysis, will receive special attention.

Matrix-solid phase dispersion

Matrix-solid phase dispersion (MSPD) was introduced in 1989 by Barker et al. *(124)* as a process that allowed the disruption of the structure of a solid matrix and its

extraction in a single treatment. In MSPD, the (semi-)solid matrix is blended with an appropriate sorbent until a dried and homogeneous mixture is obtained. This process results in the homogenous distribution of the matrix components on the sorbent surface and, in practice, can be considered a solid-solid extraction. The mixture obtained is then packed into a column (or syringe barrel) from which the analytes of interest are eluted with a suitable solvent. MSPD is also applicable to liquid or viscous matrices by simply mixing of the sample and the sorbent, and subsequent sedimentation and homogenous packing of the slurry into a column. Proper selection of sorbent and eluent can effect specific retention of impurities on the sorbent and selective elution of the target compounds. These combined clean-up effects frequently enable direct analysis of the collected extract. As an illustrative example of the efficiency of the MSPD process, Figure 5 compares the total-ion GC-MS during the analysis of selected pesticides in single insects, i.e. 40 mg *Porcellio scaber (30)*. MSPD does not require special equipment and it is feasible for field application.

The quoted features and the relative simplicity of MSPD are responsible for the wide and fast acceptation of the technique in many application fields in which it has demonstrated to be a valuable alternative to more classical exhaustive techniques, as highlighted in several reviews *(125-127)*.

MSPD miniaturisation can be achieved simply by reducing the amount of sample subjected to the analysis, with corresponding reduction of the sorbent used for dispersion. This simple operation allows to reduce the tens grams of sorbent and several hundreds of milliliters of solvent required for sample preparation by most conventional methods to around to or to less than 1g of sorbent and 5-20 mL of solvent in miniaturised MSPD. Apart from the inherent benefit when only a small amount of sample is available *(30, 128)*, sample preparation is typically completed in less than 1 h with minimum sample manipulation, something that sharply contrasts with the several hours and several treatment steps required by classical large scale procedures. The feasibility of miniaturised MSPD for the quantitative extraction and simultaneous purification of a variety of analytes have been demonstrated in a number of application studies *(126-127)*, in which the initial sample reduction has easily been compensated by the high sensitivity provided by modern instrumentation.

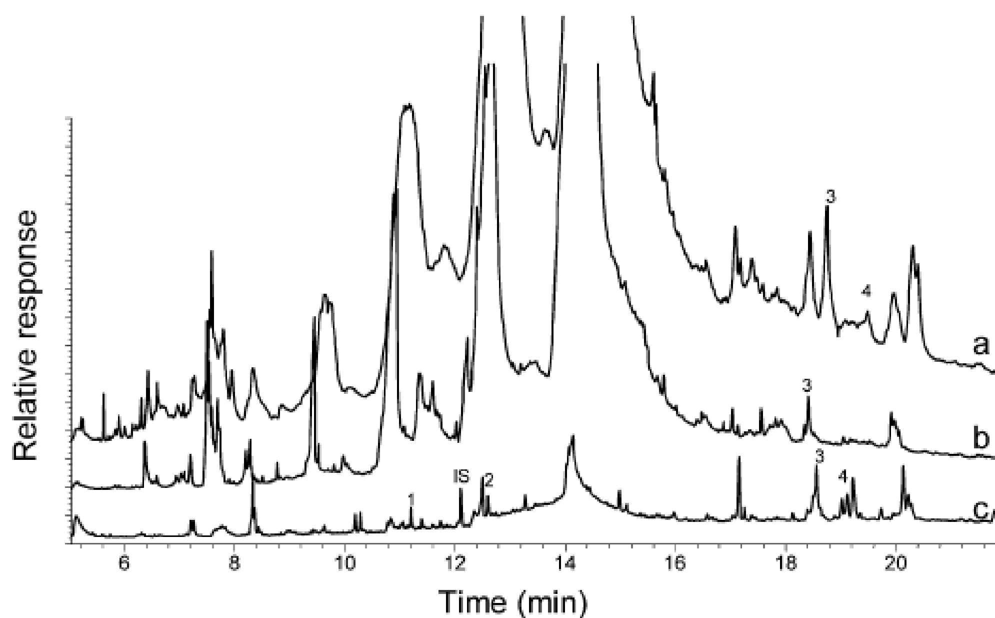

Fig. 5. Comparison of total-ion GC-MS chromatograms of extracts obtained by MSPD from insects with 100 µL (a) ethyl acetate from a C8-bonded silica/sample mixture, (b) ethyl acetate from C8/sample mixture and washing before extraction, and (c) n-hexane from silica/sample mixture. Peak assignement: (1) diazinon, (2) malathion, (3) permethrin, (4) cyfluthrin and (IS) parathionmethyl.

However, somehow surprisingly, miniaturised MSPD is still far from been considered a common practice in laboratories.

Miniaturised MSPD has been demonstrated to be a valuable alternative for the fast and accurate determination of relative abundant components, such as essential oils in herbs *(129)* and polyphenols and organic acids in tobacco *(130)*; but also trace analytes, including environmentally relevant PAHs in soil *(131)* and different classes of pesticides in non-fatty matrices, such as juice *(132)*, fruits *(133-135)*, and cereals *(133)*. For these latter types of matrices, and despite the different chemical structures and polarities of the target compounds, recoveries above 80% have frequently been reported and, when using a selective and sensitive technique for final determination (i.e., GC–MS *(132, 134)* or LC–MS *(133)*), LODs in the low-µg/g range are easily obtained even although no extra treatment of the collected extracts was carried out. As an illustrative example of the potential of the approach for the development of complete on-line (or at-line) processes, using a Prospect-type set-up, Kristenson et al. *(134)* proved that 25 mg of fruit and 100 µL of ethyl acetate sufficed for accurate

extraction (recoveries in the 83-118% range and RSDs below 13%) of a variety of triazines and organophosphorous pesticides at the MRL typically set in EU legislations (LODs in the 4-90 μg/g range).

When dealing with more complex samples, e.g. for pesticide determination in insects, more careful selection of the sorbent-solvent combination is required to perform the necessary clean-up without affecting the performance of the method (see Fig. 5 for a representative example *(30)*). The sequential elution of closely related impurities and analytes and/or performing some extra in-line purification by packing an appropriated sorbent in the bottom of the extraction column are also successful approaches for the one-step analysis of such samples *(128)*.

The analysis of trace compounds in fat-containing matrices represents a particularly difficult analysis case. In this type of determination, apolar sorbents, such C18, are usually preferred for sample dispersion and mixtures of non-polar and medium-polar solvents are used for the sequential extraction of interfering matrix components and target compounds from the column. However, this analytical strategy does not always result in the desirable complete elimination of the lipidic residues and extra treatment of the collected extracts is frequently required *(136-137)*. As an alternative to the subsequent off-line treatment of the MSPD extracts, in-line (or on-line) packing of more polar sorbents, such as Florisil *(131, 138-139)*, strong ion-exchangers *(140)* and silica modified with sulfuric acid *(10)*, have been proposed for complete fat removal so yielding ready-to-analyse extracts.

Somehow unexpectedly, the use of dispersant sorbents alternative to the typical C18 for fat removal in these types of determinations is still rather scarcely considered. However, carbon has been proved to be an efficient alternative for lipid retention during the analysis of dithiocarbamates pesticides and their main metabolites in avocado and nuts *(133)*, although in this study the widely variable structures and polarities of the studied analytes resulted in rather wide recoveries (11-96%). During the analysis of ethylene bisdithiocarbamate metabolites in almond *(141)*, lipids were hydrolised and removed from the column with 0.02 mol/L NaOH. Remaining traces of fat were selectively retained on Alumina (in a second on-line column) during the extraction step. In this study, satisfactory recoveries (76-85%), RSDs lower than 12% and LODs of 50-70 ng/g were obtained although only 200 mg of sample was used

and LC-DAD VU was selected for final determination. In this case, sand was used as sorbent support for MSPD to avoid column clogging. Satisfactory results (i.e., recoveries in the 81-130% range with RSDs of 2-12%) were also obtained when using silica modified with sulfuric acid as MSPD sorbent for fat removal of fatty animal foodstuffs containing up to 45% of lipids (w/w on a dried basis) *(10)*. Ready-to-analyse extracts were obtained when an extra layer of modified silica was packed at the bottom of the extraction column to ensure in-line removal of remaining lipidic traces. LODs as low as 0.09-3 pg/g were obtained for all 23 PCB congeners investigated using GC-microECD for final determination, which proved the feasibility of the proposed procedure for accurate determination of these trace lipofillic pollutants even although subsamples as small as 100 mg of heterogeneous matrices were used for the analysis.

<u>Enhanced fluid/solvent extraction techniques</u>

Extraction efficiency during the preparation of (semi-)solid matrices can be enhanced by heating or shaking the sample or by using as extractant a fluid or solvent with a higher diffusion rate. The latter is the basis of supercritical fluid extraction (SFE) and subcritical water extraction (SWE); the former approaches are used in pressurised liquid extraction (PLE), microwave-assisted extraction (MAE), and ultrasonic-assisted extraction.

The main benefices and limitations of SFE as an essentially solvent-free, i.e. green, and in many instances miniaturised analytical extraction technique have been discussed in previous chapters and will not be repeated here.

Pressurised liquid extraction

Since its introduction in the mid-1990s *(142)* and rapid acceptation as a US Enviromental Portection Agency (EPA) method *(143)*, PLE has experienced a fast development and it is nowadays a widely accepted extraction technique in many research fields (including procedures in which water is used as the extractant, i.e. SWE) *(144)*.In PLE, the sample, typically dispersed in a drying or inert sorbent such as sodium sulfate, Hydromatrix, or diatomaceous earth, is packed in a stainless-steel cell and, once inserted in a closed flow-through system, extracted with the selected

solvent at temperatures above its atmospheric boiling point. Because the solvent must be kept liquid during extraction, relatively high pressures are also applied. Its well documented efficiency, rapidity, and moderate solvent consumption are recognized as the main merits of this essentially analyte- and matrix-independent technique and the reasons for its widespread application. The feasibility of the approach for on-line or in-line coupling with some of the techniques revised in previous sections for subsequent fractionation and/or enrichment of the extracted analytes, an aspect particularly relevant during SWE of less polar compounds, is another valuable feature of the technique.

Despite its many attractive characteristics, the number of studies dealing with minisiturised PLE has, until now, been rather limited, probably because of the relatively large size of the smaller extraction cells of commercial systems (i.e., at least 11 mL). Some authors *(145)* have demonstrated, using such a PLE systems that, for instances, 20 mg of freeze-dried bacterial cells or 100 mg of soil sufficed for the accurate determination of phenols. However, the large dimensions of the 11-mL cell as compared to that of the sample obliged to fill the rest of extraction cell with an inert sorbent. In other cases, the remaining space has been used to pack clean-up sorbents to perform in-line purification of the PLE extacts *(146-147)*. Irrespective of the approach used, these types of arrangements result in the use of amounts of sorbents and solvents similar to those of conventional PLE applications although a much smaller sample is analysed. In other words, sample reduction does not result in the desirable greening of the analytical process.

At present, the only way to solve this problem is to design a home-made miniaturised PLE system *(5, 148-150)*. This type of set-up and a heatable 10 mm x 3.0 mm i.d. stainless-steel extraction cell enabled quantitative extraction of the 16 EPA PAHs (recoveries, 90–110%) from 50 mg soil with only 100 μL of toluene. Direct injection of 50 μL of this raw extract into a PTV system containing the so-called ATAS-A sorbent, enabled in-liner clean-up before GC–SIM-MS analysis and LODs as low as 2–9 ng/g soil for a large majority of the target compounds. Although a small amount of a very heterogeneous sample was used, the RSDs of 2-15% were similar to those found when using traditional methods for this type of determination *(5)*. The approach allowed complete sample preparation in 10 min, minimized reagents

consumption and, because of the small volume of solvent, showed a better potential for hyphenation and automation than with commercial systems. The same arrangement was subsequently used for PLE of chloroanilines from soil. Again, acceptable recoveries were obtained for most of the studied analytes (36-109%) and satisfactory RSDs (8-13%) were reported even although only 50 mg of sample were used for the determination. LODs as low as 5-50 ng/g were obtained when 20 µL of acetone:hexane (1:1, v/v) out of the 100 µL used for the extraction were injected in the GC-MS system.

The main limitation of this set-up, the lack of flexibility as regards the dimensions of the extraction cell, was overcome by using a large, heatable oven, e.g. that of a GC *(148)* or a specifically designed miniaturised oven *(5,150-151)*. The former solution has typically been adopted for SWE because of the need to insert a relatively large coil for heating the water before the extractant reaches the extraction cell *(148-149)*. The latter has been proved to be particularly interesting when the goal is the in-cell purification of the target compounds in order to obtain ready-to-analyse extracts with minimum time and reagent consumption *(150)*.

Microwave-assisted extraction

As with PLE, no miniaturised MAE system is commercially available; consequently, a very limited number of studies dealing with miniaturised MAE is reported in the literature *(152-153)*. The first attempt to develop miniaturised MAE was reported by Cresswell and Haswell *(152)*. In this study, a sediment slurry was pumped through a wide (1/8 in) PTFE tubing installed inside the MAE system at 0.75 mL/min. After passing through the microwave cavity, the slurry was in-line filtered to separate the solid particles from the liquid fraction. Analytes in the aqueous phase were then on-line preconcentrated in a C18 cartridge. The method was applied to the analysis of PAHs in certified sediment although the results were far from satisfactory (RSDs in the range 22–50%). More convincing results were reported by Ericsson and Colmsjo *(153)* who, using an essentially similar approach, proposed inserting a preheating column in front of the extraction cell in the microwave cavity and the back-elution of the target compounds from the 10 mm x 2 mm PLRP-S SPE disposable cartridge used for on-line SPE of the extracted analytes. Using this

configuration the authors demonstrated the feasibility of dynamic MAE coupled on-line with SPE for accurate determination of PAHs in a reference sediment (recoveries 88–104%, RSDs 1–10%) using only 60 mg of sample, 400 μL of MTDE for back-extraction of the analytes from the SPE cartridge and GC-PID for final determination.

Ultrasonic-assisted extraction

The use of ultrasounds for analytical applications is relatively recent. Nevertheless, some applications have already demonstrated the potential of sonication for the miniaturised, rapid, relatively inexpensive and quantitative extraction of analytes of different nature. Early papers in this field focussed in the dynamic extraction and on-line purification of, for example, Cr(VI) in soil *(154)* and organophosphate esters in air filters *(155)*, using a stainless-steel extraction cell placed in either an ultrasonic bath *(155)* or in a water bath close to an ultrasonic probe *(154)*. In both cases, a dynamic approach was preferred because the continuous transfer of the analytes from the matrix to the extractant solvent reduced the risk of analyte degradation by the high temperatures and pressures generated by the cavitation process compared with the static mode. As an example of the typical results obtained, it can be mentioned that this arrangement allowed quantitative extraction of the organophosphate esters preconcentrated from air on 25 mm binder-free A/E borosilicate glass fiber filter in 3 min with only 600 μL hexane:methyl tert-buthyl ether (7:3) and with RSDs below 8%.

Interestingly, as an alternative to the more frequent static ultrasonic bath extraction of relatively small samples (i.e., less than 0.5 g) followed by off-line column purification of the slurries *(156-157)*. Albero et al. *(158)* have recently demonstrated that the speed and efficiency of SPE of pesticides from juice can be improved by placing the SPE cartridge inside an ultrasonic bath for a preselected time. On the base of this observation, Ramos et al. *(159)* proposed the so-called ultrasonic-assisted matrix solid-phase dispersion (UA-MSPD) as an alternative sample preparation technique for the fast extraction and purification of analytes in a single, and if required miniaturised, step. The feasibility of the approach was illustrated for the simultaneous extraction and cleaning-up of selected triazines and organophosphorous

pesticides from fruits. Complete sample preparation was accomplished in only 1 min by direct immersion of the MSPD mixture (i.e., 100 mg of fruit peel dispersed in a similar amount of C8 and wet with the extraction solvent), in a sonoreactor at 50% amplitude. Recoveries above 80% and RSDs in general better than 12% were obtained for the target compounds at spiking levels similar to those set as MRLs in current EU legislations.

In our opinion, all these preliminary results indicate that sonication, alone or in combination with other previously mentioned techniques, might become an interesting analytical alternative to other more conventional leaching procedures.

4. ANALYTICAL MICRO-SYSTEMS: FROM LAB-ON-A-VALVE TO MICRO-TAS

Since its introduction in 1975 *(160)*, flow injection (FI) has experienced a fast progress as an analytical concept, rather than a technique, that effectively contributes to improve the rapidity, robustness and reliability of many (relatively simple) sample preparation operations for which it allows complete automation *(161)*. The FI concept has evolved through two new generations, namely sequential injection (SI), referred as second-generation FI *(162)*; and the so-called laboratory-on-valve (lab-on-valve, LOV), referred as third-generation FI *(163)*. The key aspect to develop SI systems was the replacement of the continuous flow used in FI by programmable flow, which allowed the digital-controlled displacement of liquids, gas and/or beads, by stopping, reversing and accelerating flow rates *(161)*. Subsequent downscaling was achieved by integrating the SI principle on a LOV platform.

The basic component of a LOV microsystem is a transparent, monolithic structure made of Perspex and mounted atop a six-port valve. The system is designed to include connecting ports, working channels and a flow-through cell, through which other individual ports are connected. This central cell is also connected with a propulsion unit, typically a syringe pump, necessary to circulate liquids through the SI-LOV system. In principle, this basic structure allows a number of chemical and physical processes, including fluidic and microcarrier bead control, sample dilution, homogeneous reaction, liquid-solid interaction and analyte preconcentration, and real-time monitoring of various reaction processes via in-cell detection with optical fibers

(161, 164). Auxiliary units (e.g., holding and mixing coils, T connections and auxiliary pumps and valves) can also be incorporated to this basic set-up so increasing the versatility of its potential applications *(161, 164)*.

One of the main features of SI-LOV as compared to other flow systems is the miniaturisation of the flow channels. While the latter operate in the mL-scale, SI-LOV allows downscaling the sample and reagent volumes to the 10-20 μL range, and effectively contributes to minimise waste generation (typically, only 100-200 μL/assay) *(165)*. (For deeper discussion regarding extra practical benefices deriving from proper choice of the LOV channel dimensions, the reader is encouraged to consult a more specialised revision *(161, 165)*). These figures make evident the potential of SI-LOV (either with or without bead injection) for the accurate, automated and green handling of minute samples. The feasibility of the approach for the on-line separation/preconcentration of selected analytes was firstly illustrated for metals, for which LODs in the ultra-trace level have been reported when the LOV system is combined with highly selective and sensitive spectroscopic- or mass spectrometric-based detectors *(161)*, and references therein. In recent years, a number of applications studies have demonstrated the potential of the LOV concept also in the bioanalytical field. Here, the continuous monitoring of relative abundant compounds, such as glucose, lactate, glycerol or ethanol, have been used for the continuous monitoring of cell cultures or enzymatic reactions *(164)*, and references therein. The incorporation of protein-coated beads in a SI-LOV has been demonstrated to be a successful alternative tool for the evaluation of antibody-protein interactions. This approach has also been used for the selective determination of target analytes (antibody or protein). The reported results illustrate the potential of this approach for automated microscale affinity chromatography. Apart from the reduction of the sample and solvent volume and the reduced number of beads used to pack the micro-column, beads can be discarded after each experiment, which contribute to increase the accuracy of the determination by avoiding cross-contamination or ghost peaks due to progressive column degradation. Again, the small volume of eluent typically obtained from LOV-based systems simplifies their direct coupling with sensitive detectors and, if required, with powerful (and essentially

miniaturised) separation techniques, namely CE and LC, for accurate determination of minor compounds in complex mixtures.

The feasibility of further miniaturisation of the systems, including complete integration of the several analytical steps (i.e., sample preparation, analyte separation and detection) in a single monolithic device has been an active subject of research during the last fifteen years *(166)*. This analytical concept yielded the idea of the so-called micrototal analytical systems (μ-TAS), also named lab-on-a-chip, and the downscaling of volumes involved to the nL-range. The several interesting achievements reported in this field since its introduction, and especially during the last decade, should certainly be considered as a prove-of-the-concept. Nowadays, a number of applications, typically involving CE as separation technique and either electrochemical, fluorescence or chemiluminiscence detection, has been reported for the determination of both ions and organic analytes in liquid matrices with LODs in the μM range *(167)*. But, newly, the treatment of solid samples is still challenged: it demands a previous (off-chip) extraction step and, due to the complexity of matrix, immunoassay-based determination. Nevertheless, using this analytical strategy, results in agreement with those obtained with reference methods have been reported, for instances, for the determination of botulinum neurotoxin A *(168)* and folic acid *(169)* in infant formula.

Although further development is still required to overcome some of the shortcomings of present μ-TAS systems, including those associated to sample introduction, slow sample transport and the micromachining of more appropriate interfaces between the different components *(170)*, the promising results obtained up to now make new achievements also be expected in this research field. Probably, in coming years, we will attend to new developments that will yield new μ-TAS generations allowing more sophisticated, integrated and greener on-site analytical determinations.

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1.3. DETERMINACIÓN INSTRUMENTAL DE COMPUESTOS ORGÁNICOS

Los principales problemas asociados al análisis instrumental de los compuestos orgánicos objeto de estudio en esta tesis doctoral son (i) el elevado número de congéneres de algunas de las familias de COPs investigadas, como es el caso de los PCBs y PBDEs; (ii) los bajos niveles a los que deben ser determinados de manera inequívoca en las muestras ambientales y de alimentos investigadas, y (iii) el hecho de que con frecuencia coexisten en el extracto final purificado analitos de estructura química afín pero con niveles de concentración diferente, pudiendo interferir unos en la identificación de otros. Todo ello hace que la selección de la técnica instrumental empleada para la determinación de los analitos en el extracto purificado tenga una especial relevancia en este tipo de análisis, con el fin de eliminar, o cuando menos minimizar, los potenciales problemas derivados de posibles coeluciones cromatográficas, en especial cuando éstas no pueden ser resueltas mediante un incremento en la selectividad del detector.

La volatilidad media-alta de la mayoría de los compuestos incluidos en este estudio y la elevada capacidad de resolución aportada por la cromatografía de gases (GC), hacen de ésta la técnica más frecuentemente empleada para la separación cromatográfica de estos analitos. La elevada sensibilidad requerida para su correcta determinación a niveles traza hace que se use acoplada a detectores sensibles y selectivos, siendo los más habituales los de captura electrónica (ECD), sobre todo en su modalidad micro (μ–ECD) por su mayor sensibilidad, y la espectrometría de masas (MS). Otros detectores, como el de llama (FID) o el fotométrico de llama (FPD), no tienen prácticamente cabida en este campo de aplicación debido a que su baja selectividad limita sus posibilidades de aplicación para el análisis de matrices tan complejas como las ambientales y los alimentos.

Los detectores de ECD se caracterizan por su elevada selectividad y sensibilidad hacia compuestos que contengan un átomo electrófilo en su estructura, por lo que resultan particularmente adecuados para el análisis de compuestos halogenados. La menor dilución experimentada por los analitos en el μ–ECD respecto al ECD convencional contribuye a reducir los LODs para estos compuestos al menos entre 10-100 veces respecto a los conseguidos con este último, por lo que desde su

introducción ha ido poco a poco desplazando a la versión original, sobre todo para el análisis de COPs en matrices ambientales. El precio asequible y facilidad de manejo y mantenimiento son otras de las ventajas de este tipo de detector.

La principal ventaja de la MS es que permite solventar algunas de limitaciones encontradas con el ECD. En particular, permite resolver, al menos en parte, los problemas de coelución del analito de interés con otro(s) compuesto(s) con átomos electrófilos, sean estos de la misma o distinta familia de xenobióticos o sean otros componentes coextraidos de la matriz, al posibilitar la aplicación de criterios adicionales de identificación basados en patrones de fragmentación y, en ocasiones, la posibilidad de extraer iones selectivos para el compuesto el interés frente a sus interferentes. La modalidad de trabajo en MS y el tipo de analizador a emplear dependerá de las necesidades de sensibilidad y selectividad requeridas por el estudio. En general, debido a las elevadas sensibilidades requeridas para el análisis de COPs en muestras ambientales, suele preferirse trabajar en modo de registro selectivo de iones (SIM) en lugar de en modo SCAN, aunque ello suponga la pérdida de la información referente a la (posible) presencia de otros contaminantes no anticipados en la muestra. Trabajando en SIM, la MS de baja resolución (LRMS) suele proporcionar sensibilidades adecuadas para la correcta determinación de los distintos COPs considerados en este trabajo (es decir, LODs en torno a la ppb), incluso utilizando el analizador más sencillo, el cuadrupolar (qMS). Otras determinaciones, sin embargo, requieren mayores sensibilidades y/o selectividades, por lo que implican requerir el empleo de analizadores más sofisticados, como los de alta resolución (HRMS) de campo magnético imprescindibles para la aplicación de los métodos oficiales de análisis de PCDD/Fs y PCBs no-*orto* sustituidos, de coste mucho más elevado. En la bibliografía, no obstante, se pueden encontrar algunos ejemplos que demuestran que la aplicación de MS en modo tándem, bien en el formato de trampa de iones, ITD(MS/MS), o con los analizadores en serie (como en la triple trampa cuadrupolar, QqQ), permite una reducción del ruido de fondo que hace posible su aplicación, incluso, para este tipo de determinaciones en ciertos substratos manteniendo los criterios de calidad establecidos en la legislación vigente.

Las bajas concentraciones a las que es necesario determinar los analitos objeto de estudio en las muestras investigadas en este trabajo hacen que el modo de inyección

seleccionado en GC sea el splitless, para asegurar que el total del extracto inyectado es introducido en la columna y llega al detector. Cuando los niveles son tan bajos que no alcanzan el LOD con el detector seleccionado, como alternativa, se suele emplear la inyección de grandes volúmenes (LVI), que permite pasar de inyectar los 1-2 µL típicos de la inyección splitless a incluso 100 µL, dependiendo del modo de trabajo. Sin embargo, este tipo de inyección requiere el uso de una instrumentación específica, no siempre disponible en los laboratorios.

Las columnas capilares utilizadas en el análisis rutinario de COPs y, en general, de compuestos orgánicos análogos mediante GC suelen tener naturaleza apolar, del tipo 100% metilpolisiloxano o, más frecuentemente, modificadas con un 5% de grupos fenilo (columnas tipo DB-5 y BPX-5). Las dimensiones habituales suelen ser de 30-60 m de longitud, con diámetros internos de 0,25 mm y espesores de fase de 0,25 µm en separaciones monodimensionales, estando la longitud de la columna condicionada sobre todo por la selectividad del detector empleado en el estudio. No obstante, se comercializan también algunas columnas especiales, desarrolladas específicamente para favorecer la separación de ciertas familias de contaminantes, como es el caso de la columna DB-EUPAH para la separación de PAHs, las de alta estabilidad térmica (por ejemplo, tipo HT-8) para la separación rápida de compuestos (semi-)volátiles, y las ultrainertes y de bajo sangrado, especiales para su uso combinado con MS. A pesar de todas estas mejoras, incluso hoy día no existe ninguna columna comercial capaz de separar, en un solo análisis, todos los congéneres de numerosas familias de xenobióticos, como por ejemplo los 209 PCBs. Como es lógico, la situación resulta aún más compleja cuando en el extracto a analizar pueden estar presentes otro tipo de analitos, afines o no, que no hayan sido eliminados durante el proceso de purificación. Cuando se trabaja con GC monodimensional, la solución a este problema suele pasar por el análisis del extracto en dos columnas de diferente polaridad, con el fin de resolver las coeluciones detectadas en una columna en otra que ofrezca un mecanismo de separación diferente. Otras alternativas son el fraccionamiento del extracto previo a su análisis cromatográfico (en general, en columnas empaquetadas o de cromatografía líquida, LC) o el empleo de detectores que ofrezcan una elevada selectividad (como la HRMS), si bien estas aproximaciones son más laboriosas y llevan asociado un mayor coste por análisis. Finalmente, es posible el empleo de

alguna de las técnicas de separación multidimensional desarrolladas en los últimos años, bien sea en su modalidad de heart-cut (MDGC) o en la de cromatografía de gases completa en dos dimensiones (GC×GC), en las que dos columnas de distinta selectividad se acoplan en serie de tal forma que o bien una parte seleccionada del extracto, en la MDGC, o bien su totalidad, en GC×GC, son secuencialmente analizadas en dos columnas que ofrecen mecanismos de separación distintos.

A continuación, se presenta una revisión bibliográfica en la que se discuten los principales avances conseguidos en relación a la aplicación de la GC×GC en análisis medioambientales y el actual estado de las investigaciones en este campo mediante una revisión de los estudios más representativos publicados desde la introducción de la técnica.

1.3.1. CROMATOGRAFÍA DE GASES COMPLETA BIDIMENSIONAL: APLICACIONES A MUESTRAS MEDIOAMBIENTALES[1]

Introduction

Environmental samples, understood here as any liquid or solid environmental matrix, are extremely complex mixtures in which a large variety of compounds with related and unrelated structures are simultaneous present at widely variable levels of concentration. A large majority of these compounds can mutually interfere during their instrumental analysis even if a highly selective separation-plus-detection technique is selected for final determination. Organic micropollutants are entrapped in these complex mixtures, but typically at very low concentrations (typically in the μg/g–pg/g range) and frequently absorbed or strongly bound to other matrix components. In addition, it is now generally accepted that, because of the several anthropogenic impacts simultaneously register by ecosystems, organic microcontaminants are usually present as complex mixtures rather than as individual entities in these samples. This is especially true for some well-known families of industrial pollutants that have been used –and enter the ecosystem webs– as mixtures of isomers in which individual components exhibit different levels of toxicity. These considerations justify the main features of the analytical procedures in use for the analysis of trace organic pollutants in environmental liquid and (semi-) solid matrices. First, the extremely low concentrations at which the target compounds should accurately be detected makes mandatory an exhaustive extraction of the analytes from the matrix to ensure their quantitative recovery and proper detection. The essentially non-selective character of this first step makes mandatory the development of a subsequent laborious –and frequently manual– multistep protocol for purification and fractionation of the target analytes, first from unrelated coextracted material to avoid matrix effect, and then from other structure-related compounds present in the sample

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[1] J.J. Ramos, M. Pena-Abaurrea, L. Ramos; Chapter 11. Comprehensive two-dimensional gas chromatography: environmental applications in "Comprehensive two-dimensional gas chromatography". Edit.: L. Ramos. Comprehensive Analytical Chemistry (CAC) Series. Volumen 55. Elsevier, The Netherlands (2009).

that can interfere with their final determination. complexity of many of the environmental extracts and the simultaneous presence of known (and unknown) compounds can yield inaccurate and inconsistent results and compromise the validity of the final result.

The extended risk of overlap and the environmental field´s interest in determining the actual concentration of a particular analyte, rather than that of a family of pollutants, explain the need for enhanced separation power. The resolution provided by gas chromatography (GC) and its feasibility for direct coupling with a number of selective and sensible detectors explain the general preference for this technique over liquid chromatography and capillary electrophoresis for the analysis of medium- to low-volatility organic pollutants. The significant simplification achieved in the (1D) GC chromatograms by adding an extra dimension either by using mass spectrometric detectors (MS) or other chromatographic-based multidimensional approaches has been demonstrated in the literature and illustrated by self-explicative chromatograms. But the overwhelming separation power of comprehensive two-dimensional gas chromatography (GC×GC) as compared to any other monodimensional and multidimensional GC-based techniques and the sensitivity enhancement achieved through the modulation process were considered especially interesting features that could help solve some of the most pressing challenges in this research area.

This chapter highlights the main advantages and remaining limitations regarding the use of GC×GC for the analysis of organic (semi-)volatile micropollutants in environmental matrices through selected applications that represent the current state of the-art in three main research fields: the analysis of organohalogenated pollutants, the determination of other non-halogenated toxic compounds, and the enantiomeric analysis of chiral contaminants.

1. ORGANOHALOGENATED POLLUTANTS

Aromatic organohalogenated pollutants

Probably the field in which GC×GC was more rapidly incorporated after its development in the petrochemical field was the analysis of organohalogenated

compounds *(1)*. The aim in these studies has usually been rather different from that of petrochemical studies. While group-type separation and fingerprinting are usually the goals of petrochemical studies, most environmental studies have been reported up to now focused on target-compound analysis. The reason is clear: even in the case of numerous families of pollutants containing isomers that are closely related structurally, such as polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs; 210 isomers) or polychlorinated biphenyls (PCBs; 209 possible isomers), variable toxic effects have been reported for the several congeners present in the mixtures *(2)*. Congener-specific determination then becomes mandatory to determine if a particular matrix accomplishes with the maximum residue levels (MRLs) set in current legislations. The extremely low concentration levels at which these particular congeners should accurately be determined in real-life samples and the inherent complexity of the extracts have typically required parallel analysis of the extracts in at least two GC column with different selectivity and, in some cases, the use of high-resolution mass spectrometry (HRMS) for final determination of the target compounds *(3)*. The enhanced separation power provided by GC×GC was immediately recognised as a possible analytical alternative to these tedious and expensive instrumental analysis protocols. Early studies involving GC×GC–HRMS reported promising results and an impressive low limit of detection (LOD) for 2,3,7,8-TCDD of about 200 at *(4)* using a conventional non-polar × polar column set. The chromatographic times, initially similar to those required in conventional 1D GC–HRMS, were reduced to less than 20 min in subsequent studies *(5)*. Despite its evident advantages, progress involving this coupling has been somehow limited *(6)*, probably because of the high price of the detector and its relatively slow acquisition speed. In fact, most of the research dealing with the analysis of 2,3,7,8-PCDD/Fs and toxic PCBs (non-*ortho*-CBs, 77, 81, 126 and 169; and mono-*ortho*-CBs, 105, 114, 118, 123, 156, 157, 167 and 189 *(7)*) has been carried out with alternative (less expensive and easy to handle) detectors, such as the flame-ionization detector (FID) *(8-9)* and electron-capture detector (ECD) *(10)* in the early stage of development of the technique, and with a clear preference for the micro electron-capture detector (μECD) and time-of-flight mass spectrometry (ToF MS) in the last decade (Table 1).

The feasibility of FID for fast determination of toxic non- and mono-*ortho*-CBs in the technical mixture Clophen A50 was demonstrated by Haglund et al. *(8)* using a smectic liquid-crystal phase as first dimension (^{1}D) and a non-polar column, BPX-5, as second dimension (^{2}D). The special selectivity of liquid-crystal phases for planar compounds resulted in a relatively fast elution of non-planar CBs from the column as compared to toxic congeners. To reduce the retention of the later analytes in this phase, a short (10 m) and narrow (0.15 mm) ^{1}D column, with a thin phase of 0.1 μm, was used. The column was also operated at a higher than usual carrier gas velocity. Altogether leaded to an elution of analytes from the ^{1}D at relatively low temperatures which, consequently, experienced a strong retention in the ^{2}D, housed in the main oven. This effect was compensated by using as short a ^{2}D column as possible (i.e., 0.4 m, corresponding to an effective length of 0.25 m). Under these conditions, temperature ramps as fast as 18ºC/min could be used without significantly affecting the 2D analyte separation but promoting an interesting reduction in the analysis time, which was then completed in only 9 min. However, the limited loading capacity of the columns used in the study resulted in peak broadening for some more abundant congeners present in the technical mixture, that is, the indicator PCBs. De Geus et al. *(10)* avoided this problem by using a longer (24 m) and broader (0.20 mm) smectic ^{1}D column. But the price here was time: a 100 min chromatographic run was required to complete the GC×GC separation of the Aroclor 1248 components. In this study, a conventional ECD was used as detector, something that partially explains the peak broadening observed in the contour plots. In a subsequent work, the authors reported a fourfold improvement (i.e., narrowing) in the peak widths by using a DB-1 × CP-WAX-52 set of short narrow columns, and controlling the temperature of the ^{2}D means of an auxiliary GC oven *(11)*]. Despite the remaining peak broadening associated with the large detector-cell volume, the general performance of the two-oven GC×GC system was demonstrated by satisfactory quantification of the seven prioritary PCBs (congeners No. 28, 52, 101, 118, 138, 153 and 180) in a cod liver reference matrix (certified concentrations in the range 42–1120 ng/g).

Table 1. Selected GC×GC applications involving the analysis of organohalogenated persistent organic pollutants. For simplicity, only optimised experimental set-ups or those providing the most conclusive results have been mentioned.

Analytes	Sample	Column combination (m×mm ID×µm d_f)	Modulator	Detector	Ref.
12 toxic and prioritary PCBs	Clophen A50	LC-50 (10×0.15×0.10) × BPX-5 (0.4×0.1×0.10)	LMCS [a]	FID	*(8)*
Aroclor 1254	standard solution	SB-Smectic (24×0.20×0.15) × Ultra 2 (5.3×0.2×0.33)	two-stage TDM [b]	ECD	*(10)*
7 prioritary PCBs, toxaphene	Aroclor 1248, narwhal blubber	DB-1 (9.7×0.18×0.40) × CP-WAX-52 (0.8×0.10×0.20)	Sweeper	ECD	*(11)*
31 PCBs, 17 PCDD/Fs	standard mixtures	DB-1 (9.0×0.20×0.33) × CP-WAX-52 (0.3×0.10×0.20)	Sweeper	µECD	*(9)*
12 toxic PCBs, 17 PCDD/Fs	cod liver	HP-1 (30×0.25×0.25) × HT-8 (1×0.1×0.10)	Sweeper	µECD	*(12)*
209 PCBs	seal blubber	DB-XLB (60×0.18×0.18) × LC-50 (2×0.15×0.10)	LMCS	µECD	*(13)*
12 toxic PCBs,17 toxic PCDD/Fs	spiked milk	DB-XLB (30×0.25×0.25) × LC-50 (0.9×0.18×0.10)	loop modulator, CO_2	µECD	*(14)*
15 toxic and prioritary PCBs	sludge	HT-5 (15×0.25×0.10) × BPX-50 (1.0×0.10×0.10)	loop modulator, LN_2	µECD	*(15)*
12 toxic PCBs, 17 toxic PCDD/Fs	spiked milk, fish oil	DB-XLB (30×0.25×0.25) × LC-50 (1.4/0.9× 0.15 ×0.10)	LMCS	µECD	*(16)*
12 toxic PCBs, 17 toxic PCDD/Fs	food and feedstuffs	DB-XLB (30×0.25×0.25) × LC-50 (0.9×0.15×0.10)	LMCS/loop modulator, CO_2	µCD	*(17)*
209 PCBs	standard solution	HT-8 (50×0.22×0.25) × BPX-50 (2.5×0.1×0.10)	quad-jet dual stage modulator, LN_2	ToF MS	*(18)*
4 non-*ortho* PCBs, 17 toxic PCDD/Fs	fly ash, sediments, vegetation, fish	Rtx-Dioxin 2 (60×0.25×0.25) × Rtx-500 (2.0×0.18×0.10)	quad-jet dual stage modulator, LN_2	ToF MS	*(19)*
17 toxic PCDD/Fs, 18 toxic and prior. PCBs	foodstuffs	Rtx-500 (40×0.18×0.10) × BPX-50 (1.5×0.10×0.10)	quad-jet dual stage modulator, LN_2	ToF MS	*(20)*

Analytes	Sample	Column combination (m×mm ID×µm d_f)	Modulator	Detector	Ref.
4 non-*ortho* PCBs, 17 toxic PCDD/Fs	standard solution	Rtx-Dioxin 2 (60×0.25×0.25) × Rtx-PCB (2.0×0.18×0.18)	quad-jet dual stage modulator, LN_2	ToF MS	*(21)*
4 non-*ortho* PCBs, 17 toxic PCDD/Fs	spiked fish oil	Rtx-Dioxin 2 (60×0.25×0.25) × Rtx-PCB (3.0×0.18×0.18)	quad-jet dual stage modulator, LN_2	ToF MS	*(22)*
38 PCBs, 11 OCPs, 12 PBDEs, 1 PBB	serum, milk	DB-1 (15×0.25×0.25) × HT-8 (1.2×0.10×0.10)	quad-jet dual stage modulator, LN_2	ToF MS	*(23)*
17 PCDD/Fs	certified fly ash, flue gas	InertCap 5MS/Sil (60×0.25×0.10) × InertCap 17MS/Sil (1.5×0.075×0.10)	loop modulator, LN_2	HRToF MS	*(24)*
209 PCBs	standard solution	BP-1 (27×0.32×0.32) × VF-23MS (1×0.10×0.10)	home-made dual-jet modulator [c]	ECNI qMS	*(25)*
17 toxic PCDD/Fs	standard solution	DB-XLB (30×0.25 ×0.25) × LC-50 (0.9×0.10×0.10)	home-made dual-jet modulator [c]	ECNI qMS	*(26)*
125 PBDEs, 7 PBDE metabolites, 6 PBBs, HCBD, TBBP-A, Me-TBBP-A	standard solution, dust	DB-1 (30×0.25×0.25) × 007-65HT (1.0×0.10×0.10)	loop modulator, CO_2	µECD	*(27)*
17 PBDEs	fish (eel)	DB-1 (30×0.25×0.25) × 007-65HT (1.0×0.10×0.10)	home-made dual-jet modulator [c]	ECNI qMS	*(26)*
26 MBPs	dolphin blubber	Rtx-5 Crossbond(10×0.18×0.20) × BPX-50 (0.70×0.10×0.10)	quad-jet dual stage modulator, LN_2	ToF MS	*(28)*
Toxaphene	technical mixture	DB-1 (10×0.25×0.25) × HT-8 (1.0×0.10×0.10)	LMCS	ToF MS	*(29)*
PCAs	technical mixture (C_{10}, 65 wt% Cl)	DB-1 (30×0.25×0.25) × 007-65HT (1.0×0.10×0.10)	home-made dual-jet modulator [c]	ECNI qMS	*(26)*
PCAs plus PCBs, PCDD/Fs, PBDEs, OCPs, PCDEs, PCNs, toxaphene, PBBs	dust	DB-1(30×0.25×0.25) × 007-6 5HT (1.0×0.10×0.10)	loop modulator, CO_2	µECD	*(30)*
PCAs	technical mixture (C_{10}, 65 wt% Cl)	DB-1 (30×0.25×0.25) × 007-65HT (1.0×0.10×0.10)	home-made dual-jet modulator [c]	ECNI qMS	*(26)*

PCAs	standard solution, technical mixtures, dust	DB-1 (30×0.25×0.25) × 007-65HT (1.0×0.1×0.10)	loop modulator, CO_2	ECNI ToF MS	*(31)*

[a] LMCS, longitudinally modulating cryogenic system; [b] TDM, thermal desorption modulator; [c] home-made dual-jet modulator *(32)*.

Further improvement was achieved when replacing the ECD (1.5-mL cell volume) by the μECD (150-μL cell volume) *(9)*. Although peak widths were still wider than those obtained with FID (10–20 μL cell volume), a significant peak shape improvement was obtained by the 10-fol reduction of the ECD cell volume. The narrowing of the peak widths also had the obvious consequence of peak sharpening, that is, enhanced sensitivity. In this pioneer study, LODs for individual PCB congeners in the 6–20 fg range (injected mass) were obtained. These rewarding results, combined with the selectivity of μECD for the analytes containing an electrophilic atom, justified its wide acceptation as GC×GC detector for the analysis of organohalogenated pollutants. Despite the short columns used in this study, the orthogonal nature of the selected column set (DB-1 × CP-WAX-52) resulted in a wide spread of the targeted PCBs in the 2D plot and, even though the separation conditions were not really optimised, a first indication of an ordered pattern. The ability of GC×GC to generate structured chromatograms was demonstrated by Korytár et al. *(12)* in a wider study involving 90 PCB congeners. In this case, two nonpolar stationary phases, HP-5 (30 m×0.32 mm, 0.25 μm) and HP-1 (30 m×0.25 mm, 0.25 μm), were selected as ^{1}D columns and used in combination with phases of different polarity (BPX-50 and Supelcowax-10) and selectivity (HT-8). Two column sets, HP-1 × HT-8 and HP-1 × Supelcowax-10, allowed a complete separation of all 12 toxic non- and mono-*ortho*-CBs from the other congeners included in the mixture. HP-1 × Supelcowax-10 provided the best separation of the PCBs investigated (84 congeners eluted free from interference with this combination), but little or no ordered structure was observed in the 2D contour plot. With HP-1 × HT-8, 78 congeners eluted as resolved peaks in clearly structured chromatograms in which PCBs were grouped together according to the degree of chlorination, while within-group position was determined by the number of *ortho*-chlorine-substituents. This type of ordered structure allowed tentative identification of PCB congeners for which the corresponding standard was not available. Consequently, this column set was preferred for further optimisation and application to the analysis of a purified cod liver extract spiked with the 17 toxic PCDD/Fs. Because of their planar structure, PCDD/Fs exhibited a strong retention in ^{2}D, eluting at retention times longer than

those of the most retained PCBs, i.e. the non- and mono-*ortho*-CBs, and separated from them. The only exception was one penta-CDD that co-eluted with PCB 169. Typical PCB structured chromatograms are shown in Figure 1. In the latter, wraparound is also visible for the hepta- and octa-CDD/F congeners.

This first work was followed by several detailed studies involving more complete sets of either columns or analytes. Using DB-XLB as first dimension, Harju et al. *(13)* evaluated five column combinations and demonstrated that the DB-XLB × SP-2340 and DB-XLB × LC-50 sets provided the most satisfactory results allowing the separation ($Rs \geq 0.5$) of, respectively, 176 and 181 of the 209 PCB congeners; and the detection of 126 of the 136 PCBs present in Aroclors 1232, 1248 and 1260 at concentrations greater than 0.05% (w/w). As a further illustration of the technique´s potential for accurate determination of individual PCBs in complex mixtures, the DB-XLB × LC-50 set was used for analysis of a purified seal blubber extract. In this case, 64 PCBs were identified by applying a peak template, although use of an internal standard was mandatory to correct the observed small retention time shifts. In another exhaustive study *(14)*, the authors concluded that, under orthogonal conditions, using DB-1 as ^{1}D and a μECD as detector, the best simultaneous separation of the 17 2,3,7,8-substituted PCDD/Fs and the 12 dioxin-like PCBs was obtained using a relatively polar phase (VF-23; absolute cyano content 70–90%) or the shape-selective LC-50 column as ^{2}D. These columns sets had the extra advantage of providing structured chromatograms. However, they failed to separate the PCDD/Fs from the matrix constituents: the targeted congeners showed up in the matrix band when analysing a purified milk extract.

This finding prevented use of these column sets for real-life applications at trace level. More importantly, it made evident that the matrix should also be considered during GC×GC method development. Complete separation of all 29 toxic analytes from each other and from the co-extracted matrix components was only achieved using DB-XLB × LC-50. This result confirmed previous observations from Harju et al. *(13)* and proved that, despite the identification potential derived from ordered structures, orthogonality should preferably be (partly) sacrificed to achieve the required separation from the sample matrix. Supporting this statement are the

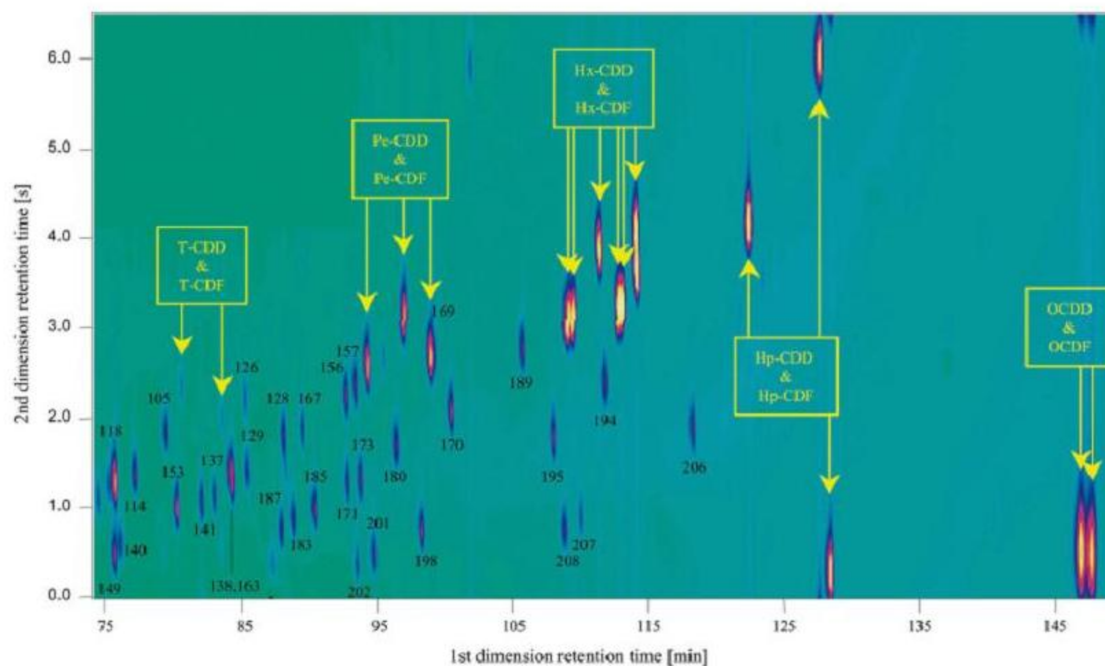

Fig. 1. Structured GC×GC–µECD contour plot of a mixture of 90 PCBs and 17 PCDD/Fs with HP-1 × HT-8 *(12)*.

satisfactory quantitative data obtained with this column set (three orders of linearity, LODs below 70 fg for dioxin-like PCBs and in the 40–150 fg range for the 2,3,7,8-PCDD/Fs, and RSDs lower than 6.5% of all target compounds).

Further investigation of this topic *(16-17)* involved a variety of spiked and non-spiked food matrices and confirmed the main conclusions of this study. Probably the most interesting point here was that the use of VF-1 as first dimension allowed the elution of the analytes at a temperature ca. 20ºC lower than that required for DB-XLB. In practice, that meant that the studied compounds had a higher retention in the ^{2}D column, LC-50. A 0.9-m-long LC-50 column sufficed then to achieved a separation for PCBs similar to that obtained with a 1.4-m-long column coupled to DB-XLB *(16)*. Unfortunately, it also resulted in co-elution of PCDD/Fs due to wraparound, even although a relatively large modulation period of 8 s was used. Because of the limitation in the maximum working temperature of the LC-50 phase, use of a faster temperature program and a secondary oven to hold the ^{2}D column did not represent practical solutions to the problem. As an alternative, the ^{2}D separation was speeded up by programming the flow of the carrier gas (1.3 mL/min (21 min), at 0.4 mL/min to 1.6 mL/min (5 min), and then at 0.4 mL/min to 1.3 mL/min).

Experimental results showed that flow programming caused retention-time instability and different retention-time shifts for the analytes and the internal standard, which made it difficult to use templates for peak identification. In addition, the high flows adversely affected the separation in ^{1}D. The comparison of the total toxic equivalent of 2,3,7,8-TCDD (TEQs) of PCDD/Fs calculated using VF-1 (30 m×0.25 m×0.25 μm) as first dimension and a 0.9 m×0.18 mm×0.15 μm LC-50 as the second column with those obtained by conventional GC–HRMS proved that, although this column set provided satisfactory results for toxic PCBs, the concentrations calculated for some PCDD/F congeners were overestimated. The separation obtained among the most toxic PCBs (i.e., congeners No. 77, 126 and 169) and the test 2,3,7,8-PCDD/Fs and (remaining) matrix components with DB-XLB × LC-50 is nicely illustrated in Figure 2, where the position of the selected internal standards (TCN and OCN) used for retention-time shift correction is also indicated. The improved separation yielded improved quantitation and demonstrated that, when properly tuned, GC×GC–μECD gives average concentrations comparable to those of the reference HRMS method. The (expected) concentration-dependent response of the μECD at concentrations close to the limit of detection (as those found when analysing nonspiked food), the baseline instability observed at those low concentrations, and the ca. tenfold higher sensitivity of GC–HRMS compared to GC×GC–μECD were suggested as possible responsible for the somehow worse, though still acceptable (below 22%), repetitiveness of the latter technique.

All these results support GC×GC–μECD as a promising alternative for screening of PCBs and PCDD/Fs in environmental samples. However, present software limitations regarding detection and quantification of compounds with low signal-to-noise ratio or close-eluting compounds made manual integration still mandatory in this type of analysis. The practical consequence is a significant increase in the final price (in terms of time) of the toxic PCB and PCDD/F analysis by GC×GC–μECD, which has been estimated as about double that involving the conventional HRMS procedure. Further technical development of μECD design is also desirable to avoid the deterioration of the chromatographic resolution caused by postcolumn band broadening in this detector.

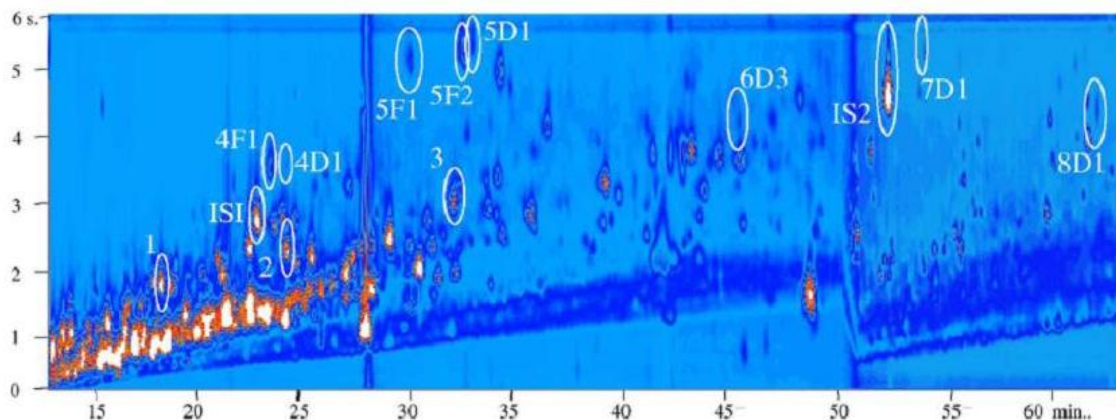

Fig. 2. GC×GC–μECD contour plot of a fish oil (non-ortho-CB and PCDD/F fraction) with DB-XLB × LC-50 *(16)*.

Commercialisation of robust and fast-scanning mass spectrometers, and in particular of ToF MS, has decisively contributed to the increased use of this technique in combination with GC×GC to yield a powerful separation-plus-detection setup providing three-dimensional separation.

The first study reporting on the use of GC×GC–ToF MS for environmental application was published in 2004 *(18)*. This work was in the line of those published at the time by other authors with μECD and reported on the relative merits of four column combinations — DB-1 × HT-8, DB-XLB × HT-8, DB-XLB × BPX-50 and HT-8 × BPX-50 — for the separation of the 209 PCB congeners. In all instances, relatively long columns (in the 50–75 m range for ^{1}D and of 2.5 m in the second dimension) and a slow ramp of temperature were used. This resulted in improved separation, but also in relatively long analysis times of up to 2.5 h. The use of thermally stable phases allowed the application of an off-set of temperature of 40ºC to the secondary oven housing the ^{2}D column. Under these conditions, wraparound was minimised and peak widths of 100–150 ms were obtained. In agreement with previous observations, the authors concluded that a satisfactory separation of the 12 toxic and 7 prioritary PCBs from any other congener present in the mixture was possible with several of the column sets assayed. However, they suggested HT-8 × BPX-50 as the best alternative. This column combination resulted in highly ordered roof-tile structures, similar to those observed for DB-1 × HT-8, but with a wider spreading of the compounds in the 2D contour plot. As a result, a clear separation of

the homologue groups into subseries according to the *ortho*-substitution level was observed *(18)*. A total of 188 PCBs were chromatographically separated with this column set. Use of the ToF MS allowed differentiation of four extra congeners, yielding a total of 192 separated peaks in 146 min. This separation is somehow better than the previously mentioned 194 congeners in 240 min on 60 m DB-XLB × 2.5 m BPX-70 *(13)* using GC×GC–μECD. However, the significant reduction of analysis time probably depended more on the substitution of DB-XLB by the high-temperature phase HT-8 as ^{1}D than on the use of ToF MS as detector.

The real potential of ToF MS, and again the need to consider the matrix effect during method development, were illustrated in a subsequent study dealing with the determination of the four non-*ortho*-PCBs and the 17 2,3,7,8-PCDD/Fs in a variety of environmental and biological samples, that is, fly ash, sediment, vegetation and fish tissue *(19)*. Using a 40 m Rtx-Dioxin2 column as first dimension, a good separation was obtained among all tested PCDD/Fs, as shown in Figure 3.A for the HxCDD/F congeners. However, the severe co-elution of these analytes, with matrix components not completely eliminated during sample preparation and having masses similar to those of the studied compounds, would have obscured their determination in 1D GC–ToF MS (see reconstructed 1D trace based on *m/z* 390 and 374 in Figure 3.B). The satisfactory separation achieved among the target analytes and these isobaric interferences using a 2 m Rtx-500 column as second dimension solved the problem and allowed their accurate determination in as complex a matrix as a fish tissue, even although unit-resolution was used. The selected column combination, Rtx-Dioxin2 × Rtx-500 allowed satisfactory resolution of all test contaminants among them and from main matrix constituents in the purified extract, except for the critical pair 2,3,7,8-TCDD and PCB 126. This coelution problem was solved by careful selection of the ion masses used for identification and quantitation for both compounds. (Later on, Hoh et al. found an alternative chromatographic solution to this problem when replacing the Rtx-500 column used in these experiments by a 2-*(21)* or 3-*(22)* m long Rtx-PCB.) Use of the isotopic dilution procedure based on ^{13}C-labelled standards helped increase the accuracy of both the identification and the quantitation processes with GC×GC–ToF MS. Thus, concentrations similar to those obtained using the

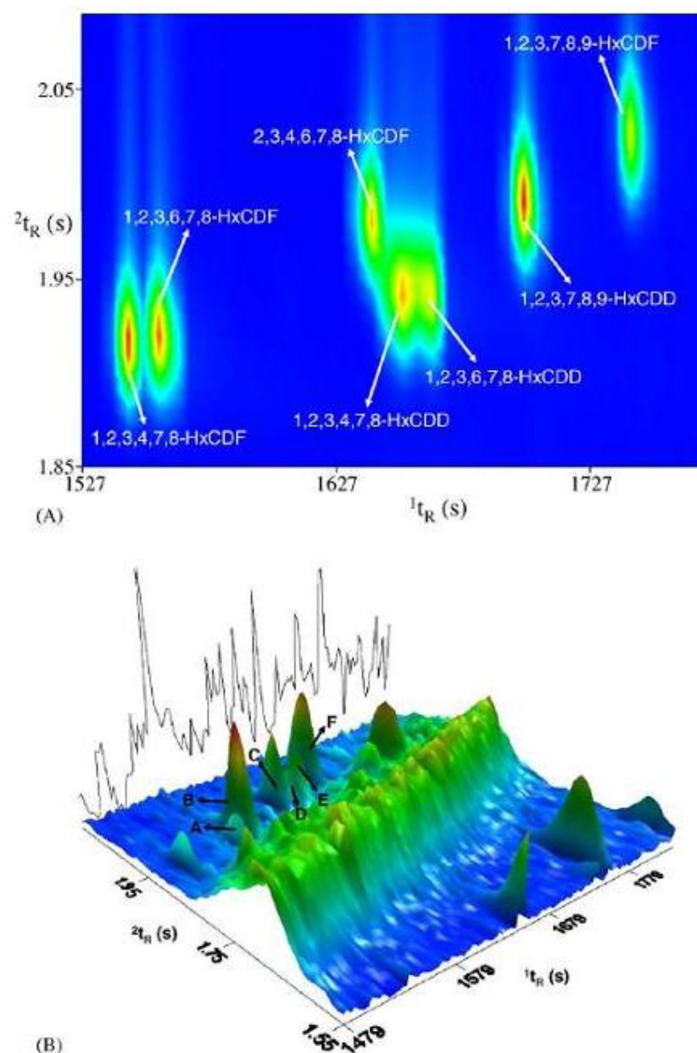

Fig. 3. (A) Extended section of a GC×GC contour plot (m/z 374+390) of a standard solution of HxCDD/Fs. (B) Extended section of the HxCDD/F region of a GC×GC shade surface plot (m/z 374+390) of a purified fraction containing PCDD/Fs isolated from a fish sample *(19)*.

conventional GC–HRMS method were reported for the 21 toxic analytes investigated. Despite the high detector voltage used in these experiments (1800 V), the lower PCCD/F levels detected in biological matrices, close to the instrumental LODs of the GC×GC–ToF MS (0.5 pg for 2,3,7,8-TCDD, while a ca. tenfold lower value of 0.04 pg is reported in GC–HRMS), compromised the application of the technique to the analysis of these particular families of pollutants, unless larger sample sizes were used. However, the higher concentration levels typically found for non-*ortho*-PCBs guaranteed their accurate determination even in these diluted and complex

matrices. hough similar total TEQs were obtained for GC–HRMS, GC coupled to an ion trap working in the tandem MS mode, GC–ITD(MS/MS), GC×GC–ToF MS, and the dioxin-responsive chemical-activated luciferase gene expression (DR-CALUX) — the latter with the exception of milk — all three techniques were found to be less repetitivity than conventional HRMS regarding the determination of individual congener concentrations.

Interestingly, in one of these works *(19)*, the authors pointed out another interesting feature of GC×GC–ToF MS. Because of the nontargeted acquisition of the ToF MS, other close-related trace organohalogenated contaminants, such as certain organochlorinated pesticides (OCPs), polybromodiphenyl ethers (PBDEs) and phthalates, were identified in the same extract used for PCB and PCDD/F analysis. This observation suggested the potential suitability of GC×GC–ToF MS for simultaneous determination of all these relevant pollutants within the same run.

In a follow-up, the authors reported on the feasibility of GC×GC–ToF MS for simultaneous measurement of selected PCBs, OCPs and relevant brominated flame retardants, such as polybrominated biphenyls (PBBs) and PBDEs, in a single chromatographic injection *(23)*. In this case, a shorter DB-5HT column (15 m) was used as ^{1}D to preserve the integrity of PBDEs, and a 1.2 m HT-8 was selected as ^{2}D for effective separation of the planar target analytes from other matrix components. The enhanced separation power provided by GC×GC, the possibility of ToF MS deconvolution, and the use of ^{13}C-labelled internal standards for quantitation, combined with the somehow higher concentrations at which these pollutants are detected in the human serum and milk samples compared to PCDD/Fs, resulted in accurate simultaneous determination of the 59 test pollutants and satisfactory comparison with results obtained with GC–HRMS. Good correlation coefficients spanned over three orders of magnitude (0.5–2000 pg/μL), instrumental LODs ranged between 0.5 and 10 pg/μL, and method LODs were in the 1–15 pg/μL range for all analytes. The reproducibility of the method was better than 11%, that is, almost as good as that of the standard HRMS method for analytes determined in nonspiked pooled human serum. These results demonstrated the suitability of GC×GC–ToF MS for environmental monitoring and, in particular, for human biomonitoring of

these families of pollutants: While analysis of the several families of pollutants tested would require three separate runs with most 1D GC-based techniques today available, one single GC×GC–ToF MS run sufficed to obtain accurate simultaneous information regarding these trace microcontaminant families.

To the best of our knowledge, at the time of writing, only one environmental application had been published involving GC×GC coupled to high-resolution ToF MS (HRToF MS) *(24)*. This study evaluates the feasibility of GC×GC–HRToF MS for accurate determination of the 17 toxic PCDD/F congeners in a certified fly ash and several flue gas samples emitted from municipal waste incinerators using a rather conventional column combination — a 60-m 5% phenyl/phenyl-methyl silicone (InsertCap 5MS/Sil) coupled to a 1.5-m 50% phenyl/phenyl-methyl silicone (InsertCap 17MS/Sil), with a very narrow inner diameter (0.075 mm) for improved separation in the second dimension. A modulation period of 3 s was set to enable 75 data points per modulation since the minimum time for acquisition by the HRToF MS was 0.04 s. Using a mass resolution of 5000 at 500 *m/z*, the author were apparently able to unambiguously determine all target compounds in a fly ash, even in the case of severe co-elution. As an example of the improved resolution power achieved with this instrument, Figure 4 shows the mass profile obtained at 40.28 min between *m/z* 335 and 346 for a fly ash crude (nonpurified) extract and demonstrated that the 337.8678 *m/z* [M+] of PeCDF was separated from 337.3844 *m/z* and 338.2026 *m/z*, which are ions probably derived from other compounds. Despite these promising preliminary results and the low instrumental LODs reported (in the 0.4–5 pg range using ^{13}C-labelled compounds), comparison of the results found for real samples with those obtained using the reference method, (i.e., GC–HRMS) indicated serious bias for specific congeners and suggested that further improvement might be necessary.

Two almost simultaneous papers reported on the feasibility of rapid-scanning quadrupole mass spectrometers (qMS) with an electron-capture negative ion (ECNI) option as detector for GC×GC analysis of PCBs *(26)* and PCDD/Fs *(25)*. In both studies, the instrument was operated in the single ion monitoring (SIM) mode. The

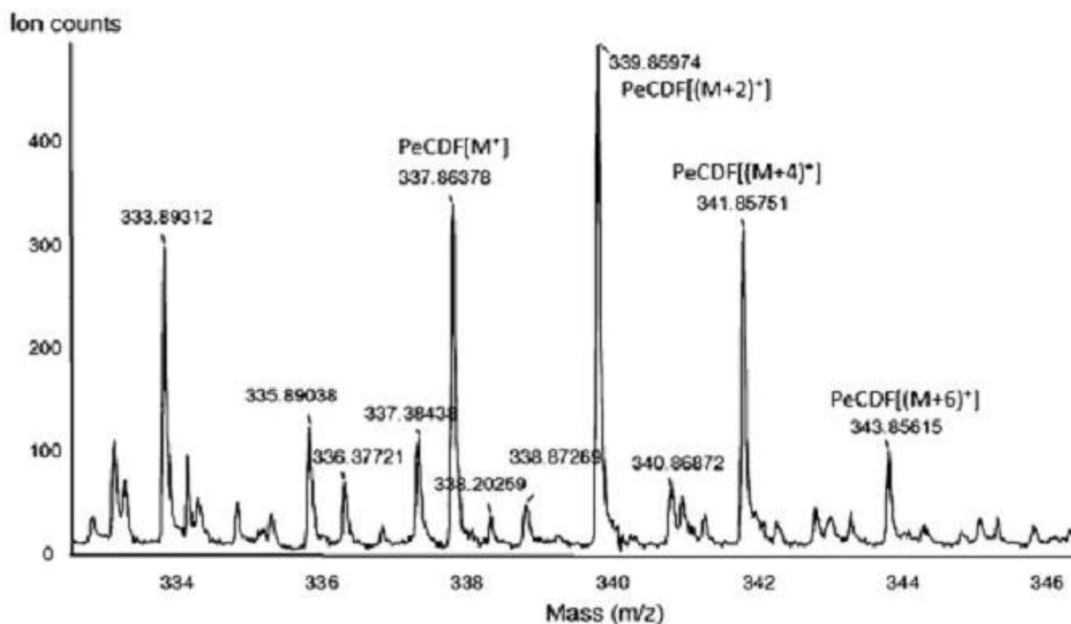

Fig. 4. GC×GC−HRToF MS mass profile of a crude fly ash extract measured from m/z 335 to 336 at 40.28 min *(24)*.

limitation in the number of scanned ions resulted in the desired 33−50 Hz acquisition rate. However, the selected mass range should still ensure that enough mass information was collected for the eluting peaks. In the case of PCBs, seven time-scheduled chromatographic windows were defined. Reported data proved that, despite the limited mass range selected, identification on the base of the mass spectrum was possible (matches of 98−99%). Satisfactory LODs below 2 pg injected mass were reported for a limited number of congeners, a value somehow higher than the 0.1−0.5 pg reported for ToF MS in *(18)*, but still acceptable for a number of real-life applications.

PCDD/F determination with GC×GC−ECNI qMS required a careful optimisation of the several experimental parameters affecting ionisation and data acquisition. In this application, only two selective ions, typically corresponding to the $[M]^-$ and $[M{-}Cl]^-$, were chosen to monitor the corresponding homologue group. Under these conditions, instrumental LODs below 100 fg, that is, in the range of those obtained using GC×GC−μECD *(14)* were obtained for the penta- to hepta-CDD/Fs. Unfortunately, the high LODs reported for OCDD (430 fg) and, especially,

for 2,3,7,8-TCDD (710 fg) limited the applicability of the technique to the analysis of real samples.

Next to PCBs and PCDD/Fs, other families of environmentally relevant aromatic organohalogenated pollutants rapidly attracted the attention of researchers working with GC×GC. Because of their widespread use and rapid increase of their environmental levels during the last decades, most attention was directed to flame retardants and, in particular, to PBDEs *(23, 27)*.

PBDEs are a numerous family of hydrophobic, persistent, ubiquitous and rather nonvolatile pollutants with molecular masses in the range 482–950. According to their chemical structure, there are 209 possible PBDE congeners, which are identified following the same numbering used for PCBs. Technical mixtures contain a limited number of components, 20–25 congeners *(33)*. However, the instrumental analysis of these pollutants is complicated by thermal degradation of the higher brominated congeners and the frequent simultaneous presence in the purified extracts of other closely related analytes *(23)*. Korytár et al. *(27)* reported a detailed study on the GC×GC separation of 125 PBDE congeners and discussed the relative merits of six column sets regarding the notorious analytical problems associated with this type of analysis. Using DB-1 and DB-XLB as first dimension, 007-65HT was preferred as second dimension over VF-23 and LC-50 because (i) it provided a better resolution among the 125 studied PBDEs (co-elution persisted for 17 pairs, while 22 co-elutions were detected with 1D GC), and (ii) it can stand with the high temperatures required to elute nona- and deca-BDEs. Regarding degradation, a severe decomposition of these four higher brominated congeners during the first-dimension run was observed, and DB-1 × 007-65HT then became the column set to choose. Figure 5 shows the apex plot of the 125 PBDEs on this column combination. Under finally proposed conditions, a satisfactory chromatographic separation was achieved for most relevant congeners in 80 min, both among them and from other relevant toxicants included in the study i.e., selected OH- and MeO-BDE metabolites, certain PBBs, and other relevant flame retardants commonly found in environmental samples, in particular hexabromocyclododecane (HBDE), tetrabromobisphenol-A (TBBP-A) and dimethyltetrabromobisphenol-A (Me-TBBP-A). Wraparound was only observed for

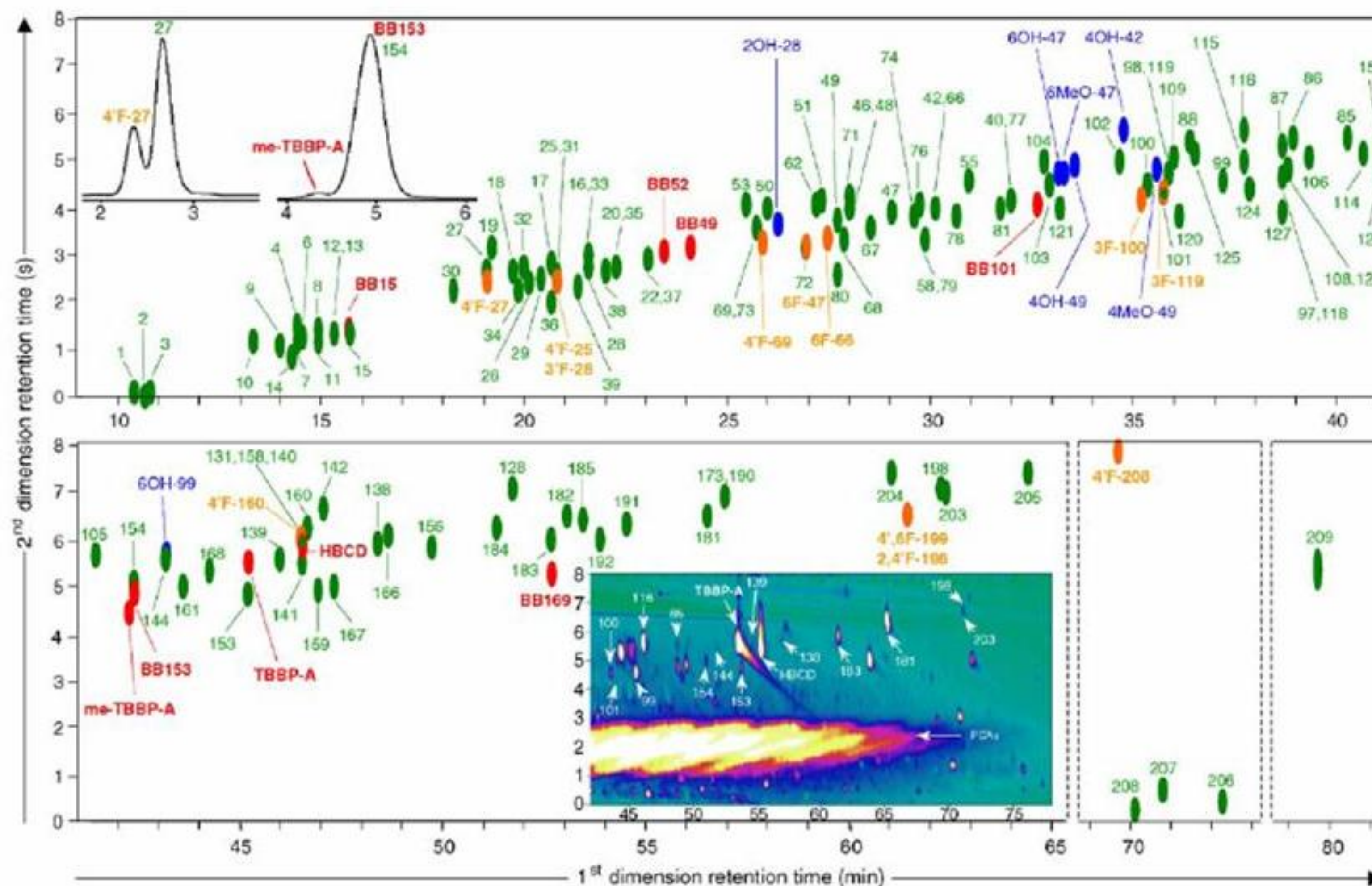

Fig. 5. Overlaid GC×GC–µECD chromatogram on DB-1 × 007-65HT column combination of PBDES (green), fluorinated PBDEs (orange), other brominated flame retardants (red) and PBDE metabolites (blue) *(27)*.

the nona- and deca-BDE congeners. The insert in the upper left-hand side of this figure demonstrates that a satisfactory separation among close eluting compounds was achieved through the whole chromatographic run. The insert in the lower right-hand side shows that successful separation was also attained between PBDE 153 and TBBP-A and that accurate determination of PBDE 153 was possible even if a high concentration of TBBP-A was present. This study proved that μECD is an appropriate detector for this type of analysis. However, use of the most selective (and sensitive) ECNI qMS in the SIM mode has demonstrated that much more (unidentified) bromine-containing compounds can actually be present in real-life extracts *(28)*. The high separation demand posed by these substrates open a new working field for GC×GC.

This point has recently been illustrated by Pangallo et at. *(28)* in their work dealing with the identification of a set of 26 new halogenated 1´methyl-1,2´-bipyrroles (MBPs) in the dolphin blubber. MBPs are a family of halogenated natural products with physical and chemical properties similar to other persistent organic pollutants, which are of great interest to environmental chemist and toxicologist *(34)*. Bioaccumulative MBPs include more than 20 congeners of mixed halogenation (containing bromine and chlorine) in addition to the perchlorinated and perbrominated isomers. The source, biosynthethic pathway, environment fate and toxicity of these compounds remain unclear, but there is now evidence of geographic-dependent accumulation in marine mammals. One of the main present limitations regarding the analysis of this group of natural products is the lack of appropriated synthetic standards. In this study *(34)*, four previously identified MBP isomers were isolated from dolphin blubber and, after purification, were used as reference and calibration standards. These four standards sufficed for tentative identification of 43 close eluting analytes as MBP isomers by GC–ECNI-qMS. Further evidence of the 28 partially halogenated MBP isomers was obtained by GC×GC–ToF MS using a 10 m Rtx-5 Crossbond column as ^{1}D and a 0.70 m BPX-50 housed in a secondary oven as ^{2}D. This orthogonal column set resulted in organised roof-tile structures in the 2D plane for the standard solution that extended to the newly found derivatives. MBP-Br_7 and its brominated congeners were found to align on none diagonal, while MBP-

Br_6Cl and congeners containing one chlorine aligned along a second, lower, parallel diagonal. Despite the (apparently) exceptional behaviour of MBP-HBr_5Cl and MBP-H_2Br_4Cl, the highly structured distribution of the target compounds provided support for tentative identification of the new analytes (Fig. 6) and demonstrate the suitability of GC×GC–ToF MS for the identification of unknown compounds.

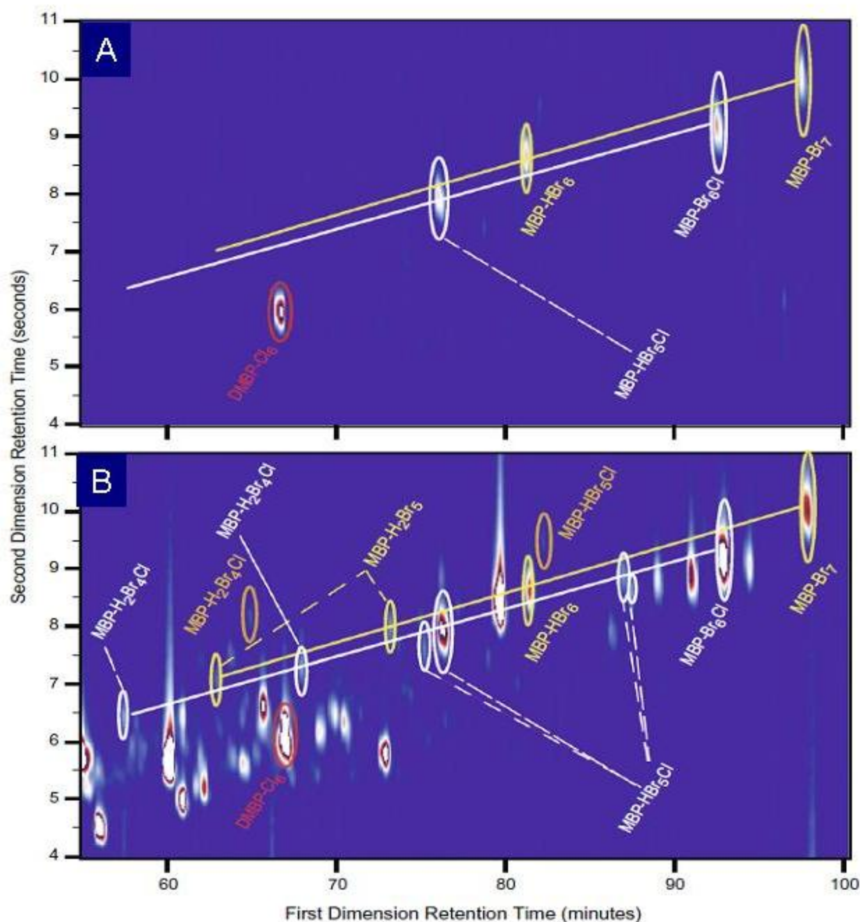

Fig. 6. Section of the GC×GC–ToF MS contour plot of (A) the total in current of MBP standards and (B) the MBPs (m/z: 496, 540, 575, 620, 654 and 698) in a dolphin blubber extract. Detected MBP peaks are indicated by a coloured circle (yellow, white and orange); the internal standard is indicated by a red circle; lines indicate the MBP containing bromine (top, yellow) and chlorine (bottom, white); peaks eluting above these lines are proposed to be MBPs (orange circle)*(28)*.

Non-aromatic organohalogenated pollutants

Technical toxaphene is a mixture of polychlorinated monoterpenes obtained by chlorination of camphene under UV irradiation. Introduced in 1945, toxaphene was mass-produced until the mid-1980s and widely used as an insecticide, especially in cotton cultures. Currely, toxaphene is considered a worldwide distributed pollutant frequently detected at significant levels in fresh-water and marine biota as a complex mixture of compounds. The main constituents of toxaphene are chlorobornanes (for which 16640 possible congeners has been calculated *(35)*) and chlorocamphenes (with 12288 possible congeners). Chlorodihydrocamphenes (with 32768 possible congeners) and chlorobornenes (or bornadienes) are considered minor components. The technical mixture also contains small amounts of other chlorinated and nonchlorinated hydrocarbons *(36)*. Although most probably not all these theoretically possible congeners are present in technical mixtures, the figures help create an impression of their complexity and explain why the number of compounds estimated to be present on them has increased as the separation power of the instrumental approach selected for the analysis increased *(29)*. Until the introduction of GC×GC, the best resolution (and highest figures, 675 congeners) was achieved by using multidimensional gas chromatography for the analysis of no less than 160 pre-separated fractions of the mixture *(37)*.

After a first attempt of use GC×GC, this time with ECD, for the separation of five bornanes *(11)*, a much more detailed study on the composition of toxaphene was reported using μECD and ToF MS as detectors *(29)*. Using a 30 m HP-1 as first dimension and a 1 m HT-8 as second dimension, the authors obtained structured chromatograms reflecting the high complexity of the toxaphene mixture and the close structural relationship of the constituent compound classes. The total analysis time was 140 min, and the number of compounds present was estimated to be over 1000. The use of 23 individual congener standards (5 chlorocamphenes and 18 chloroboranes) and ToF MS as detector confirmed that these two classes of compounds do not separated from each other in the 2D contour plot, and at the same time validated the fact that the several observed group peaks comprised congeners with the same number of chlorosubtituents with apparently no influence of the class of compound considered (Fig. 7). Use of ToF MS also helped confirm that

the mixture contained minor amounts of chlorodihydrocamphenes. In a subsequent study involving the same column combination but faster ramps of temperature, the authors also detected the presence of an extra group of compounds with roof-tile and eluting below toxaphene in the contour plot that was identified as hexa- to nona-chlorobornenes formed by thermal degradation of chlorobornanes *(30)*.

Introduced in 1932, polychlorinated *n*-alcanes (PCAs) are complex mixtures obtained by chlorination of *n*-alcanes feedstock under uncontrolled conditions using molecular chlorine at temperatures of 50–150ºC, elevated pressures and/or under UV irradiation. PCA mixtures are classified according to their carbon chain length as short-chain PCAs (C_{10}–C_{13}), medium-chain PCAs (C_{14}–C_{17}) and long-chain PCAs (>C_{17}). These mixtures, with chlorination degrees in the range of 30–70%, have been used as additives in a large variety of industrial products and are found worldwide at variable concentration levels. Today, PCAs are classified as priority and toxic substances by the major international protection agencies.

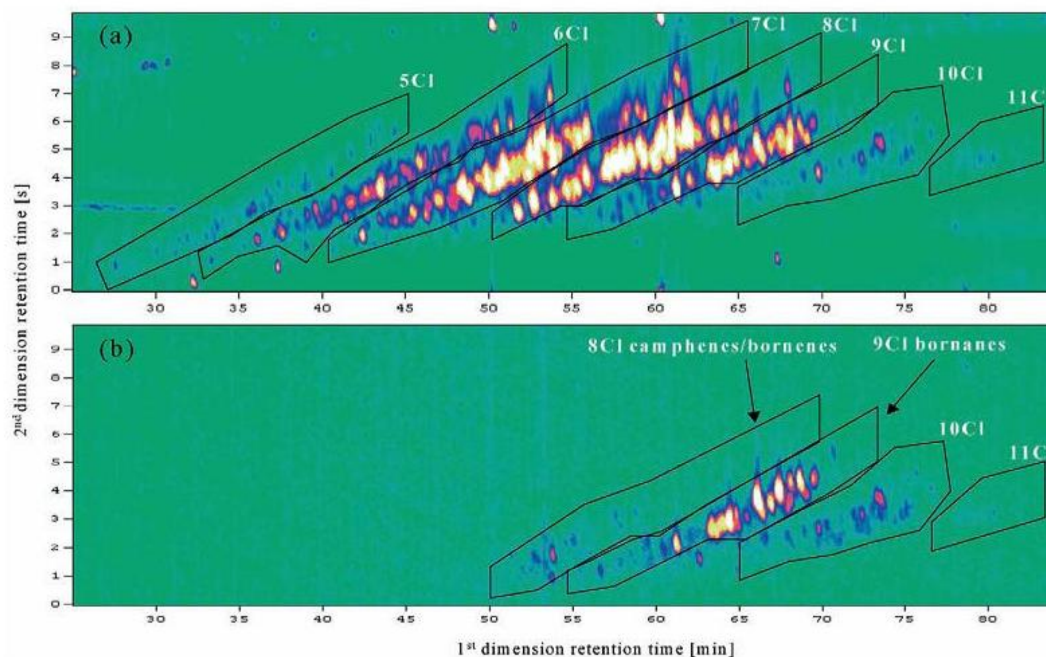

Fig. 7. GC×GC–ToF MS chromatogram of technical toxaphene. (A) Total ion chromatogram (m/z 45–550), and (B) extracted ion chromatogram for m/z 413 *(29)*.

The undetermined high number of components present in the PCA mixtures made impossible a congener-specific analysis with 1D GC regardless of the detector used, although examples involving ECD (because of its high sensitivity), qMS (mainly in the ECNI mode to avoid the excessive fragmentation produced by the EI mode) and ITD(MS/MS) (so far, probably the best alternative because of its higher selectivity) have been reported. At the time of writing, only a limited number of studies reporting on the GC×GC separation of PCAs can be found in the literature.

PCAs were included in a study *(30)* that evaluated the relative merits of five column combinations for group-type separation of 12 POP families were evaluated. The enhanced separation power and detectability provided by GC×GC–μECD, combined with the orthogonal character of most column sets assayed, resulted in structured chromatograms that allowed differentiation of several homologues bands in the technical mixture PCA-60. The complexity of this mixture became apparent through the several subgroups observed within these bands and, unfortunately, also through the partial overlapping among bands. DB-1 × 007-65HT was proposed as the best alternative for PCAs analysis because it provided the clearest homologue bands separation as well as the best separation between PCAs and other potentially interfering POP classes frequently detected in real environmental samples. Nevertheless, the results also showed that, despite the enhanced separation power provided by GC×GC and high sensitivity and selectivity achieved with the μECD used as detector in this work, a complete separation of all components present in the PCA mixtures was not possible *(30)*.

Subsequent studies tested the feasibility of alternative detectors, such as ECNI-qMS *(26)* and ToF MS *(31)* to study the composition and characteristics of short-, medium- and long-chain PCAs. Again, the complexity of the mixtures prevented from a congener-specific analysis, but some features become apparent. For instances, the analysis of a polychlorinated decanes mixture with an average chlorine content of 65 wt. % by GC×GC–ECNI-qMS using DB-1 × DB-XLB as column set resulted in a structured chromatogram in which four parallel bands were separated. On the basis of the ECNI mass spectra, these bands were assigned, respectively, to hexa- and

nona-chlorinated decanes, confirming that separation was based on the number of chlorine substituents.

Similar conclusions were obtained using ECNI-ToF MS as a detector, the same column combination and a larger number of technical mixtures, as well as 35 individual PCA standards: compounds having the same chlorine substitution pattern but different carbon chain length were ordered as more or less parallel lines in the 2D contour plot *(31)*. The closely similar polarity was suggested as the most plausible explanation for this behaviour. It was also confirmed that, for compounds with the same carbon chain, congeners having chlorine substituents on only one end of the chain had shorter retention times in the ^{2}D column than those with chlorines substituents distributed over the entire carbon chain because of their lower polarity. In addition, use of individual standards proved that some congeners (e.g., 1,1,1,3,6,8,8,8-C_8Cl_8, 2,5,6,9-$C_{10}Cl_4$, 1,2,5,6,9-$C_{10}Cl_5$ and 1,2,5,6,9,10-$C_{10}Cl_6$) can exist as a number of diasteroisomers. A detailed inspection of the homologue group structures in technical PCA mixtures revealed the existence of subgroups within the homologue bands (see Fig. 8 where there are no straight lines connecting the peak apices within each band). Overlay of the chromatograms obtained for technical short-chain (C_{10}–C_{13}) PCA mixtures (Figure 8.E) indicated that compounds having the same number of carbon-plus-chlorine atoms showed up in the same diagonal line, but also that components with carbon chains differing in at least three carbons were efficiently separated with the DB-1 × 007-65HT column combination. In other words, this column set allowed partial differentiation of short-, medium- and long-chain PCAs, as it was demonstrated for the analysis of dust extracts.

Group-type analysis

The different toxicities of the individual microcontaminants made of analyte unambiguous determination a main requirement in the environmental field. As illustrated by previous sections, this need has made that the large majority of the studies dealing with GC×GC of pollutants focus on target analyte. In this type of determination, the main requirement is that the compound(-s) of interest were sufficiently separated from each other and from the sample matrix *(38)*. However, in many of these studies, the orthogonal nature of the selected column combinations

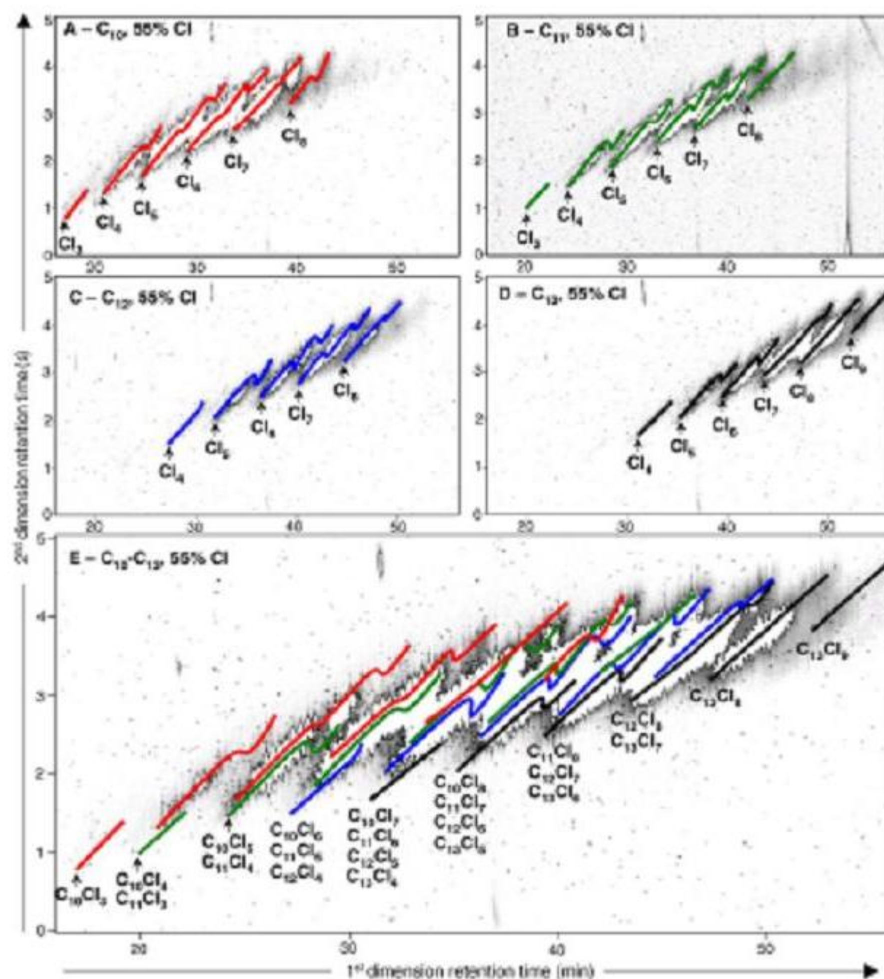

Fig. 8. GC×GC–ECNI-ToF MS chromatograms of polychlorinated (A) decanes, (B) undecanes, (C) dodecanes, (D) tridecanes and (E) C10-C13 technical mixture using obtained on DB-1 × 007-65HT column set. Lines indicate the positions of apices within the bands *(31)*.

resulted in organised chromatograms in which structurally related compounds typically elute as a band. This band-type organisation suggests the possibility of using the improved separation provided by GC×GC for fast screening of pollutants families using a group-type approach similar to that frequently used for the characterisation of petrochemical, fragrance and food samples. In this type of approach, the goal is to maximise the separation between the different component groups and among them and the sample matrix *(38)*. To achieve these requirements, if necessary, within-group separation could somehow be sacrificed because quantification is, for obvious reasons, not the main objective.

Despite the potential of this kind of group-type analysis for simultaneous fast screening of selected pollutant classes, for example, in monitoring analyses, until now the approach has somehow been overlooked. At the time of writing, only two papers have reported on the feasibility of this approach in the environmental field *(30, 39)*. In both cases, a μECD was used as detector, and closely related POP families were selected as test classes. Table 2 summarises the main conclusions of these studies.

None of the assayed column sets allowed the simultaneous and complete separation of all pollutant classes (12 POP families in *(30)* and 8 in *(39)*). However, some column combinations provided satisfactory separations among selected families and the rest of the pollutants investigated, indicating their suitability for fast isolation

Table 2. Main features observed with different column combination regarding group-type separation of aromatic and non-aromatic halogenated micropollutant families.

Column combination	Analyte class separated	Reference
DB-1 × 007-210	–	*(30)*[a]
DB-1 × HT-8	–	
DB-1 × LC-50	PCDDs, PCDFs, PCDTs and PCNs from non-planar	
DB-1 × 007-65HT	PCAs and PBDEs Br- from Cl-substituted analogue classes	
DB-1 × VF-23ms	–	
ZB-5 × HT-8	–	*(39)*[b]
ZB-5 × BPX-50	PBDEs (except in marine samples)	
ZB-5 × CW	–	
HT-8 × BPX-50	PCDDs, PCDFs and PBDEs	
DB-17 × HT-8	PCDDs, PCDFs and PCNs COPs (except in marine samples)	
DB-17 × BPX-50	toxaphene (partialy from PCBs)	
DB-17 × SW-10	–	
BP-10 × HT-8	classes with one or two rings (PCBs, PCNs, toxaphene and OCPs) from those with three aromatic rings (PCDDs, PCDFs and PBDEs) PCBs from toxaphene (partial separation)	
BP-10 × BPX-50	Toxaphene	

Analyte classes included in the study: [a]PCBs, PCDEs, PCNs, Polychlorinated dibenzothiophenes (PCDTs), PCDDs, PCDFs, PCTs, PCAs, toxaphene, OCPs, PBBs and PBDEs; [b]PCBs, PCNs, PCDDs, PCDFs, PCTs, toxaphene, OCPs and PBDEs.

and detection of these particular group classes among all other POPs. As a typical example of the results obtained, Figure 9.A shows the separation achieved among the several families investigated on DB-1 × 007-65HT *(30)*. This column set allowed a satisfactory separation of PCAs from all other studied POP classes, including some numerous groups such as toxaphene and PCTs, which were more retained in the second dimension (Fig. 9.B). Interestingly, this column combination was also found to provide a highly rewarding separation of PBDEs from other commonly overlapping POPs difficult to separate during sample clean-up, as well as a significant

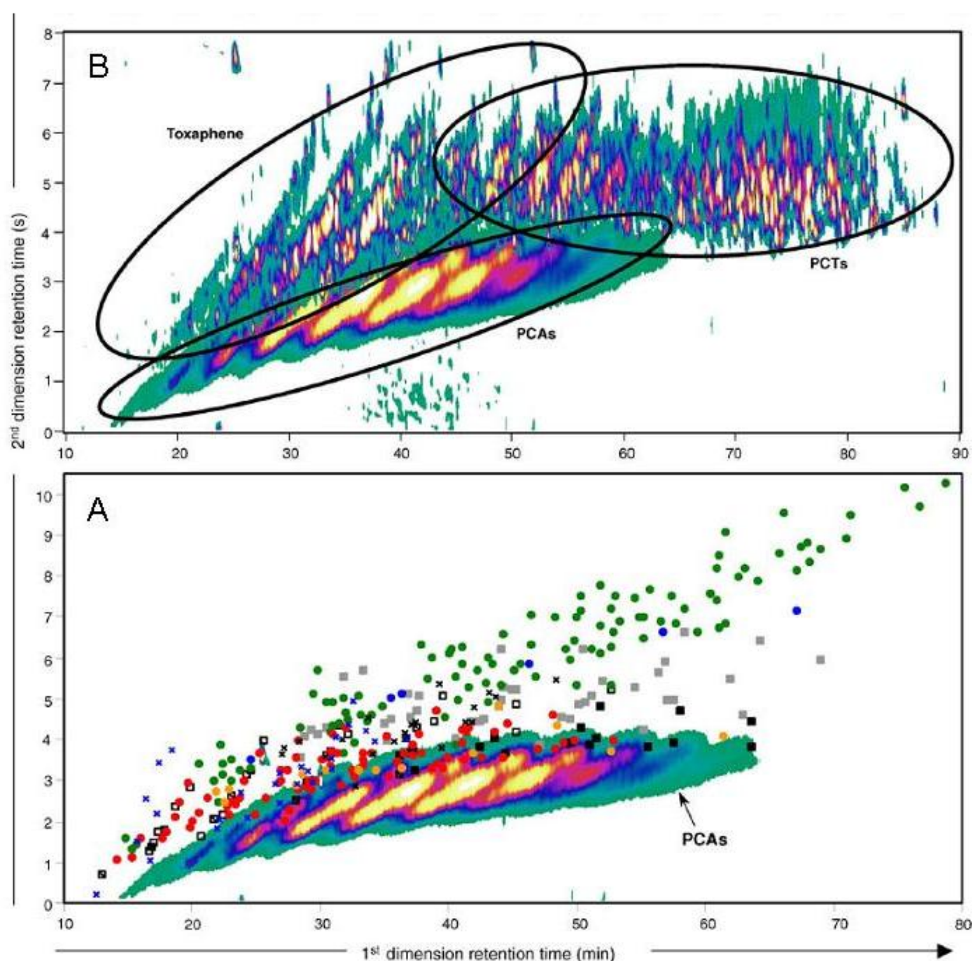

Fig. 9. (A) Overlaid GC×GC–µECD chromatogram on DB-1 × 007-65HT column set of PCBs (red circle), PBBs (blue circle), PCDEs (orange circle), PBDEs (green circle), PCDTs (grey square), PCNs (white square), PCDD/Fs (black square), OCP (blue cross) individual toxaphene standrads (black cross), and PCAs (PCA-60). (B) PCA (PCA-60), PCTs (Aroclor 5442+5460) a toxaphene technical mixture *(30)*.

separation among Br-substituted compounds and the corresponding Cl-substituted class, as shown for PBBs and PCBs. Because of the orthogonal character of most of the column combination assayed in these two studies and the high peak capacity provided by GC×GC, in many of the assayed column sets accurate within-group identification of target toxic compounds was still possible, giving the approach extra analytical potencial.

The general practicability of this group-type analysis for fast environmental screening was demonstrated in both highly purified *(30)* and non-fractionated POP extracts *(39)* obtained from a variety of naturally contaminated samples, including sediments and dust *(30)* as well as biological tissues and foodstuffs *(30)*.

2. NON-HALOGENATED POLLUTANTS

Pesticides

Contrary to that observed for other classes of pollutants and for organohalogens in particular, the determination of (nonchlorinated) pesticides has attracted only limited attention (Table 3).

In some of the early studies reporting on the GC×GC separation of pesticides, these were chosen as model analytes rather than as real analytical targets *(40,55)*. However, impressive results were obtained demonstrating the feasibility of this technique for this type of determination. As an example, Figure 10 shows the baseline separation of 17 pesticides and two internal standards, pentachlorophenol and heptadecanoic acid, achieved in 4.5 min using a 2 m-DB-1 as first column and a 0.8 m-OV-1701 as second dimension *(40)*. Using an FID as detector, on-column limits of detection in the range 2–4 pg were obtained. This result proved the practicality of the method for pesticide determination in relatively clean samples, such as human serum.

Khummueng et al. *(41)* compared the effectiveness of several column combinations for separation of N-containing fungicides in a Brussels sprout extract. With BPX-5 × BPX-50 as column set, LODs and limits of quantification (LOQs) below 74 and 250 ng/L, respectively, were obtained with nitrogen-phosphorous detector (NPD) and even lower when using a μECD. These results, combined with

Table 3. Selected GC×GC applications involving the analysis of non-halogenated pollutants. For simplicity, only optimised experimental set-ups or those providing the most conclusive results have been mentioned.

Analytes	Sample	Column combinations (m×mm ID×µm d_f)	Modulator	Detector	Ref.
17 Pesticides	human serum	DB-1 (2.0×0.25×0.25) × OV-1701 (0.8×0.1×0.05)	TDM	FID	*(40)*
9 Fungicides	Brussels sprouts	BPX-5 (30×0.25×0.25) × BPX-50 (1.0×0.15×0.15)	LMCS	NPD/µECD	*(41)*
33 Pesticides (OPPs, triazines, pyrethroids)	orange, pear, grape, apple	ZB-5 (30×0.25×0.25) × BPX-50 (0.8 × 0.10 × 0.10)	Loop modulator, LN_2	µECD	*(42)*
92 Pesticides	spiked red grapefruit	SLB-5MS (30×0.25×0.25) × Omegawax (1.0×0.10×0.10)	Loop modulator, LN_2	qMS	*(43)*
58 Pesticides	Celery, carrot	CP-SIL5 CB (15×0.25×0.25) × BPX-50 (0.8×0.10×0.10)	LMCS	ToF MS	*(44)*
20 Pesticides	Apple, peach	DB-XLB (30×0.25×0.25) × DB-17 (1.0×0.10×0.10)	Quad-jet dual stage modulator	ToF MS	*(45)*
51 Pesticides	Grape	RTX-5MS (10×0.18×0.2) × TR-50MS (1.0×0.10×0.10)	Quad-jet dual stage modulator	ToF MS	*(46)*
106 Pesticides	Feed	RTX-CL (30×0.25×0.25) × BPX-50 (2.0×0.10×0.10)	Quad-jet dual stage modulator, LN_2	ToF MS	*(47)*
14 (OPPs and OCPs)	Tobacco	Rtx-1 (30×0.25×0.25) × Rtx-200 (1.0×0.18×0.18)	Quad-jet dual stage modulator	ToF MS	*(48)*
36 Pesticides	Tea	BPX-5 (40×0.18×0.18) × SupelcoWax (2.5×0.10×0.10)	Quad-jet dual stage modulato, LN_2	ToF MS	*(49)*
24 PAHs	Soil	BPX-5 (30×0.25×0.25) × BPX-50 (1.2×0.10×0.20)	LMCS	FID	*(50)*
9 PAHs	Sediment	HP-5MS (20×0.25×0.25) × BGB-1701 (0.5×0.1×0.10)	home-made semi-rotating cryogenic modulator [a]	FID	*(51)*
PAHs, nonylphenols	Sediment	DB-5 (20×0.25×0.25) × BGB-1701 (1.0×0.10×0.10)	Dual stage modulator, CO_2	ToF MS	*(52)*
12 Nonylphenol isomers	river water	DB-5 (30×0.25×1) × SP-Wax (1.0×0.10×0.10)	Loop modulator, LN_2	qMS	*(53)*

[a] home-made semi-rotating cryogenic modulator *(54)*.

the satisfactory repeatability and reproducibility of peak response proved the potential of the GC×GC method proposed for routine analysis of fungicides in vegetables. A similar column combination, ZB-5 × BPX-50, was also found to provide the best separation among fruit matrix components and the 33 pesticides included in a recent study involving GC×GC–μECD *(42)*. As in the observations made in *(41)*, analyte compression through the modulation process resulted in extremely low LODs that ensures accurate determination at the low MRLs set in current legislations.

Despite the satisfactory results obtained with these element selective detectors, in most of the studies concerning pesticide analysis, detectors providing structural information (i.e., mass spectrometers) have been preferred (Table 3). Among them, ToF MS has been by far the most frequently used *(44-49)*.

The potential of GC×GC–ToF MS for non-target pesticide analysis was first illustrated by Dallügue et al. in 2002 *(44)*. The separation power of this three-dimensional technique was demonstrated through quite self-explicative

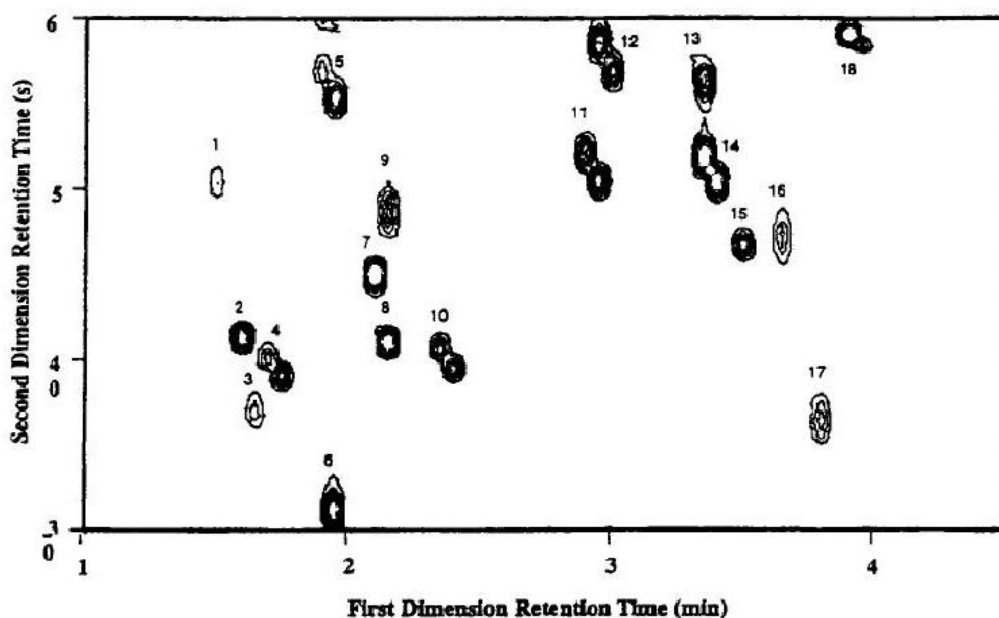

Fig. 10. GC×GC separation of a pesticide mixture. Peak identification: 1, dicamba; 2, trifluralin; 3, dicloran; 4, phorate; 5, pentachlorophenol (internal standard); 6, atrazine; 7, fonofos; 8, diazinon; 9, chlorothalonil; 10, terbufos; 11, alachlor; 12, matalaxyl; 13, malathion; 14, metalachlor; 15, DCPA; 16, captan; 17, folpet; 18, heptadecanoic acid (internal standard) *(40)*.

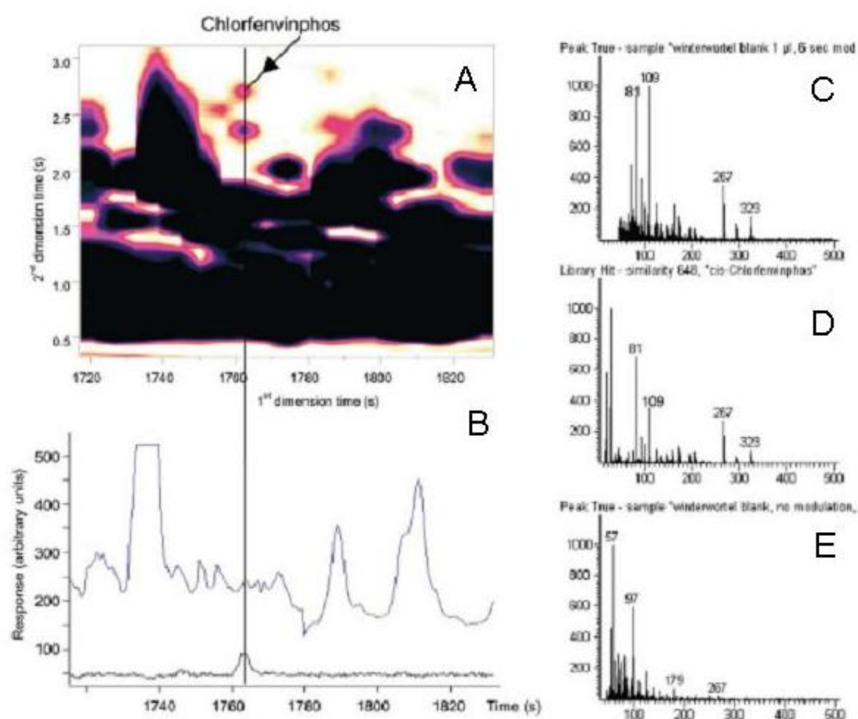

Fig. 11. GC×GC–ToF MS vs 1D GC–ToF MS for the analysis of a carrot extract. (A) GC×GC–ToF MS contour plot. (B) 1D GC–ToF MS of the region selected in (A). (C) Mass spectrum obtained after GC×GC separation showing the characteristic m/z ions of chlorfenvinphos and comparison with (D) library spectrum and (E) spectrum obtained at the same retention time with 1D GC *(44)*.

chromatograms, as those reproduced in Figure 11. This figure compares the 1D GC–ToF MS mass spectra (Fig. 11.E) obtained for a carrot containing 0.02 mg/kg of chlorfenvinphos (corresponding to 10 ng/mL in the injected extract) with that obtained using GC×GC–ToF MS (Fig. 11.C). GC×GC can separate the quantification interferences from the matrix (Fig. 11.A and B), but the qualitative identification is provided through a combination of GC×GC and spectral deconvolution (peak true). In other words, while chlorfenvinphos identification was hampered by matrix components in the 1D GC approach, accurate determination at low concentration levels was possible with the comprehensive technique.

The experimentally determined LODs of 10–30 pg for the N/P-containing pesticides included in the study lent support to this statement. In a somehow closely related study, Zrostlíková et al. *(45)* concluded that GC×GC–ToF MS can provide a 1.5- to 50-fold improvement in the LODs calculated for 20 modern pesticides as

compared to the equivalent GC–ToF MS method. The combined effect of analyte compression on the modulator, improved separation from co-extracted sample material provided by the GC×GC, and deconvolution capabilities offered by the ToF MS explains these results *(45, 48)*.

A detailed study on the influence of the different experimental parameters affecting the modulation and acquisition data processes on the detectability and final identification of pesticides with GC×GC–ToF MS can be found in *(46)*. Results demonstrated that when pesticides should accurately be determined in complex extracts, a data-acquisition rate of 100 Hz or higher should preferably be used. Van der Lee et al. *(47)* used a data-acquisition rate of 200 Hz to deal with pesticide analysis in as a complex matrix as feed. Despite the laborious multistep procedure used for cleanup of the sample, analyte co-elution with matrix material was frequently detected (Figure 12). However, the accurate mass spectra definition achieved, thanks to the high data-acquisition rate, allowed automatic identification by the software, by comparison of the spectra of all individual compounds detected in the sample against a target library. Using a representative feed and setting a similarity threshold of 600, all 106 targeted pesticides were virtually detected through automatic screening at levels of 50 μg/kg. At a level of 10 μg/kg, 73% of the analytes were still fully automated, but at lower concentration levels the number of compounds detected decreased dramatically. The GC×GC–ToF MS linearity was excellent in solvent and only slightly affected by matrix, and the LODs were in general below 20 μg/kg. Furthermore, the acquisition of full-range mass spectra provided powerful confirmation of the pesticides in the sample, even for nontarget analytes *(44, 47,48)*. This potential is in principle reinforced by the automated peak find and spectral deconvolution software capabilities, although in practice intensive manual data revision is often needed *(44, 47, 49)*.

The new generation of high-speed qMS system represents a valuable alternative to the more expensive ToF MS also in this field. In these analyzers, the mass scanning range should still be limited to a relatively narrow range (at least compared to that typically used in ToF MS experiments), which limits the possibility of subsequent analysis of archived data for unknown identification. However, examples reporting

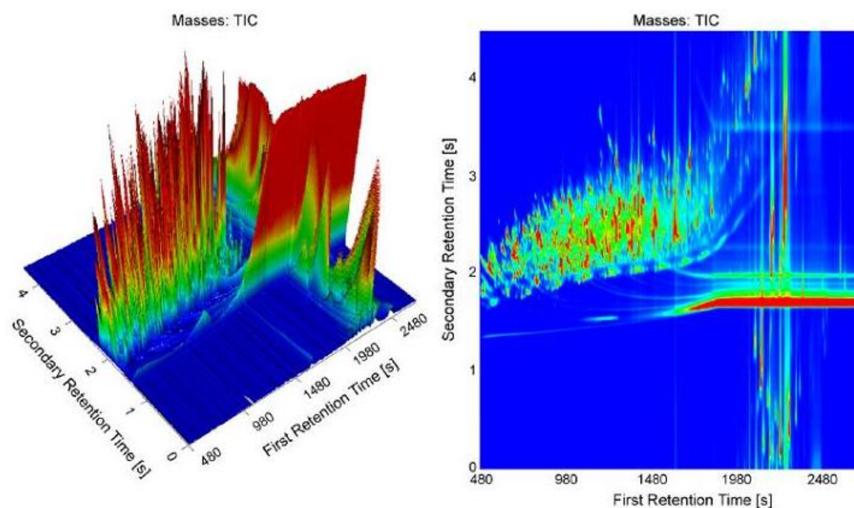

Fig. 12. Typical 3D GC×GC–ToF MS image and contour plot obtained for a purified feed sample *(47)*.

satisfactory linearity (regression coefficients better than 0.9994 in the 1.0–15 ppm range with four-point calibration curves) and LODs low enough to ensure analyte detection at the low levels set in current legislation have already been described in the literature *(43)*.

All previously revised studies focus on target analyte. Consequently, efficient separation of the investigated analytes from the sample matrix was the main concern during method development, especially when additional structural information was not available. This explains why group-type analysis has received limited attention in the pesticide field, despite the efficiency of this approach for fast screening proposes *(42)*.

Other organic pollutants

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic micropollutants containing two or more condensed rings. Apart from those cases associated with oil pollution, PAHs are produced mainly by anthropogenic combustion and are typically found in the environment as complex mixtures. However, not all components exhibited the same level of toxicity, and congener-specific determination has again become mandatory.

PAHs are a relevant class of compounds in petrochemistry. However, up to now they have apparently received rather limited attention from environmental chemists working on GC×GC. This sharp difference among the number of studies concerning PAHs in these two research areas is probably a result of the different aims and analytical requirements for both types of analyses. In the area of petrochemistry, the main interest is group-type analysis and a FID suffices for accurate determination. Meanwhile, environmental studies are focussed on target analysis, and use of a nonselective detector, such FID, can be problematic because of the low levels of the analytes and the typical high complexity of the extracts. On the other hand, although use of MS-based detectors can be desirable because they allow structural confirmation, their limited sensitivity can become a problem when analysing real-life samples with trace levels of these compounds.

The feasibility of GC×GC–FID as a screening method for the analysis of 24 environmental relevant PAHs in contaminated soils from a former gasworks site (concentrations in the 10–300 mg/Kg range) was firstly illustrated by Ong et al. *(50)*. Not surprisingly, structured chromatograms in which PAHs were grouped according to their number of aromatic rings were obtained using BPX-5 × BPX-50 as column set. All PAHs were separated among them and from other hydrocarbon classes present in the purified extract, with the exception of two pairs, benzo(b)fluorantene — benzo(k)fluorantene, and indeno(c,d)pyrene — dibenzo(a,c)anthracene. After careful optimisation of the sample preparation procedure, the clean obtained extracts allowed PAH identification by direct comparison of the ^{1}D and ^{2}D retention times in the soil extract with those found for the standards. Comparison of the GC×GC–FID method with the more conventional GC–MS from a quantitative point of view demonstrated that, although both sets of data compared reasonably well for low-molecular mass PAHs, high-molecular mass PAHs were underestimated with the comprehensive technique.

Similar conclusions were obtained in a subsequent study oriented toward the study of qualitative and quantitative aspects of GC×GC. In this case, PAHs were selected as model compounds and a FID as detector *(51)*. The quality and efficiency of the GC×GC separation were evaluated on the basis of several parameters, namely,

the peak width at peak base, asymmetry, resolution, depth of the valley between PAH and preceding matrix peak, and total retention time, using both LC purified and nonpurified sediment extracts. For quantitation, two different approaches based on peak areas and volumes were tested. Results proved that increasing the matrix amount 16-fold compared to levels in the LC cleaned sediment did not affect the separation of PAHs in terms of peak width, asymmetry and resolution. However, these large matrix amounts increased the depth of the valley between the considered PAH and the preceding matrix peak and reduced the repeatability of the retention times as well as those of the peak areas and volumes. Regarding quantitation, calibration based on peak area trended to underestimate the trace PCH levels in the sediment analysed, especially for high-molecular mass components in nonpurified extracts. Volume-based calibration resulted in more accurate results, especially when using the external calibration procedure, for both cleaned and noncleaned extracts.

Nonylphenolpolyethoxilates are commonly used nonionic surfactants. In wastewater treatment plants, these compounds degrade to a number of products, including nonylphenols. Although nonylphenols are typically detected in environmental samples as a mixture of isomers due to branching of the C-9 group, only some of these isomers have been reported to exhibit estrogenic potential. The feasibility of GC×GC for unambiguous determination of some of these isomers was investigated by Ieda et al. *(53)* using a qMS as detector. To increase the data-acquisition rate, a limited scan range (*m/z* 105 to *m/z* 170), corresponding to 24.5 Hz, was set. As an example of the potential of the technique in this field, the authors reported on the separation of 102 peaks of nonylphenols in a technical mixture. The optimised method was then applied to quantification of selected nonylphenol isomers in water river with satisfactory results, that is, linear response in the 5−100 ng/L range with correlation coefficients better than 0.994; and LODs lower than 0.7 ng/L.

3. Analysis of chiral pollutants

Previous sections have illustrated the complexity of most of the compound-specific analyses developed in the environmental field. It is easy then to figure out that the accurate determination of a possible enantiomeric enrichment of chiral pollutants is even more difficult owing to the many co-elution problems and low

concentration levels of the analytes. This difficulty could explain the somehow limited research conducted on this topic. However, its interest is clear. Industrial contaminants, such as PCBs or toxafene, are released into the environment as racemates. Therefore, a nonracemic composition of these pollutants might be evidence of selective biotransformation and/or bioaccumulation. Some studies have also pointed to different biological and toxic behaviour for each of the enantiomers *(56)*, something that can be especially relevant for pesticides exhibiting chiral properties.

Chiral analysis of trace pollutants in complex environmental samples has typically required laborious and time-consuming fractionation steps before instrumental determination of the target analytes *(57)*. Heart-cut multidimensional gas chromatography represents a valuable alternative to these approaches that efficiently contributes to reduce sample manipulation. However, it can also be rather tedious because only a limited number of target compounds that can be transferred to the second column in a single run.

The first attempt to use GC×GC for chiral separation of organic micropollutants was reported by Harju and Haglund in 2001 *(58)* (Table 4). Nine out of the 19 atropoisomeric PCBs (PCB Nos. 45, 84, 88, 91, 95, 131, 132, 135, 136, 139, 144, 149, 171, 174, 175, 176, 183, 196 and 197) *(63)* were used as test compounds. In particular, those that can be separated into enantiomers on the permethylated β-cyclodextrin column (Chirasil-Dex) selected as first-dimension column. Using a shape selective column as second dimension, LC-50, six out of nine studied atropisomeric PCBs were resolved (PCB Nos. 91, 132, 135, 136, 149 and 176) and two more (PCBs 84 and 174) were partially separated from co-eluting congeners in a mixture of 144 congeners using a μECD as detector. In a follow-up *(59)*, the authors concluded that the use of VF-23ms as second dimension instead of LC-50 yielded more satisfactory results when analysing real-life samples. With this column set and a single injection, the authors reported on the enrichment factor (EF) of five atropisomeric PCBs (PCB Nos. 91, 95, 132, 149 and 174) and simultaneously determine the concentrations of the seven prioritary and twelve toxic PCB congeners in grey-seal samples. Results demonstrated that the EFs of some PCBs deviated strongly from racemic (results

confirmed by GC×GC–ToF MS) and that ratio deviations were higher in liver than in blubber, suggesting a possible enantioselective metabolism.

Using the same column combination but with a longer first dimension (25 instead of 10 m Chiralsil-Dex column), Bordajandi et al *(60)* were able to elute free from interferences seven out of the nine atropisomeric PCBs that can be resolved into enantiomers with this phase (i.e., PCBs 84, 91, 95, 136, 149, 174 and 176). However, PCBs 135 and 132, the later being one of the most abundant in food samples, co-eluted with PCBs 82 and 141, respectively. With Chiralsil-Dex × Supelcowax, PCBs 91, 95, 132, 135, 149, 174 and 176 eluted without interference from a test mixture of 95 PCBs, but congeners 84 and 136 coeluted with PCBs 56 and 85, respectively. The combined use of these two column sets allowed accurate EFs determination for the nine atropoisomeric PCBs in milk and cheese samples. In a subsequent study *(61)*, three β-cyclodextrin-based columns (Chirasil-Dex, BGB-172 and BGB-176SE) were combined with HT-8, BPX-50 and Supelcowax-10 as second dimension and evaluated for simultaneous determination of the 19 chiral PCB enantiomers, the seven prioritary and twelve toxic PCBs. In general, the best results were obtained with those columns sets involving Supelcowax as second dimension. In addititon, these column combinations allowed prioritary and toxic congeners to be detected in real food samples free from interferences. Regarding chiral PCBs, BGD-172 × Supelcowax-10 provided the best overall separation allowing all enantiomers to be determined free from interferences (Fig. 13), with the only exception of PCB 91. Accurate determination of the EF for this particular congener was only possible on BGB-176SE × Supelcowax-10. Further confirmation of the results obtained with the comprehensive approach by heart-cut multidimensional gas chromatography analysis of the extracts proved the feasibility of the proposed method even if structural information was not available.

Table 4. Selected GC×GC applications involving the analysis of chiral pollutants. For simplicity, only optimised experimental set-ups or those providing the most conclusive results have been mentione

Analytes	Sample	Column combination (m×mm ID×µm d_f)	Modulator	Detector	Ref
9 Chiral PCBs	Standard solution	Chirasil-Dex (10×0.10×0.10) × LC-50 (1.0×0.10×0.10)	LMCS	µECD	*(58)*
9 Chiral PCBs (+ 7 prioritary and 12 toxic PCBs)	144 PCBs, seal liver and blubber	Chirasil-Dex (10×0.10×0.10) × VF-23MS (1.5×0.10× 0.10)	LMCS	µECD	*(59)*
9 Chiral PCBs	milk, cheese	Chirasil-Dex (25×0.25×0.25) × VF-23ms (1.0×0.1×0.10) Chirasil-Dex (25×0.25×0.25) × Supelcowax-10 (0.9×0.1×0.10)	loop modulator	µECD	*(60)*
19 Chiral PCBs (+ 7 prioritary and 12 toxic PCBs)	milk, cheese, salmon	BGB-172 (30×0.25×0.18) × Supelcowax-10 (1.0×0.1×0.10)	loop modulator	µECD	*(61)*
5 Chiral toxaphenes	fish oil	BGB-172 (30×0.25×0.18) × BPX-50 (2.0×0.10×0.10)	loop modulator	µECD	*(62)*

BGB-172 and BGB-176SE in combination with HT-8, BPX-50 and Supelcowax-10 were also evaluated for the enantiomeric separation of five chiral toxaphenes typically found in real-life marine samples, Parlar 26, 32, 40, 44 and 50, in nonfractionated extracts containing other POPs and using a μECD as detector *(62)*. Under these conditions, BGB-172 × BPX-50 provided the best results allowing the unambiguous determination of the EFs of the five studied toxaphenes with satisfactory repeatability and reproducibility values (RSDs lower than 11%) and with adequate LODs of 2–6 pg/μL. Again, the EF values calculated for real samples, in this case fish oil, agreed with those determined using heart-cut multidimensional gas chromatography.

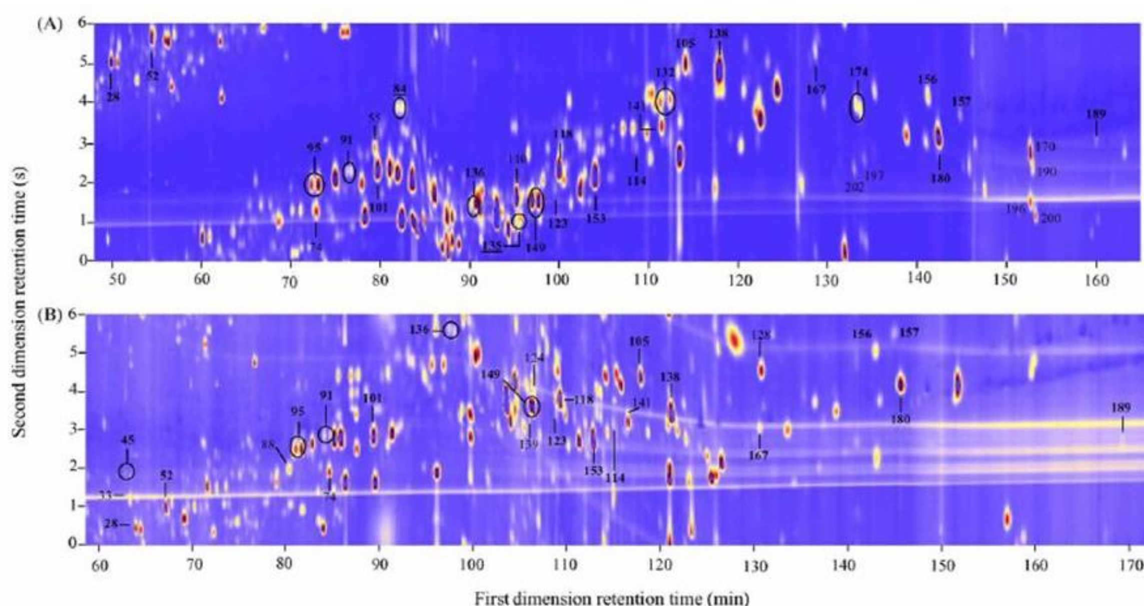

Fig. 13. GC×GC–μECD contour plots of a salmon extract on (A) Chirasil-Dex × Supelcowax-10 and (B) BGD-172 × Supelcowax-10 *(61)*.

Conclusions

The complexity of most of the micropollutant mixtures and the constant demand for enhanced separation in this research area made GC×GC immediately attract the attention of the environmental analytical chemists. Early attempts to use GC×GC to unravel the composition of numerous classes of contaminants, such as PCBs, demonstrated the potential of the technique for accurate target analysis, as far as an adequate detector was available. Such a detector was μECD, as demonstrated by the

many studies reporting on this subject since its introduction in 1990. μECD allowed a convenient data acquisition rate of 50 Hz and, more importantly, provide enough selectivity and sensitivity to allow detection of organohalogenated pollutants at the low levels typically found in the environment. Many column combinations and experimental conditions were then assayed to determine the best experimental conditions for chromatographic isolation of the target toxic congeners from other compounds belonging to the same class and to closely related chemical families that cannot completely be separated by sample preparation procedures in use. Because of their toxicity, most attention was devoted to PCBs and PCDD/Fs, and, due to the nonpolar nature of these toxicants, nonpolar × (semi-)polar columns sets providing highly structured chromatograms were initially preferred. However, subsequent application of the optimised methods to analysis of real-life samples showed the relevance of sample matrix in this type of determination. The complexity of many of the extracts analysed in this field frequently resulted in the co-elution of matrix components with the target compounds and the consequent overestimation of the concentrations of the later. Ongoing investigations demonstrate that the use of shape-selective columns, and in some cases reverse configuration, can help solve this problem. At present, GC×GC–μECD is considered a valuable analytical alternative for accurate determination of individual congeners in classes such as PCBs, PBDEs, PCNs and OCPs. Depending on the concentration levels, that is, in the case of highly contaminated samples, it can also provide satisfactory results for PCDD/Fs, however, in all cases, only after an intensive, that is, mainly manual, data processing step that seriously limits the applicability of the technique for routine and monitoring analyses.

For other more numerous classes, such as PCAs, toxaphene and PCTs, the enhanced sensitivity and separation power provided by GC×GC–μECD has contributed to gathering useful information about the several subgroups of structurally related components simultaneously present in these families. However, isomer-specific analysis is not yet possible. The introduction of ToF MS as a GC×GC detector added an extra separation dimension over to that of GC×GC by incorporating structural information and deconvolution capabilities. GC×GC–ToF

MS added extra insight into the composition of these complex mixtures, but further research is needed to unravel their composition.

Interestingly, GC×GC–ToF MS also contributed albeit (slowly) to extending the application field of GC×GC to other classes of pollutants that previously had received only limited attention, with PAHs and pesticides as prominent representative classes. Once more, the main goal in these studies has been target analysis and, consequently, separation of the tested analytes form the sample matrix the most urgent demand. The feasibility of this three-dimensional approach for accurate automated detection of a complete set of pesticides at acceptable concentration levels of 50 μg/kg in complex matrices has been demonstrated. However, at lower levels (i.e., below 10 μg/kg), the efficiency of the method decreased dramatically, and manual integration was again required. Unfortunately, the sophisticated nature of ToF MS, together with its high price, prevents the introduction of this powerful detector in many laboratories. In these cases, much less expensive and more user-friendly rapid-scanning qMS instruments provide satisfactory results using limited mass ranges of 200-250 Da –which is a sufficiently wide range for most of these target– type applications.

Regarding future trends, a specific research field in which ToF MS offers unsurpassed capacities is in the preliminary identification of new (i.e., unknown) pollutants. Here, the continuous (and complete!, *m/z* range, 5–999) structural information provided by ToF MS through the entire GC×GC chromatogram, combined with the powerful deconvolution algorithms incorporated in its advanced software, represents a distinguished feature that no doubt will be explored in coming years. The scripting capabilities recently introduced in commercial MS-based software packages could help in the use of GC×GC for fast screening of selected families of pollutants in monitoring studies. Finally, the application of chemometric approaches for efficient data interpretation and pattern recognition could also contribute to the practical implementation of this technique in environmental laboratories.

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1.4. VALIDACIÓN DE MÉTODOS ANALÍTICOS

Se define como validación de un método la confirmación mediante el suministro de evidencia objetiva de que se han cumplido los requisitos para la utilización o aplicación específica prevista. La validación de un método es un punto clave en la práctica de los análisis químicos ya que permite definir una especificación o requisito analítico y confirmar que el método propuesto es apto para lo que requiere la aplicación y por tanto los resultados obtenidos pueden considerarse fiables y fidedignos. Queda implícito en esta definición que dichos estudios se realizan con equipamientos que funcionan correctamente y por parte de un operador que sea competente en el campo de trabajo y con conocimiento suficiente de la materia sobre la que trabaja. La validación de un método es de especial relevancia cuando se desarrolla un método nuevo, cuando se está revisando uno existente para ampliar sus aplicaciones, si los controles de calidad indican que el método está cambiando con el tiempo o cuando se adapta un método utilizado en otro laboratorio y es preciso compararlo con uno propio. Así, es importante definir primero el método a usar o desarrollar para una determinada aplicación y, una vez elegido, evaluar su aptitud para dicha aplicación.

Los parámetros que deben ser evaluados en todos los procedimientos analíticos para una adecuada elección del método a usar y/o desarrollar y su posterior validación han sido establecidos por diferentes organismos nacionales e internacionales en controles oficiales En esta tesis se prestará especial atención a aquellos relacionados con el análisis de alimentos destinados al consumo animal y humano *(1, 2)* e incluyen:

Confirmación de la identidad y de la selectividad/especificidad

Confirmar la identidad es establecer que la señal producida en la medición, o que la propiedad medida que se atribuye al analito, solamente se debe a éste y no a la presencia de otro compuesto con propiedades físicas o químicas similares.

La selectividad define la aptitud de un método para determinar de manera exacta y específica el analito de interés en presencia de otros componentes de la matriz siendo totalmente específico cuando se logra medir sólo aquello que se busca detectar. Es decir, un método es específico cuando es 100% selectivo.

En los casos en los que el analito pueda presentarse en distintos formas químicas (véase el ejemplo de los compuestos metálicos, proteínas, etc.) es también importante discernir si éste se encuentra en la forma química deseada; es decir, con grado de oxidación apropiado, si está formando complejos o aleaciones, etc.

Límite de detección y límite de cuantificación

El límite de detección (LOD) se define como la mínima cantidad de analito que puede ser detectada en la muestra con un nivel de confianza especificado. Este nivel suele situarse en los sistemas cromatográficos en tres veces el valor de la desviación estándar del ruido de fondo.

El límite de cuantificación (LOQ) se define como la menor cantidad de analito que puede ser determinada cuantitativamente con una incertidumbre concreta, para un nivel de confianza dado. En general, su valor suele oscilar entre 3 y 10 veces el valor del LOD.

Intervalo de trabajo y respuesta lineal

La linealidad de un método define la aptitud de éste para obtener resultados proporcionales a la concentración del analito en la muestra objeto de estudio. Por tanto, el intervalo de respuesta lineal delimita el intervalo de concentraciones para las cuales el método permite obtener resultados proporcionales a la concentración del analito.

Los límites inferior y superior del intervalo lineal suelen estar marcados, respectivamente, por el LOD o LOQ de la técnica instrumental (o en su caso, de la metodología completa aplicada en la determinación), y por diversos factores que suelen afectar a la cuantificación en su nivel superior, en general dependientes de la respuesta del instrumento (o del procedimiento analítico completo aplicado).

Exactitud

La exactitud se define como la proximidad entre el resultado de una medición realizada con el método propuesto y el valor considerado verdadero o de referencia para un determinado analito. La exactitud se puede determinar por diferentes procedimientos aunque los más frecuentes son utilizar un material de referencia o

realizar ensayos de recuperación. A modo de referencia, cabe mencionar que según las normativas antes mencionadas para el control de PCDD/Fs y *dioxin-like* PCBs en alimentos, un método puede considerable confirmatorio para el análisis de estas sustancias cuando, tras su análisis mediante GC─HRMS, su exactitud en relación con el valor de referencia está en un intervalo ± 20 % el valor de referencia expresado en TEQs totales *(1)*.

Precisión

Expresa la proximidad entre valores obtenidos por mediciones repetidas de una magnitud en las condiciones específicas marcadas por el método adoptado. Las dos medidas de precisión más comunes son la repetitividad y la reproducibilidad, que representan los extremos de la precisión que se pueden obtener con un procedimiento analítico dado. La repetitividad expresa el tipo de variabilidad que se puede esperar cuando el método propuesto es aplicado de manera repetida al análisis de la muestra de referencia por un mismo analista, en un mismo equipo y en un periodo corto de tiempo. La reproducibilidad expresa esa misma idea de variabilidad pero con ensayos realizados por diferentes analistas, en diferentes laboratorios (y por tanto con equipos distintos) y en un tiempo más prolongado, por lo que las variaciones son mayores.

La precisión suele expresarse en términos de desviación estándar (SD) o como desviación estándar relativa (RSD) de un conjunto de medidas. Los métodos oficiales para el análisis de COPs establecen un valor máximo en la precisión de RSD<15%.

Sensibilidad

Se define como el cambio observado en la respuesta de un instrumento de medición como consecuencia de una alteración en el estímulo correspondiente. En el campo analítico, suele definirse como el cambio en la concentración de alguno de los analitos estudiados que produciría un cambio proporcional en la señal del equipo instrumental.

Robustez

La robustez mide la capacidad de un procedimiento analítico para no ser afectado por variaciones pequeñas, pero apreciables, en los parámetros experimentales, lo cual es una indicación de cuán fiable es ese método durante su uso habitual.

Referencias

1. EC, Commision Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. *Official Journal*. **2009**, *L 54*, 1.
2. EC, Commission Regulation (EC) No 1883/2006 of 19 December 2006 laying down methods of sampling and analysis for the official control of levels of dioxins and dioxin-like PCBs in certain foodstuffs. *Official Journal*. **2006**, *L 364,* 32.

Chapter – Capítulo 2

BJECTIVES

OBJETIVOS

De acuerdo con las consideraciones llevadas a cabo en la Introducción general, resulta claro que existe una gran variedad de técnicas analíticas disponibles y en uso para la extracción, purificación y concentración de contaminantes orgánicos clásicos y emergentes de muestras de interés medioambiental, pero también la actual demanda de procesos de tratamiento de muestra más sostenibles desde el punto de vista ambiental y, preferiblemente, genéricos, simplificados y miniaturizados. Así mismo, y en relación con el análisis instrumental, resulta evidente el potencial analítico de las técnicas cromatográficas multidimensionales que, con su elevado poder de resolución, permiten solventar algunos de los problemas de identificación y cuantificación fidedigna no resueltos con técnicas basadas en aproximaciones monodimensionales, pero también abordar de manera satisfactoria el análisis de extractos de elevada complejidad, como los obtenidos al aplicar metodologías simplificadas de preparación de muestra a matrices complejas. Además, estas técnicas pueden permitir el análisis simultáneo de distintas familias de contaminantes presentes en la muestra, incluyendo aquellos que, en un primer momento, no eran considerados objeto de estudio.

En base a estas consideraciones, se plantean dos **objetivos genéricos** para esta memoria de tesis. Por un lado, la evaluación de la posibilidad de simplificación y miniaturización de ciertos protocolos de preparación de muestra para el análisis de COPs en muestras (semi-)sólidas complejas de interés ambiental; y, por otro lado, la aplicación de la técnica de GC×GC para la determinación y elucidación estructural de distintas familias de compuestos orgánicos de interés presentes en extractos purificados provenientes de esas matrices.

Para alcanzar estos objetivos genéricos, se plantearon los siguientes **objetivos concretos**:

En relación con la preparación de muestras medioambientales (semi-)sólidas complejas para el análisis de COPs se propone:

- Evaluar la posibilidad de adaptar o modificar ciertas técnicas de extracción convencionales para acelerar y mejorar la eficacia del proceso de extracción.

- Desarrollar nuevos procedimientos de preparación de muestra miniaturizados que incluyan el uso de técnicas aceleradas de extracción.

- Evaluar la posibilidad de acoplar las etapas de extracción y purificación, siempre que sea posible, a fin de acortar los tiempos de análisis y minimizar la manipulación de los extractos.

- Adaptación de nuevos dispositivos miniaturizados para la purificación de extractos complejos.

En relación con la evaluación del potencial de la GC×GC para el análisis multicomponente de extractos complejos provenientes de matrices ambientales se propone:

- Aplicar la técnica de GC×GC para la separación e identificación simultánea de componentes individuales de distintas familias de contaminantes cuyo análisis fidedigno con técnicas monodimensionales no es posible.

- Cuantificar los niveles de ciertos contaminantes orgánicos individuales pertenecientes a esas familias en los extractos complejos obtenidos al aplicar las metodologías de preparación simplificadas.

- Identificación y elucidación estructural de ciertos contaminantes emergentes y compuestos de potencial interés ambiental presentes en estos extractos.

Chapter – Capítulo 3

RESULTS AND DISCUSSION

RESULTADOS Y DISCUSIÓN

Section – Sección 3.1

SAMPLE PREPARATION

PREPARACIÓN DE MUESTRA

3.1.1. ANÁLISIS DE COMPUESTOS ORGANOBROMADOS DE ORIGEN NATURAL Y ANTROPOGÉNICO EN ATUNES DEL MAR MEDITERRÁNEO[1]

Abstract

Anthropogenic compounds, such as polybrominated diphenyl ethers (PBDEs), together with naturally-produced organobromines, such as methoxylated PBDEs (MeO-PBDEs), polybrominated hexahydroxanthene derivatives (PBHDs), 2,4,6-tribromoanisole (TBA) and a mixed halogenated monoterpene (MHC-1), were measured in muscle from 26 farmed and wild bluefin tuna (*Thunnus thynnus*) caught in the Mediterranean Sea. This species is ecological attractive because of the changes of geographic habitat throughout its long lifespan which affect its feeding. PBDE concentrations were similar between tuna samples of different groups (17-149 ng g^{-1} lipid weight - lw in farmed tuna, 25-219 ng g^{-1} lw in long-line fished tuna and 26-126 ng g^{-1} lw in net-fished tuna). However, higher concentrations of naturally-produced MeO-PBDEs and PBHDs were observed in the two types of wild tuna (longline fished and net-fished) compared to farmed tuna suggesting that wild tunas come easily in contact with sources of these compounds. In all cases PBHDs presented the highest contribution to the sum of organobromines (50 % in farmed tuna and > 90 % in wild tuna).TBA was detected at low concentrations (< 6 ng g^{-1} lw), while MHC-1 was found at higher concentrations (up to 42 ng g^{-1} lw) in farmed tuna. The estimated daily ingestion of PBDEs from tuna was 830 ng PBDEs day^{-1}, regardless of the origin of the tuna. While this value is approximately 600 times lower that the minimum risk level set by the U.S. Department of Health and Human Services, it is approximately eight times higher than the total intake of PBDEs via diet, suggesting that consumption of tuna can add considerably to the total daily intake of PBDEs.

Keywords: wild, farmed, bluefin tuna, Mediterranean Sea, PBDEs, MeO-PBDEs, PBHDs, naturally-produced, organobrominated compounds, dietary intake

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[1] M. Pena-Abaurrea, L. Weijs, L. Ramos, N. Borghesi, S. Corsolini, H. Neels, R. Blust, A. Covaci. Anthropogenic and naturally-produced organobrominated compounds in bluefin tuna from the Mediterranean Sea. *Chemosphere*. **2009**, *76* (11) 1477.

INTRODUCTION

Brominated flame retardants (BRFs), such as polybrominated diphenyl ethers (PBDEs), have been the focus of an increasing number of studies during the last decade because of their potential for bioaccumulation and contamination in the environment and food webs *(1-3)* and because of their potential health effects *(4-5)*. PBDEs are synthetic compounds, commercially available as mixtures with different degree of bromination (Penta-, Octa- and Deca-BDE), used in a variety of products such as plastics, textiles and electrical equipment. In 2004, the Penta- and Octa-BDE mixtures were banned by the European Union *(6)*. The Deca-BDE mixture was banned in 2008 *(7)*. Recently (May 2009), the Penta- and Octa-BDE mixtures were added to the Stockholm Convention list.

Several structural PBDE analogues, such as the methoxylated PBDEs (MeO-PBDEs), have recently been evidenced in fish and marine mammals *(8-12)*. MeO-PBDEs are naturally-produced in the marine environment by sponges or algae *(13-14)*. More than 4000 halogenated naturally-produced compounds have been found in the marine environment *(15)*, while only a few of them, such as MeO-PBDEs, polybrominated hexahydroxanthene derivatives (PBHDs), 2,4,6-tribromoanisole (TBA) and a mixed halogenated compound (MHC-1), have been measured at high concentrations in marine organisms, including top-predators *(13, 16-20)*. Yet, only a few papers reported on concentrations of PBDEs and MeO-PBDEs in Mediterranean Sea organisms *(8, 11, 21)*.

The present work focuses on bluefin tuna (*Thunnus thynnus*) from the Mediterranean Sea, an economically and gastronomically relevant species. Tuna populations are variable according to the season, the Mediterranean area being massively populated during warmer seasons *(22-23)*. Remarkably, populations have considerably decreased in the last decades (almost by 80 %) most likely due to over-fishing by industrial fisheries *(23-25)*. The bluefin tuna is a good example of a flexible species, capable of adapting to its environment which changes rapidly due to their temporal migration. Moreover, this species is also suitable for aquaculture. They feed on crustaceans, small fishes and cephalopods in their juveniles stages, and rely on large cephalopods and pelagic fishes as adults *(23, 25)*. Because of all these

confounding factors, these animals may exhibit different bioaccumulation profiles, which make this species interesting from an ecotoxicological point of view.

This paper aims at assessing the presence of anthropogenic and naturally-produced organobrominated compounds in bluefin tuna from the Mediterranean Sea. Differences in concentrations and accumulation profiles of these brominated compounds between wild and farmed tuna were investigated. Moreover, the daily human intake of brominated compounds was estimated from the ingestion of Mediterranean tuna and its contribution to the total diet was evaluated.

2. MATERIALS AND METHODS

2.1. Sample collection and preparation

Muscle samples from 26 bluefin tuna (12 males and 14 females between 2-13 yrs old) were collected in the Mediterranean Sea (South Thyrrenian Sea) during 2003 by different fishing techniques. Twenty wild animals were used from which 10 were caught by longline fishing (baited hooks hanging from a single line) and 10 by "mattanza" technique (net chambers). Six tuna originated from a fish farming area from the same area. Fishes were dissected and muscle samples were frozen at -20 ºC until analysis.

2.2. Chemicals

All solvents used for the analysis were of SupraSolv grade (Merck, Darmstadt, Germany). Anhydrous sodium sulfate and silica gel (Merck) were washed with *n*-hexane and used after activation by heating overnight at 160 ºC. Empty polypropylene columns for clean-up (25 mL) were purchased from Alltech (Lokeren, Belgium). Nine PBDE congeners (IUPAC numbers 28, 49, 47, 66, 100, 99, 155, 154, and 153), 15 MeO-BDEs (4´-MeO-BDE 17, 2´-MeO-BDE 28, 3´-MeO-BDE 28, 4-MeO-BDE 42, 3-MeO-BDE 47, 6-MeO-BDE 47, 5-MeO-BDE 47, 4´-MeO-BDE 49, 2´-MeO-BDE 68, 4-MeO-BDE 90, 6-MeO-BDE 90, 5´-MeO-BDE 99, 6-MeO-BDE 99, 5´-MeO-BDE 100, 6-MeO-BDE 140), two PBHDs isomers (2,7-dibromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1*H*-xanthene, abbreviated as triBHD, and 2,5,7-tribromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-

1*H*-xanthene, abbreviated as tetraBHD), MHC-1 and TBA were targeted in the samples. BDE 77 and 1,2,3,4-tetrachloronaphtalene (1,2,3,4-TCN) were used as internal standard and syringe standard, respectively. Standards of PBDE and a mixture of MeO-PBDE congeners were purchased from Wellington Laboratories (Guelph, ON, Canada). Additional MeO-PBDEs were from Accustandard (New Haven, CT) and TBA was purchased from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). A mixture of triBHD and tetraBHD at 1.6 ng μL^{-1} in iso-octane and a standard of MHC-1 at 1.9 ng μL^{-1} also in iso-octane were a gift from Dr. Walter Vetter (University of Hohenheim, Germany).

2.3. Sample preparation

The analytical procedure for determination of organobrominated compounds has been previously described in *(11)*. Briefly, typically 3 g of tuna muscle were accurately weighed and grounded with sodium sulfate. The mixture was spiked with 4 ng of BDE 77 and extracted for 2 h in a hot Soxhlet (Büchi, Flawil, Switzerland) with 100 mL of *n*-hexane:acetone (3:1; v/v). The extract was evaporated and cleaned-up on acid silica (~8 g) using 20 mL of *n*-hexane and then 15 mL of dichlorometane as elution solvents. The extract was concentrated and further evaporated under a nitrogen stream to near dryness and redissolved in 100 µL of recovery standard TCN (used to calculate the recovery of the internal standard). The lipid content was determined gravimetrically on an aliquot of the extract by solvent evaporation in an oven (105 ºC, 1 h).

2.4. Analysis

For the determination of target analytes, each extract was injected in two systems. An Agilent 6890-5973 GC–MS system operating in the electron ionization (EI) mode was equipped with a 25 m × 0.22 mm × 0.25 µm HT-8 capillary column (SGE, Zulte, Belgium). The ion source, quadrupole and interface temperature were set at 230, 150 and 300 ºC, respectively. One µL of the cleaned extract was injected in cold pulsed splitless mode. Helium was used as carrier gas at constant flow (1 mL min^{-1}). The mass spectrometer was used in selected ion monitoring (SIM) mode and the two most

abundant ions from the cluster were monitored for each homologue group of compounds with a dwell time of 40 ms.

All extracts were injected also in an Agilent 6890-5973 GC–MS system operated in electron capture negative ionization (ECNI) mode and equipped with a 30 m × 0.25 mm × 0.25 μm DB-5 capillary column (SGE, Zulte, Belgium). The ion source, quadrupole and interface temperature were set at 250, 150 and 300 ºC, respectively. Helium gas was used as carrier gas at constant flow (1 mL/min). Methane was used as reacting gas. The electron multiplier voltage was set at 2200 V. One microliter of the cleaned extract was injected in solvent vent mode. The mass spectrometer was operated in SIM mode by monitoring bromine isotope ions (*m/z* 81 and 79) with a dwell time of 50 ms. Additional details can be found in *(11)*.

2.5. Quality assurance and quality control

Multi-level calibration curves in the linear response interval of the detector were created for the quantification, and good correlation ($r^2 > 0.99$) was achieved. The identification of each analyte was based on the simultaneous detection and ratio between the corresponding ions and on the relative retention times (RRTs) to the internal standard. For most compounds, results obtained by EI were used since concentrations were high enough and selectivity was improved through the monitoring of specific ions from the molecular cluster. Yet, for some compounds (e.g., BDE 28), results obtained by ECNI were used since co-elutions were not observed here. Nevertheless, results obtained for BDE 47 by EI and ECNI did not differ by more than 10 % from each other. The quality control was also performed through analysis of procedural blanks, a replicate sample and a standard reference material (SRM 1945 whale blubber which has certified values for PBDEs). For the replicate and SRM 1945, the relative standard deviations (RSDs) were < 10 % with few exceptions (e.g. BDE 153) at very low concentrations for which RSDs were < 20 %. Recoveries of the IS were between 96-120 % (mean, 106 %; RSD < 8 %). PBDEs, MeO-PBDEs, TBA, PBHDs and MHC-1 were not detected in the procedure blanks in the EI mode analysis. Nevertheless, BDE 47, BDE 100 and BDE 99, 2-MeO-BDE 28 and TBA were detected in the blanks using ECNI-MS and the mean value of each

analyte in the procedural blanks was used for subtraction. Method limits of quantification (LOQ) were set at 3 × SD obtained in the procedural blanks. LOQs ranged from 0.1 to 0.25 ng g^{-1} lw for individual PBDE and MeO-PBDE congeners, and were 1 ng g^{-1} lw for the two PBHD isomers and 0.1 ng g^{-1} lw for TBA and MHC-1.

2.6. Statistical analysis

For calculations of sums and means**,** congeners with concentrations below LOQ were replaced by a value of ½ × LOQ. Most statistical analyses were carried out using Excel 8.0 for Windows. Differences between concentrations of organobromine compounds in the three fish groups (longline, "mattanza", and farmed) were investigated by one-way ANOVA. Grubb´s test was employed to detect outliers between samples caught by the same fishing technique. Limit of statistical significance was set at 0.05.

3. RESULTS AND DISCUSSION

3.1. Lipid content

Lipid percentages differed according to the origin of the tuna samples (Table 1). Farmed tunas presented higher values probably due to a combination of an intensive diet (mostly based on herring) and a lower activity compared to their wild counterparts. A narrow range (between 27 % and 37 %, mean 32 %, RSD < 12 %) was observed for farmed tuna due to the identical conditions in which they were kept**.** A wider lipid content range was found in wild fishes with percentages ranging from 2.7 % to 30 %, but independent from tuna age, length and gender (ANOVA, all $p > 0.05$).

3.2. PBDEs

The investigated PBDE congeners were detected in all three fish groups: **farmed**, longline fished **(wild 1)** and net-fished tunas **(wild 2)**. The PBDE profiles were similar in all three cases with BDE 47 being the predominant congener (mean 44 %,

Table 1. Lipid percent (on wet weight basis) of bluefin tuna analyzed in the present study. Groups are based upon age and gender.

n	Age (yrs)	Gender	Lipid percentage (%)			
			Mean	**SD**	**Median**	**Range**
Wild tuna						
3	2	F	14	12	11	2.4-27
2	2	M	7.4	0.2	7.4	7.3-7.5
2	3	F	6.1	4.8	6.1	2.7-9.5
3	3	M	8.2	3.4	7.1	5.5-12
2	4-6	F	20	7	20	15-27
3	4-6	M	7.5	3.7	9.4	3.2-9.8
2	6-9	F	13	8	13	6.8-19
1	10-13	M	29	-	-	-
2	>13	M	27	5	27	23-30
Farmed tuna						
5	unknown	F	31	4	31	27-37
1	unknown	M	35	-	-	-

RSD < 3 %), followed by BDE 100 (mean 19 %, RSD < 4 %) and BDE 99 and 154 (mean 9 %, RSD < 5 %) (Fig. 1). Total PBDE concentrations (sum of PBDEs) varied largely between samples of the same group (17-149 ng g^{-1} lw in farmed tuna, 25-219 ng g^{-1} lw in 'wild 1' tuna and 26-126 ng g^{-1} lw in 'wild 2' tuna) (Table 2). However, no statistical significant differences were found between PBDE levels in the three tuna groups (ANOVA, all $p > 0.05$). An outlier was detected in the wild 1 tuna group (Grubbs's test) and removed from further calculations. No associations were found between morphologic characteristics, i.e. gender, age and length, and the total content of PBDEs.

In a similar study, identical PBDE profiles were found for farmed and wild salmon from the Pacific Ocean, with BDE 47 being the most predominant congener (> 50 % of total PBDEs), and other PBDEs varying in the order BDE 99 > BDE 100 > BDE 49 > BDE 154 *(26)*. However, median concentrations found in farmed salmon were almost ten times higher than those of the wild salmon, an effect that was not observed in the present study. PBDE concentrations in salmon were also lower than those found in tuna, ranging from 1 to 3 ng g^{-1} ww in farmed salmon, and from 0.1 to 0.3 ng g^{-1} ww in wild salmon.

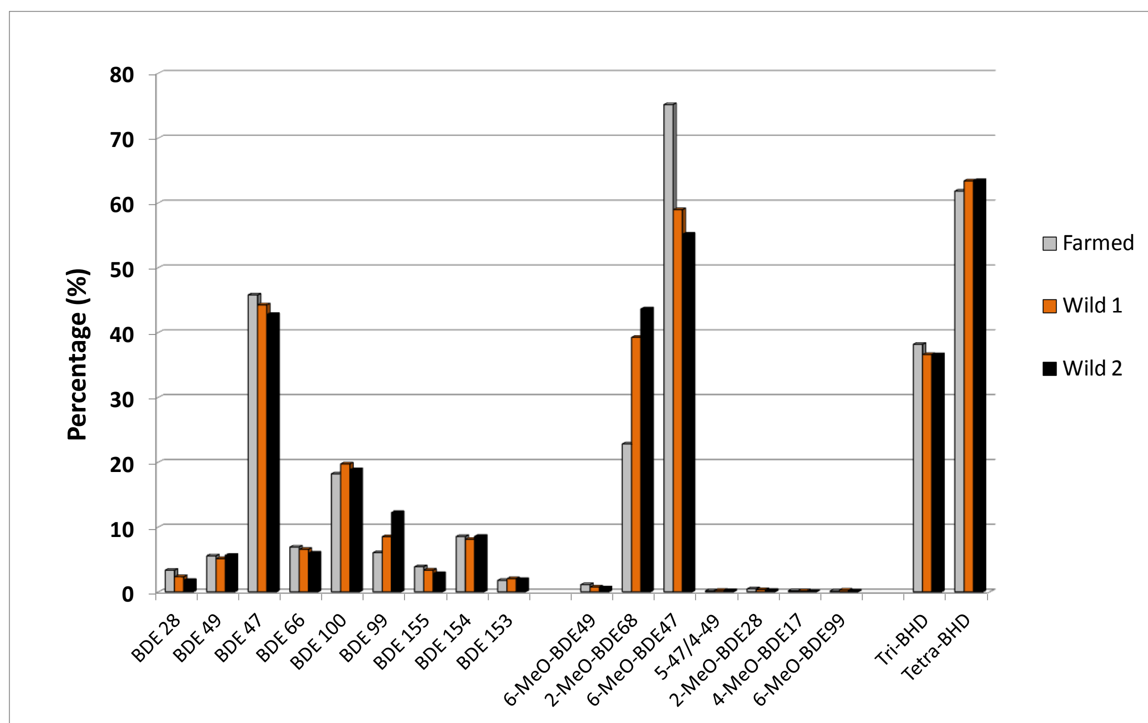

Fig. 1. Profiles for PBDEs, MeO-PBDEs and PBHDs in the three fish groups. (Wild 1 = longline fished tuna, Wild 2 = net-fished tuna).

PBDE concentrations found in tuna samples from the Mediterranean Sea were similar to, or higher than those found in skipjack tuna (*Katsuwonus pelamos)* from open sea areas worldwide (East Asia, Brazil, Seychelles) *(27)*, which ranged from 1.8 to 53 ng g^{-1} lw. BDE 47 was also the most abundant congener, although residue patterns varied in the order BDE 154 > BDE 100 > BDE 99. Similar PBDE congener profiles to those found in tuna were measured in marine mammals (whales and dolphins) from the Mediterranean Sea, although at higher levels (70-8100 ng g^{-1} lw) *(8)*. Lower PBDE levels were found in tuna from Catalonian supermarkets *(28)* and in swordfish (*Xiphias gladius*) from Mediterranean Sea *(21)*, 71-1503 pg g^{-1} ww and 4-1108 pg g^{-1} ww, respectively, compared to the current study (1-52 ng g^{-1} ww, Table 1, after conversion using lipid percentage). In both cases, tetra-BDEs also contributed with more than 50 % to the tri- to octa-PBDEs mixtures.

Table 2. Concentrations of organobrominated compounds (ng/g lipid weight) in muscle of Bluefin tuna (*Thunnus thynnus)* from the Mediterranean Sea.

		Farmed tuna (n=6)				**Wild 1 (n=10)**				**Wild 2 (n=10)**			
	LOQ	**Mean**	**SD**	**Median**	**Range**	**Mean**	**SD**	**Median**	**Range**	**Mean**	**SD**	**Median**	**Range**
Lipids (%)		32	4	32	27-37	17	10	17	3.2-30	9	7	7	2.7-27
BDE 28	*0.1*	2.3	2.3	1.2	0.4-5.3	1.9	1.9	1.1	0.5-2.4	1.0	0.5	0.8	0.5-2.2
BDE 49	*0.1*	3.9	3.7	2.1	0.9-8.8	3.5	2.1	2.6	1.4-8.1	3.2	1.7	2.6	1.9-7.0
BDE 47	*0.25*	33	32.3	16	7.7-75	34	29	26	11-109	25	14	23	11-53
BDE 66	*0.1*	3.8	2.3	3.3	1.3-6.8	5.3	5.5	4.0	1.3-20	3.7	3.1	2.7	<0.1-9.9
BDE 100	*0.1*	13	12.5	6.0	3.0-30	15	11	11	4.3-40	11	5.7	9.4	4.5-22
BDE 99	*0.25*	3.8	3.1	3.1	0.8-7	5.7	3.6	3.7	2.8-13	7.2	4.2	6.5	2.3-17
BDE 155	*0.2*	2.3	1.9	1.3	0.7-5	2.3	1.3	1.9	0.7-5.4	1.7	1.1	1.4	<0.2-3.7
BDE 154	*0.2*	5.1	4.1	3.0	1.6-11	5.6	3.4	4.9	1.9-14	4.9	2.6	5.1	1.7-11
BDE 153	*0.2*	1.1	0.8	1.0	0.3-2	1.3	0.6	1.1	0.5-2.5	1.0	0.6	1.0	<0.2-2.1
Sum PBDEs		**68**	**63**	**37**	**17-149**	**74**	**57**	**58**	**25-219**	**58**	**31**	**53**	**26-126**
6-MeO-BDE 49	*0.1*	0.7	0.3	0.6	0.5-1	0.9	0.3	1.0	0.5-1.4	1.0	0.8	1.0	<0.1-2.8
2´-MeO-BDE 68	*0.25*	15	5.2	16	8.2-20	49	19	45	23-80	77	66	53	25-250
6-MeO-BDE 47	*0.25*	47	7	50	38-54	82	49	57	37-163	89	59	74	44-246
5- MeO-BDE 47 + 4- MeO-BDE 49	*0.1*	0.1	0.0	0.1	<0.1	0.3	0.1	0.3	<0.25-0.4	0.3	0.3	0.2	<0.1-1.1
2-MeO-BDE 28	*0.1*	0.3	0.0	0.3	0.2-0.4	0.5	0.3	0.4	<0.25-1.1	0.3	0.2	0.4	<0.1-0.6
4-MeO-BDE 17	*0.1*	0.1	0.1	0.1	<0.1-0.2	0.2	0.2	0.1	<0.25-0.5	0.1	0.0	0.1	<0.1
6-MeO-BDE 99	*0.1*	0.1	0.0	0.1	<0.1	0.3	0.3	0.2	<0.25-0.9	0.3	0.5	0.1	<0.1
Sum MeO-PBDEs		**63**	**12**	**68**	**47-72**	**134**	**56**	**110**	**73-248**	**167**	**126**	**134**	**69-503**
Tri-BHD	*1*	47	13.3	43	37-73	1195	758	1229	117-2199	1802	1034	1652	854-4443
Tetra-BHD	*1*	144	213	58	40-578	2385	1982	1892	183-6187	3440	2894	2391	1713-11170
Sum PBHDs		**191**	**226**	**101**	**78-651**	**3580**	**2689**	**3157**	**300-8269**	**5241**	**3884**	**4029**	**2678-15610**
TBA	*0.1*	0.8	0.2	0.8	0.4-1.0	3.1	1.6	3.5	0.8-5.2	4.2	1.3	4.1	2.0-6.4
MHC-1	*0.1*	42	21	49	<0.1-54	26	15	24	<0.1-47	30	14	27	15-66

For congeners with concentrations below LOQ, the mean concentrations were calculated using a value of ½ × LOQ

3.3. MeO-PBDEs

Only eight out of the targeted 15 MeO-PBDE congeners were found at measurable levels in the studied tuna samples (Table 2). MeO-PBDE profiles were similar in the three investigated groups, with 2´-MeO-BDE 68 and 6-MeO-BDE 47 contributing with almost 98 % of the sum MeO-PBDEs, which agrees with reported literature for marine species *(9, 11, 29)*. A shift in the ratio 2´-MeO-BDE 68 to 6-MeO-BDE 47 was observed between wild and farmed tunas. In the first case, ratio was close to 2:3, whereas in farmed tuna the ratio dropped to 1:3, meaning that a higher proportion of 2´-MeO-BDE 68 was present in farmed tuna (t-test, $p < 0.05$). As suggested by Vetter *(30)*, a higher contribution of 2´-MeO-BDE 68 would indicate sponges as the dominant source of MeO-PBDEs, while a higher proportion of 6-MeO-BDE 47 would point to algae as the principal source of MeO-PBDEs. Our results support this hypothesis and this is explained by the restriction in mobility of farmed tuna and, as a consequence, a reduced direct contact with sponges. The dominance of 6-MeO-BDE 47 in wild tuna agrees with the congener pattern observed in marine species from the Northern Hemisphere *(9, 11, 29, 31)*. Concentrations of MeO-PBDEs in tuna samples were in the same range as PBDE levels and varied from 47 to 74 ng g^{-1} lw in farmed tuna, from 73 to 248 ng g^{-1} lw in wild 1 tuna, and from 69 to 503 ng g^{-1} lw in wild 2 tuna (Table 2). Significant differences between MeO-PBDE concentrations were found between farmed and two wild groups of tuna (ANOVA, $p < 0.05$).

The literature on MeO-PBDEs in fish from different locations is rather scarce and only a few reports of these compounds in other aquatic animals are available. Petterson et al. *(8)* measured MeO-PBDE levels in whales and dolphins from the Mediterranean Sea and found them similar to or slightly higher (range 3-808 ng g^{-1} lw) than those in tuna. Congeners 2´-MeO-BDE 68 and 6-MeO-BDE 47 were also dominant (> 95 % of the total content of MeO-PBDEs). Lower levels of MeO-PBDEs were found in two deep-sea fish species from the Mediterranean Sea (averages 6.5 and 28.9 ng g^{-1} lw, respectively) *(11)*, while a large variation in the concentrations of MeO-PBDEs was observed in a Canadian Arctic marine food web *(32)*. While MeO-PBDE congeners were not detected in lower organisms and

sediments, several studies have suggested that they can biomagnify in aquatic food chains, reaching higher concentrations in top-predators *(12, 32)*.

3.4. PBHDs

PBHDs have recently been reported as naturally-produced brominated compounds originating from sponges *(11, 18-19, 33)*. PBHD congeners detected in this study showed the highest concentrations among all brominated compounds measured. While the total PBHD contribution represented around 50 % of the sum of organobromine compounds in farmed tuna, PBHDs represented for more than 90 % of the total content in wild tuna (Fig. 2.), probably due to the higher interaction of these animals with natural sources (e.g., sponges). PBHD concentrations differed largely between farmed and wild tunas, with the concentrations in the wild tuna being one order of magnitude higher than those in farmed tuna (Table 2).

Hiebl et al. *(18)* found that several fish species in aquaculture (salmon, sea bass and gilt head bream) presented higher levels of PBHDs than the corresponding wild fishes, which is the opposite trend to the present study. This could be associated with the different sampled areas in the former study. The farmed fish investigated in *(18)* originated mostly from the Mediterranean Sea, New Zealand and Norway and had concentrations ranging between 1 and 144 ng g^{-1} lw, being lower than those found in farmed tuna of the present study.

PBHDs have been found in the Mediterranean sponge (*Scalarispongia scalaris)* and in different marine species, such as sea bass (< 5-120 ng g^{-1} lw), sardines (410 ng g^{-1} lw) and anchovies (850 ng g^{-1} lw) *(18-19)*, all of these fish constituting an important part of the diet of the bluefin tuna. In most of these samples, the PBHD profile was different than the one observed in tuna, with triBHD being more abundant than tetraBHD. The abundance of tetraBHD could be an indication of either metabolic breakdown of triBHD in tuna, a higher bioaccumulation capacity for tetraBHD, or a particular diet habit, as already suggested for PBHDs in Mediterranean deep-sea fish species *(11)*. Bluefin tuna may feed on zoobenthos (benthic crustaceans, mollusks and cephalopods *(34)*), but there are no data on a direct predation of sponge. Tuna might

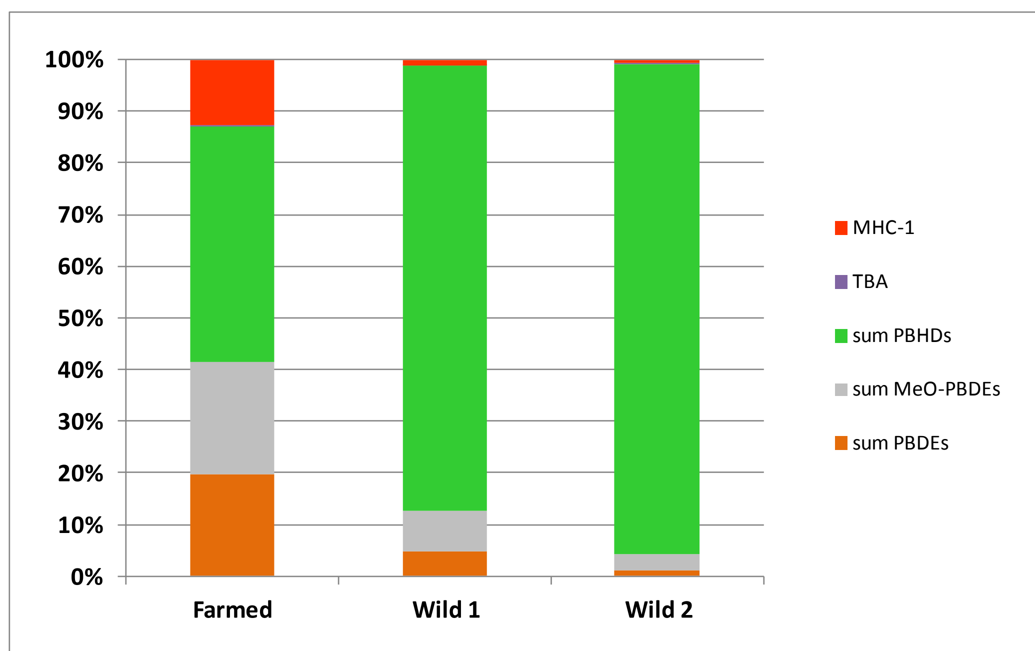

Fig. 2. Organobrominated compound distribution (expressed in percentage) in the three studied fish groups. (Wild 1 = longline fished tuna, Wild 2 = net-fished tuna).

bioaccumulate PBHDs from the environment in relation to the natural background levels in seawater, or they might biomagnify these naturally-produced brominated compounds through consumption of prey which contain PBHDs. In general, tuna are voracious predators feeding at a higher trophic level respect to the species studied by Hiebl et al. *(18)*. Feeding habits may be responsible for the different PBHD profiles found in tuna and its prey *(11)*.

3.5. TBA and MHC-1

These two organobrominated compounds were detected in all investigated tuna samples. Concentrations of TBA, which derives from the natural methylation of tribromophenol *(35-36)* mostly produced by algae *(37)*, in Mediterranean bluefin tunas ranged from levels found in farmed fishes (0.4-1.0 ng g^{-1} lw) to higher levels measured in wild tunas (2-6.4 ng g^{-1} lw). These results suggest that there is little contact between algae and tuna. Higher concentrations of TBA were found in marine samples *(18)* with lower levels in free-living species (gilt head bream, 2 ng g^{-1} lw) and higher concentrations in sea bass and salmon (from 6 to 90 ng g^{-1} lw) reaching 250 ng g^{-1} lw in green-lipped mussel from New Zealand. Only low amounts of TBA were found in deep-sea fishes from the Mediterranean Sea (<0.3 ng g^{-1} lw) *(11)*.

MHC-1 levels detected in farmed tuna were slightly lower than those of PBDEs and MeO-PBDEs (mean value, 42 ng g^{-1} lw). In contrast, mean concentrations found in longline and net-fished tunas were 26 and 30 ng g^{-1} lw, respectively, representing < 1 % of the total sum of organobromines. Covaci et al. *(11)* found lower MHC-1 concentrations in two deep-sea fishes from the Mediterranean Sea (9.7 and 0.6 ng g^{-1} lw, respectively). The same behavior was observed in other Mediterranean fishes, such as sea bass (14 ng/g lw), gilt head bream (1 ng g^{-1} lw) and sardines (3 ng g^{-1} lw) *(18)*. Similar to TBA, higher MHC-1 levels were observed in fishes from different habitats worldwide (940 ng g^{-1} lw in salmon from an aquaculture area in Faeroe Islands) *(18)*. This suggests that natural sources of TBA and MHC-1 are scarce in the Mediterranean Sea.

3.6. Estimated daily ingestion (EDI)

The significant contribution of fish and seafood to the dietary BFR intake in humans (~1/3 of the total *(28)*), combined with the relevance of bluefin tuna in a suitable diet, emphasizes the necessity to control and monitor the presence of these pollutants in the diet. The minimum risk level (MRL) for PBDEs in humans has been set by the U.S. Department of Health and Human Services at 7 μg kg^{-1} body wt day^{-1} *(4)* or 490 μg PBDEs day^{-1} for an adult of 70 kg.

By using the estimated daily fish consumption in Catalonia (Spain) set in 2003 at 68 g fish per day by a standard adult man (70 kg body weight) *(38)* and assuming that tuna is the only fish eaten, the average EDI (considering the mean of sum PBDEs) was 12 ng kg^{-1} body wt day^{-1} (or 830 ng PBDEs day^{-1}). Mediterranean tuna is a common food item in the diet of Catalonians, but is not the only fish species eaten, so this value is probably an overestimation of reality. This result is approximately 600-fold lower that the MRL established by Agency for Toxic Substances and Disease Registration (ATSDR). While there is no risk for human health as defined by ATSDR, the total PBDE ingested via tuna consumption is approximately 8 times higher than the total PBDE through diet *(28)*, suggesting that consumption of tuna can add considerably to the total intake of PBDEs. However, the ATSDR limit does not take into account any other brominated compounds which can be present in much higher

concentrations than PBDEs in wild tuna and for which no toxicological data are available. According to data from the present study, the average intake of MeO-PBDEs and PBHDs through tuna consumption is 2.2 and 34.8 μg day^{-1}, respectively.

Conclusions

Similar profiles of anthropogenic and naturally-produced organobrominated compounds were observed in farmed and wild tunas from the Mediterranean Sea. Naturally-produced compounds were more abundant and varied largely between these two groups, suggesting that wild tunas may easily come in contact with possible sources, e.g. sponges. Results also indicate that the estimated daily ingestion of PBDEs through tuna consumption do not pose a risk to human health. Yet, tuna contains many other toxic halogenated chemicals, which might show an unpredictable synergetic effect for consumers.

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3.1.2. EXTRACCIÓN SELECTIVA CON LÍQUIDOS PRESURIZADOS DE BIFENILOS POLICLORADOS Y POLIBROMODIFENIL ÉTERES DE PIENSOS DE ACUICULTURA[1]

Abstract

A new miniaturised pressurised liquid extraction (PLE) with *in-cell* purification method has been developed for the simultaneous determination of endogenous prioritary and toxic polychlorinated biphenyls (PCBs) and environmentally relevant tri- to deca-brominated diphenyl ethers (PBDEs) in different feed matrices. The proposed methodology combined the use of matrix solid phase dispersion (MSPD) with PLE for enhanced extraction efficiency. Once the parameters affecting the extraction were optimised (i.e., sorbent:matrix ratio, volume and nature of the extraction solvent, PLE working mode, extraction time and temperature, and amount of clean-up sorbents), the collected purified extracts were directly analysed by gas chromatograph with electron capture micro detector (GC–μECD) for PCB determination and by gas chromatograph with negative chemical ionization-mass spectrometry (GC–NCI-qMS) for PBDE analysis. A gas chromatography system coupled to ion trap detector working in tandem mode (GC–ITD(MS/MS) was used for final PCB confirmation during method validation. Sample treatment consisted of 2 sequential 7-min static PLE with *n*-hexane and *n*-hexane:dichloromethane (1:1, v/v). Only 8 mL of the organic solvents and 3.5 g of sorbents sufficed for complete treatment of 0.25 g of feed sample. Repeatability and recovery for the complete PLE with *in-cell* purification method were evaluated at two spiking levels, 0.4 and 4 ng/g wet weight. Recoveries in the range 60-120% were obtained for PCBs, while those of PBDEs ranged from 86% to 114% for most of the target analytes. The relative standard deviations (RSDs) were in general lower than 20%. The optimised procedure was finally applied to the determination of the target PCBs and PBDEs in a variety of feed samples.

Keywords: Selective pressurised liquid extraction, PCBs, PBDEs, feed analysis

[1] M. Pena-Abaurrea, J.J. Ramos, M.J. González, L. Ramos. Selective pressurised liquid extraction of polychlorinated biphenyls and polybrominated diphenlys ethers from feedstuffs. *In preparation.*

1. INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) are recognised as worldwide distributed contaminants and there is an increasing interest for determining their concentration levels in environmental matrices samples *(1)*. Due to their non-polar and lipophilic character, in many instances, sample preparation methods similar to those previously validated for close related microcontaminants, such as polychlorinated biphenyls (PCBs) and/or polychlorinated dibenzo-*p*-dioxins and furans (PCDDs/PCDFs), have been used for the extraction and clean-up of PBDEs and, when required, final fractionation of the investigated families of pollutants when dealing with complex fat containing matrices *(2-4)*. In general, these conventional analytical methods are expensive in terms of solvent, sorbent and time consumption, use to involve much manipulation of the extracts and, because of the lack of method reoptimisation, in some cases, have resulted in rather unsatisfactory results for the high brominated congeners, i.e. hepta- to deca-BDEs *(5-6)*.

Pressurised liquid extraction (PLE) is an analyte- and matrix-independent technique that, despite its exhaustive nature, uses to provide cleaner extracts than more conventional, but widely accepted, time-consuming classical procedures used for the extraction of persistent organic micropollutants from fat-containing matrices. Careful optimisation of the parameters affecting the efficiency and selectivity of the PLE process, combined with an appropriate *in-cell* clean-up strategy, has been demonstrated to be a valuable analytical approach able to generate clean extracts ready for instrumental analysis *(7-11)*. However, in most of these studies, relatively large amounts of sample, solvents and sorbents have been used, although the improved detectability provided by most modern instrumental techniques finally resulted in the analysis of only a small fraction of the purified extract. According to this consideration, scaling down of the sample treatment process without affecting the accuracy of the determination should be possible. In the case of the PLE technique, such an alternative have been explored by using commercial system with smaller extraction cells (cell volume below 11 mL) or by using home-made miniaturised set-ups *(9, 12-13)*. None of these studies dealt with the analysis of feedstuffs, a matrix for which only a limited number of PLE application studies focussing on the determination of PCBs can be found in the literature *(10-11)*.

This paper describes a new miniaturised PLE-based method for fast simultaneous determination of PCBs and PBDEs in feed samples. Once optimised, the proposed method allowed an exhaustive extraction with simultaneous *in-cell* purification of feed samples with minimum sample manipulation and little solvent, sorbents and time consumption. The method, which was combined at-line with gas chromatography–micro electron capture detection (GC–μECD) and with gas chromatography coupled to ion trap detector working in tandem mode (GC–ITD(MS/MS) for PCB analysis and with gas chromatography–negative chemical ionization-mass spectrometry (GC–NCI-qMS) for PBDE determination, was tested for the analysis of environmentally relevant PCBs and PBDEs in real-life feed samples. These results were compared to those obtained when analysing the same matrices with an also miniaturised but more conventional sample preparation methodology previously validated for the analysis of fat-rich biological matrices *(14)*.

2. MATERIALS AND METHODS

2.1. Chemicals and samples

Acetone, dichloromethane (DCM) and iso-octane were of pestipur quality and were purchased from SDS (Peypin, France). *n*-Hexane was purchased from Merck (Darmstadt, Germany). Sulphuric acid was of pro-analysis quality (Merck). Anhydrous sodium sulphate was obtained from J.T. Baker (Deventer, The Netherlands) and Silica gel 60 from Merck.

The 23 PCB congeners studied (PCBs 28, 52, 77, 95, 101, 105, 114, 118, 123, 126, 132, 138, 149, 153, 156, 157, 167, 169, 170, 180, 183, 189 and 194) were selected because of their toxicity *(15)* and relative abundance in environmental samples *(16)*. A working stock solution was prepared from individual PCB standards (Dr. Ehrenstorfer, Augsburg, Germany) containing 1000 pg/μl of each compound in isooctane. This solution was used for further dilution and, when required, spiking of the samples. 1,2,3,4-Tetrachloronaphtalene (TCN, Dr. Ehrenstorfer) and PCB 209 were used as syringe standards for PCB determination by GC–μECD and added to the final extracts just before the chromatographic analysis to correct any possible injection and chromatographic fluctuation. Recovery labelled standards of the 13

most toxic PCB congeners ($^{13}C_{12}$-labelled PCBs 77, 101, 105, 114, 118, 126, 153, 156, 157, 167, 169, 180 and 189, Wellington Laboratories, Ontario, Canada) were added to the matrix solid phase dispersion (MSPD) mixture before any sample treatment in confirmatory experiments involving GC–ITD(MS/MS) *(17)*.

15 PBDEs including from three to ten bromo-substituted congeners (PBDEs 17, 28, 47, 66, 85, 99, 100, 153, 154, 183, 184, 191, 196, 197 and 209) were selected among those frequently detected in environment samples and considered in the present study *(2)*. A working stock solution was prepared from individual PBDE standards (Dr. Ehrenstorfer) containing 1000 pg/µl of each compound in isooctane and used for further dilution and, when required, spiking of the samples. ^{13}C-labelled PBDE 139 standard (Wellington, Ontario, Canada) was added to the purified extracts before GC–NCI-MS analysis as syringe standard.

The investigated samples included two (under development) vegetal-based feeds for aquiculture use (potato- and pea-based feedstuffs), three standard aquiculture feeds provided by a commercial company (Aquac. I, II and III), and a commercial feed for cold water fishes purchased in a supermarket in Madrid, Spain (Goldfish feed) (Table 1). All samples were conserved in a dried atmosphere and protected from light until analysis.

2.2. Selective PLE procedure

A previously validated method for miniaturised selective PLE of PCBs from fatty food matrices *(9)* has now been modified and adapted for the simultaneous determination of PCBs and PBDEs in aquiculture feed matrices. Variables affecting the efficiency of the PLE process (such as nature and temperature of the extraction solvent(-s), solvent(-s) volume and extraction time) and fat removal (amount of silica sorbents used for the dispersion of the sample and for final purification of the extracts) were re-evaluated and re-optimised. Once optimised, a typical experiment consisted on MSPD of a representative portion of the feed sample, ca. 1.0 g, on similar amounts of Na_2SO_4 and silica modified with 44% (ww) of sulphuric acid (SiO_2-HSO_4). After blending and homogenisation in a glass mortar, 0.750 g of the resulting homogeneous mixture, corresponding to 0.250 g of sample, was packed in a stainless steel extraction cell. Then, different layers of silica sorbents were also packed

in the cell to allow simultaneous clean-up of the PLE extracts (see Figure 1 for final sorbent disposition). The packed cell was installed in a miniaturised home-made PLE system *(18)* and *n*-hexane was pumped at 0.4 mL/min to fill the extraction cell and lines. After pressurisation at 10.5 MPa, a first static PLE was performed for 7 min at 50 ºC. Afterwards, the solvent was completely replaced by a mixture of *n*-hexane:DCM (1:1, v/v) and a second 7 min static PLE at 50 ºC was again carried out. Finally, some fresh solvent (i.e., *n*-hexane:DCM, 1:1, v/v) was flushed through the cell to ensure proper purging of the sample, the clean-up sorbents and the lines. The eluates from both PLE cycles were jointly collected, concentrated under a gentle nitrogen current and subjected to instrumental analysis by the corresponding technique. Procedural blanks were prepared following the same procedure as for feedstuffs but replacing the feed matrix by bare sand prewashed with the extraction solvents. No background interferences was found to be introduced by the methodology proposed.

The performance of the proposed selective PLE method was evaluated by analysing the PCB and PBDE contents in a real non-spiked feed sample and by subsequent comparison of these results with those obtained when using an already validated more conventional analytical procedure based on MSPD of the sample and subsequent fat removal described elsewhere *(14)*. Otherwise specified, all experiments were carried out in triplicate.

2.3. Intrumental Analysis

Determination of the selected PCBs in the purified extracts was performed by GC–µECD (HP 6890 Series, Hewlett-Packard, Palo Alto, CA, USA). Samples were injected in the hot splitless mode (1 µL, 270 ºC, splitless time 1.0 min) in a capillary BPX-5 column (60 m × 0.25 mm i.d.× 0.25 µm film thickness) purchased from SGE (Melbourne, Australia). The column temperature was programmed from 80 ºC (2 min) to 250 ºC (50 min) at a rate of 30 ºC/min and then to 270 ºC (10 min) at 5 ºC/min. Nitrogen was used as carrier gas (constant flow, 1.5 mL/min) and as make-up gas (30 mL/min). The detector temperature was set at 300 ºC. Confirmation of the investigated PCB congeners was carried out using a GC (CP-3800, Varian, CA, USA) equipped with an ion trap MS detector (Saturn 2000, Varian) working in the MS/MS

mode, GC–ITD(MS/MS) under the experimental conditions described elsewhere *(17)*.

PBDE determination was performed by GC–NCI-qMS (HP 6890 Series, Hewlett-Packard, Palo Alto, CA). The source and transfer lines temperatures were set at 150 ºC and 300 ºC, respectively. Chromatographic conditions were carefully optimised to avoid PBDE 209 degradation during analysis *(19)*. Samples were injected in the hot splitless mode (1 µl, 270 ºC, pulsed splitless time 4.0 min, P pulse, 5 psi) in a capillary DB-5 column (15 m × 0.20 mm i.d × 0.20 µm film thickness) purchased from J&W Scientific (USA). The column temperature was programmed from 120 ºC (4.2 min) to 200 ºC at 30 ºC/min, heated to 275 ºC at 5 ºC/min, then to 300 ºC (10 min) at 40 ºC/min and finally to 310 ºC (2 min) at 10 ºC/min. Helium was used as carrier gas (constant flow, 1.5 mL/min).

3. Results and discussion

3.1. Characterization of the samples

Samples were characterized in terms of lipid and humidity content. Humidity was gravimetrically calculated by heating the samples during 48 h at 105 ºC until constant weight. The lipid content was determined by extracting the fat content of 0.5 g of the selected sample with 10 mL of acetone:*n*-hexane (1:1; v/v). The resulting fat extract was evaporated under a gentle nitrogen stream until eliminating the organic solvent and the lipid content was then gravimetrically determinate. Results of these experiments are summarised in Table 1.

The potato-based feed sample was used for method development and validation because of its intermediate fat and humidity contents in comparison to the other feedstuffs investigated.

Table 1: Lipid and humidity content (%, weight base) in the investigated feed matrices.

Matrix	Potato-based	Pea-based	Goldfish feed	Aquac. I	Aquac. II	Aquac. III
Lipid content	20	20	5	28	26	26
Humidity	4	3	5	6	4	6

3.2. Optimisation of the PLE procedure

On the case of previously reported results using the miniaturised PLE system used in the present study *(9)*, the goal of this part of the study was to determine the appropriate cell size to ensure easy and reproducible packing of the sample plus purification sorbents as well as proper analyte determination. After several experiments, a 4.75-mL extraction cell was selected for subsequent studies as it allowed the packing of 0.75 mg of the MSPD, (i.e. 0.25 g of sample) and 2 g of acidic silica (SiO_2-HSO_4) between two layers of 0.5 g of neutral silica just above the previous MSPD layer for complete *in-cell* fat removal (Fig. 1).

One of the main experimental parameters affecting the efficiency of the PLE process is the solvent nature and this was, consequently, the first variable studied. *n*-Hexane has been demonstrated to provide quantitative recoveries during PLE of PCBs from fat-rich matrices *(8-9)*. However, this solvent can easily result in incomplete recovery of the high brominated PBDE congeners *(2)*. The use of more selective solvents, such as DCM or mixtures containing this solvent, becomes especially advisable when the goal is to ensure the quantitative displacement of PBDEs from highly sorptive matrices with minimum solvent consumption *(2, 8, 11)*. In the present study, a sequential extraction of the target analytes with two solvents, *n*-hexane and a mixture of *n*-hexane:DCM (1:1; v/v), was proposed. Thereby, the method consisted of two consecutive static PLE cycles with complete solvent removal among them followed by a dynamic step using n-hexane:DCM to ensure complete solvent removal from the cell and the lines. The solvent flow was set as 0.4 mL/min, the extraction temperature at 50 ºC and the extraction pressure at 10.5 MPa *(9)*. The eluents from the cells were separately collected to determine the efficiency for each extraction cycle. In a typical experiment, *n*-hexane was initially pumped for 8.5 min to fill the extraction cell and lines. Then, the upper valve was closed to pressurise the system. After a 7-min static PLE, the solvent was completely replaced by *n*-hexane:DCM and the eluent from the cell (approx. 3 mL) collected in a vial. A new 7-min static extraction was carried out under similar experimental conditions.

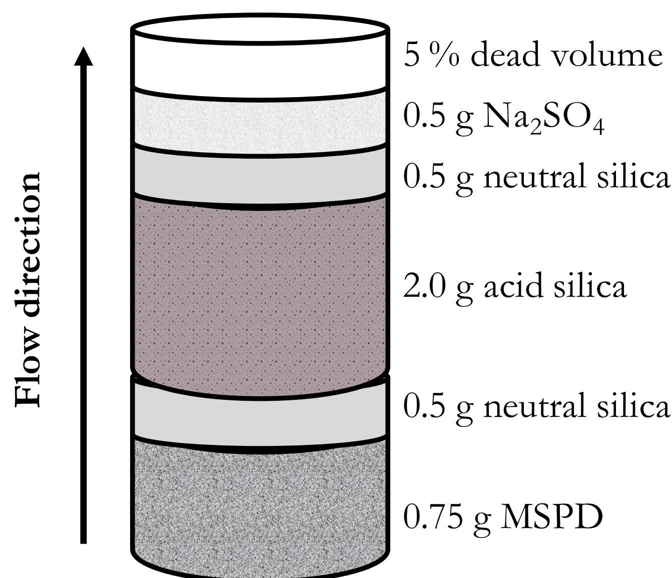

Fig. 1: Final arrangement of the PLE extraction cell.

For optimisation purposes, a third static PLE using *n*-hexane:DCM was carried out. The three ready-to-analyse extracts were separately collected, concentrated under a gentle nitrogen stream and reconstituted in the corresponding syringe standards (20 μL of TCN and PCB # 209 in isooctane) and analysed by GC–μECD. As a typical example of the results obtained in this part of the study, Figure 2 summarizes the concentrations found in the three collected fractions for some selected PCBs. Most of the investigated PCBs were quantitatively eluted in the first fraction (> 70 % of the total PCB content). Nevertheless, a significant fraction of the analytes was found to elute in the second fraction. This was especially the case for high chlorinated PCBs, e.g. see PCBs 153, 167 and 180. The appropriate divergent behaviour observed for PCB 95 looked to be due to coelution of the target analyte with an interference. Amounts detected in the third fraction accounted for less than 10 % of the total extracted concentration for a large majority of the investigated congeners. Therefore, according to these considerations, the final extraction conditions were set as follows: two 7-min static PLE cycles with *n*-hexane and *n*-hexane:DCM, respectively, followed by a dynamic extraction step of 10 min with *n*-hexane:DCM.

The extraction pressure was not separately optimised in the present study as it has been demonstrated not to affect the PLE efficiency as far as it was high enough to keep the extraction solvent as a liquid during the extraction step *(20)*. A extraction pressure of 10.5 MPa was found to fulfil this requirement at the relatively soft

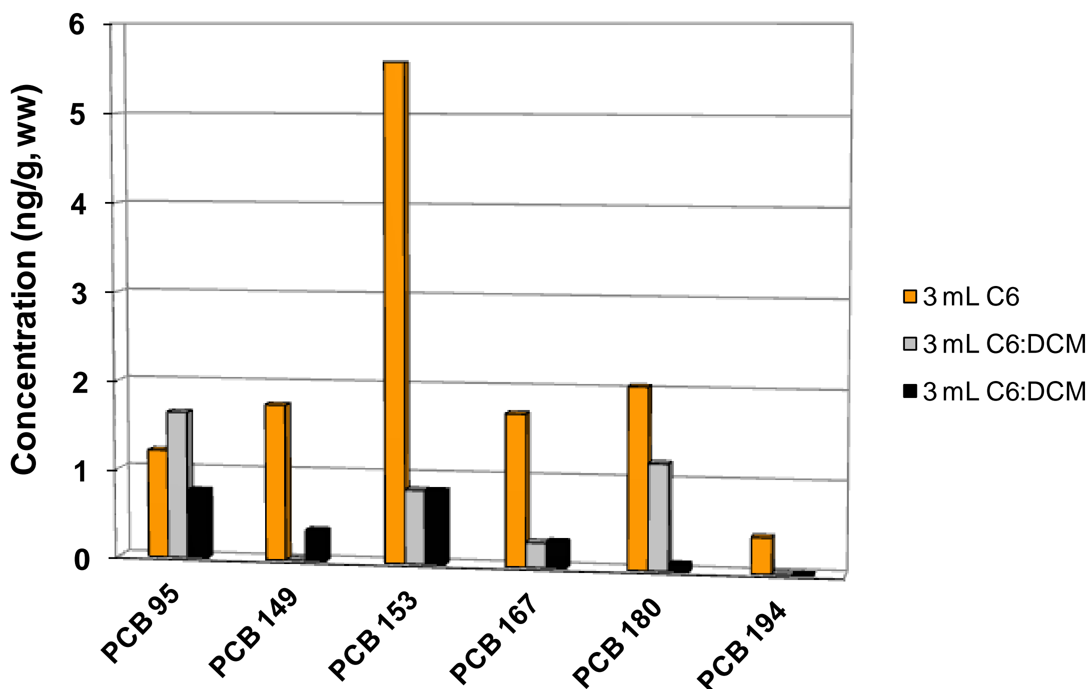

Fig. 2: Influence of the nature and volume of the extraction solvent in the PLE efficiency for selected PCB congeners. PCB concentrations (ng/g, ww) in the different eluted fractions. C6: *n*-hexane.

temperature range used in this study and to allow the easy control with the Valco® valves used in the PLE system.

A new set of experiments was then carried out to evaluate the influence of the extraction temperature in the PLE efficiency. Independent experiments were carried out at three test extraction temperatures of 50, 70 and 100 ºC using the extraction protocol previously described, but, in this case the resulting purified extracts were jointly collected. After concentration under a gentle nitrogen stream, the extracts were re-constituted in the corresponding syringe standards and analysed by GC–µECD for PCB quantification. In general, essentially similar endogenous PCB levels were found in the collected extracts at the three investigated temperatures. The apparently higher recoveries achieved for some low chlorinated congeners at high temperatures of 70 and 100 ºC seemed to be actually associated to coelution with other matrix components that were increasingly eluted as the extraction temperature raised, and that were not completely eliminated during the *in-cell* purification treatments. This finding, which is clearly illustrated in Figure 3 by the observed rise of the background baseline in the first part of the chromatogram, agreed with previous observations in

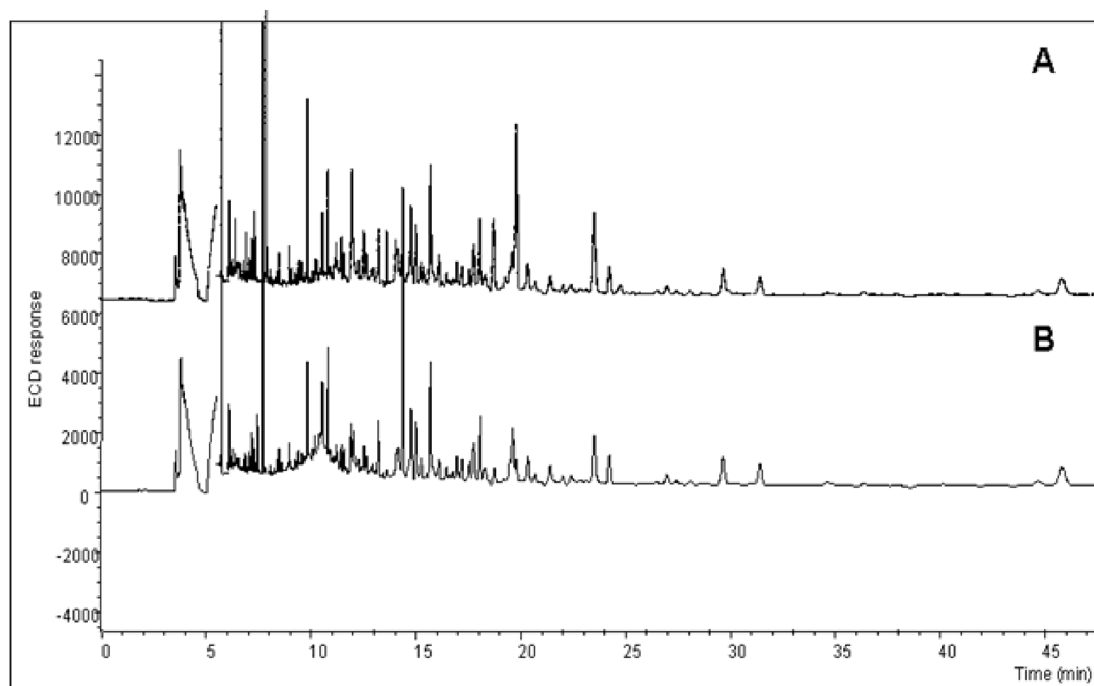

Fig. 3. Influence of the extraction temperature in the PLE efficiency. PLE was performed at (A) 50 ºC and (B) 70 ºC.

studies dealing with the selective PLE of micropollutants from complex fat-containing samples *(9)*, and compromised the accurate determination of the some more volatile congeners investigated. According to these considerations, an extraction temperature of 50 ºC, which provided satisfactory recoveries of the investigated compounds with improved selectivity and minimized the sample manipulation by avoiding the need for further purification of the PLE extracts, was selected for subsequent experiments.

Considering the chemical similarity existing among the two families of pollutants investigated, PCBs and PBDEs, and the modification already introduced in the extraction procedure to enhance the selectivity of the extraction solvent for PBDEs, *(2, 7-8, 21)*, no extra re-optimisation was carried out in the proposed PLE methodology, which was directly applied to the simultaneous determination of PCBs and PBDEs in the test feed.

3.3. Method validation and application

The analytical performance of the proposed miniaturised PLE procedure for the determination of the two families of target compounds was initially evaluated by

analysing the test feedstuffs at two spiking levels (0.4 and 4 ng/g ww) of each PCB and PBDE. Realistic spiking levels were selected to evaluate the feasibility of the proposed PLE-based procedure for the analysis of contaminated feedstuffs without any further modification. The spiking levels were set on the base of the European MRLs for PCBs in feed samples *(22)*. Standards were added on the top of the sample in the extraction cell, which corresponds to the bottom of the chromatographic column packed in the extraction cell as positioned in the PLE *(9, 23)*. Two purified extracts were obtained per experiment and collected in the same vial for subsequent GC–μECD (for PCBs) and GC–NCI-qMS (for PBDEs) analysis. Relevant analytical data are summarized in Table 2 and 3.

Recoveries in the 61-123 % range were obtained for most of the target PCBs at the low investigated fortification level (mean value, 87 % with relative standard deviations, RSDs, in all instances below 18 %). Only PCB 126 and 183 showed recoveries out of this range with values of 47 and 130 %, respectively, although with still satisfactory repeatibilities (RSDs of 12 and 2 %, respectively). Essentially similar results were obtained at the higher assayed spiking level of 4 ng/g: recoveries in the 60-121 % range (mean value, 85 %) and RSDs lower than 20 % except for PCB 114 (recovery, 58 %; RSD, 12 %) and PCB 157 (recovery, 31 %; RSD, 2 %). The un-expected low recovery obtained for PCB 157 was due to coelution with an interference from the silica used for *in-cell* clean-up of the extracts that was not possible to eliminate by further off-line purification of the extracts. In general, the obtained recoveries were similar to or slightly higher than those found in close related studies dealing with the determination of PCBs in feedstuffs by using either selective PLE *(10-11)* or conventional multistep analytical sample treatment protocols *(24-25)*. Results were also similar to those reported for methods involving miniaturised selective PLE of PCBs from food and biota samples *(9)* or large scale commercially available PLE systems *(7-8)*. In addition, the low limits of detections (LODs), in the range 0.01-0.2 ng/g ww, as calculated for the real-life non-spiked reference feed with the exception of PCBs 28 and 52, for which substantially higher values were obtained

Table 2. Recoveries (%) and RSDs (%) as calculated for the investigated PCBs at the two investigated spiking levels of 0.4 and 4 ng/g ww (n=3), and limits of detection (LODs) as calculated for real-life samples (ng/g ww) with GC–µECD.

PCB No	**LOD (ng/g)**	**Recovery (RSD)**		
		Sp. level (ng/g)	**0.4**	**4**
28	0.1		67 (8)	72 (13)
52	0.2		99 (15)	65 (15)
95	0.05		92 (5)	60 (10)
101	0.03		97 (7)	61 (19)
77	NA[a]		75 (6)	68 (20)
149	0.04		110 (4)	62 (17)
123	0.01		61 (15)	96 (6)
118	0.05		86 (6)	82 (8)
114	0.07		123 (18)	58 (13)
153	0.06		118 (3)	72 (8)
132	0.02		75 (18)	83 (7)
105	0.01		88 (13)	99 (9)
138	0.03		103 (1)	85 (10)
126	NA		47 (12)	78 (13)
183	0.02		130 (2)	88 (9)
167	0.04		80 (14)	85 (10)
156	0.01		71 (13)	92 (8)
157	0.02		71 (15)	31 (11)
180	0.01		87 (7)	121 (11)
169	NA		68 (9)	110 (9)
170	0.03		91 (11)	99 (7)
189	0.01		81 (11)	112 (8)
194	0.03		90 (3)	94 (10)

[a] NA, not analysed.

(0.1 and 0.2 ng/g, respectively), proved the practicability of the method for the determination of the investigated PCB congeners even if only 0.25 g of the sample were used for the determination and a GC–µECD was selected for final determination.

Satisfactory results were also obtained for PBDEs, for which even better recoveries were obtained due to the high selectivity of the technique used for final separation-plus-detection; i.e. GC–NCI-qMS. In this case, recoveries ranging from 90 to 114 % (mean value, 103 %) and RSDs below 20 % were obtained at the lower spiking level of 0.4 ng/g. Values out of this range were only obtained for PBDE 209 (recovery, 145 %; RSD, 20 %). However, satisfactory results were obtained for all

Table 3. Recoveries (%) (RSDs, %) as calculated for the studied PBDE congeners at the two investigated fortified levels, 0.4 and 4 ng/g, ww (n=3), and LODs as determined for real-life samples (ng/g, ww) with GC–NCI(MS/MS).

PBDE No	LOD (ng/g)	Recovery (RSD)		
		Sp. level (ng/g)	0.4	4
17	0.02		98 (13)	107 (5)
28	0.01		98 (12)	114 (6)
47	0.03		110 (11)	111 (9)
66	0.02		95 (7)	109 (8)
100	0.03		114 (17)	102 (9)
99	0.02		107 (13)	104 (11)
85	0.002		90 (8)	99 (15)
154	0.006		92 (20)	100 (13)
153	0.006		95 (12)	93 (17)
184	0.002		105 (13)	90 (16)
183	0.007		96 (9)	92 (15)
191	0.01		97 (6)	90 (15)
197	0.005		103 (7)	93 (13)
196	0.009		103 (9)	86 (18)
209	0.04		145 (20)	100 (27)

target PBDEs at the higher spiking level assayed, with recoveries in the 86-104 % range (mean value, 99 %) and RSDs lower than 18 % (except for PBDE 209 for which a RSD of 27 % was obtained). These recovery values were slightly better than those reported in other studies dealing with the analysis of PBDEs in feedstuffs by using conventional room temperature sample preparation methodologies, such as MSPD *(25-26)*, and are in the range of those obtained when using enhanced extraction techniques, such as supercritical fluid extraction method *(27)*, a result that probably indicates that convenience of using enhanced extraction techniques for the effective extraction of these microcontaminants from highly sorptive techniques, such as feedstuffs. Our results were also in the range of those obtained by other authors using selective PLE-based approaches for PBDE determination in food matrices *(5, 7-8, 21)* and miniaturised MSPD with on-line clean up for the analysis of these analytes in biota *(28)*. Again, the experimentally determined LODs as calculated for a real-life matrix (the reference feedstuff) were low enough (0.002-0.04 ng/g wet weight, ww) to guaranty accurate determination of the target analytes at the low levels typically found in real samples even though as a small sample size as 0.25 g was used.

Final validation of the developed miniaturised selective PLE method was carried out by comparison among the endogenous PCB concentration calculated for the reference feed by using this procedure and those obtained with a more conventional procedure previously validated in our working group for the analysis of PCBs in fatty matrices *(14)*. This reference method combined the use of MSPD with on-line purification of the extracts by packing appropriated sorbents at the bottom of the extraction column. Because of the intended comparison between both methodologies, the experimental conditions were kept as similar as possible to those proposed for selective PLE. Thereby, 0.75 g of a MSPD mixture containing the feed sample, Na_2SO_4 and acid silica (1:1:1 on a weight base, w:w:w) was packed in a glass column above 2 g of acidic silica packed between two layers of 0.5 g of neutral silica each. A thin layer of anhydrous Na_2SO_4 was packed on the top of the column. Once prepared, columns were placed in a vacuum SPE-12G system and extracted using a two-step protocol involving two 7-min static sequential extractions with *n*-hexane and *n*-hexane:DCM (1:1, v/v) at ca. 0.4 mL/min. Again, extracts were jointly collected, concentrated, re-constituted in the corresponding syringe standards and directly subjected to GC–μECD analysis. Figure 4 compares the concentrations found for selected PCB congeners with both methodologies. Non-unexpectedly, the higher pressure and temperatures applied in the PLE procedure resulted in an enhanced extraction efficiency as compared to results obtained under environmental conditions, a result that agreed with previous observations *(9, 29)*, and that becomes especially evident when dealing with the analysis of highly sorptive matrices, such as feedstuffs. On the other hand, the use of enhanced extraction conditions also uses to result in co-extractions of other matrix components that can interfere in the final determination of the target analytes. In this study that was the case of PCBs 28, 52, 101, 114 and 157 when using ECD as detector. In such cases, the use of more selective detectors, such as ITD(MS/MS), becomes highly advisable. Thereby, a new set of experiments were carried out. In these experiments, the MSPD mixture was spiked with a ^{13}C-labelled PCB standard solution containing the 13 most toxic PCB congeners before any treatment. Samples were extracted and simultaneously purified using the proposed selective PLE method and the collected extracts were directly

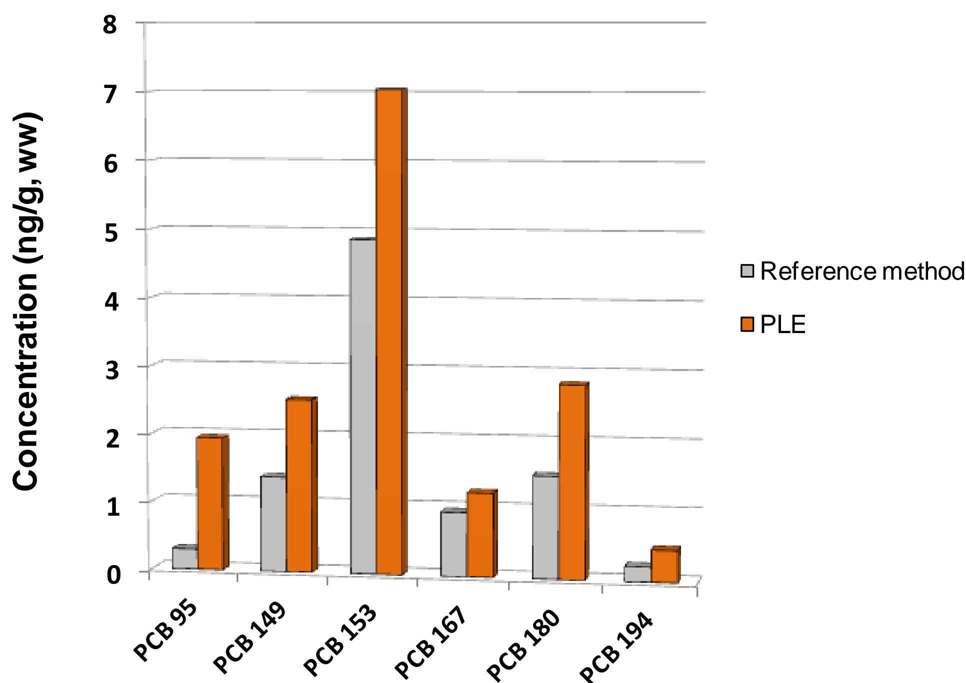

Fig. 4. Comparison of the endogenous PCB concentrations (ng/g; ww) detected in the reference feed with the optimised selective PLE method and a previously validated procedure.

analysed by GC–ITD(MS/MS). Under experimental conditions used for these instrumental determinations *(17)*, the previously mentioned coelutions affecting the determination of early eluting prioritary and toxic congeners were solved. The improved selectivity offered by this technique also made possible accurate determination of the three *non-ortho*-substituted PCB congeners 77, 126 and 169. Interestingly, although PCBs 126 and 169 were found at levels below the instrumental LOD (< 0.01 ng/g), PCB 77 was detected at 0.345 ng/g ww (RSDs, 4 %). These results contributed to demonstrate the efficiency of the selective PLE method developed for the quantitative extraction and simultaneous *in-cell* purification of PCBs and PBDEs from complex and highly sorptive matrices, such as feedstuffs and, so, the validity of the proposed methodology as a valuable analytical alternative to conventional multistep procedures involving larger amount of sample and reagents and longer analytical times.

Finally, as an extra illustration of the feasibility of the complete analytical methodology for the intended determination, the proposed miniaturised PLE method was applied to the analysis of selected environmentally relevant PCBs and PBDEs in

different feed matrices. Samples were spiked with the corresponding ^{13}C-labelled PCB standards, prepared as previously described, and extracted according to the PLE-based methodology developed. The purified extracts were sequentially analysed by GC–ITD(MS/MS) and GC–NCI(MS/MS) for PCB and PBDE determination, respectively. Table 4 summarizes the concentrations detected for the two investigated families of pollutants in different real-life feedstuffs. In general, the commercial feed showed the lowest PCB concentrations while not significant differences in the total PCB content could be observed between the alternative (vegetal-based) feeds and the conventional (standard aquiculture) feeds. Total toxic equivalents of 2,3,7,8-tetrachloro-*p*-dioxin (TEQs) were calculated according to the European Directive 2006/13/EC which sets 7.0 ng TEQs/feed kg (once standardised to a 12% of humidity and calculated for the sum of dioxins, furans and *dioxin-like* PCBs) as maximum residue level (MRL) allowed in animal feedings *(22)*. All studied feedstuffs showed PCBs TEQ levels two- to three-folds lower than the official MRL set for feed matrices (Table 4).

Concerning PBDEs, the vegetal-based feed showed lower contents than the standard aquiculture feedstuffs although in both cases many of the investigated congeners were found at level below their corresponding LOD. Anyway, the total PBDE concentrations calculated for these samples were in the range of those reported in similar studies involving selective PLE of PBDEs *(8, 21)*. As compared to these previous studies, probably the most noticeable difference was the relatively high concentration found in the present work for the PBDE 209 in the three standard aquiculture feeds in which this congener contributed for around 50% of the total PBDE amount (a finding that however agreed with the results reported in *(26)*). Although at present, there is no regulation regarding MRLs of PBDEs in feedstuffs, the levels found in the investigated feeds were low enough to consider that these compounds did not pose any real health problem. In general, PCB and PBDE concentrations detected in this study were in the range of those found in similar studies for fatty feed matrices *(10, 25-27, 30)*. Berntssen et al. described significant differences between conventional and alternative feeds, where fish oil and meal were substituted by plant ingredients and krill, and where the burden of PBDEs and PCBs

Table 4: Endogenous PCB and PBDEs concentrations (ng/g, ww) for the different feedstuffs investigated after applying the optimised PLE with subsequently GC─ITD(MS/MS) and GC─NCI-qMS analysis, respectively. Total toxic equivalent of PCBs (TEQ_{PCBs}) as calculated for each feedstuff (ng TEF PCB WHO/kg).

	Concentration (ng/g, ww)					
	Conventional			**Alternative**		**Commercial**
	Aquac. I	**Aquac. II**	**Aquac. III**	**Potato-based**	**Pea-based**	**Goldfish feed**
PCBs						
CB 28	28.66	38.24	35.78	0.394	30.95	8.34
CB 52	5.74	3.30	21.40	3.53	4.94	<0.2
CB 95	7.11	2.75	9.88	1.94	17.55	6.05
CB 101	3.42	<0.3	3.78	2.21	3.69	<0.3
CB 77	0.614	0.573	0.843	0.345	0.473	
CB 149	3.65	0.35	4.03	2.53	2.63	0.96
CB 123	0.50	0.58	0.35	<0.01	<0.01	<0.01
CB 118	2.79	0.36	2.96	2.13	1.33	0.59
CB 114	<0.1	<0.1	<0.1	0.144	<0.1	<0.1
CB 153	14.33	0.92	10.48	7.04	6.19	2.19
CB 132	2.58	0.57	1.75	1.07	1.60	0.75
CB 105	0.06	<0.01	0.28	0.18	0.27	<0.01
CB 138	5.77	1.42	4.77	2.41	3.96	1.26
CB 126	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
CB 183	0.71	<0.02	0.42	0.54	0.03	<0.02
CB 167	0.74	<0.04	0.83	0.08	<0.04	<0.04
CB 156	<0.01	<0.01	<0.01	0.17	<0.01	<0.01
CB 157	<0.06	<0.06	<0.06	0.575	<0.06	<0.06
CB 180	7.07	0.04	2.22	2.82	1.75	0.28
CB 169	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
CB 170	1.91	0.25	0.54	1.14	0.83	0.30
CB 189	0.16	0.29	0.15	<0.01	<0.01	<0.01
CB 194	0.98	0.47	0.23	0.47	0.39	<0.03
∑PCBs	86.18	49.54	99.85	29.72	76.11	20.72
TEQ_{PCBs}	**$7.45 \cdot 10^{-2}$**	**$5.88 \cdot 10^{-2}$**	**$9.72 \cdot 10^{-2}$**	**$4.68 \cdot 10^{-2}$**	**$5.07 \cdot 10^{-2}$**	**$5.09 \cdot 10^{-3}$**
PBDEs						
BDE 17	<0.02	<0.02	<0.02	0.03	<0.02	<0.02
BDE 28	<0.01	<0.01	<0.01	<0.01	0.02	<0.01
BDE 47	0.37	0.82	0.44	0.46	0.25	0.14
BDE 66	<0.02	<0.02	<0.02	0.03	0.02	<0.02
BDE 100	<0.03	0.11	<0.03	0.10	0.07	0.03
BDE 99	0.06	0.14	0.10	0.09	0.04	0.02
BDE 85	<0.002	<0.002	<0.002	<0.002	0.009	<0.002
BDE 154	0.014	0.025	0.210	0.10	0.016	<0.006
BDE 153	<0.006	<0.006	<0.006	<0.006	0.018	<0.006
BDE 184	<0.002	<0.006	<0.006	<0.002	<0.002	<0.002
BDE 183	<0.007	<0.007	<0.007	<0.007	0.007	<0.007
BDE 191	<0.01	0.21	<0.01	<0.01	<0.01	<0.01
BDE 197	<0.005	0.152	<0.005	<0.005	<0.005	<0.005
BDE 196	0.18	<0.01	<0.01	<0.01	<0.01	<0.01
BDE 209	1.20	1.87	1.09	0.52	0.06	0.08
∑PBDEs	**1.88**	**3.368**	**1.903**	**1.356**	**0.534**	**0.319**

could be reduced up to 65 % *(30)*. Even if lower PBDE concentrations have been found in the present study for the alternative vegetal-based feeds, a similar conclusion cannot be withdrawn here for the under-development feedstuffs, and possibly due to the feed base ingredients.

Conclusions

A new miniaturised PLE with *in-cell* purification method has been developed for the simultaneous analysis of PCBs and PBDEs in feed matrices of different nature. The proposed methodology allowed quantitative recoveries of the selected PCBs and PBDEs from a model feed matrix and accurate determination of the target compounds even if only 0.25 g of the sample were used. Interestingly, sample size miniaturisation resulted in significant reduction of the amount of the solvents and sorbents required for sample preparation. Thereby, sample treatment was completed with only 8 mL of the organic solvents and 3.5 g of sorbents and 45 min sufficed to obtain ready-to-analyse extracts. The application of the developed method to the analysis of several conventional and alternative vegetal-based feeds contributed to further demonstrate the validity of the approach for the intended determination of PCBs and PBDEs in fat-rich matrices.

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3.1.3. EXTRACCIÓN CON LÍQUIDOS PRESURIZADOS MINIATURIZADA DE BIFENILOS POLICLORADOS EN SEDIMENTOS [1]

Abstract

In this study, miniaturised selective pressurised liquid extraction (s-PLE) has been used for the analysis of prioritary and toxic polychlorinated biphenyls (PCBs) in sediments. The validated methodology consisted of two 10-min static PLE cycles followed by a dynamic flush of the extraction cell and the lines with *n*-hexane:dichloromethane (1:1, v/v) at 100 ºC and 10.5 MPa. Under finally proposed experimental conditions, sample preparation is accomplished in 45 min, in a single step, with minimal sample manipulation, and with minimum sample (0.5 g), solvent (20 mL) and sorbent (5 g) consumption. The collected purified extracts were analysed by gas chromatography coupled to either micro-electron capture detector (GC–µECD) or ion trap detector working in the tandem mass spectrometry mode, GC–ITD(MS/MS), and results obtained in both sets of analyses compared. The proposed analytical methodology showed suitable dynamic linear responses in the test range of 0.5-10 ng/g dried weight (in general, correlation coefficients higher than 0.98) and satisfactory average recoveries (in the range 90-106 % for GC–µECD and 84-102 % for GC–ITD(MS/MS)) and reproducibilities (relative standard deviations below 15 % for most of the target compounds). The feasibility of the proposed methodology for accurate analyte determination (in the case of GC–ITD(MS/MS) or fast screening (in the case of GC–µECD) was demonstrated by the analysis of environmentally relevant PCBs in the certified reference freshwater harbor sediment BCR-535.

Keywords: Selective pressurised liquid extraction, polychlorinated biphenyls, sediment analysis

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[1] M. Pena-Abaurrea, M.J. González, L. Ramos. Miniaturised pressurised liquid extraction of polychlorinated biphenyls from sediments. *In preparation*

1. INTRODUCTION

Polychlorinated biphenyls (PCBs) are well-known worldwide distributed microcontaminants. Despite the decades of PCB use and production, their hydrophobicity and high physicochemical stability make possible to find even nowadays measurable levels of these compounds in all types of abiotic and biotic matrices *(1-4)* and the monitoring of their residual environmental levels still mandatory. From this point of view, sediments are considered an important source of information regarding the distribution of pollution in the aquatic ecosystems, where they act as a sink for non-polar analytes.

Conventional analytical methodologies in use for PCB analysis in complex environmental abiotic (semi-)solid matrices, such as sediments, are typically laborious and expensive in terms of time, sorbent and solvent consumption due to the several (usually manual) analytical steps required for exhaustive extraction of the analytes, separation from co-extracted matrix components and, when necessary, for further isolation of PCBs from other chemically-related compounds usually present in these samples *(5-6)*. The low levels at which PCBs are typically detected in these types of matrices, especially when sampled from pristine areas, added an extra difficulty to the analysis.

Pressurised liquid extraction (PLE) is a relatively novel, but well-established and accepted, exhaustive and relatively matrix-independent extraction technique that has already demonstrated its feasibility for the analysis of a number of micropollutants and minor compounds in a variety of (semi-)solid abiotic matrices, including sediments and soils *(7-9)*. When analysing such as complex samples, the high temperatures and pressures used during the PLE process improve solvent penetration through the matrix pores and so enhance the extraction efficiency, which can then be accomplished in shorter times as compared to other more conventional extraction techniques. In addition, when combined with a suitable *in-cell* purification strategy, ready-to-analyse extracts can be obtained. In these cases, PLE is so-called selective PLE (s-PLE) and the analyte extraction and purification is performed in a single (simultaneous) step and the collected extracts are frequently subjected to final instrumental analysis without any further treatment but concentration *(9-12)*.

Miniaturisation is a clear trend in many application areas, including the environmental analytical chemistry *(13)*. The many efforts carried out in this research field in the last decades have resulted, in the case of sample preparation, in the development of a number of extraction and preconcentration techniques that have resulted especially advantageous for the (often unattended) treatment of liquid and viscous matrices with minimum sample and reagents consumption. The development of equivalent approaches for the treatment of semi-solid and solid samples has been, from far, much more limited, due, in most instances, to the inherent difficulty of the extraction step for such as sorptive matrices and the general lack of appropriate (devoted) instrumentation. Thereby, for PLE, the smallest extraction cell available in commercial instruments is 11-mL, which in most instances have resulted in the use of amounts of solvents and sorbents in the range of those required for the treatment of conventional size-samples, even if as an small amount of sample as a few mg was used (*(14)* and references therein) (Dionex® has recently introduced in the market extraction cells of 1- and 5-mL). Up to now, this problem has been solved by the design and setting-up of home-made miniaturised PLE systems in which extraction cells with a more suitable size were inserted. The suitability of the approach for accurate determination of trace contaminants in complex matrices have been demonstrated in a number of representative examples dealing with the analysis of both biotic *(15-16)* and abiotic *(17-19)* matrices and typically involving 50-500 mg of sample and solvent consumptions of a few μL-mL.

In this study, a previously reported (conventional size) s-PLE-based methodology developed for the analysis of environmentally relevant polybrominated diphenyl ethers (PBDEs) in sediments *(10)* has been scaled down and re-optimised for the miniaturised s-PLE determination of relevant and toxic PCB congeners in sediments. The original 22-mL extraction cell used in the original procedure has been replaced by a size-adapted stainless-steel cell of 4.75 mL which allowed a better use of the inner cell volume, prevented from the use of inert materials to fill the void space and so contributing to an optimal solvent utilization, reducing the cost per analysis and waste generation, and greening the sample preparation process. The performance of the novel approach, which was combined at-line with gas chromatography coupled to either micro-electron capture detection (GC–μECD) for method development or

with an ion trap detector working in the tandem mass spectrometry mode (GC–ITD(MS/MS)) for final confirmation, was tested for the determination of prioritary and toxic PCBs in a non-spiked sediment from a pristine area. Final validation was carried out by evaluation of the levels of the target compounds in a certified harbor sediment (BCR-535).

2. Materials and methods

2.1. Chemical reagents

All solvents used in the analysis were of pestipur quality. Dichloromethane and isooctane were purchased from SDS (Peypin, France); *n*-hexane and acetone were from Merck (Darmstadt, Germany). Alumina 60 (0.063-0.20 mm) and power Cu (< 63 μm) were obtained from Merck, and anhydrous sodium sulfate from J.T. Baker (Deventer, The Netherlands). Alumina and sodium sulphate were prewashed before use with methanol and dichloromethane (puriss for analysis quality). Alumina was activated by heating at 130 ºC for 12 h before using. Cupper was also activated before use by washing with HCl 1M (Merck), Milli–Q water (purified in a Milli-Q system, Millipore, Bedford, MA, USA) and acetone.

The 23 PCB congeners studied (see Table 1 below) were selected because of their toxicity and relative abundance in environmental samples *(20)*. A working stock solution was prepared from individual PCB standards (Dr. Ehrenstorfer, Augsburg, Germany) containing 1000 pg/μl of each compound in isooctane. This solution was used for further dilution and, when required, spiking of the samples. Standard solutions were conserved in the fridge at 5 ºC until use. 1,2,3,4-Tetrachloronaphtalene (TCN, Ehrenstorfer) and PCB 209 were used as syringe standards and added to the final extracts just before PCB determination by GC–μECD. ^{13}C-Labelled standards of the 13 most toxic PCB congeners were added to the sediment samples before treatment when using GC–ITD(MS/MS) for final determination.

2.2. Samples

A sediment from a non-contaminated area, the Carlos Anwandter Sanctuary (Valdivia, Chile), was used for method development. Once collected, this test sample

was dried by heating at 100 ºC until constant weight, and conserved in a glass vessel, at room temperature in the dark, until analysis.

A certified freshwater harbor sediment (BCR-536, IRMM, Geel, Belgium) was used for method validation. This certified reference material was stored at 18 ºC until analysis. No water elimination process was required before treatment.

2.3. Miniaturised s-PLE

In the present study, a s-PLE method previously optimised for the determination of PBDEs in sediments *(10)* has been scaled-down and adapted for the analysis of PCBs. After optimisation, a typical experiment consisted of the dispersion of 1 g of the test sediment accurately weighed on a mixture containing 2 g of activated Alumina plus 2 g of power copper (weight ratio in the final mixture, 1:2:2, w:w:w) until a dried homogeneous mixture was obtained. Then, 2.5 g of the resulting mixture (corresponding to 500 mg of the test sample) were packed in the home-made stainless-steel extraction cell (75 mm × 9.0 mm i.d. × 10.2 mm o.d.) on top of a layer of anhydrous sodium sulphate. Then, 3 g of Alumina and another thin layer of sodium sulphate were packed on top of the matrix solid-phase dispersion (MSPD) mixture. This sample and clean-up sorbents disposition allow to fill ca. 95 % of the total extraction cell volume. The cell was then installed in the miniaturised home-made PLE instrument *(15, 21)* for sample treatment. s-PLE was performed by two 10-min static extraction cycles with *n*-hexane:dichloromethane (1:1, v/v) at 10.5 MPa and 100 ºC, which were followed by two dynamic steps allowing complete solvent removal. In all instances, the extraction solvent was pumped at a constant flow of 0.5 mL/min. Eluates from both s-PLE cycles were jointly collected (ca. 20 mL), concentrated under a gentle nitrogen stream and reconstituted in 20 µL of the corresponding syringe standards and subjected to instrumental analysis without any further treatment.

The suitability of the proposed s-PLE method for PCB extraction was preliminary evaluated by analysing the test sample spiked at four levels in the range 0.5-10 ng/g dried weigth (dw), corresponding to PCB concentrations typically found in real-life sediments. Recovery and repeatability of the optimised methodology were also evaluated at these spiking levels. In all instances, samples were spiked before any

analytical treatment by adding the proper amount of PCBs dissolved in *n*-hexane to the sample and by allowing to stand the homogenised mixture until complete solvent evaporation. Definitive evaluation of the PLE feasibility for PCB extraction was carried out by determining the target compounds in a non-spiked sample and by analysing a certified harbor sediment (BCR-535). Otherwise specified, experiments were carried out in triplicate.

Procedural blanks were prepared according to the same analytical procedure described for sediment but in which the sample has been replaced by cleaned see sand. Regular analyses to check for contamination throughout the analytical procedure, showed no presence of the analytes of interest.

2.4. Instrumental analysis

Determination of the selected PCBs in the purified extracts during method development was carried out by GC–μECD. In this case, the concentrated extracts were injected on a HP 6890 Series GC–μECD (Hewlett-Packard, Palo Alto, CA, USA) in the hot splitless mode (1 μl; 270 ºC; splitless time 1.0 min) equipped with a capillary BPX-5 column (60 m, 0.25 mm i.d., 0.25 μm film thickness) purchased from SGE (Melbourne, Australia). The column temperature was programmed from 80 ºC (2 min) to 250 ºC (50 min) at a rate of 30 ºC/min, and then to 270 ºC (10 min) at 5 ºC/min. Nitrogen was used as carrier gas (constant flow, 1.5 mL/min) and as make-up gas (30 mL/min). The detector temperature was set at 300 ºC.

Determination of the individual target PCBs during method validation and application to the analysis of the certified reference material (CRM) was carried out by GC–ITD(MS/MS) (CP-3800 & Saturn 2000, Varian, CA, USA). In these experiments, the concentrated extracts were injected in the splitless mode (4 μL at 0.5 μL/s) and the inlet temperature programmed from 100 ºC (2 min) to 300 ºC at 200 ºC/min; the splitless time was 2 min. A VF-5MS capillary column (50 m × 0.25 mm × 0.25 μm) purchased from Varian (CA, USA) was used for the chromatographic separation with the oven temperature program as follows: 60 ºC (3 min) to 200 ºC (3 min) at 30 ºC/min, then to 230 ºC (15 min) at 3 ºC/min and then to 270 ºC (15 min) at 5 ºC/min. Helium was used as carrier gas (constant flow, 1.0 mL/min). The transfer line and the ion trap temperatures were set at 305 and 250 ºC, respectively.

Further details concerning ionization and detection conditions have been described elsewhere *(22)*.

3. Results and discussion

3.1. Optimisation of the miniaturised s-PLE method

As previously mentioned, the proposed sample preparation protocol is based on a previously optimised s-PLE methodology for the analysis of PBDEs in sediments involving a commercial PLE system and a 22-mL extraction cell *(10)*. The ratio of the sample:Alumina:powder copper in the MSPD mixture was maintained similar to that of the original large scale procedure but the amounts were half of those used for PBDE determination. Also the amount of Alumina packed in the cell for analyte purification was reduced to 3 g. More importantly, the use of a 4.75 mL cell prevented from packing an extra inert support (i.e., hydromatrix) to fill the remaining empty part of extraction cell, which allowed a significant reduction of the volume of the solvent required for quantitative extraction of the target compounds from the matrix. Figure 1 shows a schematic of the finally proposed arrangement for miniaturised s-PLE of PCBs from sediments.

The nature of the extraction solvent has, for obvious reasons, a profound effect on the efficiency and selectivity of the PLE process. *n*-Hexane has been proved to provide quantitative extraction recoveries for the analysis of persistent organic pollutants (POPs) from environmental matrices of different nature *(14, 16-17, 23-24)*. The use of more polar solvents (or mixtures containing polar solvents) is usually recommended for the analysis of highly sorptive matrices *(9, 12, 14, 25)*. However, these solvents can also yield to dirtier extracts due to more exhaustive extraction. In the analysis of POPs, the use of extraction solvent mixtures containing a non-polar solvent, such as n-hexane or n-heptane, and a more selective solvent like dichloromethane has usually sufficed to obtain quantitative extractions with cleaner extracts *(10-11, 25)*. Therefore, a mixture of *n*-hexane:dichloromethane (1:1, v/v) was selected as extraction solvent in the present study on the base of results previously published by other authors dealing with similar application studies *(11, 26)* and our

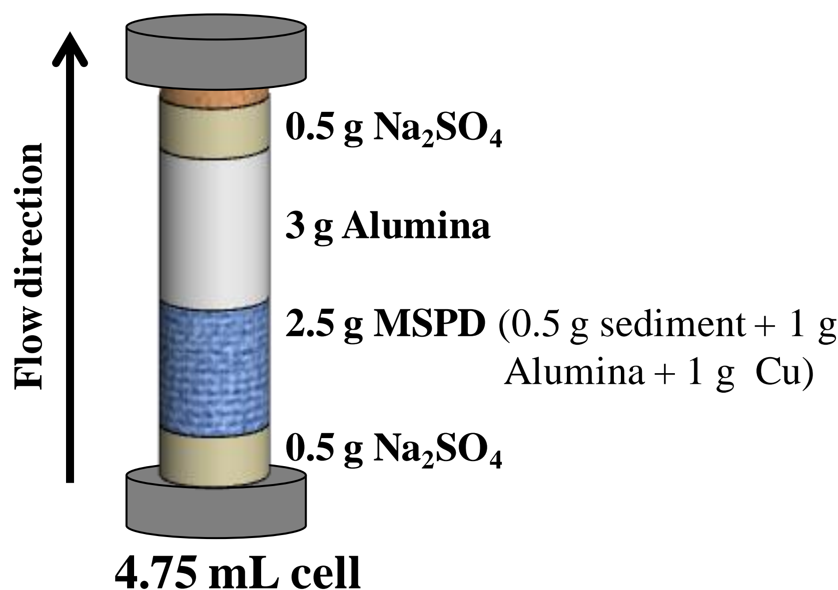

Fig. 1. Packing of the miniaturised extraction cell.

own experience *(16)*. The somehow standard experimental conditions selected for PLE, 100 ºC and 10.5 MPa, were chosen also on the base of previously reported procedures for the extraction of the non-polar microcontaminants from highly sorptive abiotic matrices, such as soils and sediments *(10-12, 25, 27)*, and again our own experience *(17)*. The solvent flow was set at 0.5 mL/min to ensure proper and uniform sample wetting and solvent removal under the proposed experimental conditions *(15)*. Preliminary experiments were carried out to determine the total amount of extraction solvent required to complete the two 10-min static PLE cycles sufficed for analyte extraction and to ensure complete solvent removal at the finally set working temperature of 100 ºC. After several experiments, it was determine to use a cold trap (i.e., an ice bath) for proper trapping of the most volatile PCB congeners investigated in the collection vial during cell depressurisation and initial solvent removal, and to use a solvent volume equivalent to approximately two times the extraction cell volume, i.e. ca. 10 mL, per extraction cycle. The relatively long dynamic step required for solvent exchange in between static extraction cycles ensured that the extraction solvent reached the preselected extraction temperature before the pressurisation of the system started, something that contributed to avoid overpressure inside the cell during PLE and to improve the reproducibility of the extraction

process *(15)*. The two purified PLE extracts generated during s-PLE were jointly collected (total solvent consumption, ca. 20 mL per sample), concentrated under a gentle nitrogen stream until incipient dryness, reconstituted in the corresponding syringe standard solutions and subjected to instrumental separation-plus-detection using the technique selected in each case.

The analytical performance of the at-line s-PLE plus GC–μECD procedure for real-life samples was preliminary evaluated by analysing a non-contaminated test sediment spiked at four different levels (0.5, 1.0, 5.0 and 10 ng/g dw of each PCB). The zero level, corresponding to the endogenous PCB levels detected in the test sediment, was also included in this study. Four separate analyses were carried out for each of the four spiking levels and for the zero level. In all instances, samples were extracted following the s-PLE method previously described and extracts from the two static cycles were jointly collected in a single vial. Two of the extracts were analysed by GC–μECD and the other two by GC–ITD(MS/MS). In the latter case, ^{13}C-labelled PCBs were added to the sediments before any sample treatment. Relevant analytical data are summarised in Table 1.

The total analytical procedure proposed showed good linearity over the whole test range for most of the target compounds with regression coefficients better than 0.985 for GC–μECD and above 0.987 for GC–ITD(MS/MS). Slightly lower, but still satisfactory, regression coefficients in the 0.980-0.985 range were obtained for some of the high halogenated PCB congeners considered in the study (i.e., PCBs 170, 180 and 194) with both procedures. This trend agreed with previous observations in studies dealing with both miniaturised *(15, 18)* and conventional *(28)* PLE of PCBs from similar or close-related environmental matrices.

The efficiency of the s-PLE as well as the repeatability of the two proposed miniaturised methodologies were evaluated at the four spiking levels studied (each experiment was done in duplicate). The relative standard deviation (RSD) data, which were essentially the same irrespective of the PCB level investigated, were 15 % or better for a large majority of the target analytes (see Table 1 for average RSD values). Recoveries were calculated by comparison of the PCB concentrations found in the

Table 1. Analytical performance of the s-PLE plus GC–µECD and GC–ITD(MS/MS). Correlations coefficients (r^2) as calculated in the tested range of 0.5-10 ng/g dw. Average recoveries (%) and RSD (%) calculated at the several spiking levels investigated. LODs (ng/g, dw) as calculated for the test sediment.

	GC–µECD					**GC–ITD(MS/MS)**				
PCB No	**r^2**	**Range**	**Rec.[a] (%)**	**RSD (%)**	**LOD (ng/g)**	**r^2**	**Range**	**Rec. (%)**	**RSD (%)**	**LOD (ng/g)**
28	0.983	79-104	91	12	0.8	0.975	73-105	84	15	0.6
52	0.982	79-123	101	18	0.9	0.987	85-104	93	8	0.2
95	0.991	85-103	96	8	1	NA[b]				
101	0.998	96-122	106	11	0.1	0.995	89-102	97	5	0.02
77	0.993	91-123	102	14	0.4	0.998	94-114	100	8	0.005
149	0.998	94-110	102	7	0.2	NA				
123	0.996	89-111	98	9	0.5	0.996	91-112	100	8	0.002
118	0.996	91-106	97	6	0.2	0.999	96-102	99	2	0.04
114	0.993	84-125	102	15	0.2	0.999	96-111	102	6	0.007
153	0.987	81-103	91	9	0.2	0.993	88-103	94	6	0.01
132	0.985	81-117	99	15	0.1	NA				
105	0.991	79-115	100	14	0.2	0.996	88-107	98	7	0.01
138	0.990	84-118	100	14	0.2	0.993	85-106	98	9	0.02
126	0.994	88-123	103	14	0.1	0.997	90-106	99	6	0.02
183	0.996	89-107	100	8	0.4	NA				
167	0.998	87-125	104	15	0.2	0.999	97-103	100	2	0.008
156	0.994	90-107	97	8	0.2	0.998	90-106	100	6	0.02
157	0.994	89-116	106	12	Int[c]	0.999	95-110	100	6	0.008
180	0.991	85-105	94	10	0.3	0.983	83-104	100	6	0.02
169	0.992	89-124	101	16	0.9	0.998	89-104	101	9	0.008
170	0.983	84-111	96	14	0.4	0.980	88-107	95	11	0.008
189	0.992	86-112	99	11	0.3	0.996	90-117	101	12	0.008
194	0.981	79-104	90	12	0.1	0.979	88-113	102	10	0.008

[a] Rec., average recovery
[b] NA, Not analysed
[c] LOD was not determined because of an interference

spiked sediments after s-PLE under finally proposed extraction conditions with those quantified in the s-PLE extract obtained for the non-contaminated sample and subsequently spiked at the corresponding supplementation level. Again, essentially similar recoveries were obtained at the four spiking levels assayed for all PCB congeners. PCB recoveries, as determined at the four spiking concentration levels evaluated, are summarized in Table 1. Average recoveries in the 90-106 % range were calculated using GC–µECD for final determination; while those determined using GC–ITD(MS/MS) ranged from 84 % to 102 %. The mutual agreement observed

among values calculated at the four spiking levels investigated irrespective of the detector used illustrated the robustness of the proposed miniaturised s-PLE method over the 0.5-10 ng/g dw range tested and demonstrated its practicality for the analysis of both non-polluted and highly polluted sediments under similar experimental conditions even though only 500 mg of the homogenized sample were used for determination. These results were also similar to *(11, 23, 28-29)* or better *(24)* than other previously reported for similar application studies involving conventional (i.e., large scale) PLE systems with both *in-cell (10-11, 23)* or *off-line (24, 28-29)* purification and involving larger amounts of sample and solvents. However, non-unexpectedly, when using GC–μECD for final determination of the target compounds, interference due to co-elution with matrix components was observed for specific early eluting PCB congeners (e.g., PCB 28, 52, and 101) and the use of matrix-matched calibration became advisable. The improved selectivity offered by GC–ITD(MS/MS) solved the problem. This point became also evident through the experimentally determined limits of detection (LODs) calculated with both instrumental techniques. LODs (S/N, 3:1), as calculated for the non-spiked test sediment, in the 0.1-1 ng/g dw range were obtained when using GC–μECD for final determination; while substantially lower values in the 0.005 to 0.6 ng/g dw range were obtained when the same extract was analysed by GC–ITD(MS/MS). In general, the latter LOD values were in the range to those previously reported in similar studies dealing with PLE of PCB from complex environmental solid matrices and using GC–MS-based approaches for final instrumental determination *(18, 29)*. Interestingly, they are also in the range of those values reported in studies involving highly selective detectors, such as high resolution mass spectrometry, for final determination (LODs of 0.12-0.56 pg/g *(25)*). Therefore, on the base of all these results one can conclude that the proposed s-PLE plus GC–ITD(MS/MS) methodology shows fully satisfactory performance under conditions typically encountered in environmental PCB analysis, while the use of GC–μECD can still be fully satisfactory for the fast and cost-effective screening of these microcontaminantes in sediments.

3.2. Method validation

The proposed miniaturised s-PLE procedure was finally applied to the analysis of relevant PCBs in a certified freshwater harbor sediment (BCR-535) for further validation of the developed analytical methodology. Table 2 compares the results obtained when using GC–µECD and GC–ITD(MS/MS) for final instrumental determination of the target analytes with the certified PCB concentrations. Analyses were carried out in quintuplicate to evaluate the repeatability of the proposed methods. In agreement with previous observations, a satisfactory agreement was observed among certified PCB levels and concentrations calculated using GC–ITD(MS/MS) for final instrumental determination, with calculated recoveries in the 71-102 % range and RSDs better than 12 %, with only exception of PCB 52 (RSD, 21 %). These recovery and RSDs values are in the range of those typically reported by other authors dealing with the determination of PCBs in certified sediment matrices using conventional size PLE systems *(9, 11, 30)*. Although more divergent results were obtained when using GC–µECD for separation-plus-detection of the investigated analytes (recoveries in the 63-108 % range, although with notable exception such as those of congeners 28 -133 %-, 156 -39 %- and 170 -55 %-), the still satisfactory reproducibility of the complete s-PLE plus GC–µECD, with RSDs

Table 2. Comparison of the certified PCB levels (µg/Kg) in the CRM BCR-535 with those calculated by applying the proposed miniaturized s-PLE in combination with either GC–µECD and GC–TD(MS/MS) analysis (n=5).

		GC–µECD		**GC–ITD(MS/MS)**	
PCB No	**Certified value range**	**Exp. value (RSD, %)**	**Recov. (%)**	**Exp. value (RSD, %)**	**Recov .(%)**
28	44 ± 5	65 (7)	133	30 (13)	76
52	38 ± 5	47 (5)	108	32 (21)	97
105	3.5 ± 0.6	1.8 (13)	62	2.6 (7)	88
118	27.5 ± 2.2	16.9 (8)	67	18.4 (5)	73
138	27 ± 5	14 (7)	64	22 (12)	102
153	50 ± 4	29 (8)	63	53 (7)	98
156	3.0 ± 0.4	1.0 (7)	39	2.4 (5)	92
170	13.4 ± 1.4	6.6 (7)	55	8.5 (2)	71
180	22.4 ± 2.1	14.2 (6)	70	23.7 (4)	97

lower than 13 % for all tested PCBs, support the validity of the approach for screening purposes.

Finally, as an example of the typical results obtained in this study, Figure 2 shows the chromatograms obtained after s-PLE of 500 mg of the certified harbor sediment using GC–μECD and GC–ITD(MS/MS) for final instrumental analysis. The clean chromatograms obtained in both cases demonstrated both the selectivity of the PLE process under the experimental conditions proposed and the detectability of the investigated analytes even if an as small amount of sample as 500 mg was used for the

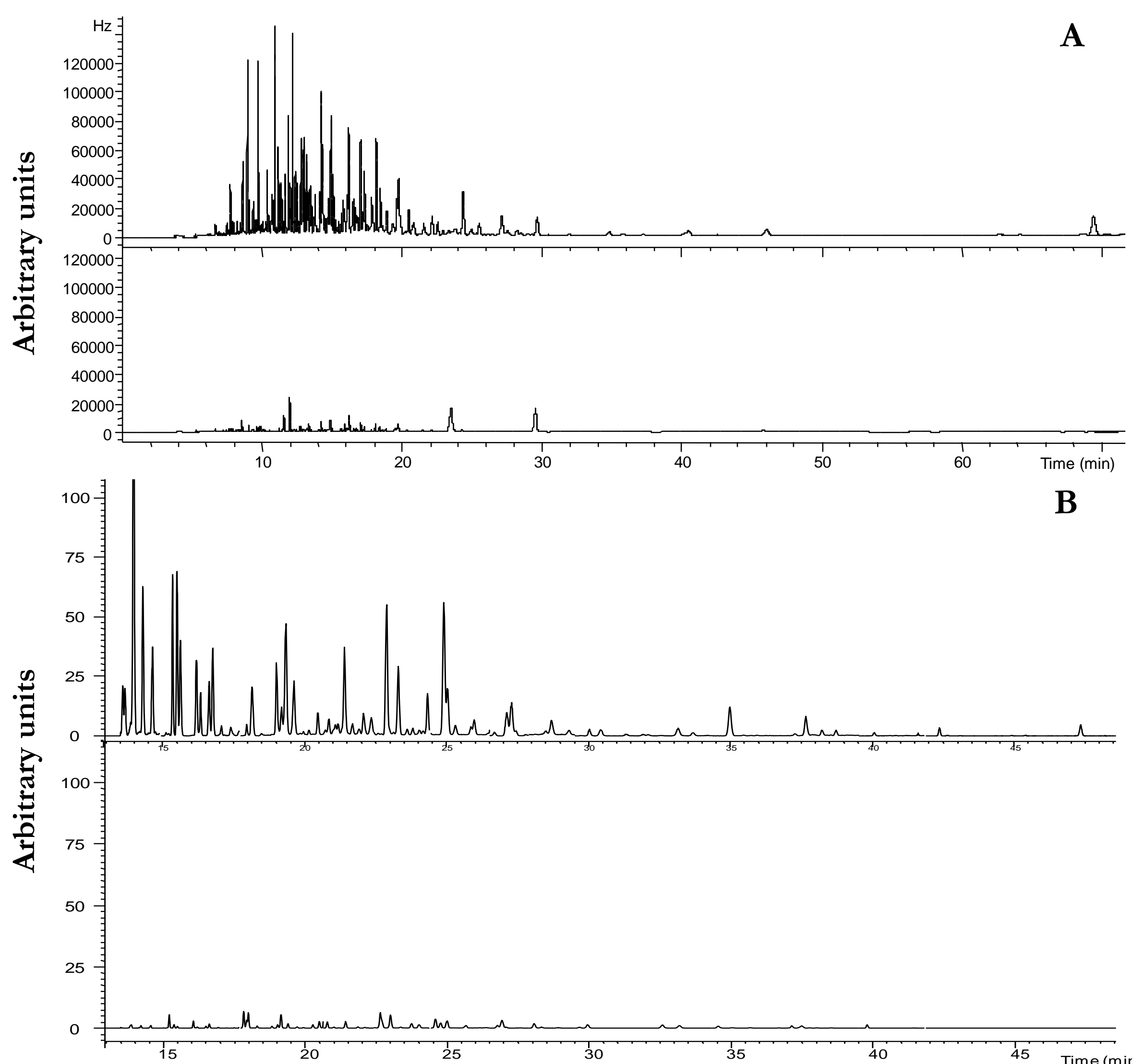

Fig. 2. Typical chromatograms obtained for the certified harbour sediment (upper chromatogram) and the corresponding procedural blanks (bottom) after miniaturised s-PLE combined at-line with (A) GC–μECD and (B) GC–ITD(MS/MS).

analysis; contributing to further illustrate the potential of the proposed method for the fast and cost-effective determination of environmentally relevant PCBs in complex solid matrices, such as sediments.

Conclusions

A miniaturised PLE with *in-cell* purification method has been proposed for the analysis of prioritary and toxic PCB congeners in sediments. Once optimised, the complete analytical method showed a satisfactory dynamic linear response in the evaluated range of 0.5-10 ng/g dw (levels determined in the non-spiked test sample were considered as zero value), with correlation coefficients in general better than 0.98, and average recovery values in the range 90-106 % when using GC–μECD for final determination and ranging from 84 % to 102 % when using for GC–ITD(MS/MS). These results for spiked samples were further confirmed by the analysis of a certified harbor sediment which demonstrate the feasibility of the s-PLE plus GC–ITD(MS/MS) procedure proposed for the accurate determination of the target analytes at the low level typically encountered in real-life samples. The satisfactory repeatability obtained for the s-PLE plus GC–μECD approach (RSDs lower than 15 % for most of the target compounds) supported the suitability of this second methodology for screening purposes.

Compared with conventional PLE procedures, the present approach reduces sample volumes to about 500 mg, and solvent consumption to 20 mL rather than 50–200 mL. The reduced volume of rather volatile extraction solvents used in the study, effectively contribute to shorten the concentration step typically required in these types of analyses for appropriate analyte detection, at least indeed large volume injection was used. The cleanness of the collected s-PLE extracts allowed direct GC analysis without any further purification. Even so, the LODs for a large majority of the target analytes were 0.1-1 ng/g dw with GC–μECD and substantially lower (0.005-06 ng/g dw) for GC–ITD(MS/MS) demonstrating the practicality of the method for the analysis of relevant PCBs at the levels typically encountered in real-life sediments.

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3.1.4. EXTRACCIÓN ASISTIDA CON ULTRASONIDOS COMBINADA CON PURIFICACIÓN EN PUNTAS DE PIPETA DESECHABLES PARA EL ANÁLISIS DE BIFENILOS POLICLORADOS EN MUESTRAS BIOLÓGICAS DE TAMAÑO LIMITADO[1]

Abstract

The use of solid-phase extraction pipette tip (also so-called disposable pipette extraction, DPX) has been evaluated for the purification of environmentally relevant polychlorinated biphenyls (PCBs) in fatty extracts obtained by ultrasound-assisted extraction with a probe from small amounts of biological tissues. Complete sample treatment was carried out in a few minutes with minimal sample manipulation and reagents consumption (i.e., 1.5 mL of n-hexane and 0.8 g of acidic silica). The performance of the proposed methodology for the intended determination was firstly evaluated by determination of the endogenous PCB levels in an internal reference material. The determined concentrations showed a good agreement with those found when applying a more conventional sample preparation procedure previously validated (recoveries, as compared to levels determined using the latter method, were in the 85-123% range for a large majority of the congeners and relative standard deviations, below 14%). Results obtained for the analysis of food samples with reference PCB levels and certified reference materials NIST 1945 and 1947 demonstrated that, when combined with gas chromatography coupled to ion trap mass spectrometry working in the tandem mode, GC-ITD(MS/MS), the proposed methodology allowed accurate determination of most of the investigated PCBs and that 50 mg of sample sufficed for the screening of, even, less abundant PCB toxic congeners.

Keywords: Sample preparation, miniaturisation, biological samples analysis, polychlorinated biphenyls

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[1] M. Pena-Abaurrea, V. S. García de la Torre, L. Ramos, Ultrasound assisted extraction combined with disposable pipette purification for the determination of polychlorinated biphenyls in small size biological samples. *In preparation.*

1. Introduction

The determination of trace microcontaminants in size-limited biological samples is a challenge in the environmental field where, similarly to other research areas, the development of faster, more cost-effective and greener sample preparation protocols is an actual demand. Two analytical strategies have typically been followed to fulfill these requirements: the adaptation of previously developed conventional large-scale methodologies, and the set-up of novel analytical techniques *(1)*. Regarding the latter approach, a variety of new miniaturised extraction techniques have been introduced during the last two decades and their feasibility for the analysis of micropollutants in aqueous and relatively pristine liquid samples demonstrated through a number of illustrative application studies (see e.g. *(2-4)* and references therein). However, the attempts to apply these techniques to the analysis of (semi-)solid complex matrices, such as those containing large amounts of lipids, has been much more limited and their success widely variable *(1)*. Apart from the higher difficulty associated to the extraction of the target analytes from these types of matrices, the need for subsequent elimination of co-extracted matrix components before instrumental separation-plus-detection remains as a relatively difficult task, frequently carried out as a separate and manual additional sample treatment step. Efforts carried out in this field during the last decades have resulted in the development of several (frequently miniaturised) sorbent-based techniques, such as the nowadays well-established solid-phase microextraction and stir-bar-sorptive extraction, or the more recently introduced but already widely accepted dispersive solid-phase extraction, with the QuEChERS method as its most representative example *(5)*. However, among the different analytical strategies available for sample clean-up, solid-phase extraction (SPE) is from far the most profusely used technique in the laboratories. The various commercialised formats, the wide variety of sorbents available and the possibility of performing it automatically can be mentioned as main reasons.

Solid-phase extraction pipette tip (also named as disposable pipette extraction, DPX) is a recently introduced SPE-based device in which a small amount of SPE sorbent is loosely placed inside a pipette tip fitted with a screen at the bottom narrow end of the tip and a barrier near the top *(6)*. In a typical DPX experiment, the liquid sample (or extract) is aspirated into the void pipette space between the screen and

barrier. Subsequent air aspiration results in a thorough mixing of the solvent with the SPE sorbent, so allowing a fast and very efficient extraction. As compared to other SPE formats, the DPX efficiency is based on the equilibration time following the mixing of the sample solutions with the sorbent and consequently the process is flow rate independent. In addition, the small amount of sorbent used allows for smaller volumes of elution solvents *(6)* which are typically subjected to instrumental analysis without any further treatment. Although some other DPX formats have been used for the purification of extracts in biological-orientated studies, up to know, DPX has mainly been applied to the determination of drugs in human fluids *(6-7)* and for the multiresidue analysis of pesticides in fruit and vegetable extracts *(8-9)*.

Sample preparation protocols in use for the determination of polychlorinated biphenyls (PCBs) in (semi-)solid biological samples are typically complex highly manipulative procedures involving several separate treatments for exhaustive extraction of the target compounds from the matrix structure and subsequent isolation from co-extracted material and, in most instances, final fractionation from other close-relate micropollutants. In general, these analytical procedures involve relatively large amounts of sample (i.e., 10-100 g) and so of sorbents and solvents. Attempts to miniaturised and, when possible, simplify these sample preparation protocols are still rather limited in the literature *(10)*. The lack of appropriate instrumentation, which in some instances has forced the design and setting-up of devoted home-made instrumentation *(10-11)*, can be pointed out as a possible explanation. Commercially available ultrasounds probes, however, represent a per se miniaturised device. These types of systems have been profusely used in the biological field for years. However, although several studies have already demonstrated the feasibility of ultrasound-based approaches for the efficient extraction of trace micropollutants from biotic samples *(12-14)*, to the best of our knowledge, the application of ultrasonic probes in environmental studies is still limited.

In this study, an ultrasonic-assisted extraction-based method followed by SPE disposable pipette tip purification is proposed for the determination of environmentally relevant PCBs in minute solid biological matrices. The different parameters affecting the DPX step (namely, nature and amount of the extraction sorbent, DPX clean-up protocol) have been optimised for at-line coupling with the

previous extraction step, which was performed according to previously validated conditions *(11)*. The performance of the novel sample preparation approach, which was combined with either gas chromatography-micro electron capture detector, GC–μECD, for method development, or with gas chromatography-ion trap mass spectrometry working in the tandem mode, GC–ITD(MS/MS), once the method was optimised, was tested by the analysis of prioritary and toxic PCBs in non-contaminated samples. Final validation was carried out by the analysis of appropriated certified reference materials (CRMs).

2. MATERIAL AND METHODS

2.1. Reagents and chemicals

n-Hexane and isooctane used for sample preparation were of pestipur quality and purchased from Merck (Darmstadt, Germany) and SDS (Peypin, France), respectively. Silica gel, 60 mesh (Merck), was pre-washed with methanol and dichlorometane and used after activation at 100 ºC for 48 h. Acidic silica gel (44%, w/w) was prepared as described in *(15)* with sulphuric acid of pro analysis quality (Merck). Anhydrous sodium sulphate, Na_2SO_4, (J.T. Baker, Deventer, The Netherlands) was pre-washed with methanol and dichlorometane and conserved in a heater at 60 ºC until use.

22 PCB congeners (IUPAC No 28, 52, 95, 101, 77, 123, 118, 114, 153, 132, 105, 138, 126, 183, 167, 156, 157, 180, 169, 170, 189 and 194) were selected for study because of their environmental relevance and toxicity *(16-17)*. A working standard solution containing 500 pg/μL of each congener was prepared from individual and mix-PCB standards (Dr. Ehrenstorfer, Augsburg, Germany). This stock solution was used for further dilution and, when required, spiking of the samples. A solution containing 25 pg/μL of 1,2,3,4-tetrachloronaphtalene (TCN, Ehrenstorfer) and 37.6 pg/μL of PCB # 209 was used as internal standard for PCB determination by GC–μECD and added to samples before any treatment. ^{13}C-labelled standards of the 13 most toxic PCB congeners (Wellington Laboratories) were used as internal

standards when using GC–ITD(MS/MS) for final determination of the target compounds.

2.2. Samples

Method development was carried out by analysing a non-contaminated common two-banded sea bream from Huelva (Spain) used as an internal reference material in our laboratory *(18)*. Two extra foodstuffs, i.e. chicken meat and salmon, from interlaboratory exercises and for which consensus concentration levels of PCBs are known, and two CRMs, whale blubbler (NIST 1945) and fish tissue from the Michigan Lake (NIST 1947), were used for method validation. All samples were preserved at –20 ºC until analysis and, then, freeze-dried and homogenised before any treatment. Fat content in the investigated samples was determined according to the Smedes method *(19)*, and was calculated to be 6 % (weight:weight, w:w, on a freeze-dried basis) for common two-banded sea bream, 7.09 % for chicken meat, 12.12 % for salmon, and 22.78 % for the egg yolk.

2.3. Sample preparation

Samples were extracted according to a miniaturised ultrasound-based method previously validated in our working group *(11)* which provided a satisfactory linear response for a large majority of the investigated PCBs in the evaluated range of 5-1000 ng/g, with average recoveries in the 90-107% range and a repeatability lower than 15% for most of the target analytes. (Relevant analytical data concerning this ultrasonic probe-based extraction methodologies can be found in the Annex section). Briefly, in a typical experiment, 50 mg of the investigated matrix were placed in an 1.5-mL Eppendorf (Deltalab, Barcelona, Spain) and extracted for 40 s (i.e., 20 pulses of 2 s) with 150 µL of *n*-hexane using a 2-mm ultrasonic titanium probe (130 Vibra Cell, Sonics, Newtown, USA) operated at 130 W and 20 kHz. The supernatant was separated by centrifugation during 2 min at 14000 rpm (MiniSpin Eppendorf centrifuge, Eppendorf, Hamburg, Germany) and slowly aspirated with a micropipette (Gilson tipe, Labbox Labware, Mataró, Spain) into a 5-mL polypropylene tip (Labbox labware) modified to contain the clean-up sorbent (acidic silica, 44%, w:w). A low volume of air was also aspirated to maximize the extract-sorbent interaction. After 10

s, the pre-purified extract was eluted onto a new (clean) Eppendorf. Due to the high lipid content of some of the investigated matrices, a second DPX-based purification step was incorporated to the finally proposed purification protocol to ensure complete fat removal from these particularly complex matrices. Thereby, a new pipette tip was installed in the micropipette and the purification step was repeated following the previously described protocol. The final volume of the collected clean extract was adjusted to 50 μL by evaporation to incipient dryness under a gentle nitrogen stream and reconstituted in isooctane and subjected to final instrumental analysis without any further treatment.

During method development, the levels of the endogenous PCBs determined in the internal reference material used for method development were compared with those determined when analysing the same sample using a more conventional sample preparation method previously validated in our group for the determination of PCBs in foodstuffs *(10)*. In brief, in this method 150 mg of a MSPD mixture containing the tested sample, acid silica and sodium sulphate (1:1:1, w:w:w) were homogenised, packed in a 3 mL glass column containing a layer of acidic silica, placed in a vacuum SPE-12G system (J.T. Baker, Deventer, The Netherlands) and eluted with 8 mL of n-hexane at 0.5 mL/min. The ready-to-analyse extract was collected in a vial, concentrated under a gentle nitrogen current and reconstituted in 50 μL of isooctane for GC–μECD analysis.

In all instances, procedural blanks were analysed to check for any contamination during the analytical process. Blanks were prepared as before explained but without sample. No background interferences affecting the determination of the target compounds was found to be introduced by the methodology proposed. Otherwise specified, experiments were carried out in quadruplicate.

2.4. Instrumental analysis

GC–μECD (HP 6890 Series, Hewlett-Packard, Palo Alto, USA) was used for the instrumental separation-plus detection of the target compounds during method development. Samples were injected in the hot splitless mode (1 μL; 270 °C; splitless time, 1.0 min) in a capillary DB-5 column (60 m × 0.25 mm inner diameter × 0.25 μm film thickness) purchased from SGE (Melboune, Australia). The column

temperature was programmed from 80 ºC (2 min) to 185 ºC (3 min) at a rate of 30 ºC/min, then to 230 °C (10 min) at 1.5 ºC/min, and raising 270 ºC (10 min) at 5 ºC/min. Nitrogen was used as carrier gas (constant flow, 1.5 mL/min) and make-up gas (30 mL/min). The detector temperature was set at 300 ºC.

Samples analysed during final method optimisation and validation were analysed in a GC (CP-3800, Varian, CA, USA) equipped with an ion trap MS detector (Saturn 2000, Varian) working in the MS/MS mode. Extracts were injected in the splitless mode (4 μL at 0.5 μL/s) and the inlet temperature programmed from 100 ºC (2 min) to 300 ºC at 200 ºC/min with a 2 min splitless time. A VF-5MS capillary column (50 m × 0.25 mm × 0.25 μm) purchased from Varian (CA, USA) was used for the chromatographic separation with the oven temperature program as following: 60 ºC (3 min) to 200 ºC (3 min) at 30 ºC /min, then to 230 ºC (15 min) at 3 ºC/min and then to 270 ºC (15 min) at 5 ºC /min. Helium was used as carrier gas (constant flow, 1.0 mL/min). The transfer line and the ion trap temperatures were 305 ºC and 250 ºC, respectively. Further details concerning ionization and detection conditions for the target compounds have been described elsewhere *(20).*

3. Results and discussion

3.1. Method optimisation

Preliminary experiments were carried out to ensure a proper purification of the fatty extract resulting from the extraction step. In these assays, increasing amounts of acidic silica in the 0.4-0.8 g range were loosely placed inside the 5-mL pipette tip, in between frits. The maximum amount of acidic silica to be placed in the tip was experimentally determined as that allowing complete loading of sample extract obtained after ultrasonic extraction in the pipette tip, ca. 1.3 mL, and still fast and proper interaction of the extract with the sorbent inside the tip. This maximum amount was finally set as 0.8 g, corresponding to ca. two thirds of the remaining volume in between tip frits.

Due to the high lipidic content of some of the test matrices considered in the study, experiments carried out with 0.4 g and 0.6 g of sorbent were clearly insufficient for complete fat removal even after two successive treatments. Thereby, 0.8 g of

acidic silica were used in subsequent experiments. This amount of sorbent sufficed to eliminate a large majority of the co-extracted fat even for extracts obtained from samples containing as large amounts of lipids as 22 % (w/w). Despite the efficiency of the proposed purification method, complete removal of the remaining fat traces in the sample extract before instrumental analysis was mandatory to preserve the integrity of the GC column. Thereby, an extra clean-up treatment with a new pipette tip was incorporated to the sample preparation protocol. Due to the easy and fast handling of samples with the proposed clean-up pipette-based approach, in order to establish an straightforward sample preparation protocol that could be applied to a large variety of fatty samples without modification, this second treatment was systematically applied to all investigated matrices.

The feasibility of the complete sample preparation procedure proposed for the determination of endogenous PCBs in fatty biological tissues was evaluated by analysing a non-contaminated common two-banded sea bream samples, which is used as an internal reference material in our laboratory. In this part of the study, GC–μECD was used for the instrumental determination of the levels of the target compounds in the pipette tip purified extracts. As shown in Figure 1, the repeatability, evaluated as relative standard deviation (RSD, %; n=4), was found to be lower than 14 % for all target compounds, with the only exception of PCBs 95 and 105, for which slightly higher values were obtained (RSDs, 16% and 20%, respectively). In general, these RSD values were only slightly higher than those reported for DPX of spiked pesticides from less complex matrices (i.e., fruit and vegetables), which would demonstrate the straightforward nature of the proposed purification method.

Figure 1 also summarises the endogenous PCB levels determined with the ultrasounds extraction-plus-DPX purification method proposed and those obtained when analysing the same internal reference material using the reference method previously described *(10)*. In general, a good agreement was found between both sets of values. The concentrations determined using the new miniaturised sample preparation method were determined to be in the 85-123% range of those obtained with the reference method, which demonstrated the feasibility of this novel procedure for the determination of environmentally relevant PCBs in complex matrices even if

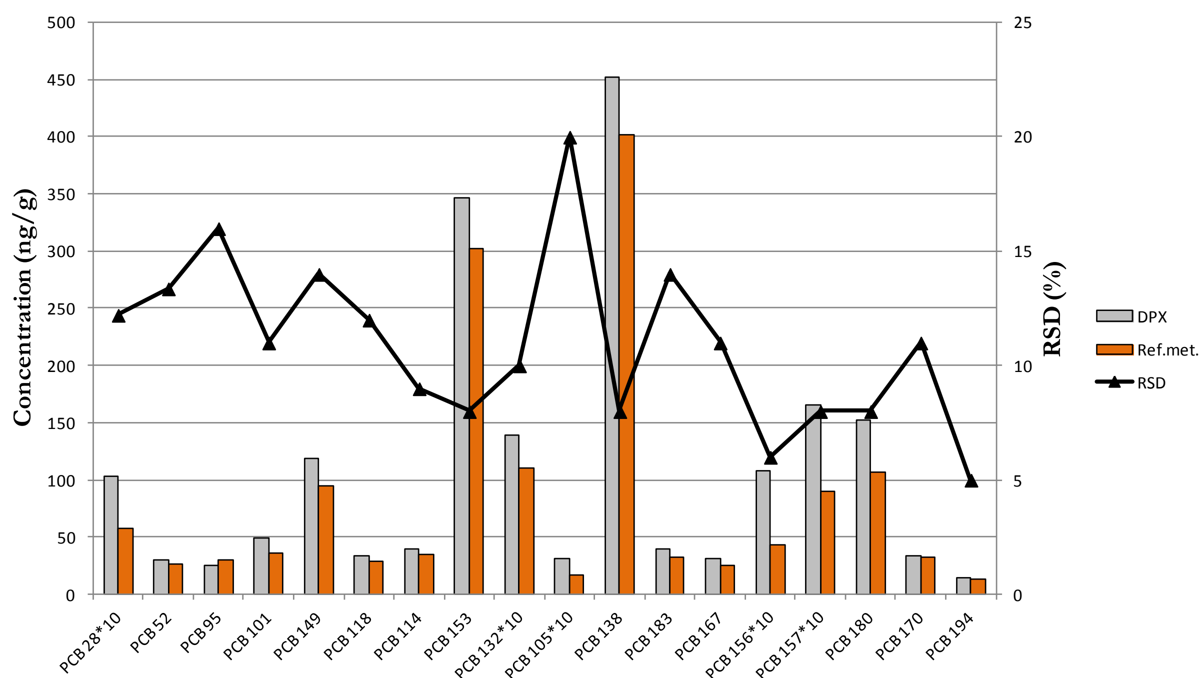

Fig. 1. PCB levels (ng/g dried weight) detected in the reference matrix after applying the DPX methodology and the reference protocol. RSDs (%) of the DPX method as calculated for the inter-day analysis in the investigated compounds.

only 50 mg were used for analysis. Slightly higher values were only obtained for congeners No. 28, 101, 105, 156 and 157 due to coelution with chromatographic interferences when using GC–μECD for final determination. To avoid this type of shortcoming, GC–ITD(MS/MS) was used for subsequent method validation.

As a typical example of the results obtained in this part of the study, Figure 2 compares the GC–microECD chromatograms obtained for the test sample when analysed by the reference method and the DPX-based procedure proposed here. Despite the simplicity of the latter approach, essentially similarly clean chromatograms were obtained in both cases, which was considered a further illustration of the potential of the procedure for the intended determination.

3.2. Method validation

The feasibility of the proposed miniaturised sample preparation method for the determination of prioritary and toxic PCBs in complex solid biotic samples was further evaluated by the analysis of a salmon and a chicken meat obtained from an interlaboratory exercise (Interlaboratory comparison on dioxins in food, Norwegian

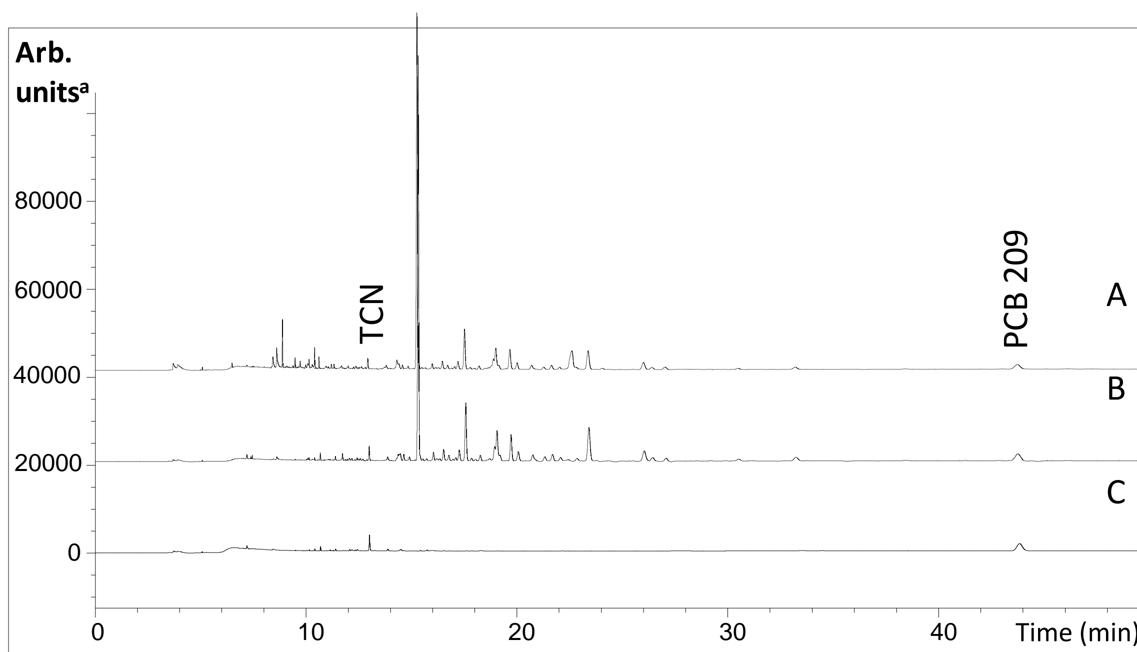

Fig. 2. Typical chromatograms obtained for the test sample after applying (A) the reference methodology, (B) the DPX-based method, and (C) its corresponding procedural blank, using GC–μECD. [a] Arb. Units, arbitrary units.

Institute of Public Health, 2007). The consensus concentrations set in these samples for the investigated PCBs are summarised in Table 1. These consensus levels were compared with those determined when applying the novel DPX-based method proposed here and the previously described reference method to the analysis of these matrices and GC–ITD(MS/MS) for final separation-plus-detection of the target compounds (Table 1). Despite the low concentrations of the investigated PCBs in the chicken meat (in the pg/g fresh weigth range) and the small amount of sample used for determination (50 mg), a good agreement was observed among the three sets of data for most of the investigated congeners. Regarding the DPX-based method, the consensus values were in the range of the experimentally determined concentrations (i.e., C_{sample} ± absolute standard deviation, SD) for twelve out of sixteen congeners considered in the study. Interestingly, as in the case of PCB 77, rather accurate concentrations were determined for some analytes detected at levels close to the limit of quantification (as calculated for the real sample), which could be an useful feature when using the proposed method for fast screening of less abundant congeners. There is no obvious explanation for the relatively low values obtained for PCBs 105, 114 and 118 using this analytical approach. Essentially similar results were obtained

Table 1. PCB consensus concentrations (pg/g, fresh weight) in chicken and salmon meat, levels found after miniaturised-UAE combined with DPX purification (absolute standard deviation, SD), and limits of detection (LODs) as calculated for real samples with GC–ITD(MS/MS) (n=4).

PCB #	Chicken meat Cons. value	Ref method	DPX method	LOD	Salmon Cons. value	Ref method	DPX method	LOD
28	109	*109*[a] (7)	*107* (5)	98	159	*162* (4)	*159* (21)	131
52	1562	1658 (117)	1399 (201)	138	310	*325* (39)	*305* (49)	211
101	3910	3392 (388)	2690 (192)	89	707	687 (13)	365 (49)	80
77	6.5	<LOD	*9.5* (1.1)	8	13	*16* (1)	<LOD	10[b]
123	43	*37* (1)	*43* (2)	22	9.7	*9.4* (1.2)	*10* (1)	7
118	3004	2290 (152)	2245 (93)	91	597	592 (22)	358 (14)	10
114	66	57 (9)	45 (3)	6	12	*13* (0.2)	*11* (1)	6
153	6362	5329 (828)	6108 (78)	118	1279	1277 (68)	1018 (113)	54
105	1169	802 (38)	891 (35)	29	214	211 (12)	143 (8)	49
138	5739	4810 (230)	5864 (139)	152	928	921 (57)	1033 (43)	45
126	2.5	<LOD	*2.43* (0.7)	2	4.1	<LOD	<LOD	10[a]
167	265	164 (5)	248 (7)	66	40	*38* (3)	*42* (6)	48
156	662	470 (19)	655 (17)	140	64	*64* (5)	*66* (9)	52
157	91	67 (8)	99 (9)	36	16	*17* (1)	*16* (3)	7
169	0.19	<LOD	<LOD	30[a]	0.53	<LOD	<LOD	30[a]
170	-	1443 (90)	2454 (118)	25	-	227 (35)	226 (24)	44
180	3685	3211 (97)	3682 (77)	130	397	391 (35)	382 (25)	34
189	78	63 (9)	74 (5)	23	6.1	*7.5* (0.9)	*6.3* (0.8)	5
194	-	393 (14)	584 (24)	55	-	<LOD	<LOD	70[a]

[a] Italic values are only indicated for screening purposes because concentrations are in the LOD – LOQ range.
[b] LOD as calculated for standard solutions.

when applying the reference method to the analysis of chicken meat. However, in this case, a relatively poor concordance was observed among concentrations calculated for the high chlorinated PCBs 156, 157 and 167, which could be associated to the softer extraction conditions applied in this analytical procedure.

Satisfactory results were also obtained when analysing the salmon sample with the reference and the DPX-based methods. In general, results obtained for this matrix were similar or better than those obtained in the previous experiments with meat chicken. Thereby, when analysing the salmon sample using the DPX-based method, concentration values out of the tolerance range were only found for PCBs 101, 105 and 118, which exhibited deviations between the experimentally determined concentrations and the consensus values in range 10-20 %.

The results obtained for the analysis of these two samples and the low limits of detection (LODs) determined for them (in general, in the 2-152 pg/g range) supported the practically of the proposed analytical approach for the analysis of relevant PCBs at the levels typically found in real-life samples.

Final validation of the proposed DPX-based method was carried out by the analysis of two CRMs, a fish tissue from the Michigan Lake and a whale blubber (NIST 1947 and 1945, respectively). Table 2 compares the certified concentrations for these two samples with those determined by applying the novel miniaturised sample preparation procedure proposed in this study with final GC–ITD(MS/MS) determination. Satisfactory results were obtained for a large majority of the investigated PCB in both matrices and especially in whale blubber, for which discrepancies were only observed for PCBs 52, 180 and 194. The former was due to coelution with an interference while no obvious explanation exits at present

Table 2. Certified PCB concentrations (ng/g, fresh weight) in whale blubber (NIST 1945) and fish tissue (NIST 1947), PCB levels found after miniaturised DPX-based method (absolute standard deviation, SD) and LODs (ng/g, fresh weight) as calculated for both real-life matrix with GC–ITD(MS/MS (n=4).

	Whale blubber			**Fish tissue**		
PCB #	**Cert.value**	**DPP method**	**LOD**	**Cert.value**	**DPP method**	**LOD**
28	13.1 (1.1)	13.4 (1.6)	0.65	14.1 (1)	15.9 (0.7)	0.55
52	40.7 (1.3)	56.4(7.4)	3.07	36.4 (4.3)	45.1 (8.0)	0.85
101	78 (12)	90.4 (7.3)	3.60	90.8 (0.3)	97.2 (7.6)	1.04
77	0.416 (0.061)	*0.402*[a] (0.05)	0.30	-	2.84 (0.2)	0.25
123	-	*0.82* (0.1)	0.70	-	2.65 (0.1)	0.49
118	76.5 (2.9)	69.9 (7.2)	2.72	112 (6)	88.8 (9.2)	1.29
114	-	1.6 (0.2)	0.40		4.08 (0.2)	0.79
153	228 (10)	231.0 (35.9)	17.76	201 (3)	220.1 (18.2)	1.80
105	28.6 (1.2)	22.2 (3.3)	0.43	50.3 (3.7)	43.8 (3.5)	0.83
138	146 (13)	162.2 (16.1)	1.59	162 (6.9)	166.5 (14.9)	5.25
126	0.182 (0.048)	*0.11* (0.01)	0.11	-	*0.82* (0.2)	0.82
167	-	5.9 (1.0)	0.18	-	6.29 (0.6)	1.50
156	11.4 (0.9)	12.8 (2.2)	0.32	13.3 (0.9)	12.0 (0.6)	0.80
157	3.27 (0.67)	2.4 (0.3)	0.74	4.08 (0.77)	3.28 (0.4)	1.17
169	0.158 (0.041)	*0.062* (0.003)	0.06		<LOD	0.05
170	42.6 (2.2)	42.9 (5.9)	0.68	29.2 (2.4)	33.8(1.1)	0.07
180	138 (10)	207.3 (28.6)	0.37	80.8 (5)	108.5 (7.2)	0.40
189	-	1.1 (0.1)	0.05	-	1.68 (0.2)	0.15
194	53.5 (5.2)	27.4 (7.2)	0.11	13.2 (0.9)	16.9 (1.5)	0.86

[a] Italic values are only indicated for screening purposes because concentrations are in the LOD – LOQ range.

for the differences observed for the two latter congeners. In general, the results obtained in this part of the study supported the idea that 50 mg of sample sufficed for accurate determination of a large majority of the relevant PCBs at levels typically found in non-contaminated matrices as well as for the fast screening of the less abundant toxic congeners.

Finally, Figure 3 compares the GC–ITD(MS/MS) chromatograms obtained when analysing the fish tissue (NIST 1947) by the reference method and by the DPX-based approach and demonstrates that, despite its simplicity, the latter method provided quantitative recoveries of the investigated analytes and clean chromatograms similar to those obtained when applying more conventional sample preparation procedures.

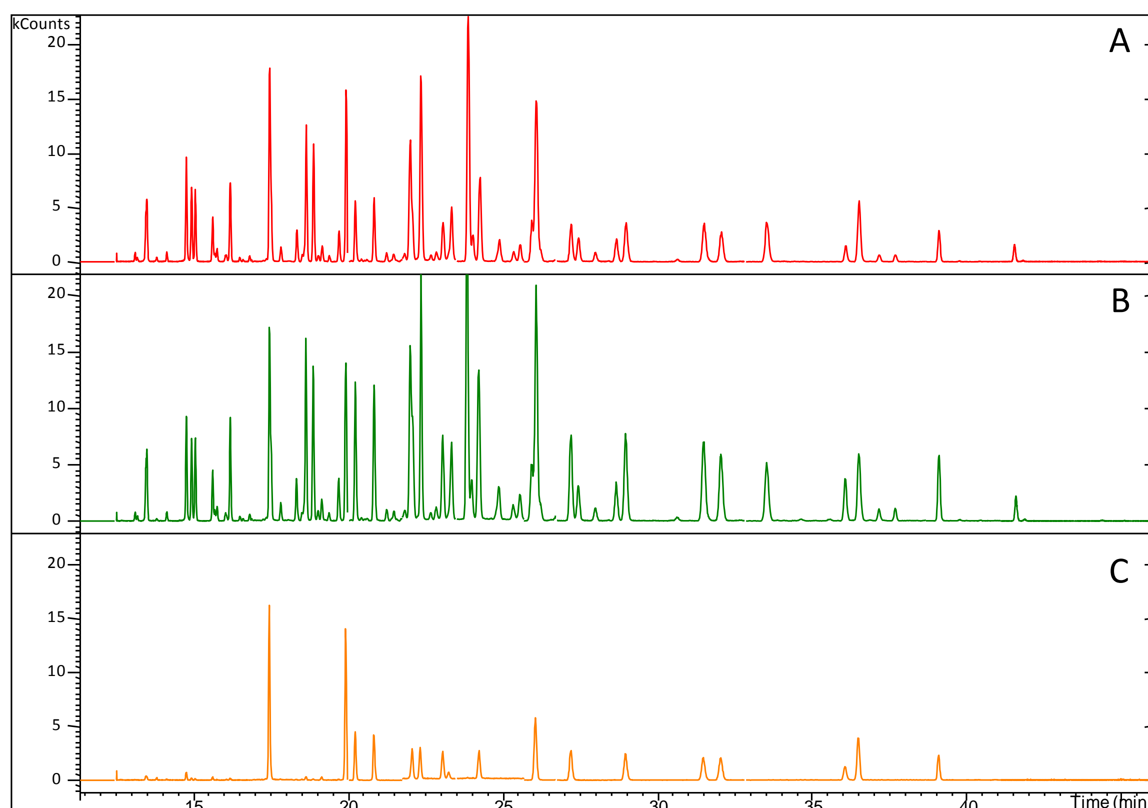

Fig. 3. Chromatograms obtained for the certified fish tissue with (A) the reference protocol, (B) the DPX methodology and (C) its corresponding procedural blank, and final GC–ITD(MS/MS) analysis (Arbitrary units, KCounts).

Conclusions

Analytical protocols in use for routine control and monitoring of trace pollutants in complex solid environmental and food samples are usually large-scale multistep

produces involving separate treatments for extraction and purification/fractionation of the target compounds from coextracted material and close-related compounds. In general, these analytical protocols are tedious, involve relatively large amounts of sample and so are expensive in terms of reagents and time consumption. The development of faster, more cost-effective and environmentally friendly procedures in this field is consequently advisable. The application of size-reduced methods is also highly advisable when dealing with the analysis of size-limited samples. As a consequence, in the last decades miniaturisation has becoming a clear trend also in the environmental field.

This study reports on the feasibility of miniaturised ultrasounds-assisted extraction combined with DPX for the analysis of relevant PCBs in small amounts of biological tissues. Results obtained for the analysis of the levels of endogenous PCBs in non-contaminated matrices demonstrated the practicality of the approach for the determination of the target compounds in a wide variety of solid fat-containing matrices when combined with GC–ITD(MS/MS) for final determination. As a whole, the proposed analytical method represents a fast, simple and cost-effective alternative to large-scale conventional protocols in use for this type of determination. Due to its features, the proposed method for sample preparation has potential for complete automation, which makes to consider it a valuable alternative particularly suitable for the fast analysis/screening of the target compounds in routine analyses.

Acknowledgement

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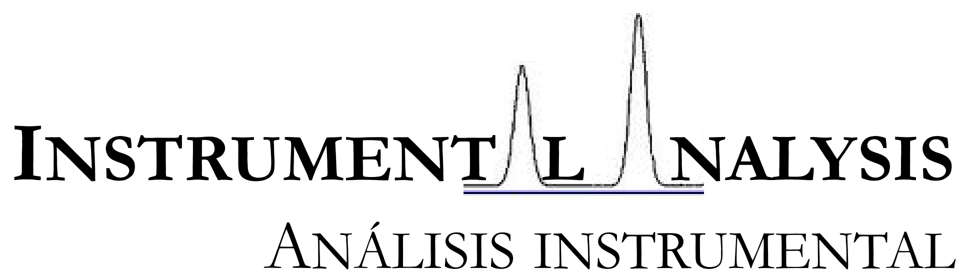
Section – Sección 3.2



Los estudios presentados en la sección anterior han demostrado que la elevada capacidad de resolución que ofrecen los actuales sistemas de GC suele ser suficiente para la determinación inequívoca de los contaminantes orgánicos traza investigados en la presente memoria, aún cuando se encuentren en extractos de elevada complejidad, bien por provenir de procedimientos genéricos de preparación de muestra, bien por los extremadamente bajos niveles a los que deben ser detectados por haber sido obtenidos aplicando procedimientos de tratamiento miniaturizados. En estos últimos casos, el acoplamiento de la GC a detectores selectivos, en especial los MS en sus distintas modalidades, han demostrado ser una alternativa analítica perfectamente válida para buena parte de las aplicaciones propuestas en esta memoria. Sin embargo, la resolución tanto cromatográfica como espectral de ciertos compuestos requieren de resoluciones superiores a las ofrecidas por esas técnicas. En estos casos, las técnicas cromatográficas multidimensiones y, en especial bidimensionales, representan una alternativa analítica de enorme potencial, en especial cuando se emplean en combinación con un detector que ofrezca la posibilidad de confirmación estructural, como es el caso de la GC×GC–ToF MS.

En esta segunda parte del trabajo se plantea aprovechar la elevada capacidad de resolución e identificación ofrecida por la GC×GC–ToF MS para proponer soluciones a problemas analíticos que difícilmente podrían ser resueltos empleando técnicas de GC monodimensionales. Un ejemplo claro de ello es el estudio abordado en la **Sección 3.2.1**, en la que los músculos de atunes obtenidos en la Sección 3.1.1 fueron analizados mediante GC×GC–ToF MS con el fin de profundizar en la caracterización de la presencia de contaminantes organobromados en los mismos. Dadas las características de la metodología empleada en la Sección 3.1.1, el objetivo de aquel estudio fue la evaluación de la presencia en las muestras de ciertos compuestos organobromados seleccionados (*target analysis*). El objetivo en la presente sección es abordar un estudio de caracterización amplia, que incluyera compuestos preseleccionados, pero también otros de los que no se dispusiera de patrones o de información previa, incluyendo desconocidos (*non-target analysis*). Así, la primera parte del estudio consistió en la optimización de las condiciones cromatográficas de separación en GC×GC para la separación completa y simultánea de ciertas familias de

compuestos organobromados, PBDEs, MeO-PBDEs, TBA, MHC-1 y PBHDs, en músculo de atunes del Mediterráneo, prestando especial atención a los problemas de coelución detectados en el estudio precedente con cromatografía monodimensional. Empleando HT-8 × BPX-50 como combinación de columnas y en las condiciones instrumentales finalmente propuestas, se consiguió, en un único análisis, una separación total de los 26 analitos preseleccionados, tanto entre sí como de otros componentes presentes en el extracto. También se conseguían resolver los problemas de coelución que afectaban a la determinación de dos pares de isómeros de MeO-PBDEs al emplear técnicas monodimensionales. Además, la capacidad de la técnica de GC×GC para generar cromatogramas estructurados en las condiciones de trabajo, en combinación con la información estructural proporcionada por el ToF MS, permitió la identificación tentativa en las muestras de atunes de diferentes isómeros de PBDEs, diMeO-PBDEs, diMeO-PBBs y PBBs de los que no se disponían de patrones en el laboratorio. Por último, se identificaron algunos isómeros de PBHDs que no habiendo sido descritos con anterioridad y cuya estructura fue confirmada por GC–QqQ MS.

El trabajo presentado en la **Sección 3.2.2** representa el primer ejemplo descrito en la bibliografía de evaluación de la técnica de GC×GC–ToF MS para la separación y posterior cuantificación de los 15+1 EU PAHs en sedimentos. Estudios previos ya habían expuesto los problemas de separación que existen para ciertos isómeros de PAHs cuando se analizan mediante técnicas monodimensionales. Por ello, en este estudio se probaron dos combinaciones de columnas que permitiesen la separación completa de esos pares y grupos críticos de PAHs. De las dos combinaciones de columnas evaluadas, DB-5 × BPX-50 y HT-8 × BPX-50, se comprobó que la primera proporcionaba, una vez optimizadas las condiciones de separación y modulación, una mejor separación entre los pares/grupos críticos, tanto en disoluciones patrón como en muestras. En las condiciones de separación propuestas, era posible además la determinación inequívoca de dibenzo[*a,c*]antraceno que se resolvía de su isómero dibenzo[*a,h*]antraceno; mientras que para el trifenileno se conseguía una resolución parcial del criseno. El conjunto de la metodología de preparación de muestra combinada con GC×GC–ToF MS se validó mediante su aplicación al material de referencia BCR-535. Finalmente, su aplicación al análisis de sedimentos recogidos en

la bahía de Cádiz permitió identificar varias estaciones afectadas por diferentes procesos contaminantes, alcanzándose en alguna de ellas niveles para ciertos isómeros de PAHs susceptibles de ocasionar daños biológicos en especies acuáticas de la zona investigada. Por último, en este trabajo se discute la posibilidad de emplear la representación de burbujas normalizadas para la inspección rápida de los resultados obtenidos al analizar muestras reales y para la identificación tentativa del potencial origen de los PAHs detectados en base a su perfil.

3.2.1. Cromatografía de gases bidimensional acoplada a espectrometría de masas con analizador de tiempo de vuelo para la identificación de compuestos organobromados en atunes[1]

Abstract

This study evaluates comprehensive two-dimensional gas chromatography (GC×GC) coupled to time-of-flight mass spectrometry (GC×GC–ToF MS) for the simultaneous analysis of several classes of organobromines (OBs), including polybrominated diphenyl ether (PBDEs), polybrominated biphenyl (PBBs), methoxylated PBDEs (MeO-PBDEs), several halogenated naturally-produced compounds (HNPs) and eight novel brominated flame retardants (NBFRs), polybrominated hexahydroxanthene derivates (PBHDs), 2,4,6-tribromoanisole and a mixed halogenated compound (MHC-1), in bluefin tuna muscles. The proposed methodology maximised separation of both within and among OB families, and among these and other halogenated micropollutants detected in these samples and co-extracted matrix components. Special attention has been paid to solve co-elution problems observed during the analysis of OBs with one-dimensional GC-based techniques. Satisfactory separation among several relevant PBDEs and MeO-PBDEs has been obtained allowing their unambiguous determination in a single run. Additional studies were conducted to identify selected NBFRs and HNPs. 2,4-Dibromoanisole, a dibromophenol isomer, and hexabromobenzene were identified in the investigated samples. Several new tri- and tetra-BHD derivates were also identified, indicating that these compounds could apparently exist as structured families in nature. In addition, a tetrabrominated diMeO-biphenyl and two tetrabrominated diMeO-BDEs were also tentatively identified.

Keywords: GC×GC, ToF MS, organobromines, emergent contaminants, identification, fish analysis

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[1] M. Pena-Abaurrea, A. Covaci, L. Ramos. Comprehensive two-dimensional gas chromatography-Time-of-Flight mass spectrometry for the identification of organobrominated compounds in Bluefin tuna. *J. Chromatogr. A*. **2011**, *1218* (39) 6995.

1. INTRODUCTION

The feasibility of one-dimensional gas chromatography (GC) systems, especially when combined with MS detectors, for unambiguous determination of relevant congeners within complex families, such as polychlorinated biphenyls (PCBs) *(1)*, polybrominated diphenyl ethers (PBDEs) *(2)* and polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs) *(3)* has been demonstrated. In these studies, either an exhaustive clean-up and fractionation of the extracts or several GC runs *(4)* may be required for complete resolution of all target analytes, especially when different classes of compounds have to be determined simultaneously. As a consequence, most of these studies are target-orientated and information concerning the possible presence of other relevant known and especially unknown micropollutants is lost. In this context, comprehensive two-dimensional gas chromatography (GC×GC), in particular when combined with time-of-flight mass spectrometry (GC×GC–ToF MS), can be considered an useful analytical tool simultaneously contributing to sample treatment simplification and preserving complete information regarding non-targeted compounds *(5-7)*. The feasibility of the technique to provide structured chromatograms is an additional feature that efficiently contributes to the identification of analytes and families of analytes for which standards are not available and so to the (virtual) identification of unknown compounds when only mass spectral information is available.

PBDEs have largely been investigated during the last decades because of their potential adverse effects in the environment and in humans *(8-11)*. Some PBDE-related compounds, such as methoxylated PBDEs (MeO-PBDEs), are also increasingly being evidenced at levels similar to those of PBDEs in marine matrices, including top-predators *(12-14)*. MeO-PBDEs can be formed by direct PBDE metabolism *(15)*, but they can also have a natural origin *(16)*. More than 4000 halogenated naturally-produced (HNPs) compounds have been recognised to date. However, only a reduced number of these compounds have been identified at measurable levels in environmental matrices, most frequently in the marine environment *(17-19)*. In fact, the polybrominated hexahydroxanthene derivates (PBHDs), 2,4,6-tribromoanisole (TBA), a mixed halogenated monoterpene

compound (MHC-1) and MeO-PBDEs are among the most frequently HNPs detected in marine biota *(20)*.

Up to now, the number of studies dealing with the simultaneous screening of different OB families is rather small *(4, 21-23)*. These studies have considered new brominated flame retardants (NBFRs), methoxylated brominated biphenyl congeners (MeO-PBBs), methoxylated phenoxyanisoles (mono and di-MeO-PBDEs) and several organohalogenated methyl and dimethyl bipyrroles (MBP and DBPs) *(24-25)*. These databases provided a useful tool for the tentative identification of compounds for which standards are not available. To the best of our knowledge, reports compiling extensive information related to the elucidation of these new organohalogens, i.e. reporting elution order, proposed analytical structure and mass spectrum information, are scarce in the literature *(4, 22, 26)*.

Bluefin tuna (*Thunnus thynnus*) is an ecotoxicologically relevant species due to its particular feeding and migrations habits *(27)*. In this species, the quantity and variety of its feed intake directly depends on the mobility and age of the individuals *(28)*. In a previous paper, the presence of different anthropogenic and naturally-produced OBs in tuna samples was reported *(20)*. The present work is focused on the complete separation of all these OBs of different origins via GC×GC–ToF MS paying special attention to the chromatographic resolution of critical pairs coeluting in one-dimensional GC-based analyses. Tentative identification of other NBFRs and elucidation of some no previously described HNPs detected in the investigated tuna samples was also carried out.

2. MATERIAL AND METHODS

2.1. Sample collection and preparation

Bluefin tunas (n=26) were collected during 2003 in the Mediterranean Sea. Samples included 12 males and 14 females, with ages ranging 2-13 years old. Tunas of wild and aquaculture origin were included in the present study. Samples were preserved and prepared as detailed by Pena-Abaurrea et al. *(20)*. In brief, 3 g of muscle were extracted in an automated Soxhlet for 2 h with 100 mL of *n*-

hexane:acetone (3:1, v/v). Fatty extracts were purified by elution through acid silica, concentrated until incipient dryness under a slightly nitrogen stream and reconstituted in 100 µL *iso*-octane containing the corresponding syringe standard (1,2,3,4-tetrachloronaphtalene, TCN). BDE-77 was used as internal standard for analysis quality control.

2.2. Chemicals

PBDEs (# 28, 49, 47, 66, 100, 66, 99, 155, 154 and 153) were purchased from Wellington Laboratories (Guelph, ON, Canada), MeO-PBDEs (2-MeO-BDE 3, 2´-MeO-BDE 7, 3´-MeO-BDE 7, 4´-MeO-BDE 17, 2´-MeO-BDE 28, 3´-MeO-BDE 28, 3-MeO-BDE 47, 6-MeO-BDE 47, 5-MeO-BDE 47, 4´-MeO-BDE 49, 2´-MeO-BDE 68, 4-MeO-BDE 90 and 6-MeO-BDE 99) were from Wellington Laboratories and Accustandard (New Haven, CT) and TBA was obtained from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). 2,7-Dibromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1*H*-xanthene (tri-BHD), 2,5,7-tribromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1*H*-xanthene (tetra-BHD) and (1*R*,2*S*,4*R*,5*R*,1´*E*)-2-bromo-1-bromomethyl-1,4-dichloro-5-(2´-chloroethenyl)-5-methyl-cyclohexane (MHC-1) standards were kindly provided by Prof. W. Vetter (University of Hohenheim, Germany). A PCB standard solution (# 28, 52, 95, 101, 77, 149, 123, 118, 114, 153, 132, 105, 138, 126, 183, 167, 156, 157, 180, 169, 170, 189 and 194; purchased from Dr. Ehrenstorfer Laboratories), two PBDE mix standards (BDE-MXA containing PBDEs # 47, 99 and 153 and BDE-MXB containing PBDEs # 28, 154 and 183; from Wellington Laboratories, Ontario, Canada) were used for method development.

2.3. GC×GC analysis

Identification of the studied OBs in the purified extracts was performed using a Pegasus IV (Leco Corp., St. Joseph, MI) consisting of a modified Agilent 6890 GC and a ToF-based mass spectrometer with electron ionization. Chromatographic conditions were optimised to ensure complete separation of the target compounds from co-extracted matrix components and from other organohalogens usually

presented in this type of matrix in a single chromatographic run. Once optimised, samples were injected in the hot splitless mode (1 μL, 300 ºC, splitless time 2.0 min) in a HT-8 × BPX-50 column setup (30 m × 0.25 mm i.d. × 0.25 μm film thickness and 1.6 m × 0.1 mm × 0.1 μm, respectively). Both columns were purchased from SGE (Melbourne, Australia). The temperature in the first oven was programmed from 80 ºC (2.5 min) to 190 ºC at a rate of 15 ºC/min and then to 300 ºC (20 min) at 3 ºC/min. The second dimension oven was programmed to follow the main oven but with an initial temperature of 110 ºC (2.5 min). The oven was then heated to 210 ºC at a rate of 15 ºC/min and raised to 300 ºC (20 min) at 3ºC/min. Helium was used as carrier gas (constant pressure, 31 psi). A nitrogen quad-jet dual-stage cryogenic modulator was used for sample focusing and injection in the second dimension column. The temperature of the modulator was set 40 ºC above that of the secondary oven. Final hot and cold pulses were optimised as follows: two 0.60 s hot pulses with 1.90 s cold pulses between stages. The transfer line temperature was set at 295 ºC.

2.4. ToF MS detection

The ion source temperature was set at 250 ºC. MS detection (electron ionization was performed in full scan; the ion energy was 70 eV, the voltage was 1800 eV and the data acquisition rate 100 Hz. Standards spectra were acquired in the *m/z* 50 to 750 range using ChromaToF software. These spectra were used to create a user library. For real-life samples, the acquisition ranged *m/z* 200-750.

2.5 GC–QqQ MS confirmation

Confirmation of PBHDs and di-MeO-PBDEs in the purified extracts was performed by using an Agilent 7000 GC-MSMS system using a HP-5ms column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). Extracts were injected in the hot pressure pulsed splitless mode (1 μL, 295 ºC, splitless time 1.25 min, time pulse 1.25 min, pressure pulse 20 psi). The oven temperature was programmed from 90 ºC (1.25 min) to 300 ºC (10 min) at a rate of 15 ºC/min. Helium was used as carrier gas at constant flow (1 mL/min). The transfer line temperature was set at 300 ºC. The ion source temperature of the MS was set at 230 ºC and electron ionization was used at

an ion energy of 70 eV. Acquisition was done in multiple reaction monitoring (MRM) using the most intense transitions. Collision energy was 10 V and the dwell time was 50 ms.

3. RESULTS AND DISCUSSION

3.1. GC×GC–ToF MS separation of selected major organobromines

On the basis of previous investigation *(6)*, HT-8 × BPX-50 was selected as column set. In this column combination, compounds containing bromines showed a stronger retention in the second dimension that those containing chlorines, which resulted in an adequate separation among both types of families in the contour plot and, in particular, among PCBs and PBDEs. Several experiments were carried out in an attempt to minimise the broadening observed in the peaks of some high molecular weight OBs in this column set and by keeping the initially proposed conditions. An increase in the final temperature of the main oven from 300 ºC to 310 ºC did not have the expected narrowing effect for these analytes and, on the contrary, resulted in column bleeding. Increasing the temperature of the transfer line above 295 ºC (investigated range, 245-305 ºC) had a similar effect. Consequently, the experimental conditions were kept similar to those described in *(6)*. Regarding the injection conditions, the application of pulse pressures of 20-51 psi during 1.5 min did not appear to modify the efficiency of this step as compared to results obtained by 2.0 min splitless injection at 300 ºC. Therefore, these latter injection conditions were selected for subsequent experiments. Finally, a modulation period of 6 s with a 0.6 s hot pulse were proposed as optimum because these conditions allowed proper focusing and reinjection of the analytes eluting from the first dimension into the second one, preserved in all instances the separation achieved in the first column and avoided wrap-around.

The PBDE standard solution, two different MeO-PBDE stocks containing 13 congeners each, a mixture including the tri-BHD and tetra-BHD isomers and the individual MHC-1 and TBA standards were then separately injected onto the GC×GC–ToF MS system under finally selected experimental conditions. The

respective retention times of the compounds were exported to Microsoft Office Excel to reconstruct the bidimensional contour plot shown in Fig. 1. In this figure, the investigated OBs have been grouped in three families to facilitate their identification: PBDEs, MeO-PBDEs and HNPs (which include the PBHD isomers, MHC-1 and TBA). The retention times of the investigated analytes and the characteristic masses of these analytes are summarised in Table 1. The 26 OBs considered in this part of the study were satisfactorily separated from each other in a single chromatographic run. Besides, wrap-around was not apparent, which can be considered a convenient feature of the optimised method for further application to real-life samples.

As somehow expected *(6)*, HNPs eluted in the same region of the contour plot as PBDEs and were completely separated from other related (chlorinated) persistent pollutants. MHC-1 and TBA eluted within the first 30 min of the chromatographic run, while MeO-PBDEs eluted at longer retention times than the corresponding PBDEs in both dimensions.

Interestingly, some critical analyte pairs that typically co-elute in one-dimensional GC-based approaches *(20)* were satisfactorily separated on HT-8 × BPX-50 under the proposed experimental conditions. Congeners 4´-MeO-BDE 17, 3´-MeO-BDE 28 and BDE 49 have been found to co-elute on a DB-5 column *(20)*. Using HT-8 × BPX-50, congeners 4´-MeO-BDE 17 and 3´-MeO-BDE 28 were separated from each other and from BDE 49, as shown in Fig. 1 B.2. for a tuna sample. Although 4´-MeO-BDE 17 was not detected in the investigated tunas (Fig. 1 B.2), the analysis of standards demonstrated the satisfactory chromatographic separation provided by the developed GC×GC method (Fig. 1 B.1). Similarly, BDE 100, 4´-MeO-BDE 49 and 5´-MeO-BDE 47, which have been found to co-elute in one-dimensional GC–MS, were also separated on HT-8 × BPX-50 (Fig. 1 C.1). Typical examples of tuna samples in which 4´-MeO-BDE 49 and 5´-MeO-BDE 47 were individually detected are presented in Fig 1 C.2 and C.3, respectively. Interestingly, these two PBDE metabolites were found to accumulate dissimilarly in females and males. These congeners showed measurable levels only in 7 out of the 26 investigated tunas and, in these samples, 4´-MeO-BDE 49 was either the only detected congener or the one

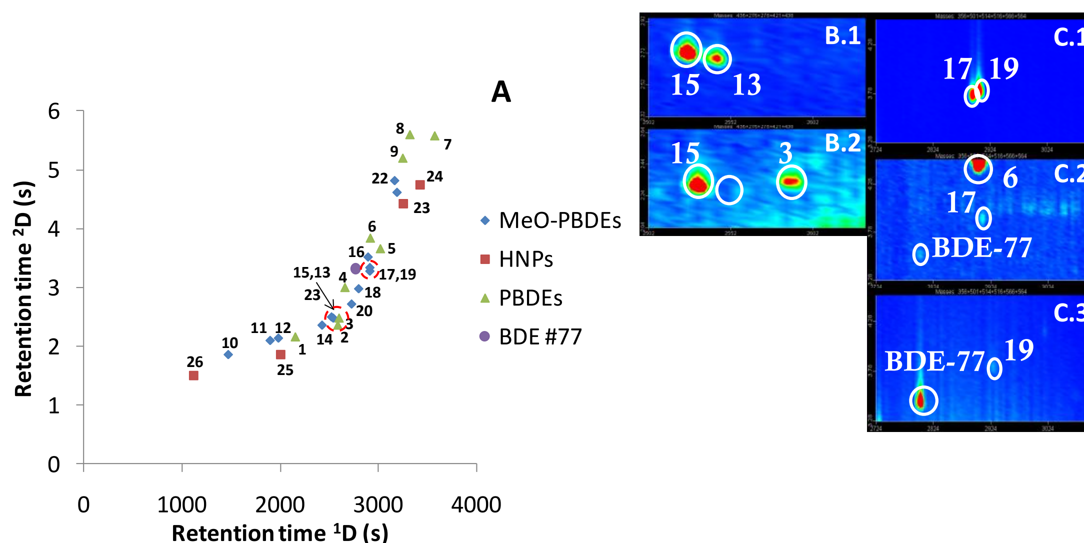

Fig. 1. (A) Reconstructed chromatogram of the 26 studied OBs under finally proposed GC×GC–ToF MS conditions. Separation obtained between 4´-MeO-BDE 17 and 3´-MeO-BDE 28 (B.1) in a standard solution and (B.2) in a real tuna sample. (C) Separation obtained for 4´-MeO-BDE 49, 5´-MeO-BDE 47 and BDE 100 (C.1) in a standard solution and (C.2 and C.3) in two real tuna samples. See Table 1 for peak identification.

found at higher concentration. In addition, 5 out of these samples were female. Congener 5´-MeO-BDE 47 was only found in one female tuna. This particular trend could be associated to a special metabolism pathway for males, but further elucidation is outside the scope of the present study.

Apart from this observation, in general, similar accumulation patterns were found for PBDEs, MeO-PBDEs and the studied HNPs (tri- and tetra-BHD isomers, TBA and MHC-1) in all investigated tunas. Nevertheless, some characteristic trends were observed. In wild tuna individuals, the highest PBDE and MeO-PBDE levels were found in the oldest individuals (i.e., those more than 4 years old), while HNP levels were similar in all individuals, irrespective of their age *(20)*. In addition, MeO-PBDEs and HNPs showed higher levels in wild than in farmed tunas, a result that agrees with previous observations *(17)*, and it is probably a consequence of their direct exposure to the natural sources and higher mobility.

Due to the feasibility of HT-8 × BPX-50 to generate roof-tile subclasses, a number of isomers belonging to the several investigated OB families were detected in the high-level samples, i.e. in general, those with the highest fat content. Fig. 2 A and

Table 1. Peak identification number, retention time in the first and second dimension and characteristic *m/z* ions of the individual OB standards included in the study.

	Peak No.	^1t_R, ^2t_R (s)	*m/z*
PBDEs			
BDE 28	1	2160, 2.16	406, 408
BDE 47	2	2604, 2.48	484, 486
BDE 49	3	2592, 2.36	484, 486
BDE 66	4	2664, 3.00	484, 486
BDE 99	5	3024, 3.66	564, 566
BDE 100	6	2922, 3.84	564, 566
BDE 153	7	3576, 5.58	642, 644
BDE 154	8	3324, 5.60	642, 644
BDE 155	9	3252, 5.20	642, 644
BDE 77 (IS)[a]		2772, 3.32	484, 486
MeO-PBDEs			
2´-MeO-BDE 3	10	1476, 1.86	278, 280
2´-MeO-BDE 7	11	1902, 2.10	356, 358
3´-MeO-BDE 7	12	1986, 2.14	356, 358
4´-MeO-BDE 17	13	2544, 2.48	436, 438
2´-MeO-BDE 28	14	2430, 2.36	436, 438
3´-MeO-BDE 28	15	2526, 2.50	436, 438
3-MeO-BDE 47	16	2898, 3.52	516, 518
5´-MeO-BDE 47	17	2916,3.28	514, 516
6-MeO-BDE 47	18	2802, 2.98	514, 516
4´-MeO-BDE 49	19	2916, 3.34	514, 516
2´-MeO-BDE 68	20	2730, 2.72	516, 518
4-MeO-BDE 90	21	3192, 4.62	594, 596
6-MeO-BDE 99	22	3168, 4.82	594, 596
PBHDs			
Tri-BHD	23	2400, 2.66	466, 468
Tetra-BHD	24	3258, 4.42	546, 548
MHC-1	25	2004, 1.86	247, 396
TBA	26	1122, 1.50	344, 346

[a]IS, internal standard

B show a zoomed area of the fragmentograms obtained for a wild tuna after extracting the *m/z* values selective for PBDEs and PBHDs. The roof-tile structure observed for PBDEs (Fig. 2 A) would allow the tentative identification of some additional tetra- and penta-BDE isomers on the base of previously reported data

concerning PBDE elution order in different stationary GC phases with GC and GC×GC systems *(29-30)*. Similarly, such a roof-tile structure, in combination with MS information, allowed the identification of several new tri- and tetra-BHD isomers in tuna muscle (white circles in Fig. 2 B). To the best of our knowledge, this is the first time that tri- and tetra-BHD isomers other than 2,7-dibromo-4a-bromomethyl and 2,5,7-tribromo-4a-bromomethyl BHD derivates (indicated with a yellow circle in Fig. 2 B) have been identified in real-life samples. The new compounds showed mass spectra similar to those registered for the two available standards (typical examples are shown in Fig. 2 C and D) and to those described in the literature for PBHDs *(18)* demonstrating, in general, a good conservation of the ion ratios among the main detected ion fragments. Differences in the ion ratios were only observed for early eluting isomers, for which the characteristic ion $[M\text{-}Br]^+$ was predominant. This tentative identification was further confirmed by GC–QqQ MS analysis (Fig. 3). These results would suggest that PBHDs could exist in nature as families of isomers similarly to that observed for other organohalogenated analyte classes.

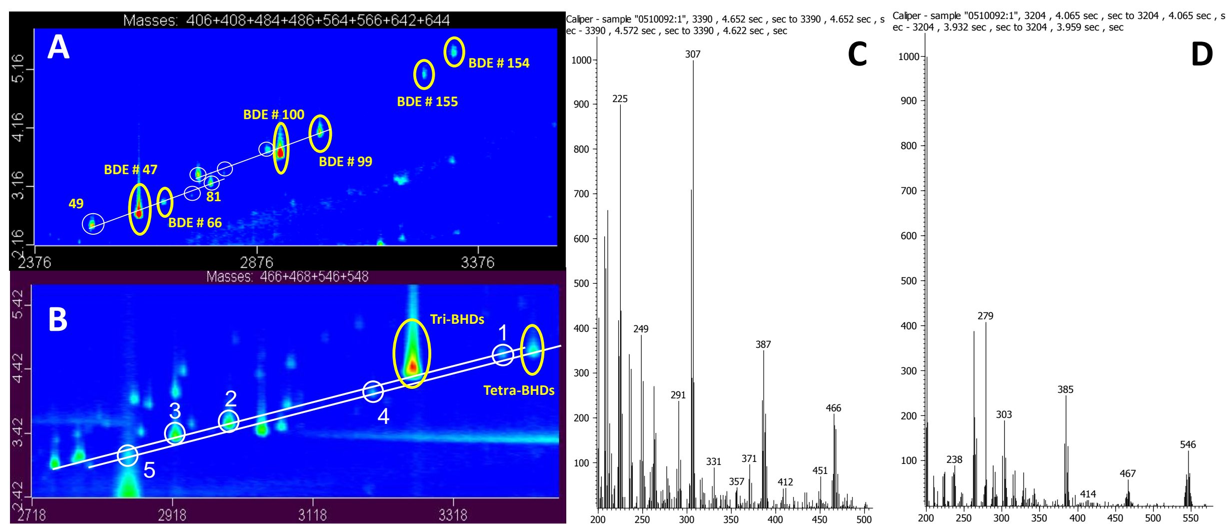

Fig. 2. Elucidation of emergent OBs: (A) tentative identification of PBDE isomers after extracting selective *m/z* ions, (B) roof-tile structure observed for PBHD isomers, and mass spectra of tentative (C) tri-BHD (peak no.1), and (D) tetra-BHD isomers (peak no. 4) as acquired for real-life samples.

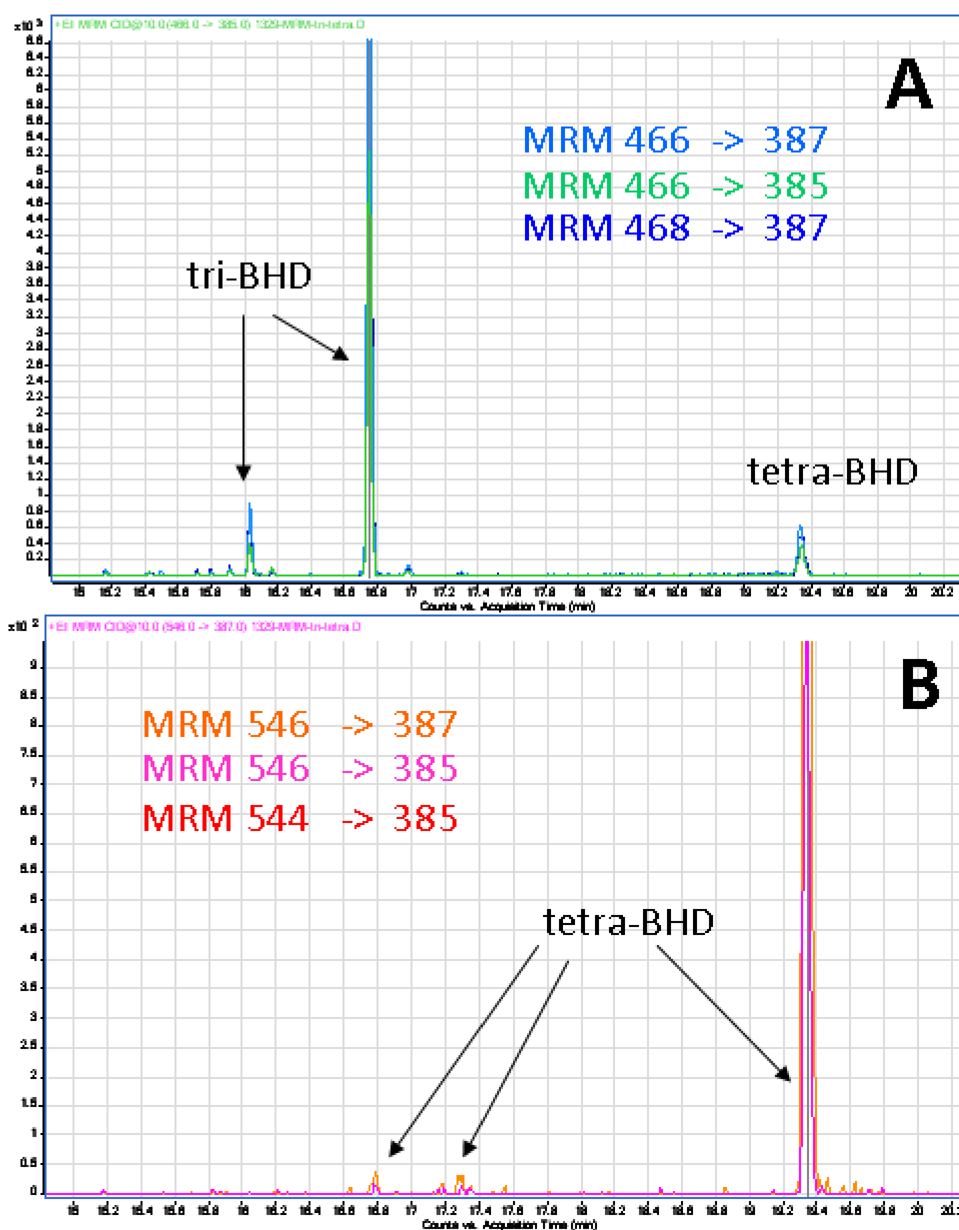

Fig. 3. Confirmation of the tentatively identified minor (A) tri-BHD and (B) tetra-BHD isomers in tuna samples by GC–QqQ MS.

3.2. Identification of other organobromines

Additional experiments were carried out to investigate the presence of other anthropogenic and naturally-produced OBs in the investigated tuna samples. A recent review about the analysis, environmental fate and behaviour of new BFRs (NBFRs) was used to select the target compounds *(21)*. Eight NBFRs, including hexabromobenzene (HBB), pentabromoethylbenzene (PBEB), pentabromotoluene (PBT), tetrabromobisphenol A diallyl ether (TBBPA-DAE), tetrabromophthalic

anhydride (TPA), 2,4,6-tribromophenol (TBP), 2,4,6-tribromophenol allyl ether (ATE) and 2,4,6-tribromophenol-2-bromoallyl-ether (BATE), were considered.

Analytes were investigated on the base of their retention time, their chromatographic behavior, by comparison of the registered mass spectra with those reported in the literature (when available) and by mass spectrum interpretation.

Some of these NBFRs (e.g., PBT, PBEB, TBBPA-BAE, TPA, TBP, ATE and BATE) were not found at detectable levels in the studied samples. However, a noticeable peak became visible in the contour plot when extracting *m/z* 551.5, corresponding to the HBB molecular ion. Both the retention times (analyte peak eluted just before PBDE # 47) and the corresponding acquired mass spectra, with a characteristic fragmentation pattern indicating successive bromines losses, matched with those expected for HBB *(22)*. The peak was consequently assigned as HBB (Table 2).

A structured group of compounds were observed when selecting *m/z* 463.7 (molecular ion of TPA). A careful inspection of the mass spectra confirmed that the detected analytes were actually tri-BHD isomers (see previous Section 3.1. and Fig. 2 B) and that TPA was not detected in the investigated samples. Similarly, when extracting *m/z* 624, corresponding to TBBPA-DBPE, a group of roof-tiled analytes were observed in the contour-plot in a region close to the tri- and tetra-BHDs previously detected (Fig. 4). Mass spectra interpretation allowed the identification of these compounds as hexabrominated biphenyls, hexa-BBs (Fig. 4 B). Extraction of the PBB homologue characteristic masses confirmed the presence of mono- to nona-BB congeners. As an example of the typical results obtained, the roof-tile structure observed for tetra- to hepta-BB is shown in Fig. 4 A.

Additionally, two extra HNPs, a DBP isomer and 2,4-DBA, were detected in the studied samples (Table 2). Their relative elution times as compared to those of other positively identified OBs *(22)* and their mass spectra confirmed the nature of these analytes, which eluted within the first 15 min of the chromatographic run.

Finally, two families of natural brominated dimethoxy-derivatives were evaluated. Polybrominated dimethoxybiphenyls (PBDMBs) have recently been identified in marine mammals from Australia *(31)*. On the base of mass spectrometric data

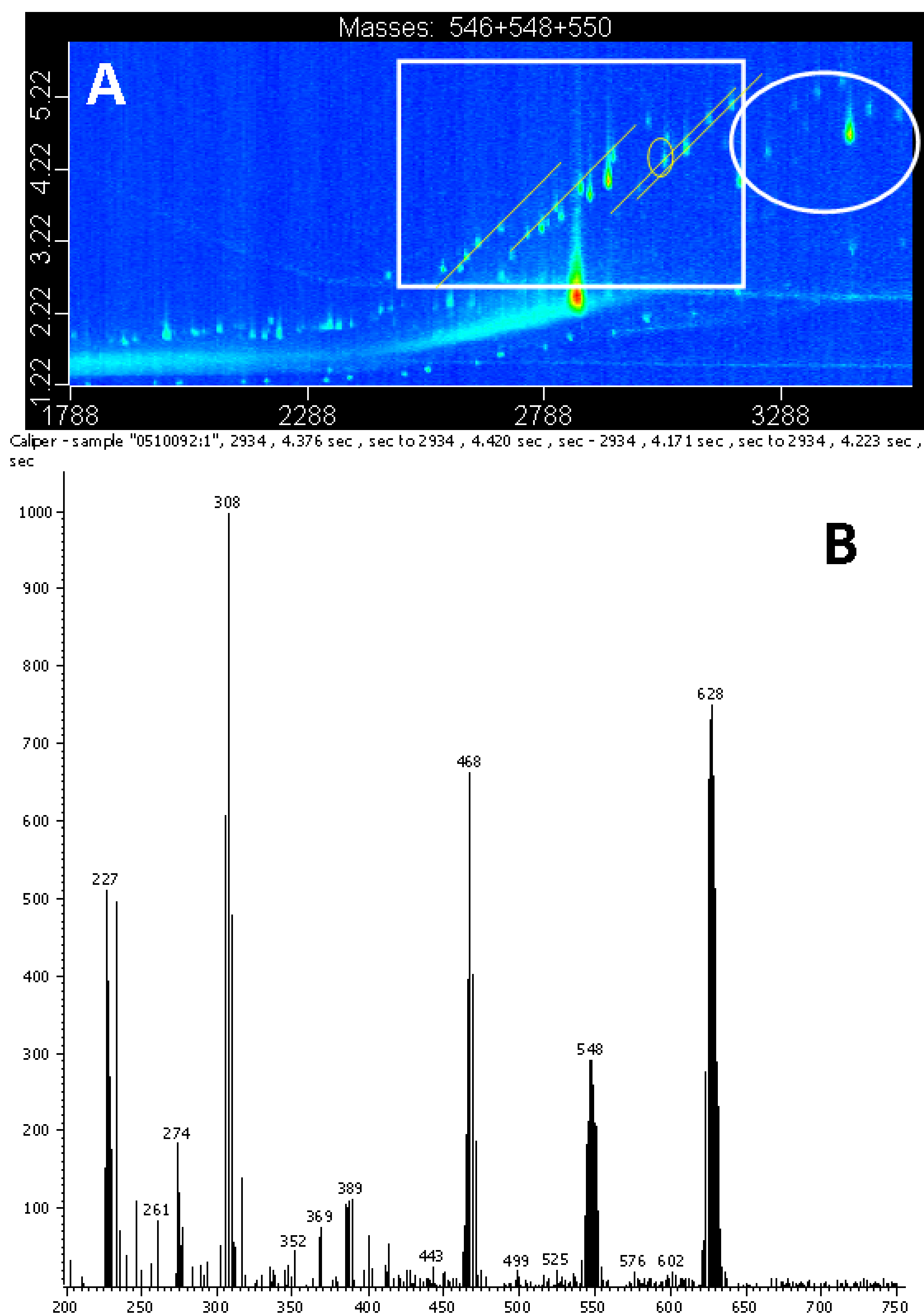

Fig. 4. (A) Roof-tile structure observed for PBBs with indication of the elution area for this family, and (B) typical mass spectrum obtained for an hexa-brominated isomer (indicated with a yellow circle in the contour-plot). The white circle shows the elution are for tri- and tetra-BHDs.

Table 2. Retention time in the first and second dimension, characteristic *m/z* ions, and name, acronym, molecular formula and assigned class of emergent OBs detected in the investigated tuna extracts.

$^{1}t_{r}$ (s)	$^{2}t_{r}$ (s)	*m/z*	Compound name	Acronym	Molecular formula	Type of compound	Ref
816	1.22	252	Dibromophenol isomer	DBP	$C_4H_4Br_2O$	HNPs	*(22)*
948	1.32	266	2,4-dibromoanisole	24-DBA	$C_7H_6Br_2O$	HNPs	*(22)*
2574	2.83	551.5	Hexabromobenzene	HBB	C_6H_6	BFR	*(21)*
2742	3.10	530	2,2´-dimethoxy-3,3´,5,5´-tetrabromobiphenyl	2,2´-diMeO-BB 80	$C_{14}H_{10}Br_4O_2$	HNPs	*(26,31)*
2886	3.88	546	Tetrabrominated dimethoxy-biphenyl ether isomer	Tetra-diMeO-BDE	$C_{14}H_{10}Br_4O_3$	HNPs	-
2922	4.70	546	3,5-dibromo-2-(3´,5´-dibromo-2´-methoxy) phenoxyanisole	2,6´-diMeO-BDE 68	$C_{14}H_{10}Br_4O_3$	HNPs	*(22)*

reported in that study and relative retention time information published in *(22, 26)*, the 2,2´-dimethoxy-3,3´,5,5´-tetrabromobiphenyl (2,2´-diMeO-BB 80) was tentatively identified in the investigated tuna extracts. It should be noted that this congener was found at concentration levels in the range of those of other OBs, such as PBDEs and their most abundant methoxylated derivates. Fig. 5 A shows the zoomed contour plot area where 2,2´-diMeO-BB 80 was identified. Its mass spectrum exhibited the molecular ion, together with a number of characteristic ions (e.g., $[M\text{-}Br]^{+}$, $[M\text{-}Br\text{-}CH_3]^{+}$, $[M\text{-}Br\text{-}OCH_3]^{+}$), as shown in Fig. 5 C. This compound was further confirmed by GC–QqQ MS (Fig. 6). None of the mono-, di- or tri-BDMB isomers found by other authors in marine matrices *(31)* were detected in our samples. A non-identified brominated compound, with an apparent molecular ion similar to that of tetra di-MeO-BB, was found to elute close to this compound (Fig. 5 A). However, GC–QqQ MS analysis confirmed that this later eluting analyte did not belong to this class.

The same procedure was followed to investigate the presence of 3,5-dibromo-2-(3´,5´-dibromo,2´-methoxy)phenoxyanisole (2´,6-diMeO-BDE 68), which has previously been reported in marine samples *(4, 32)*. This isomer was also detected in the studied tuna muscles from the Mediterranean Sea (Fig. 5 B).

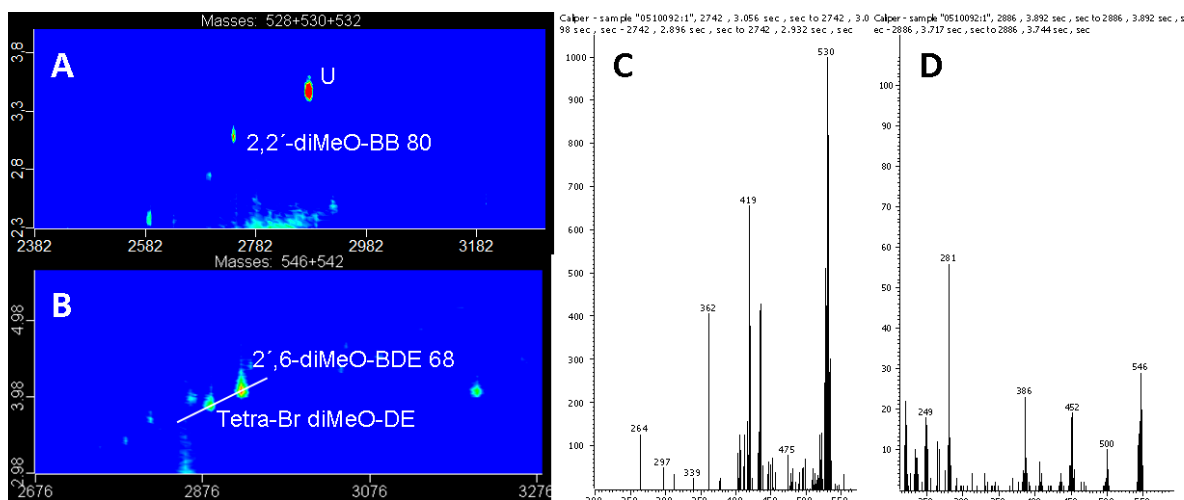

Fig.5. Zoom of the contour-plot areas where (A) tetrabrominated-DMB (2,2´-diMeO-BB 80), and (B) tetrabrominated-DMDEs eluted, and mass spectrum of (C) the tentatively identified 2,2´-diMeO-BB 80 and (D) the non-identified tetra-BDMDE congener. U, unidentified brominated compound.

Interestingly, another compound, with an identical mass spectra was identified (Fig. 5 D) and tentatively assigned as a tetra-diMeO-BDE isomer (Fig. 5 B). No data concerning the elution or identity of this new compound have been found in the literature. However, GC–QqQ MS experiments appear to confirm the identity of these compounds (Fig. 6).

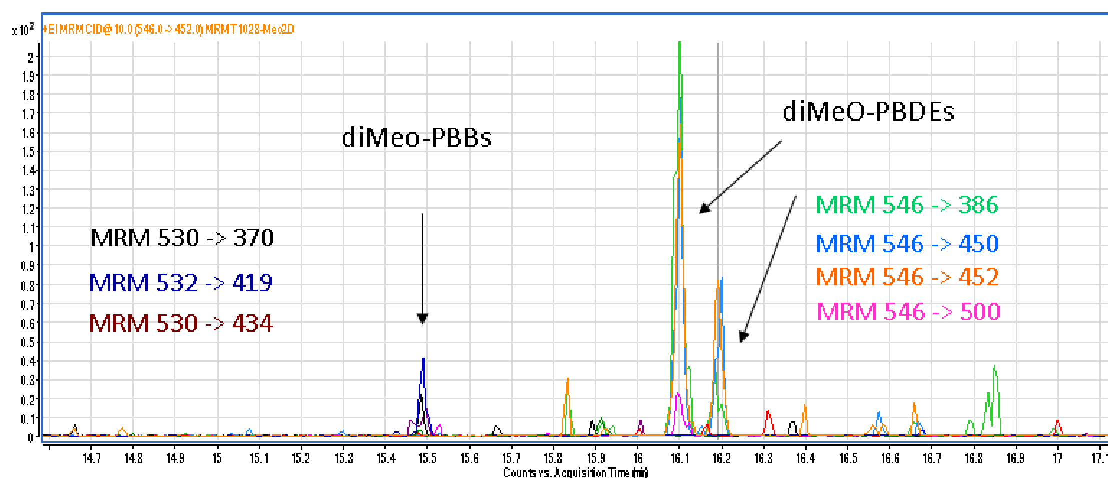

Fig. 6. Confirmation of the tentatively identified diMeO-PBDEs isomers at 16.10 and 16.19 min and a diMeO-PBB isomer at 15.49 min in tuna samples by GC–QqQ MS.

Conclusions

GC×GC–ToF MS has been used for the first time for simultaneous determination of selected environmentally relevant OB families and several NBFRs. The proposed methodology allowed the simultaneous determination of the 26 major OBs, which included PBDEs, MeO-PBDEs and other HNPs, such as PBHD isomers, MHC-1 and TBA. More importantly, the enhanced separation achieved using HT-8 × BPX-50 allowed to obtain detailed information regarding profiles of these micropollutants in tuna samples and unambiguous determination of several critical pairs of analytes coeluting in one-dimensional GC-based approaches.

The mass spectrum identification feasibility offered by GC×GC–ToF MS was employed for the screening of selected NBFRs. Among them, only DBA, DBP and HBB were detected in the tested tuna samples. Regarding HNPs, 2,2´-diMeO-BB 80 and 2´,6-diMeO-BDE 68, two isomers already reported in marine samples, were also detected here. Finally, the extra identification power derived from the use of ToF MS as detector allowed the tentative identification of several tri- and tetra-BHDs and one tetra-DMBDE not previously described in the literature. Subsequent complementary mass spectra confirmation by GC–QqQMS further supports the identification of these OBs.

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3.2.2. EVALUACIÓN DE LA CROMATOGRAFÍA DE GASES BIDIMENSIONAL ACOPLADA A ESPECTROMETRÍA DE MASAS CON ANALIZADOR DE TIEMPO DE VUELO PARA EL ANÁLISIS DE HIDROCARBUROS AROMÁTICOS POLICÍCLICOS EN SEDIMENTOS[1]

Abstract

This study evaluates the feasibility of comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC–ToF MS) for the determination of the 15+1 EU PAHs in sediments. Experimental variables affecting the injection, chromatographic separation and analytical detection of the analytes have systematically been optimised. Under finally proposed conditions, a satisfactory resolution among critical pairs/groups of PAHs, including benz[*a*]anthracene-cyclopenta[*cd*]pyrene-chrysene, the three benzofluoranthene isomers, indeno[*1,2,3-cd*]pyrene-dibenz[*a,h*]anthracene (DahA), and DahA from dibenz[*a,c*]anthracene, has been achieved using DB-5 × BPX-50 as column combination with a run time of 1 h. The feasibility of the method for the analysis of real-life samples has been demonstrated by accurate determination of relevant target PAHs in a certified reference material (harbour sediment BCR-535) and by successful application to sediments sampled from a relevant protected area located in the South of Spain. The low limit of detections (LODs) obtained for most of the targeted PAHs (in the 5.7-60 μg/kg range, as calculated for real samples) guarantied accurate quantification of the target compounds at the low levels expected in this type of pristine matrices. The strong retention experienced by the heaviest dibenzopyrene isomers included in the study resulted in relatively high LODs for these analytes which, however, were detected at concentration levels above the corresponding LOD in some of the analysed sediments. In addition, the enhanced identification power provided by GC×GC–ToF MS for the identification of non-target analytes allowed the tentative identification of a group of polynuclear aromatic thiophenes in some of

[1] M.Pena-Abaurrea, F. Ye, J. Blasco, L. Ramos. Evaluation of comprehensive two-dimensional gas chromatography-time-of-flight-mass spectrometry for the analysis of polycyclic aromatic hydrocarbons in sediments. *Submitted.*

the test samples. Finally, the potential of the use of normalised bubble plots for the fast screening of the potential PAH sources has been demonstrated.

Keywords: GC×GC–ToF MS, PAHs, Sediment analysis

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are worldwide distributed organic micropollutants mainly originated in combustion processes *(1)*. Many PAHs have been classified as either possible or probable carcinogenic for humans by the International Agency for Research on Cancer (IARC) *(2)*, although similarly to other contaminants their toxicity is compound-dependent. Benzo[*a*]pyrene (BaP) was the first PAH to go under regulation after the first evidences of carcinogenesis *(3-4)*. However, legislations were slowly extended to other PAHs as the evidence of their toxicity increased. Present regulations refer to as different matrices as smoke flavouring condensates *(5)*, ambient air *(4)*, foodstuffs *(4-6)*, as well as different types of environmental biotic and abiotic matrices *(7)*. Among the latter, soils and sediments have largely been used for PAH control and monitoring in the environment *(8-10)*. In most of these environmental studies, the so-called 16 Environmental Protection Agency (EPA) PAH list has been used *(10-11)*. Despite the popularity of this EPA list, in recent years, some additional PAHs, including benzo[*c*]fluorene (BcF), cyclopenta[*cd*]pyrene (CCP), 5-methylchrysene (5-MC), benzo[*j*]fluoranthene (BjF), and most of the dibenzopyrenes (DaxP), have attracted especial attention. These compounds have been included in recent Scientific Committee on Food (SCF) recommendations and European Union (EU) regulations *(7)*, where they have replaced the most volatile PAHs considered in the EPA list. Although this 15+1 EU PAH list is mainly orientated to the analysis of foodstuffs and related matrices (e.g., smoke condensates), the consideration of these new (less volatile) compounds arose some challenges from the analytical point of view demanding the development of new methodologies *(12-14)*.

Liquid chromatography with fluorescence detection (LC–FLD) and gas chromatography coupled to mass spectrometry (GC–MS) are techniques most frequently used for the instrumental determination of PAHs in complex food and

environmental samples *(13, 15)*. Both techniques have their own advantages and limitations. LC-FLD allows the separation of some isomers that are difficult to separate by GC and, generally speaking, provides a better performance for the heaviest PAHs as it avoids the discrimination problems observed for these analytes during GC injection *(14, 16)*. GC-MS provides superior resolution capabilities, improved sensitivity and the possibility of further chemical structure confirmation. However, some isomers or structurally related compounds should be chromatographically separated before detection for proper identification and quantification. Critical coelutions in the most commonly used GC stationary phases for EPA PAH monitoring (namely, 5% and 50% phenyl-methyl polysiloxane) include CCP, benz[*a*]anthracene (BaA) and chrysene (Chr) (molecular weight, MW, 226, 228); benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF) and BjF (MW, 252); and indeno[*1,2,3-cd*]pyrene (IcdP), benzo[*ghi*]perylene (BghiP) and dibenz[*a,h*]anthracene (DahA) (MW, 276, 278) *(14)*. Neither these GC phases nor any other commercially available stationary phases provide a complete and simultaneous separation among these compounds *(11, 14, 17)*, something that has frequently resulted in bias and overestimation of the reported concentrations *(13)*.

Under these circumstances, the use of comprehensive two-dimensional gas chromatography (GC×GC), which takes advance of the combined used of two GC phases with different selectivity *(18)*, can be advantageous. However, up to now, only a limited number of studies concerning GC×GC analysis of individual environmentally relevant PAHs have been published *(19-22)*. A possible explanation could be the convenience of using MS (i.e., in this case, preferably time-of-flight mass spectrometry, ToF MS) instead of flame ionization detection as GC×GC detector for this type of target-orientated trace analysis in complex environmental and food matrices. Another one could be the difficulty of reliability integration and quantification *(20-21)*.

This study evaluates for the first time the feasibility of GC×GC–ToF MS for the analysis of the 15+1 EU PAHs in complex environmental matrices, such as sediments. Once optimised, the proposed method has been applied to the determination of the target compounds in sediments from an ecologically relevant area subjected to different types of pollution inputs in order to evaluate the possible

influence of divergent matrix components in the identification and quantitation of the test analytes. The possibility of using GC×GC–ToF MS as a tool for the fast screening of PAHs in complex matrices and for the recognition of distribution patterns associated to different PAH sources have been evaluated.

2. Materials and methods

2.1. Chemicals and sample preparation

A working stock solution was prepared from the PAH mix 183 (Dr. Ehrenstorfer Laboratories, Augsburg, Germany) containing 1000 ng/mL of each compound in isooctane. This solution was used for further dilution for method development and optimisation. In brief, sample preparation consisted of the microwave assisted extraction (MAE) of 0.5 g of the investigated sediment sample with 30 mL of a 1:1 (v/v) mixture of *n*-hexane:acetone at 115 ºC for 15 minutes at 100% power. The cooled extract was filtered through a paper filter (20-25 μm, Grade 41, Ashless circles 90 mm, Whatman Schleicher&Schuell, Maidstone, UK). 1-mL of this filtered extract was purified by elution through an Isolute PAH HC solid-phase extraction cartridge (1g/6 mL, Biotage, Uppsala, Sweden). The target analytes were eluted from the cartridge with 6 mL of *n*-hexane:isopropanol (1:0.034, v/v), concentrated under a gentle nitrogen stream and reconstituted in isooctane for final instrumental analysis. A certified reference material (CRM), harbour sediment BCR-535 (IRMM, Geel, Belgium), was used for validation of the optimised GC×GC–ToF MS methodology.

Sediments were collected from the Sancti-Petri Channel (Cadiz, Spain) during the spring of 2004. This is a shallow (3-6 m deep) 18-km channel located within the Natural Park of Cadiz Bay which connects the southern part of the bay with the Atlantic Ocean. Sediment samples were taken at 17 stations (ST) located along the channel in order to characterize the possible PAH inputs in the channel (Fig. 1). Sampling points included a discharge area for untreated domestic water of a 88,000 inhabitants town that theoretically ceased four months before sampling (ST 3), an area under the influence of the Chiclana river and suspected of receiving uncontrolled dumps from a close industrial park (ST 8), an area close to a small leisure harbour and so influenced by boat traffic and activities (ST 13), and a reference point located

nearer to the sea and with intense water renovation (ST 17). Sediments were collected with a van Veen grab, preserved at 4 °C until transfer to the laboratory, and then frozen at -20 °C until analysis. Sediments were freeze-dried and sieved (63 μm mesh) before sample preparation.

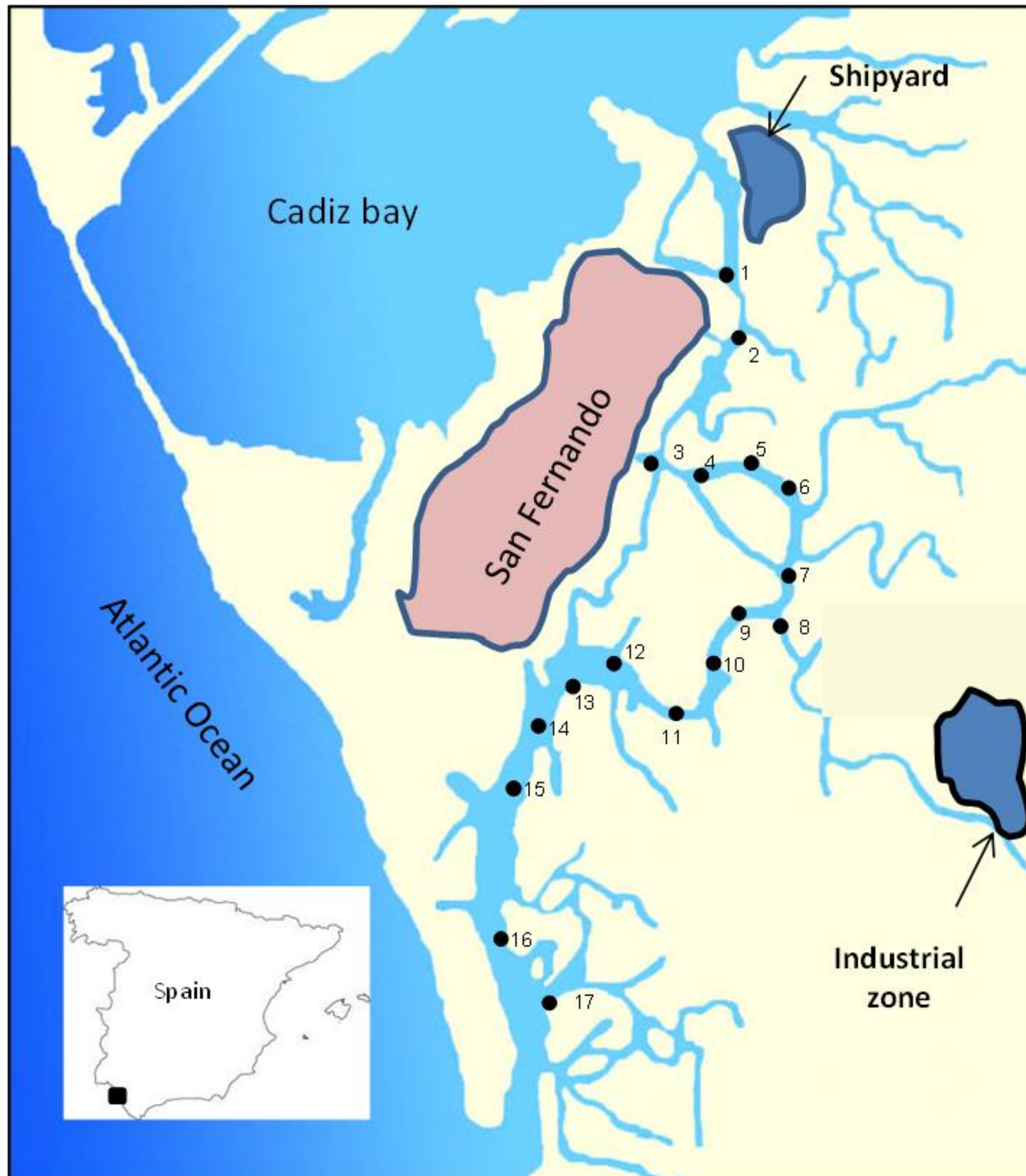

Fig. 1. Map of the surrounding area of Sancti-Petri Channel and the stations sampled in the present study.

2.2 GC×GC–ToF MS analysis

Identification of the studied PAHs in the purified extracts was performed using a Pegasus 4D (Leco Corp., St. Joseph, MI) consisting of a modified Agilent 6890 GC and a ToF MS with electron ionization. Chromatographic conditions were optimised to ensure complete separation of the target compounds from other compounds and co-extracted matrix components that could interfere in the identification and quantitation of the target analytes. Two non-polar × mid-polar column sets were assayed: DB-5 (30 m × 0.25 mm i.d. × 0.25 μm film thickness) × BPX-50 (1.9 m × 0.1 mm × 0.1 μm film thickness) and HT-8 (30 m × 0.25 mm i.d. × 0.25 μm film thickness) × BPX-50 (1.6 m × 0.1 mm × 0.1 μm film thickness). All columns were purchased from SGE (Melbourne, Australia). Once optimised, the proposed method consisted on sample injection in the hot splitless mode (1 μL, 300 ºC, splitless time 1.0 min) in the DB-5 × BPX-50 colum set-up. The temperature in the main oven was programmed from 60 ºC (1 min) to 270 ºC (2 min) at a rate of 10 ºC/min and then to 315 ºC (20 min) at 3 ºC/min. The second dimension oven was programmed to track the main oven but with an off-set of 30 ºC, except for the final temperature ramp: that is, from 90 ºC (1 min) to 300 ˚C (2 min) at a rate of 10 ºC/min and then to 320 ºC (20 min) at 3 ºC/min. Helium was used as carrier gas at a head-column pressure of 25.8 psi. A nitrogen quad-jet dual-stage cryogenic modulator was used for sample focusing and injection in the second dimension column. The temperature of the modulator was set 30 ºC above that of the main oven. The modulation period (P_M) was optimised to minimise coelutions between critical analyte pairs/groups and among target compounds and other co-extracted matrix components. A P_M of 5 s with a 0.6 s hot jet pulse was finally selected. The transfer line temperature was set at 275 ºC.

The ion source temperature was set at 250 ˚C. MS detection was performed in full scan in the *m/z* 50–500 range; the energy of ionizing electrons was 70 eV, the voltage of the multiplier was 1500 eV and the data acquisition rate 50 Hz. ChromaToF 3.32 was used for data acquisition and quantification of the samples. This software allowed automated baseline correction and peak area and volume determination. Quantitation was carried out by external calibration and data were fitted using a linear model. Calibration lines were constructed in the 100-2000 pg/μL range for each target

compound (four calibration levels; two separate analyses at each level). All calibration lines showed good linearity ($r^2 > 0.993$, Table 1) in the evaluated calibration range.

3. Results and discussion

3.1. Optimisation and validation of the GC×GC–ToF MS methodology

Challenging groups of PAHs in terms of separation on DB-5-type columns include the pair IcdP-DahA and the two groups BbF-BjF-BkF and CCP-BaA-Chr. The separation of these groups in mid-polar GC phases has also revealed to be a difficult task *(14, 17)*. Interestingly, the change in the phase polarity promotes also some changes in the elution order of the analytes in these phases as compared to that observed in non-polar ones. According to this observation, two different columns sets were selected and evaluated in the present work: DB-5 × BPX-50 and

Table 1. Calibration table for the 15+1 EU PAHs under the finally optimised GC×GC conditions.

Compound	Acr.[a]	Id. No[b]	MW[c]	1tR, 2tR[d] (s)	Calibration curve	$r^{2,e}$
Benzo[*c*]fluorene	BcF	1	216	1465, 2.580	$y= 187\cdot x - 1.30\cdot 10^4$	0.999
Benzo[*a*]anthracene	BaA	2	228	1625, 3.240	$y= 183\cdot x - 3.62\cdot 10^4$	0.993
Cyclopenta[*cd*]pyrene	CCP	3	226	1630, 3.460	$y= 182\cdot x - 1.52\cdot 10^4$	0.997
Chrysene	Chr	4	228	1635, 3.360	$y= 205\cdot x - 1.75\cdot 10^4$	0.998
5-Methylchrysene	5-MC	5	242	1745, 3.740	$y= 110\cdot x - 7.19\cdot 10^3$	0.999
Benzo[*b*]fluoranthene	BbF	6	252	1900, 4.580	$y= 134\cdot x - 1.17\cdot 10^4$	0.997
Benzo[*j*]fluoranthene	BjF	7	252	1900, 4.720	$y= 111\cdot x - 1.30\cdot 10^4$	0.996
Benzo[*k*]fluoranthene	BkF	8	252	1905, 4.620	$y= 187\cdot x - 1.04\cdot 10^4$	0.999
Benzo[*a*]pyrene	BaP	9	252	1990, 0.560	$y= 110\cdot x - 1.03\cdot 10^4$	0.999
Indeno[1,2,3-*cd*]pyrene	IcdP	10	276	2295, 4.100	$y= 103\cdot x - 1.06\cdot 10^4$	0.999
Dibenzo[*a,h*]anthracene	DahA	11	278	2300, 4.100	$y= 68.4\cdot x - 1.57\cdot 10^4$	0.994
Benzo[*ghi*]perylene	BghiP	12	276	2365, 0.720	$y= 86.6\cdot x - 8.90\cdot 10^3$	0.999
Dibenzo[*a,l*]pyrene	DalP	13	302	2725, 3.360	$y= 21.5\cdot x - 9.45\cdot 10^3$	0.995
Dibenzo[*a,e*]pyrene	DaeP	14	302	2865, 1.240	$y= 8.69\cdot x - 4.00\cdot 10^2$	0.995
Dibenzo[*a,i*]pyrene	DaiP	15	302	2920, 2.940	$y= 19.8\cdot x - 1.54\cdot 10^4$	0.994
Dibenzo[*a,h*]pyrene	DahP	16	302	2960, 4.140	$y= 11.8\cdot x - 5.30\cdot 10^2$	0.998

[a] Acr., acronym.
[b] Id. No., Identification number.
[c] MW, molecular weight (u.m.a).
[d] t_R, retention times as determined by the program on the base of the maximum modulation.
[e] r^2, correlation coefficient as calculated for a linear calibration fit.

HT-8 × BPX-50. The former has been the column combination used in previous studies dealing with the analysis of environmentally relevant PAHs of variable molecular weight *(19, 21)*, while more polar phases (i.e., 14% cyano-86% dimethylpolysiloxane) have only been used as second dimension column in studies focusing in the analysis of selected relatively volatile PAHs *(20, 22)*.

Preliminary experiments were carried out to optimise the chromatographic separation of the target compounds in the first dimension using a standard solution containing the sixteen targeted PAHs (1 ng/μL) and DB-5 × BPX-50 as column combination with non-modulated transfer. The goal of this part of the study was to minimise the chromatographic run time and peak broadening for the heaviest PAHs without compromising the chromatographic performance and/or peak resolution for critical groups of compounds. Several temperature programs starting with a relatively sharp temperature ramp (i.e., either 8 ºC/min or 10 ºC/min), which however did not affect the separation of early eluting PAHs, followed by a relatively slow ramp of temperature (3 ºC/min) up to a final temperature of either 310 ºC or 315 ºC were tested. Peak broadening was observed for the late eluting PAHs when the final oven temperature was set at 310 ºC due to the strong interaction of these higher molecular weight compounds with the chromatographic phases. As expected, this effect was temperature-dependent. Therefore, the final temperature of the ramp was set at 315 ºC for subsequent experiments.

The P_M was then optimised. P_M values of 5-10 s were evaluated, using in all instances a hot jet pulse of 0.6 s. As an illustration of the typical results obtained under the different assayed conditions, Figure 2 shows the two-dimensional chromatograms obtained with DB-5 × BPX-50 and HT-8 × BPX-50 at selected P_M of 5 and 10 s. DB-5 × BPX-50 provided a satisfactory separation of Chr from BaA and CCP (m/z, 226 and 228) in the first dimension, the two latter compounds being separated in the second dimension when using a P_M of 5 s and a hot-jet pulse of 0.6 s (Fig. 2.A). Under these experimental conditions, a satisfactory separation was also achieved among the three benzofluoranthene homologues (m/z, 252), and between IcdP and DahA (m/z, 276 and 278), which were separated in the first dimension. However, wraparound was observed for mid- to less-volatile PAHs. This shortcoming, that has already been observed by other authors *(19)*, was reduced by

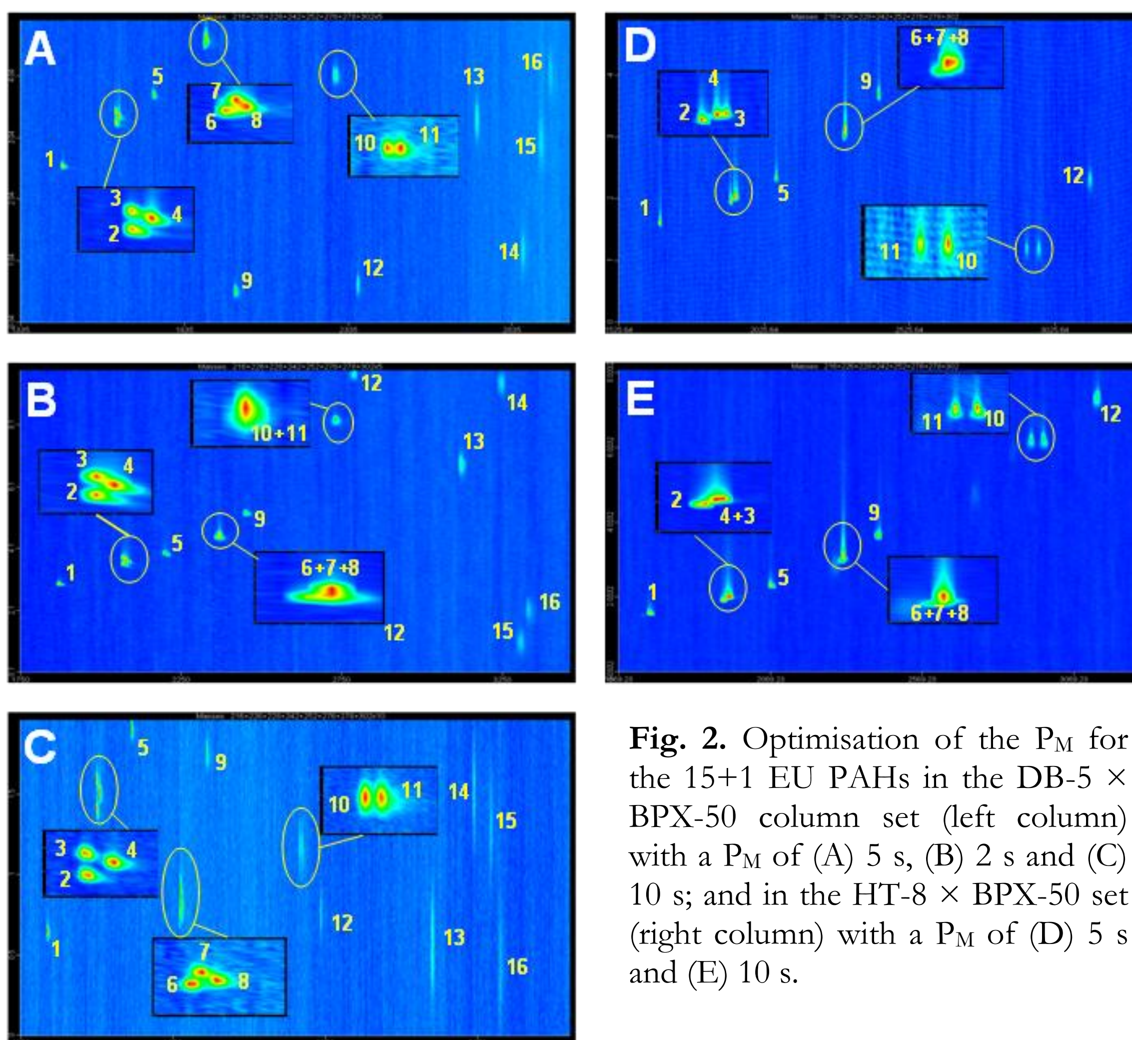

Fig. 2. Optimisation of the P_M for the 15+1 EU PAHs in the DB-5 × BPX-50 column set (left column) with a P_M of (A) 5 s, (B) 2 s and (C) 10 s; and in the HT-8 × BPX-50 set (right column) with a P_M of (D) 5 s and (E) 10 s.

increasing the P_M, which simultaneously contributed to reduce broadening for the less volatile dibenzopyrene isomers in the second dimension. However, P_M longer than 5 s also resulted in the progressive mixing in the modulator of CCP and Chr, the benzofluoranthene homologues and the pair IcdP and DahA already separated in the first dimension. The longer the P_M the most evident the mixing effect, that resulted in a complete coelution of these analytes when using a P_M of 10 s (Fig. 2.B). Under these conditions, the extra selectivity power added by the use of ToF MS as detector did not contribute to improve the situation as trace m/z 226 of CCP interfered in the determination of Chr, trace m/z 276 of IcdP interfered in the determination of DahA, and the identification of the three benzofluoranthenes became simply impossible.

As expected, the use of P_M shorter than 5 s resulted in a somehow improved separation of major critical groups investigated (see Figure 2.C for results obtained at

a P_M of 2 s). However, it also contributed to increase broadening of the PAH peaks in the second dimension, and undesirable effect that negatively affected the detectability of the target compounds by increasing their respective limits of detection (LODs). Moreover, at such as short P_M, wraparound leaded to coelution of the target analytes with other co-extracted matrix components that were satisfactorily separated from the investigated PAHs in the second dimension at P_M of 5 s. The use of ToF MS as detector partially contributes to solve interference problems due to coelution with analytes with different mass weight. However, this type of coelution has been reported to have a negative effect on the repeatability of the second dimension retention times, and on that of peak areas and volumes, which decrease *(20)*.

The use of HT-8 × BPX-50 as column combination yielded essentially similar conclusions, that is, longer P_M resulted in reduced broadening of the chromatographic peaks in the second dimension and contributed to reduce wraparound. It was also found that a P_M of 5 s preserved the separation obtained in the first dimension among BaA, CCP and Chr, and between IcdP and DahA (Fig. 2.D). However, the use of longer P_M newly resulted on mixing of CCP and Chr on the modulator, with no further separation on the second dimension due to the close retention times of these compounds in the stationary phase (Fig. 2.E). In addition, no separation was achieved among the benzofluoranthene homologues under any of the several assayed conditions with this column combination. Dibenzopyrenes experienced a strong retention on HT-8 that enlarged the run time (even when a final temperature of 320 ^{o}C was set in the main oven). This, combined with the severe broadening of these isomers in the second dimension, resulted in almost impracticable analysis conditions at the low concentration levels expected for these analytes in real-life samples. Therefore, DB-5 × BPX-50 and a P_M of 5 s were selected for subsequent experiments.

Then, the modulation conditions were optimised. In all instances, the modulator temperature was kept 30 °C above the temperature of the main oven. The use of an off-set of temperature of 40 °C above that of the first dimension oven did not yield any real improvement regarding broadening of the less volatile PAHs investigated in the second dimension and resulted in column bleeding due to the relatively high final working temperatures used in the present study. Under these conditions (P_M, 5 s;

modulator temperature offset, 30 °C), the influence of the hot/cold pulse duration in the modulation process was investigated. Hot jet pulse durations in the 0.3-1.5 s (5 levels), corresponding to cold jet pulses in the 2.2-1.0 s range, were assayed. The use of cold jet pulses as long as 2.2 s resulted again in mixing of some compounds already separated in the first dimension in the modulator, i.e. CCP and Chr and the three benzofluoranthenes. The rather short hot jet pulse duration used in this experiment was also responsible for a slow reinjection of the eluent from the first column onto the second one, which resulted in the peak tailing in the second dimension. Increasing the hot jet duration contributed to reduce this problem. Under these conditions, the separation among BbF and BjF and BkF was also slightly improved, but that of IcdP and DahA was not preserved.

Final validation of the proposed GC×GC–ToF MS methodology was carried out by the analysis of the certified harbour sediment BCR-535, which were prepared according to the procedure described in the Materials and Methods section. Once purified, 1 μL of this sediment was analysed under finally proposed conditions by GC×GC–ToF MS and the target analytes quantified by external calibration (Table 1). Although reference concentrations were only available for some of the investigated PAHs, a satisfactory agreement was found among these values and those calculated using the proposed methodology (Table 2). More specifically, results demonstrated that BaA was successfully separated from CCP and Chr and that none of the latter PAHs interfered in the determination of the former analyte under the experimentally proposed conditions. Satisfactory results were also obtained for IcdP, which was assumed as an evidence of its successful isolation from DahA. The same consideration applied for BbF (reference concentration, 2.29 mg/kg vs calculated concentration, 2.26). Although the concentration calculated for BkF was slightly lower than the reference value (0.904 ± 0.009 and 1.09 ± 0.15 mg/kg, respectively; i.e., deviation below 3%), the global result obtained for the three benzofluoranthenes were still satisfactory considering the wide dispersion observed in the results typically reported for these analytes *(13)*. Interestingly, a careful inspection of the fragmentograms corresponding to m/z 276 and 278 showed the improved resolution

Table 2. PAH levels found in the CRM and in the investigated sediment samples. Experimental LODs (S/N, 3:1) as determined in real sediments.

Compound	**LOD (µg/kg)**	**BCR-535 (mg/kg)**		**BCR-535 (mg/kg)**		**Sediment samples (µg/kg)**		
		CV[a]	**Unc.**[b]	**Mean (n=2)**	**SD** [c]	**Range**	**Median**	**SD**
Benzo[*c*]fluorene	10			0.44	0.060	<LOD-426	16.9	114
Benzo[*a*]anthracene	6.9	1.54	0.10	1.51	0.160	9.3-810	40.8	210
Cyclopenta[*cd*]pyrene	5.7			0.46	0.070	<LOD-173	14.8	45.0
Chrysene	9.8			2.62	0.160	14.1-980	52.5	245
5-Methylchrysene	31			0.13	0.003	<LOD-333	8.04	78.9
Benzo[*b*]fluoranthene	22	2.29	0.15	2.26	0.120	<LOD-580	42.2	162
Benzo[*j*]fluoranthene	27			1.31	0.113	<LOD-465	27.8	130
Benzo[*k*]fluoranthene	13	1.09	0.15	0.90	0.009	<LOD-394	20.0	112
Benzo[*a*]pyrene	31	1.16	0.10	1.07	0.070	<LOD-652	37.5	173
Indeno[1,2,3-*cd*]pyrene	60	1.56	0.14	1.41	0.003	<LOD-642	38.4	199
Dibenzo[*a,h*]anthracene	91			0.52	0.003	<LOD-517	0.0	125
Benzo[*ghi*]perylene	57			1.61	0.001	<LOD-483	34.7	146
Dibenzo[*a,l*]pyrene	95			0.44	0.0001	<LOD-359	74.2	88.5
Dibenzo[*a,e*]pyrene	561			0.76	0.030	<LOD-1110	0.0	278
Dibenzo[*a,i*]pyrene	632			ND[d]	-	<LOD	0.0	113
Dibenzo[*a,h*]pyrene	1000			ND	-	ND	-	-

[a] CV, Certified Value
[b] Unc., uncertainty calculated as the half-width of 95% of CV
[c] SD, Standard Deviation
[d] ND, Not Detected

and identification power provided by the GC×GC–ToF MS, which look to allow the separation of different dibenzoanthracene isomers, including that of DahA from dibenzo[*a,c*]anthracene, DacA (Fig. 3). (Tentative analyte identification based on results from *(14)*).

It should also be highlighted that consistently low LODs in the 5.7-60 μg/kg range (as calculated for real-life samples) were obtained for most of the targeted PAHs. This improved detectability should guaranty accurate determination of the investigated compounds at the low levels typically found in non-contaminated real-life sediments, as those earlier measured in the same area and from other protected regions *(23-25)*. Relatively high LODs were only obtained for the three late eluting dibenzopyrenes. These higher LOD values can mainly be associated to peak broadening due to their strong interaction of these compounds with the mid-polar stationary phase. Although such an interaction should be reduced by the use of relatively short narrow-bore and thin film columns *(17)* in combination with optimised chromatographic and modulation conditions, results demonstrated that the determination of the heaviest dibenzopyerenes by GC-based techniques remains a difficult task. The use of chromatographic stationary phases with a high thermal stability can be suggested as the most plausible alternative solution to this problem.

3.2. Application to the analysis of sediments

The practicability of the proposed method for the determination of the target PAHs in complex real matrices was evaluated by the analysis of 17 sediments collected from a non-contaminated protected area located in the Southwest of Spain (Fig. 1). Figure 4 shows the typical GC×GC–ToF MS TIC (Figure 4.A) and reconstructed fragmentograms obtained for sediments with relatively high (Figure 4.B) and low (Fig. 4.C) PAH levels. For both sediments, the target compounds appeared in the contour plot properly separated from remaining matrix compounds as well as from other co-extracted analytes that would interfere the determination of mid- to less-volatile PAHs in monodimensional systems even if using an MS system as detector. The improved resolution power offered by GC×GC, in combination with careful chromatographic conditions optimisation and ToF MS identification, effectively contributed to solve interferences due to chromatographic coelution with

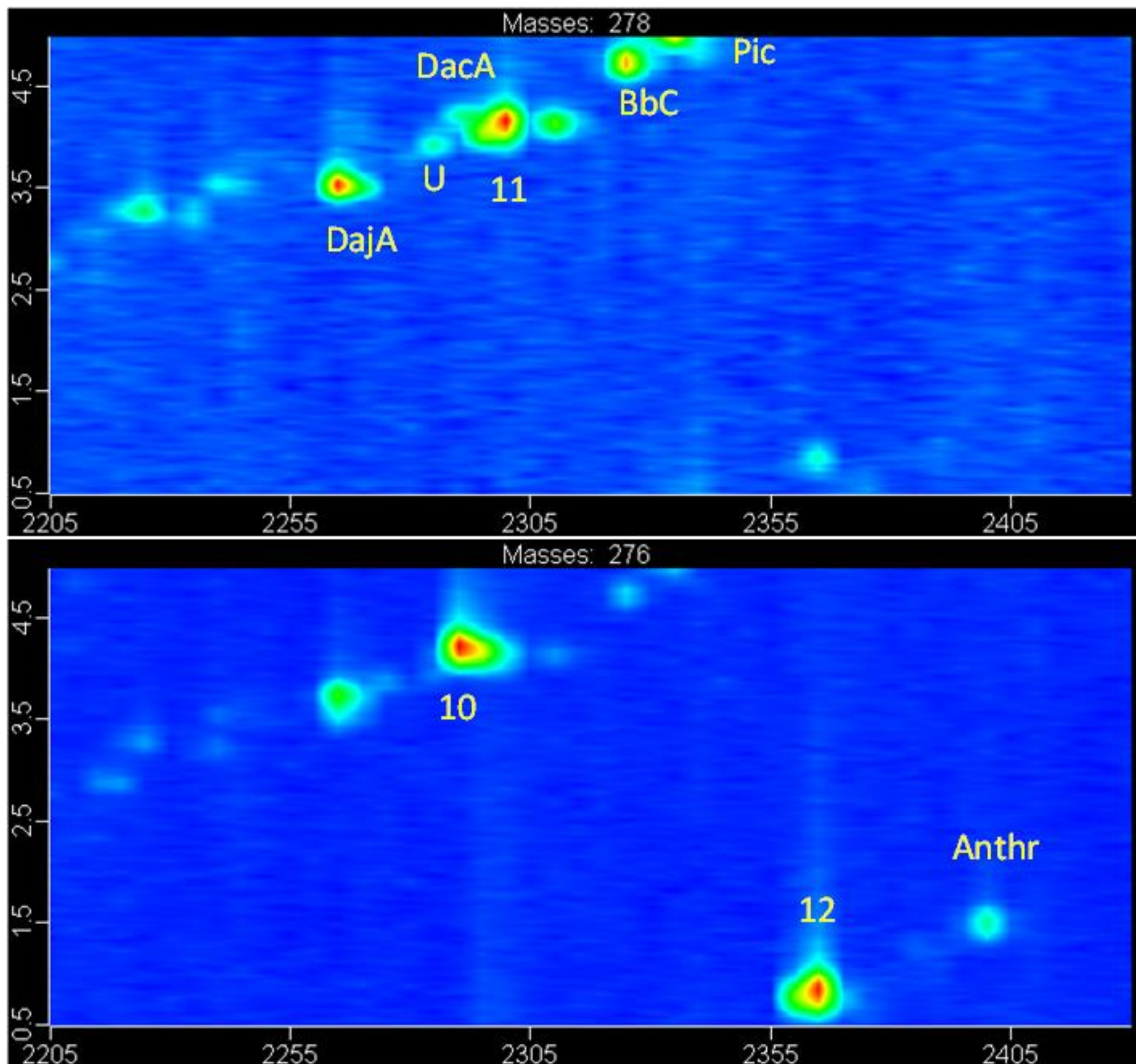

Fig. 3. Fragmentograms obtained for CRM-535 (A) m/z 278, and (B) m/z 276 with indication of the tentative identification of different non-target PAH.
DajA, dibenzo[*a,j*]anthracene; DacA, dibenzo[*a,c*]anthracene; BbC, benzo[*b*]chrysene; Pic, picene; Anthr, anthrathrene; U, unkown.

compounds with or fragments with nominal masses similar to those of the test PAHs (Fig. 4.B) allowing accurate identification and quantitation of the studied analytes. As illustrated in Figure 4.A, such a determination would have not been possible with any other type of detector.

Table 2 summarises the concentration ranges (on a dry weight basis) determined for the different investigated PAHs in the superficial sediments sampled along the Sancti-Petri channel, while total concentrations are graphically reported in Figure 5.A. Total PAH contents ranged from 97.8 to 6840 μg/kg, corresponding to stations 9 and 12, respectively. Maximum PAH levels were found in stations 3 and 12, which are

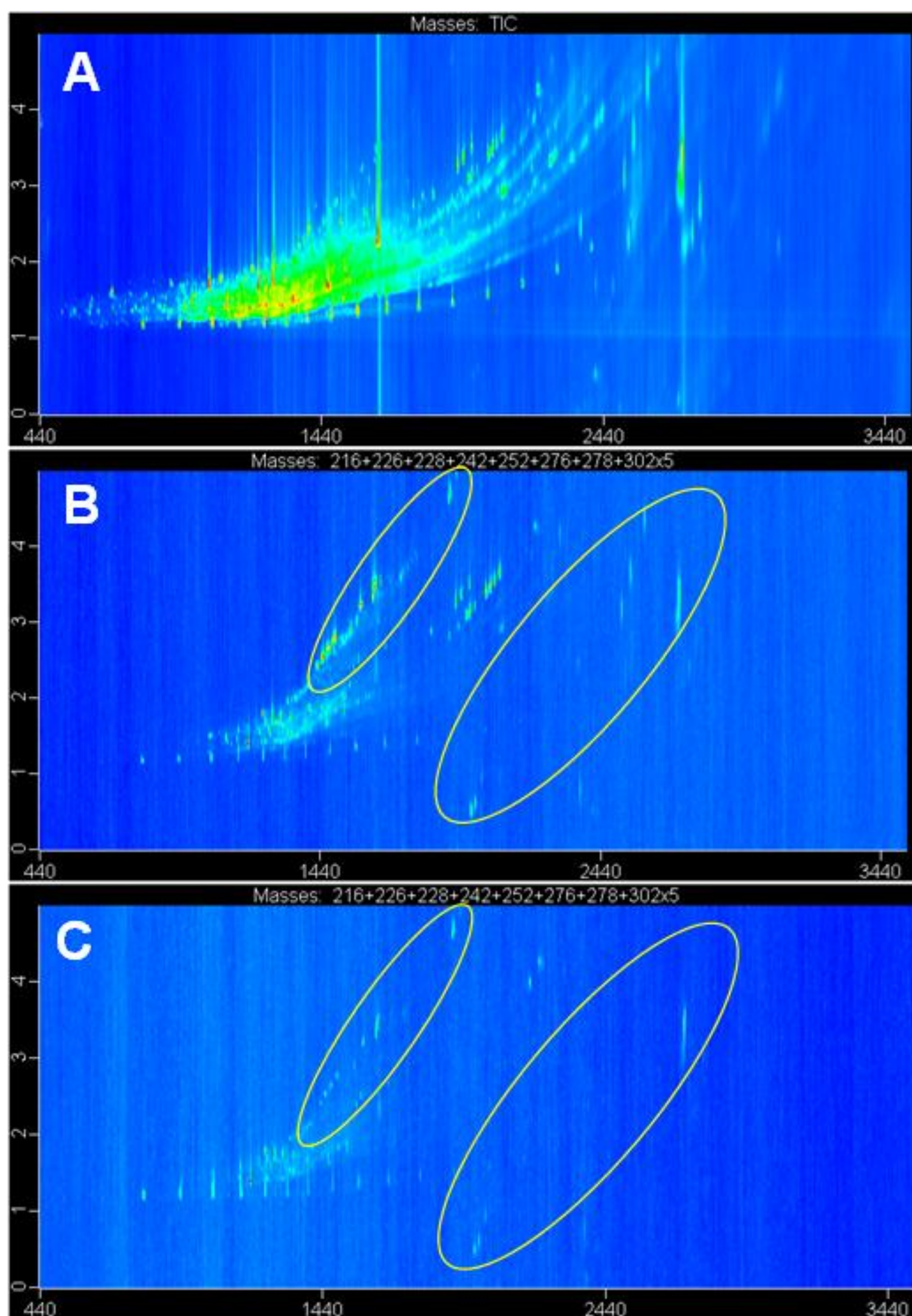

Fig. 4. (A) Typical total ion chromatogram (TIC) obtained for the sediment (ST 8) and fragmentograms corresponding to (B) the same sample, and to another sample with low PAH contents (ST 4). The position of investigated PAHs in the contour plot are indicated with yellow circles.

under the influence of San Fernando, the largest city in the area. More specifically, station 3 have received for many years the impact of the untreated urban effluents

dumped from San Fernando, a city with a population of 88.000 inhabitants at the sampling time, while station 12 is in the vicinity of an area with boat traffic and activities. Stations 1, located close to a shipyard, and station 8 , placed at the mouth of Chiclana river and under the influence of an industrial park located upstream this

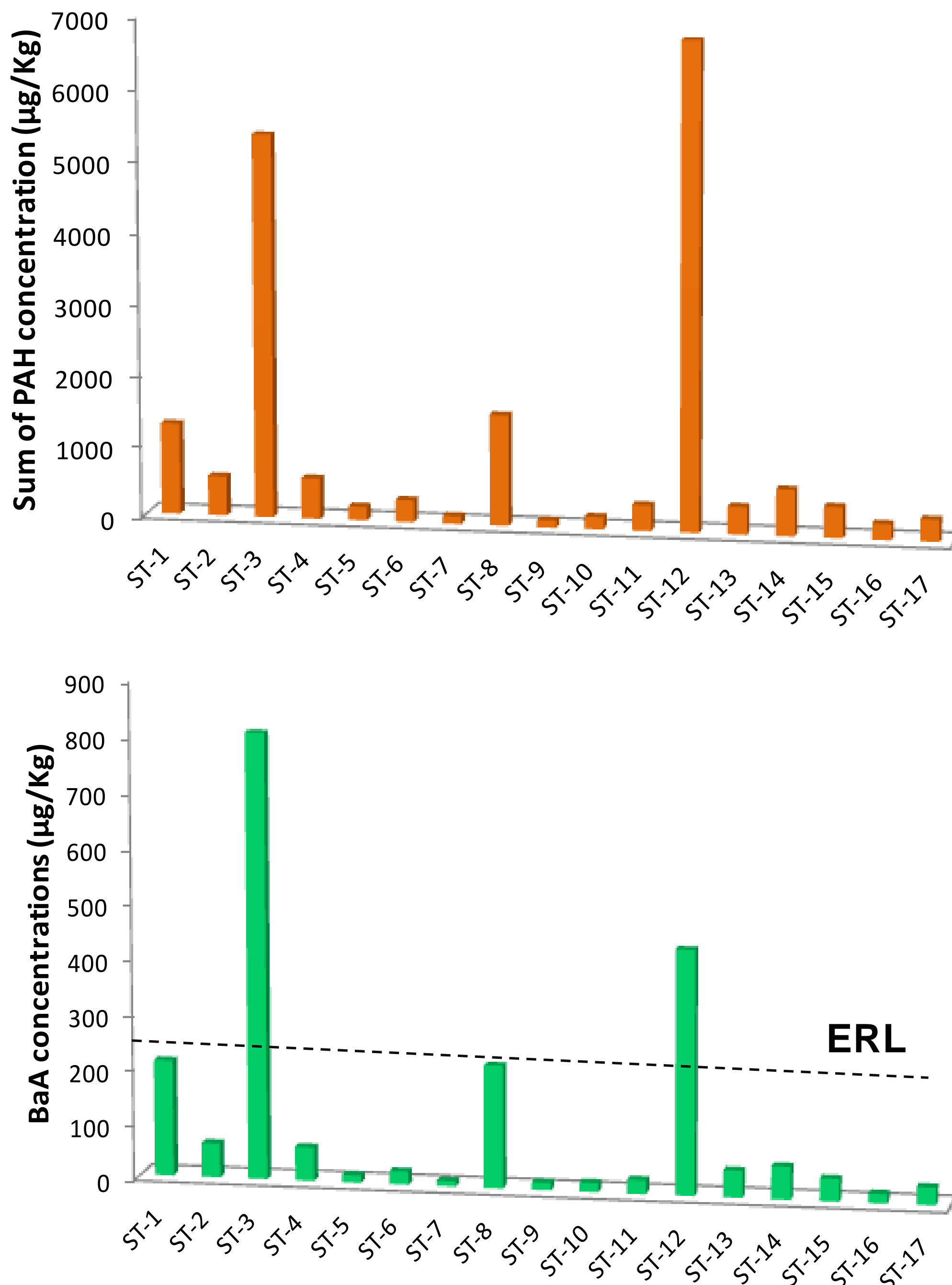

Fig. 5. Distribution of (A) sum of PAHs and (B) BaA in the sampled stations (ST) with indication of the ERL for this PAH. See Figure 1 for ST location.

river, showed the following highest PAH contents. The measured concentration of the investigated PAHs were compared with the effect range low (ERL) and the effect range median (ERM) guidelines values *(26)*. In the most contaminated sampling stations, the mid-volatile PAHs investigated showed concentrations intermediate among those of the ERL and the ERM values, meaning that some constituents of these samples can occasionally pose biological impairments. As a typical example of these results, Figure 5.B shows the occurrence of BaA in the different points sampled along the Sancti-Petri Channel. This PAH contributed in all samples to 10-15% of the total PAH content. Only stations 3 and 12 showed BaA concentrations above the ERL level (261 μg/kg). The ERM guideline values were not exceeded in any of the analysed sediments. Regarding the heaviest PAHs investigated, DalP was detected in most of the analysed sediments, at levels ranging from LOD to 359 μg/kg. Meanwhile, DaeP was only detected at ST 12, although at a level of 1110 μg/kg.

All these results demonstrated the feasibility of the proposed GC×GC–ToF MS method for the intended determination and that the experimental conditions finally proposed in the present study allowed proper analyte recognition as well as quantification even for as a complex matrix as sediments. As previously indicated, the improved chromatographic resolution provided by GC×GC in combination with the extra identification capability offered by ToF MS allowed even the separation of some PAHs that typically coelute in real-life samples, such as DacA and DahA (Fig. 3). However, under the experimental conditions proposed here, only a partial separation between Chr and triphenylene was achieved. On the other hand, the use of GC×GC–ToF MS allowed the inspection of the complete contour plot and so the tentative identification of other non-target analytes present in the test sample. Interestingly, in this study, polynuclear aromatic thiophenes (m/z 134, 148, 184, 198 and 234) were detected in some of the analysed sediments. In particular, these compounds appeared in the contour pot as a roof-tile structure in a region comprised between 1540-1660 s in the first dimension and 3-4 s in the second dimension. These analytes were tentatively identified on the base of the mass spectra information and were clearly visible in samples collected at ST 1-4, but aslo detected at lower concentration levels in ST 8 and 12.

The influence of the several activities developed in the studied area became also evident through the PAH profiles observed in the different sampled stations. Coke emissions are typically associated to profiles enriched in high molecular weight (i.e., containing five- to six-rings) PAHs; meanwhile, low temperature pyrolitic processes, such as coal combustions, are usually dominated by low molecular weight PAHs *(27-28)*. These differences can easily be visualised by direct comparison of bubble plots in which the size of the PAH peaks have been normalised by that of a reference analyte present at a higher concentration level (Fig. 6). Thereby, in the contour plot of station 3, the largest bubbles are concentrated at the beginning of the chromatogram, demonstrating a dominance of pyrolitic, i.e. urban and domestic, PAH inputs (Fig. 6.A). The increasing influence of other industrial activities became evident in the contour plot of station 12 (Fig. 6.B) by an increase of the bubble size corresponding to mid- and high-volatile PAHs (MW > 252) as compared to that observed in the previous figure. Figure 6.C, corresponding to station 8, shows the typical bubble profile obtained for a station with intermediate concentration levels and a non-specific contamination input.

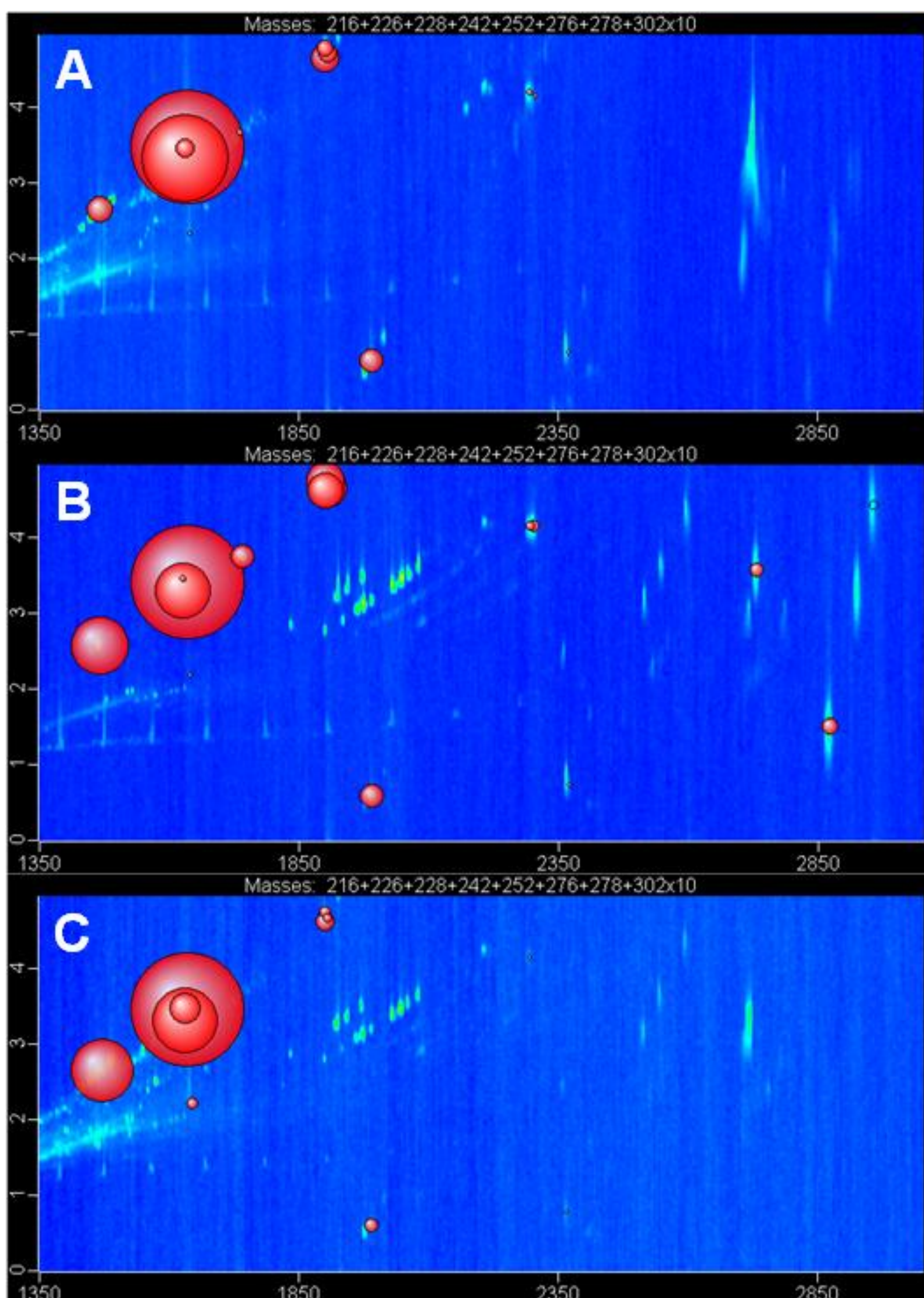

Fig. 6. Bubble plots obtained for (A) ST 3, (B) ST 12 and (C) ST 8.

4. Conclusions

A GC×GC–ToF MS method has been optimised for the identification and quantification of the 15+1 EU PAHs in complex environmental matrices such as sediments. The improved separation provided by GC×GC in combination with the

additional confirmation capabilities added by the use of ToF MS as detector resulted in a useful and powerful analytical approach allowing unambiguous determination of the target compounds in these complex samples. Once optimised, the developed method allowed a satisfactory separation of difficult pairs/group of PAHs, including BaA-CCP-Chr, BbF-BjF-BkF and IcdP-DahA, but also of other typically PAH pairs which are usually undistinguishable even using MS detection, such as DacA and DahA. Partial separation was also achieved between Chr and triphenylene. However, the strong retention experienced by the heaviest dibenzopyrene isomers included in the study resulted in relatively high LODs for these analytes.

Application of the developed methodology to real sediments sampled along the Sancti Petri Channel demonstrated that this protected area is affected by different contamination processes, which can easily be identified by the changes in both the levels of the targeted PAHs and in their associated profiles. Regarding this latter aspect, the feasibility of using normalised bubble plots for the fast screening of the potential PAH inputs has been demonstrated.

Acknowledgments

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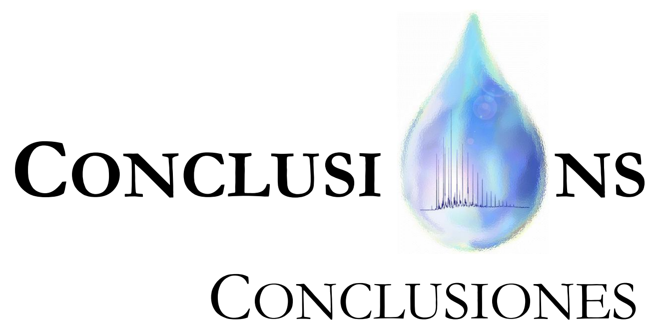
Chapter – Capítulo 4



CONCLUSIONS

CONCLUSIONES

The results obtained in this work allow to conclude that:

1. Miniaturisation is a valuable analytical alternative for sample preparation, even when the goal is the determination of trace organic pollutants in complex matrices, such as solid environmental and fat-containing biotic samples. Miniaturisation is probably the most suitable analytical approach when dealing with the analysis of size-limited samples.

2. Scaling down the sample preparation methods in use for trace organic pollutants determination results in several advantages, one of the most relevant being the possibility of coupling and/or combining of the several treatments involved. This turns in faster and more cost-effective and environmental friendly analytical methodologies eventually allowing complete sample preparation, i.e. extraction-plus-purification, to be developed in a single step.

3. The sample preparation methods proposed in this work could represent a valuable alternative to conventional procedures in use for the determination of organic micropollutants in different types of samples because they allow a significant reduction in the reagent and solvent consumption, and in the analytical time response, while keeping the level of quality demanded for this type of determination. These features make us to consider these miniaturised sample preparation methods as particularly suitable for monitoring and routine control of the investigated pollutants in environmental and food samples. In particular:

3.1. The application of hot Soxhlet allowed quantitative extraction of the target organobromines from tuna muscles in shorter times than other conventional large-scale extraction techniques, providing recoveries of the target compounds in the 96-120 % range, with RSDs below 8%, and LODs below 0.25 ng/g fat weigth when using GC−NCI-MS for final determination.

3.2. The combined use of MSPD with miniaturised selective PLE provided quantitative recoveries in the range 60–120 % and 86-114 % of the investigated PCBs and PBDEs, respectively, from feedstuffs (RSDs < 20%), and LODs low enough to ensure proper determination of the target compounds by GC–ITD(MS/MS) and GC–NCI-qMS, respectively, even if only 250 mg of sample were used for the analysis. This procedure allowed to reduce the total solvent consumption to 8.0 mL and to complete the sample treatment in only 20 min.

3.3. MSPD combined with miniaturised PLE and packing of the clean-up sorbents in the extraction cell has been demonstrated to be a fast and cost-effective approach for the quantitative extraction (recoveries in the 90-106 % range; RSDs, in general, lower than 15%) with simultaneous *in-cell* purification of PCBs from sediments. Ready-to-analyse extracts were obtained in only 45 min and with a solvent consumption of 20 mL. When combined with GC–ITD(MS/MS), the proposed methodology allowed the accurate determination of the investigated prioritary and toxic PCB congeners at low μg/kg levels.

3.4. The use of UAE combined with dispersive SPE in disposable tips for fat removal allowed simple and extremely fast extraction and purification of PCBs from size-reduced biological tissues while maintaining adequate analytical performance (recoveries in the 85-123 % range; RSDs lower than 14% for a large majority of the studied PCB congeners). When combined with a selective instrumental technique for final determination, such as GC–ITD(MS/MS), the proposed approach allowed proper determination of a large majority of environmentally relevant PCBs, even if an as small amount of sample as 50 mg were used for the determination.

4. The high separation power of GC×GC and the possibility of obtaining structured chromatograms and, when combined with ToF MS as detector, structural information, make this technique to be considered as an ideal alternative for the fast simultaneous screening of different families of pollutants obtained from complex

environmental extracts. It is also an ideal analytical alternative for non-target analysis in these types of extracts. Concerning this point, it can be highlighted that:

4.1. GC×GC-ToF MS using HT-8 × BPX-50 as column combination has been demonstrated to allow the simultaneous accurate determination of the 26 target organobromines considered in this work. The roof-tile chromatograms obtained with this column set allowed tentative identification of some extra compounds for which standards were not available. The extra possibility of structural confirmation made possible the identification of some PBHD isomers for the first time.

4.2. GC×GC–ToF MS using DB-5 × BPX-50 as column combination allowed the unambiguous determination of the 15+1 EU PAHs in sediments as well as the satisfactory resolution of other critical coeluting pairs. Normalised bubble plots have been suggested as a valuable visualisation alternative for the fast inspection of PAH profiles in sediments and related matrices, as well as for the tentative identification of their potential sources.

A partir de los resultados presentados en esta memoria es posible concluir que:

1. La miniaturización es una alternativa analítica valiosa en el campo de la preparación de muestra incluso si el objetivo es la determinación de contaminantes orgánicos a niveles traza en matrices sólidas complejas, como las ambientales o las bióticas con contenidos medios-altos de lípidos. La miniaturización es también probablemente la mejor alternativa analítica cuando el objetivo es el tratamiento de muestras de tamaño limitado.

2. La reducción del tamaño de los métodos de preparación de muestra en uso para la determinación de trazas de contaminantes orgánicos lleva asociada diversas ventajas. Una de las más relevantes es posibilitar el acoplamiento de las diferentes etapas involucradas en estos tratamientos, lo que a su vez da lugar a una simplificación del proceso global, que en ocasiones, permite unificar en una sola etapa todos los tratamientos. La mayor capacidad de respuesta, el menor coste y la menor generación de residuos son ventajas adicionales asociadas a la miniaturización.

3. Los métodos de preparación de muestra propuestos en esta memoria pueden ser considerados alternativas analíticas válidas a los procedimientos convencionales en uso para la determinación de microcontaminantes orgánicos en distintos tipos de muestras, ya que permiten una reducción significativa en el consumo de reactivos y disolventes respecto a aquellos, así como una mayor capacidad de respuesta, pero manteniendo los estándares de calidad exigidos en estas determinaciones analíticas. Estas características hacen pensar que pueden ser particularmente adecuados para estudios de monitorización y control rutinario de los contaminantes investigados en muestras ambientales y alimentos. En particular:

3.1. La aplicación de la técnica de hot Soxhlet permitió la extracción cuantitativa de los compuestos organobromados objeto de estudio de músculos de atunes en tiempos significativamente más cortos que los requeridos por otras técnicas de extracción más convencionales, pero proporcionando recuperaciones de los analitos en el intervalo 96-120 %, con RSDs inferiores a 8 % y LODs inferiores a 0,25

ng/g en peso graso cuando se empleaba GC–NCI-MS para la determinación instrumental de los analitos.

3.2. El uso combinado de MSPD con PLE miniaturizada y selectiva proporcionó recuperaciones cuantitativas (60-120 % en el caso de los PCBs y 86-114 % para los PBDEs; RSDs inferiores al 20 % en ambos casos) de los analitos extraídos de piensos de peces, y LODs suficientemente bajos como las garantizar la adecuada detección de los compuestos mediante GC–ITD(MS/MS) y GC–NCI-qMS, respectivamente, incluso si la cantidad de muestra empleada era de sólo 250 mg. El procedimiento se completaba en tan sólo 20 min con un consumo total de 8 mL de disolvente.

3.3. El uso de MSPD combinado con PLE miniaturizada y selectiva demostró ser una aproximación analítica rápida y eficaz para la extracción cuantitativa de PCBs de sedimentos (recuperaciones en el intervalo 90-106 %; RSDs inferiores al 15 %) que permitía la purificación de los extractos en la misma celda de extracción. En tan sólo 45 min y con 20 mL de disolvente se obtenían extractos listos para su análisis. Combinado con GC-ITD(MS/MS), este procedimiento de preparación de muestra permitía la adecuada determinación de PCBs prioritarios y tóxicos a niveles de μg/kg.

3.4. El uso de la sonda de ultrasonidos en combinación con la SPE dispersiva en puntas de pipeta para la eliminación de la grasa permitía la extracción y purificación de PCBs de muestras de tejidos biológicos de reducido tamaño de manera sencilla y rápida, pero garantizando una adecuada determinación de los analitos (85-123 %; RSDs, inferiores a 14 %). Combinado con GC–ITD(MS/MS) para la determinación final de los analitos, el método propuesto permitía el correcto análisis de una gran mayoría de los PCBs más relevantes desde el punto de vista ambiental, incluso empleando cantidades de muestra de tan sólo 50 mg.

4. La elevada capacidad de resolución de la técnica de GC×GC y la posibilidad de obtener cromatogramas estructurados y, cuando se combina con ToF MS,

información estructural, permiten considerar a esta técnica como una alternativa instrumental ideal para el screening simultáneo y rápido de diferentes familias de contaminantes en extractos ambientales complejos. Es también una técnica ideal para abordar estudios no orientados (non-target). En relación con estos aspectos, cabe destacar que:

4.1. La GC×GC–ToF MS con HT-8 × BPX-50 como combinación de columnas permitió la determinación simultánea e inequívoca de los 26 compuestos organobromados considerados en esta memoria. Los cromatogramas estructurados obtenidos con esta combinación de columnas en las condiciones de trabajo optimizadas, hicieron posible la identificación tentativa de algunos analitos adicionales de los que no se disponía de patrones. Además, disponer de información estructural adicional posibilitó la identificación tentativa de algunos isómeros no descritos de PBHDs.

4.2. La GC×GC–ToF MS con DB-5 × BPX-50 como combinación de columnas permitió la determinación inequívoca de los 15+1 PAHs de la EU en sedimentos, así como las resolución satisfactoria de algunos otros pares críticos. El uso de plots de burbujas normalizadas puede ser una herramienta útil para la visualización rápida de los perfiles de PAHs en sedimentos y matrices relacionadas, y para la identificación tentativa de sus posibles orígenes.

ANNEX

ANEXO

Table A.1. Performance of the new miniaturised USE-based procedure.

PCB No	t_r (min)	Linear Regression[a]	$r^{2, b}$	Rec. LFL[c] (%) (RSD, %)[d]	Rec. HFL[e] (%) (RSD, %)	LOD[f] (ng/g)
28	11.92	y=13.2x-58.5	0.971	49[g] (5)	99 (19)	12
52	12.48	y=12.8x-9.2	0.978	100 (18)	103 (16)	27
95	13.97	y=11.6x-37.1	0.993	111 (6)	107 (15)	20
101	14.59	y=11x-33.2	0.998	85 (6)	107 (13)	5
77	16.10	y=9.7x-35.6	0.992	80 (4)	91 (17)	6
149	16.68	y=9.5x+31.1	0.996	118 (6)	103 (12)	5
123	16.77	y=10.4x-27.5	0.994	104 (4)	101 (14)	4
118	16.94	y=10.5x-4.7	0.994	110 (1)	102 (13)	5
114	17.47	y=6.2x+48.3	0.976	123 (10)	93 (9)	3
153	17.77	y=9.6x+208.8	0.994	115 (3)	104 (10)	7
132	18.04	y=8.7x-7.6	0.996	121 (13)	99 (17)	4
105	18.23	y=10.2x-26.8	0.995	85 (11)	95 (18)	2
138	19.28	y=9.6+75.6	0.998	103 (1)	98 (11)	8
126	20.08	y=9.9-34.9	0.995	99 (4)	95 (14)	6
183	20.33	y=9.4x-5.4	0.997	81 (11)	96 (12)	6
167	21.02	y=10x-8.2	0.998	96 (5)	96 (11)	12
156	22.73	y=9.9x-26.3	0.995	96 (4)	93 (12)	8
157	23.11	y=9.5x-15.2	0.998	-[g]	95 (13)	8
180	23.77	y=9.4x+101	0.997	115 (4)	96 (10)	7
169	26.01	y=9.2x-19.6	0.997	89 (4)	90 (14)	4
170	26.44	y=8.5x+23.9	0.998	100 (5)	93 (12)	3
189	29.67	y=9.2x-17.9	0.997	89 (4)	92 (14)	5
194	33.87	y=9.1x-10	0.998	84 (5)	92 (13)	6

[a] Dynamic linear regression in the 5-1000 ng/g range
[b] Regression coefficient
[c] Recovery at the lowest fortification level (LFL) investigated, 5 pg/μL (n=4).
[d] Relative standard deviation at 5 ng/g (n=4)
[e] Average recovery as calculated for the high fortification levels (HFL) investigated, i.e. 100, 500 and 1000 ng/g (n=4). Average RSDs at 100, 500 and 1000 ng/g (n=4)
[f] Experimentally determined LOD (*S/N*, 3:1) for samples (sample size, 2 mg)
[g] PCB determination was affected by coelution with an interference

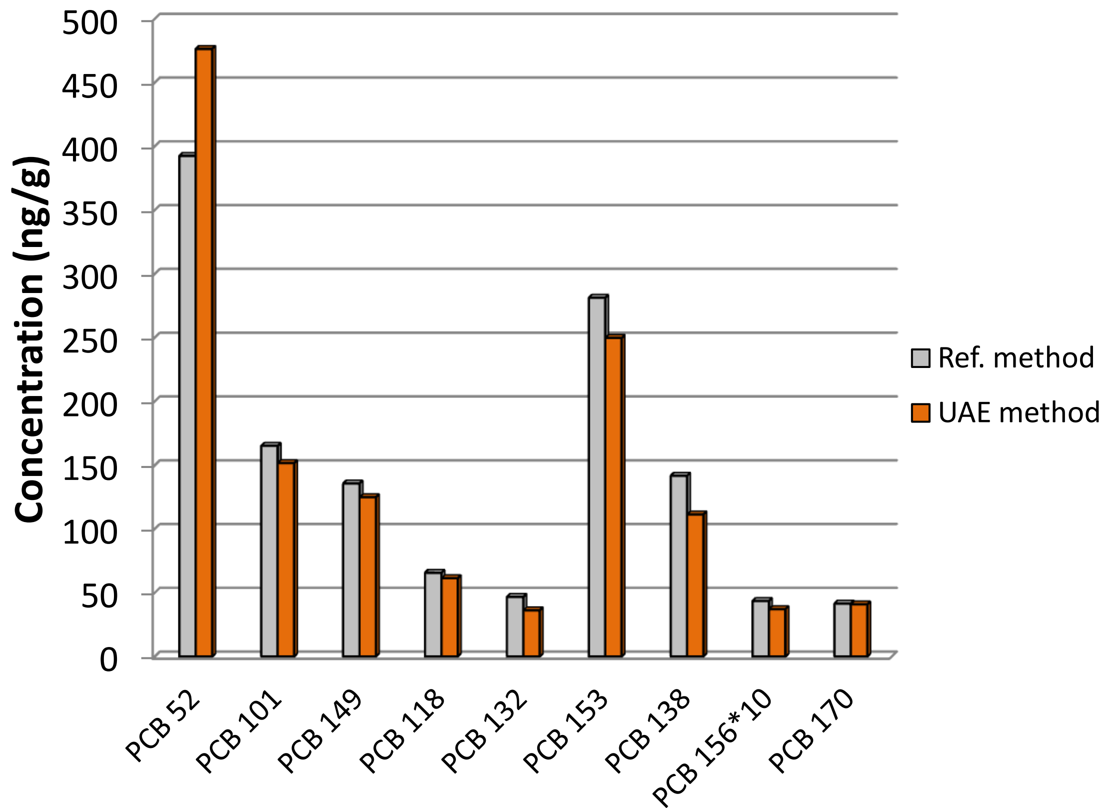


Fig. A.1. Concentrations (ng/g, wet weight) detected for selected PCB congeners in the two-common sea-bream after applying the reference method (grey columns) and the proposed UAE-based method (orange columns). GC–µECD has been used for final instrumental determination.

List of publications

Lista de publicaciones

<u>**Lista de publicaciones incluidas en revistas SCI**</u>

- **Pena-Abaurrea, M.**; Weijs, L.; Ramos, L.; Borghesi, N.; Corsolini, S.; Neels, H.; Blust, R.; Covaci, A., Anthropogenic and naturally-produced organobrominated compounds in bluefin tuna from the Mediterranean Sea. *Chemosphere*. **2009**, *76* (11) 1477.
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- **Pena-Abaurrea, M.**; Ramos, J.J.; González, M.J.; Ramos, L., Simultaneous selective pressurised liquid extraction of polychlorinated biphenyls and polybrominated diphenyls ethers from feedstuffs. (In preparation).
- **Pena-Abaurrea, M.**; Ramos, J.J.; González, M.J.; Ramos, L., Selective pressurised liquid extraction of polychlorinated biphenyls from sediments. (In preparation).
- **Pena-Abaurrea, M.**; Sanz-Landaluze, J.; Cámara, C.; Ramos, L., Determination of polychlorinated biphenyls and polybrominated diphenyls ethers in size- limited biological samples. (In preparation).
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- **Pena-Abaurrea, M.**; Ramos, J.J.; González, M.J.; Ramos, L., Simultaneous selective pressurised liquid extraction of polychlorinated biphenyls and polybrominated diphenyls ethers from feedstuffs. *Organohalogen Compounds*. **2008**, *70,* 1748.
- **Pena-Abaurrea, M.**; Weijs, L.; Ramos, L.; Borghesi, N.; Corsolini, S.; Neels, H.; Blust, R.; Covaci, A., Anthropogenic and naturally-produced organobrominated compounds in Bluefin tuna from the Mediterranean Sea. *Organohalogen Compounds*. **2009**, *71*, 1481.
- **Pena-Abaurrea, M.**; Covaci, A.; Ramos, L., GC×GC–ToF MS for the analysis of anthropogenic and naturally produced organobrominated compounds. *Organohalogen Compounds*. **2011**, *73*, 198.
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- Ramos, J.J.; **Pena-Abaurrea, M.**; Ramos, L., Chapter 11: Comprehensive two-dimensional gas chromatography: environmental applications, *in* "Comprehensive two dimensional chromatography". Comprehensive Analytical Chemistry (CAC) Series. Elsevier. The Netherlands, **2009**. ISBN-13: 978-0-444-53237-4.
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