

Análisis de retardantes de llama halogenados emergentes y su impacto en el medio ambiente y en humanos

Paula Guerra Gómez

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Universidad de Barcelona
Departamento de Química Analítica

**ANÁLISIS DE RETARDANTES DE LLAMA
HALOGENADOS EMERGENTES Y SU IMPACTO EN EL
MEDIO AMBIENTE Y EN HUMANOS**

Paula Guerra Gómez
Marzo 2011



Universidad de Barcelona
Facultad de Química
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Tesis Doctoral

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Paula Guerra Gómez
Dept. de Química Ambiental
IDAEA-CSIC, Barcelona

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por
Paula Guerra Gómez

Tutora:

Director:

Co-Directora:

Dra. Encarnación
Moyano Morcillo
Profesor Titular del
Departamento de Química
Analítica
Facultad de Química
Universidad de Barcelona

Dr. Damià Barceló i
Cullerés
Profesor de Investigación
Departamento de Química
Ambiental
IDAEA-CSIC

Dra. Ethel Eljarrat
Esebag
Científico Titular
Departamento de Química
Ambiental
IDAEA-CSIC

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*“Hay una fuerza motriz más poderosa que el vapor,
la electricidad y la energía atómica: la voluntad”*
Albert Einstein.

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ÍNDICE GENERAL

CAPÍTULO 1: INTRODUCCIÓN GENERAL Y OBJETIVOS_____19

1.1. Retardantes de llama_____21

1.2. Compuestos analizados_____22

1.3. Dinámica y distribución en el medio ambiente_____27

1.3.1. Matrices abióticas_____27

1.3.2. Matrices bióticas_____32

1.3.3. Biodisponibilidad y bioacumulación_____36

1.4. Exposición humana_____39

1.4.1. Ingestión de polvo_____39

1.4.2. Inhalación de aire_____44

1.4.3. Ingestión de alimentos_____46

1.4.4. Grupos de riesgo_____51

1.5. Toxicidad_____54

1.6. Análisis químico de retardantes de llama_____57

1.6.1. Preparación de muestra_____57

1.6.2. Análisis instrumental_____62

1.7. Validación de los métodos analíticos_____66

Artículo científico #1: Recent approaches for selected halogenated flame retardant determinations by means of LC-MS_____71

1.8. Justificación de la tesis_____94

1.9. Objetivos Generales_____95

CAPÍTULO 2: DESARROLLO DE MÉTODOS ANALÍTICOS_____97

2.1. Introducción_____99

2.2. Desarrollo de metodología_____101

Artículo Científico #2: Identification and trace level determination of brominated flame retardants by LC-quadrupole linear ion trap (QqLIT) mass spectrometry_____105

Artículo Científico #3: Simultaneous determination of hexabromocyclododecane, tetrabromobisphenol A, and related compounds in sewage sludge and sediment samples from Ebro River basin (Spain)_____117

Artículo Científico #4: Enantiomeric specific determination of hexabromocyclododecane by LC-quadrupole lineal ion trap (QqLIT) mass spectrometry in sediment samples_____127

2.3. Discusión_____137

CAPITULO 3: IMPACTO MEDIOAMBIENTAL	141
3.1. Introducción	143
<i>Artículo Científico #5: Transfer of hexabromocyclododecane from industrial effluents to sediments and biota: Case study in Cinca River (Spain)</i>	151
<i>Artículo Científico #6: Analysis and occurrence of emerging brominated flame retardants in the Llobregat River basin</i>	161
<i>Artículo Científico #7: Dechlorane Plus and related compounds in peregrine falcon (Falco peregrinus) eggs from Canada and Spain</i>	167
<i>Artículo Científico #8: Concentrations of emerging halogenated flame retardants, including enantiomeric fractions of HBCD, in peregrine falcon (Falco peregrinus) eggs from Canada and Spain. Enviado</i>	177
3.2. Discusión	198
CAPITULO 4: EXPOSICIÓN HUMANA	205
4.1. Introducción	207
<i>Artículo Científico #9: Diastereoisomer- and enantiomer- specific determination of hexabromocyclododecane in fish oil for food and feed</i>	211
<i>Artículo Científico #10: Hexabromocyclododecane in human breast milk: Levels and enantiomeric patterns</i>	219
<i>Artículo Científico #11: Temporal trends of brominated flame retardants in Australia: 1993-2009. Enviado</i>	229
4.2. Discusión	251
CONCLUSIONES GENERALES	255
REFERENCIAS	
ANEXOS	
A.1. Lista de publicaciones presentadas en esta tesis	
A.2. Lista de publicaciones no presentadas en esta tesis	
A.3. Lista de publicaciones en revistas no incluídas en SCI realizadas durante esta tesis	
A.4. Contribuciones a congresos	

ÍNDICE DE FIGURAS

Figura 1-1. Estructura general de los PBDEs.

Figura 1-2. Representación esquemática de los 16 posibles estereoisómeros del 1,2,5,6,9,10-hexabromociclododecano. Configuraciones absolutas de seis pares de enantiómeros (1a/b, 2a/b, 5a/b, 6a/b, 7a/b, 8a/b) y cuatro mesoformas (3, 4, 9 y 10) (Heeb et al., 2007).

Figura 1-3. Estructura molecular del TBBPA.

Figura 1-4. Estructura molecular del Declorane Plus.

Figura 1-5. Diagrama de una cadena alimentaria del ártico canadiense (Tomy et al., 2008b)

Figura 1-6. Concentración mediana de la suma de PBDEs y BDE-209, por países.

Figura 1-7. Concentración mediana de HBCD en muestras de polvo interior por países.

Figura 1-8. Concentración promedio de a) HBCD y b) TBBPA en distintos ambientes interiores.

Figura 1-9. Comparación de la exposición diaria de a) adultos y b) niños a HBCD a través de la ingestión de polvo por países.

Figura 1-10. Concentración promedio de a) HBCD y b) TBBPA en aire interior del Reino Unido.

Figura 1-11. Concentración de PBDE en alimentos de distintos países.

Figura 1-12. Distribución (%) de PBDEs en alimentos de a) España y b) EE.UU.

Figura 1-13. Contribución relativa (%) de varias especies marinas a la exposición de consumidores holandeses a HBCD.

Figura 1-14. Comparación de la exposición diaria a TBBPA en el Reino Unido.

Figura 1-15. Intervalos de concentración (ng/g lw) de HBCD y TBBPA en leche materna de distintos países.

Figura 1-16. Fórmula estructural de hormonas tiroideas.

Figura 1-17. Comparación del a) consumo de disolvente y el b) tiempo de extracción utilizando distintas técnicas de extracción.

Figura 2-1. Ejemplo de la separación cromatográfica de HBCD por GC-MS utilizando una columna DB5-MS.

Figura 2-2. Separación cromatográfica de α -, β - y γ -HBCD (100 pg/ μ L) utilizando UPLC-QqQ-MS.

Figura 2-3. Cromatograma obtenido para una mezcla de estándares de α -, β - y γ -HBCD con a) agua y metanol, b) agua, metanol y acetonitrilo en la fase móvil.

Figura 2-4. Cromatograma a) muestra de sedimento previo a la purificación y b) muestra de lodo de EDAR posterior al tratamiento de purificación por SPE.

Figura 2-5. Ejemplo de la distribución de TBBPA y sus compuestos relacionados en una muestra de lodo de EDAR.

Figura 3-1. Estructura molecular del a) PBEB, b) HBB y c) DBDPE.

Figura 3-2. Estructura molecular del a) Mirex, b) DPMA, c) Cl₁₀DP, d) Cl₁₁DP, e) Dec 602, f) Dec 603, g) Dec 604, h) BB-153, i) HCDBCO, j) BEHTBP, k) OBIND, l) BTBPE.

Figura 3-3. Distribución de los diastereoisómeros de HBCD en peces y sedimento de Monzón.

Figura 4-1. Concentración promedio de HBCD (ng/g lw) en diferentes países

Figura 4-2. Tendencia temporal de HBCD en Japón y Suecia.

Figura 4-3. Gráfico de EFs de α -HBCD y γ -HBCD en leche materna.

Figura 4-4. EFs de HBCD en muestras de leche materna de Australia

ÍNDICE DE TABLAS

Tabla 1-1. Resumen de niveles de HBCD, TBBPA y DP en matrices abióticas.

Tabla 1-2. Resumen de los niveles (ng/g lw) encontrados en muestras bióticas terrestres y acuáticas.

Tabla 1-3. Comparación de distintas técnicas de detección para BFRs.

Tabla 2-1. Comparación de los LOD (pg inyectados) obtenidos en los diferentes instrumentos utilizados.

Tabla 3-1. HBCD (ng/g dw) en sedimentos del río Cinca

Tabla 3-2. Comparación de concentraciones de BFRs en sedimentos de España.

Tabla 3-3. Resumen de niveles de HFRs encontrados en huevos de halcón peregrino.

Tabla 3-4. Niveles de HBCD diastereoisomérico y EF de α -HBCD en huevos de halcón peregrino.

Tabla 3-5. Niveles de HFRs en huevos de halcón peregrino.

Tabla 4-1. Concentración de HBCD (ng/g) en aceites de pescado.

Tabla 4-2. Valores de EDI de HBCD debido al consumo de suplementos alimenticios

ÍNDICE DE ACRÓNIMOS Y ABREVIATURAS

ABS	Acrilonitrilo-butadieno-estireno (ABS, del inglés <i>Acrylonitrile butadiene styrene</i>)
APCI	Ionización química a presión atmosférica (del inglés, <i>Atmospheric pressure chemical ionization</i>)
APPI	Fotoionización a presión atmosférica (del inglés, <i>Atmospheric pressure photoionization</i>)
BB-153	2,2',4,4',5,5'-hexabromobifenilo
BEHTBP	Bis(2-etil-1hexil)tetrabromoftalato (del inglés, <i>Bis(2-ethyl-1hexyl)tetrabromophthalate</i>)
BFR	Retardante de llama bromado (del inglés, <i>Brominated flame retardant</i>)
BPA	Bisfenol A (del inglés, <i>Bisphenol A</i>)
BSEF	Foro ambiental de la ciencia del bromo (del inglés <i>bromine science of environment forum</i>)
bw	peso corporal (del inglés <i>body weight</i>)
CCIW	Canada centre for inland waters
Cl ₁₀ DP	Decacloropentaciclooctadecadieno
Cl ₁₁ DP	Undecacloropentaciclooctadecadieno
CRM	Material de referencia certificado (del inglés, <i>Certified reference material</i>)
CHE	Confederación hidrográfica del Ebro
DBDPE	Decabromodifeniletano (del inglés, <i>Decabromodiphenylethane</i>)
DCM	Diclorometano
Dec 602	Declorane 602
Dec 603	Declorane 603
Dec 604	Declorane 604
DiBBPA	Dibromobisfenol A (del inglés, <i>dibromobisphenol A</i>)
DP	Declorane plus
DPMA	Hexaclorotriciclotridecadieno
DSL	lista nacional de sustancias de Canadá (del inglés, <i>domestic substances list</i>)
dw	peso seco (del inglés, <i>dry weight</i>)
ECNI	Ionización negativa con captura de electrones (del inglés, <i>electron capture negative ionization</i>)
EDAR	Estación depuradora de aguas residuales
EDI	Valor de ingesta diaria (del inglés, <i>estimated daily intake</i>)

Índices

EF	Fracción enantiomérica (del inglés, <i>enantiomeric fraction</i>)
EHTeBB	2-etilhexil-2,3,4,5-tetrabromobenzoato
EI	Impacto electrónico (del inglés, <i>electron impact</i>)
EPA	Agencia de Protección del Medio Ambiente de EE.UU. (del inglés <i>environmental protection agency</i>)
ESI	Ionización por electrospray (del inglés, <i>Electrospray ionization</i>)
EURACHEM	Foro para la química analítica europea (del inglés, <i>european analytical chemistry</i>)
<i>f</i> anti	Fracción enantiomérica anti
FD	Fracción disuelta
FP	Fracción particulada
FR	Retardante de llama (del inglés, <i>flame retardant</i>)
GC	Cromatografía de gases (del inglés, <i>Gas chromatography</i>)
GPC	Cromatografía de permeación en gel (del inglés, <i>Gel permeation chromatography</i>)
HBB	Hexabromobenceno
HBCD	Hexabromociclododecano
HCDBCO	Hexaclorociclopentenil-dibromociclooctano
HCH	Hexaclorociclohexano
HFR	Retardante de llama halogenado (del inglés, <i>halogenated flame retardant</i>)
HR	alta resolución (del inglés, <i>high resolution</i>)
HRMS	Espectrometría de masas con alta resolución (del inglés <i>High resolution mass spectrometry</i>)
IUPAC	Unión para la química pura y aplicada (<i>union of pure and applied chemistry</i>)
IT	Trampa de iones (del inglés, <i>ion trap</i>)
K _{OW}	Coefficiente de partición octanol-agua (del inglés, <i>octanol-water partition coefficient</i>)
LC	Cromatografía líquida (del inglés, <i>liquid chromatography</i>)
LR	baja resolución (del inglés, <i>low resolution</i>)
LRM	material de referencia de laboratorio (del inglés, <i>laboratory reference materials</i>)
lw	peso lipídico (del inglés, <i>lipid weight</i>)
LLE	Extracción líquido-líquido (del inglés, <i>liquid-liquid extraction</i>)
MAE	Extracción asistida por microondas (del inglés, <i>microwave assisted extraction</i>)
MonoBBPA	Monobromobisfenol A (del inglés, <i>Monobromobisphenol A</i>)

Índices

MS	Espectrometría de masas (del inglés, <i>mass spectrometry</i>)
MS-MS	Espectrometría de masas en tándem
NCI	Ionización química negativa (del inglés, <i>negative chemical ionization</i>)
OBIND	Octabromotrimetilfenilindano
OH-PBDEs	PBDEs hidroxilados
PBB	Polybrominated biphenyls
PBDE	Polibromodifenil éteres (del inglés, <i>Polybrominated diphenyl ether</i>)
PBEB	Pentabromoetilbenceno
PCB	Policlorobifenilo
PLE	Extracción con líquidos presurizados (del inglés, <i>pressurized liquid extraction</i>)
POP	Compuesto orgánico persistente (del inglés, <i>persistent organic pollutant</i>)
QqLIT	Cuadrupolo acoplado a una trampa de iones lineal (del inglés, <i>quadrupole lineal ion trap</i>)
QqQ	Triple cuadrupolo
QqTOF	Cuadrupolo acoplado a tiempo de vuelo (del inglés <i>quadrupole time of flight</i>)
RSD	Desviación estándar relativa (del inglés, <i>relative standard deviation</i>)
SFE	Extracción con fluido supercrítico (del inglés, <i>supercritical fluid extraction</i>)
SIM	Selected ion monitoring
SPE	Extracción en fase sólida (del inglés, <i>solid-phase extraction</i>)
SRM	Selected reaction monitoring
T4	Tiroxina
TBBPA	Tetrabromobisfenol A (del inglés, <i>Tetrabromobisphenol-A</i>)
TBCD	Tetrabromocyclododecadienes
TBECH	1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane
TriBBPA	Tribromobisfenol A (del inglés, <i>tribromobisphenol A</i>)
UAE	Extracción asistida por ultrasonidos (del inglés, <i>ultrasonic-assisted extraction</i>)
UPLC	Cromatografía líquida de ultra eficacia (del inglés, <i>ultra performance liquid cromatography</i>)
ww	peso húmedo (del inglés, <i>wet weight</i>)

CAPÍTULO 1: INTRODUCCIÓN GENERAL Y OBJETIVOS

1. INTRODUCCIÓN GENERAL

1.1. Retardantes de llama

Los incendios son una fuente importante de daños a la propiedad, de pérdida de vidas y de gasto de dineros tanto públicos como privados. Por ello, desde tiempos remotos el ser humano ha buscado la forma de disminuir la flamabilidad de ciertos materiales, por ejemplo los egipcios (450 AC.) para utilizaban una mezcla de alumbre para reducir la flamabilidad de la madera, mientras que los romanos (200 AC.) lo hacían con una mezcla de alumbre y vinagre (Alaee et al., 2003).

Hoy en día, con el fin de prevenir la generación de incendios y cumplir con las normas de seguridad establecidas, se utilizan sustancias químicas que se agregan o reaccionan con materiales combustibles para aumentar su resistencia al fuego (Darnierud 2003). Así, los retardantes de llama (FR, del inglés *flame retardant*) se aplican a materiales tales como plásticos, maderas, papel y textiles.

Los recientes avances en la tecnología han dado lugar a un aumento en el uso de polímeros sintéticos en productos para el hogar y la oficina, como computadoras y equipos electrónicos, contribuyendo al aumento del riesgo de incendios en edificios comerciales y residenciales. Este hecho ha llevado al desarrollo de varios tipos de FR, producidos a partir de compuestos inorgánicos, tales como los hidróxidos de aluminio y magnesio, o utilizando derivados orgánicos como ingredientes activos (nitrógeno, cloro, fósforo, y bromo), para aplicar en estos productos poliméricos.

En el proceso de combustión tiene lugar una reacción entre un combustible y el oxígeno. Hay cuatro pasos involucrados en el proceso de combustión: el precalentamiento, la volatilización / descomposición, combustión y propagación (Troitzch 1990). El FR debe inhibir o reprimir una etapa particular de este proceso. Dependiendo de su modo de acción, los FR pueden actuar química y/o físicamente tanto en la fase sólida, líquida o gaseosa. Durante el proceso de combustión se producen radicales libres, elementos esenciales para la propagación de la llama y es sobre los que actúan los retardantes de llama halogenados (HFR, del inglés *halogenated flame retardants*), ya que los halógenos son muy eficaces en la captura de radicales libres, ayudando a evitar la propagación de las llamas. La eficiencia de captura de radicales libres por parte de los halógenos es $I > Br > Cl > F$ (Wilkie y Morgan 2010). Sin embargo, no todos los compuestos orgánicos halogenados son adecuados para ser utilizados en la producción de FR. En consecuencia, sólo los compuestos organoclorados y organobromados son adecuados para utilizar en los FR. Sin embargo, debido a que los compuestos organobromados tienen una eficiencia de captura más alta y la temperatura de descomposición más baja, los BFRs (BFR, del inglés *brominated flame retardants*) son más utilizados que sus homólogos organoclorados. Actualmente, la demanda del mercado por FR se

estima en alrededor de 2,3 millones de dólares correspondientes a más de 1,2 millones de toneladas, de las cuales alrededor del 36% son BFR.

De todos los posibles FR, al comenzar la presente tesis nos centramos en el análisis de BFR aunque a medida que se desarrollaba se vio la necesidad de estudiar algunos FR clorados, por lo tanto a lo largo de este manuscrito cuando sea necesario incluir tanto a compuestos organobromados como organoclorados, los generalizaremos como HFR.

1.2. Compuestos analizados

En este apartado se explicarán las propiedades fisicoquímicas y aplicaciones de los FR a los cuales nos hemos dedicado en esta tesis. También se señalará la legislación existente que regula el uso de los FR en estudio.

Polibromodifenil éteres. Los polibromodifenil éteres (PBDEs, del inglés *polybrominated diphenyl ether*) contienen 10 átomos de hidrógeno, cualquiera de los cuales pueden ser intercambiados por bromo, pudiendo existir un total de 209 posibles congéneres (de Boer et al., 2000) (Figura 1-1). La nomenclatura que se ha adoptado es la misma que introdujeron Ballschmiter y Zell (1980) para los diferentes congéneres de los bifenilos policlorados (PCBs, del inglés *polychlorinated biphenyls*).

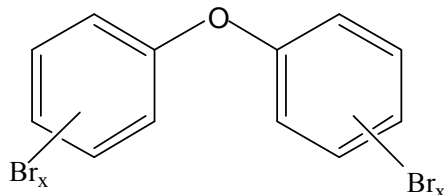


Figura 1-1. Estructura general de los PBDEs.

Los PBDEs se producen comercialmente mediante la bromación directa de difenil éter en presencia de tribromuro de aluminio o hierro como catalizadores, resultando en una mezcla de PBDEs con diversos grados de bromación.

Los tres principales productos comerciales son las formulaciones PentaBDE, OctaBDE y DecaBDE. Usualmente la mezcla de PentaBDE se compone de 24-37% de tetra-BDE, 50-60% de penta-BDE y 4-8% de hexa-BDE. En el caso de la formulación de OctaBDE consiste en una mezcla de 10-12% de hexa-BDE, 44% de hepta-BDE, 31-35% de octa-BDE, 10-11% de nona-BDE, y menos del 1% de deca-BDE; y el DecaBDE contiene más de un 97% de BDE-209 con el resto principalmente nona-BDE y una cantidad muy pequeña de octa-BDE (Esch 1994).

A pesar de que la aparición de estos compuestos en el medio ambiente tuvo lugar a finales de 1970, no se comenzó el estudio en profundidad de su impacto en el medio ambiente y la salud humana 1998, cuando Norén et al. (Norén y Meironyté 2000) encontraron que la concentración de PBDEs analizados en leche materna humana fue en aumento, duplicándose cada cinco años entre 1972 y 1997, mientras que los niveles de otros contaminantes orgánicos persistentes disminuyeron en las mismas muestras. Posteriormente, se han realizado múltiples estudios con el objetivo de analizar estos compuestos en diversas matrices ambientales.

En el año 2001 la Unión Europea anunció la prohibición del uso de las formulaciones PentaBDE y OctaBDE a partir de 2003. Sin embargo, el uso y los efectos ambientales de DecaBDE son controvertidos. La inclusión de una sentencia reciente del Tribunal Europeo de Justicia, revocó la exención de DecaBDE concedida por la Comisión Europea (Bureau 2010). Por otro lado, en los EE.UU., ha sido prohibida la producción de PentaBDE y OctaBDE (Schechter et al., 2010) y, a partir del año 2013 la industria ha acordado el cese de la producción de DecaBDE (Hess 2009).

Hexabromociclododecano. El hexabromociclododecano (HBCD) se utiliza principalmente como FR en espumas de poliestireno, textiles y equipamiento electrónico doméstico (Covaci et al., 2006; Law et al., 2006c). En los últimos años el uso de HBCD ($C_{12}H_{18}Br_6$) ha aumentado pues se utiliza en el reemplazo de otras formulaciones de BFRs, principalmente PBDEs que han sido prohibidos en Europa.

Industrialmente el HBCD se produce por la adición de bromo al *cis-trans-trans*-1,5,9, ciclododecatrino. Este proceso lleva teóricamente a la obtención de una mezcla de 16 estereoisómeros, seis pares de enantiómeros y cuatro mesoformas (Figura 1-2) (Heeb et al., 2007). Usualmente la mezcla técnica contiene tres isómeros: α -, β - y γ -HBCD donde normalmente, el isómero γ - es el más abundante (75-89%), seguido del α - (10-13%) y luego el β - (1-12%) (Covaci et al., 2006; Law et al., 2005), aunque también se han encontrado el δ - y el ϵ -HBCD en proporciones menores al 0,5% (Heeb et al., 2007). Se debe tener en cuenta que las diferencias estructurales de los estereoisómeros individuales de HBCD pueden ser las responsables de sus diferentes propiedades como polaridad, momento dipolar y solubilidad en agua. Por ejemplo, la solubilidad en agua del α -, β - y γ -HBCD es 48,8; 14,7 y 2,1 $\mu g/L$, respectivamente (Tomy et al., 2004). Estas características podrían ser las responsables de las desiguales velocidades de absorción biológica y metabólica que presentan los diastereoisómeros de HBCD, pudiendo explicar las variaciones observadas en el comportamiento del HBCD en el medio ambiente.

Al igual que los PBDEs, el HBCD puede ser incorporado al medio ambiente por diferentes vías, como son la emisión durante la producción de BFRs o la manufactura de productos que contengan

FR, además de poder ser liberados durante el uso de productos de consumo que lo contengan o durante su disposición final y/o eliminación (Tomy et al., 2005). Por otro lado, es importante considerar que el HBCD al ser un FR del tipo aditivo, no forma enlaces covalentes con el material sobre el que actúa, por ello existe el riesgo de que migre desde el producto durante su uso o eliminación hacia el medio ambiente (Covaci et al., 2006).

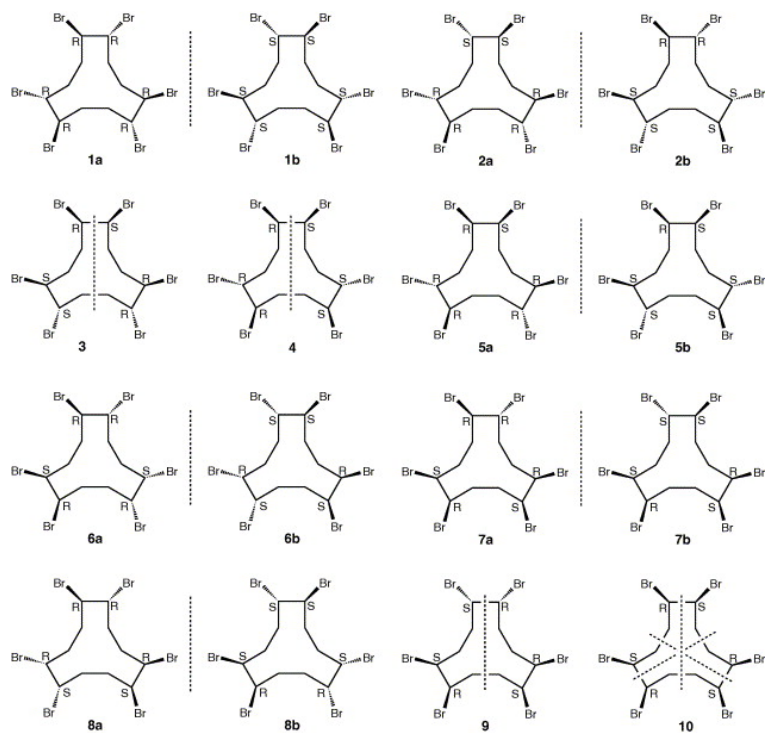


Figura 1-2. Representación esquemática de los 16 posibles estereoisómeros del 1,2,5,6,9,10-hexabromociclododecano. Configuraciones absolutas de seis pares de enantiómeros (1a/b, 2a/b, 5a/b, 6a/b, 7a/b, 8a/b) y cuatro mesoformas (3, 4, 9 y 10) (Heeb et al., 2007).

Las propiedades fisicoquímicas del HBCD son similares a algunos PBDEs y otros compuestos orgánicos persistentes. De hecho, HBCD es lipofílico y potencialmente bioacumulable; ha sido detectado en distintas matrices ambientales además de animales acuáticos y/o terrestres (Law et al., 2008; Morris et al., 2006; Tomy et al., 2004; Tomy et al., 2005; Tomy et al., 2008b; Yu et al., 2008a; Yu et al., 2008b; Zegers et al., 2005). Según la mayoría de estudios realizados, se ha podido establecer que el perfil diastereoisomérico de HBCD en sedimentos es semejante al de la formulación comercial, donde el isómero más abundante es el γ -HBCD, mientras que en muestras de biota, el α -HBCD domina el perfil (Covaci et al., 2006; Law et al., 2006c; Law et al., 2006b; Tomy et al., 2004). Hay que tener en cuenta que a temperaturas superiores a 160 °C se puede

producir un reordenamiento de los estereoisómeros, resultando en una mezcla de 78% de α -, 13% de β - y 9% de γ -HBCD (Barontini et al., 2001). Este fenómeno puede ocurrir durante la producción y eliminación de materiales que contienen HBCD y, de esta forma la abundancia relativa de los estereoisómeros puede diferir de la presente en la mezcla comercial.

Tetrabromobisfenol A. El Tetrabromobisfenol A (TBBPA, del inglés *tetrabromobisphenol A*) se produce industrialmente mediante la bromación de bisfenol A (BPA, del inglés *bisphenol A*) en presencia de un solvente, como por ejemplo metanol. Durante los años 1999 y 2004 el consumo a nivel mundial de TBBPA ($C_{15}H_{12}Br_4O_2$) (Figura 1-3) fue de 120 a 170 mil toneladas, lo que lo convierte en el BFR más utilizado (Covaci et al., 2009). El TBBPA se utiliza como FR principalmente en las resinas del tipo epoxi y acrilonitrilo-butadieno-estireno (ABS, del inglés *Acrylonitrile butadiene styrene*), polímeros de uso común en aparatos electrónicos y de entretenimiento (televisores, ordenadores y equipos de automatización de oficinas), además de laminados de circuitos impresos (BSEF 2009).

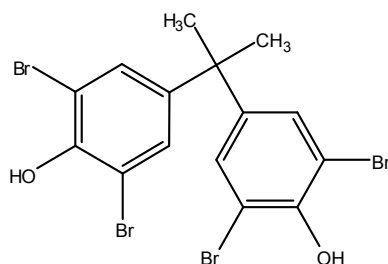


Figura 1-3. Estructura molecular del TBBPA.

Cerca de un 90% del TBBPA se emplea como BFR del tipo reactivo (~ 90%) en la fabricación de resinas epoxi, fenólicas y policarbonato, mientras que el resto se utiliza como un BFR del tipo aditivo (Nakajima et al., 2009). El TBBPA se produce solo en EE.UU., Israel y Japón (Covaci et al., 2009); sin embargo, el TBBPA puede ser importado como un producto primario o en productos que lo contienen.

El TBBPA difiere de los anteriores BFR debido a la presencia de dos grupos hidroxilo, que le confieren características de ácido débil. Los valores estimados de pK_{a1} y pK_{a2} son 7,5 y 8,5, respectivamente; esto indica que en un medio neutro, una parte importante del TBBPA se encuentra en su forma disociada (Covaci et al., 2009; Esch 1995). Debido a su baja solubilidad en agua (0,72 mg/l) y valor de log del coeficiente de partición octanol-agua (K_{ow} del inglés *octanol-water partition coefficient*) (4,5), se espera que el TBBPA en el medio ambiente se encuentre asociado al material

particulado en suspensión así como en los sedimentos (Chu et al., 2005; Saint-Louis y Pelletier 2004).

Es importante tener en cuenta que el TBBPA también se utiliza como intermediario para la producción de diferentes derivados del TBBPA (Esch 1995). Los grupos fenólicos pueden ser fácilmente utilizados para la derivatización y subsecuente modificación para formar éteres, como son TBBPA dialiléter, diglicidiléter y 2,3-dibromopropiléter.

Aunque el TBBPA se produce en volúmenes mucho más altos que otros BFR, no hay legislación que restrinja el uso de TBBPA en EE.UU. ni en Europa.

Declorane Plus. El Declorane Plus (DP) es un FR clorado ($C_{18}H_{12}Cl_{12}$) que ha sido fabricado por Oxychem (Niagara Falls, NY) durante más de 40 años (Figura 1-4). Solo recientemente se ha detectado en el medio ambiente, así como en matrices biológicas. El DP es una sustancia química con una producción estimada que oscila entre 500-5.000 toneladas por año desde 1986 y se vende en todo el mundo (Gauthier y Letcher 2009). Este compuesto sustituye al Declorane ($C_{10}Cl_{12}$), o Mirex, que fue prohibido en la década de los 70 debido a sus efectos ambientales y toxicológicos. El DP también aparece en la lista nacional de sustancias de Canadá (DSL, del inglés *domestic substances list*), pero no está clasificado como de alto riesgo en términos de bioacumulación, debido a su elevada masa molecular (648Da) y alto log Kow (9,3) (Ren et al., 2008).

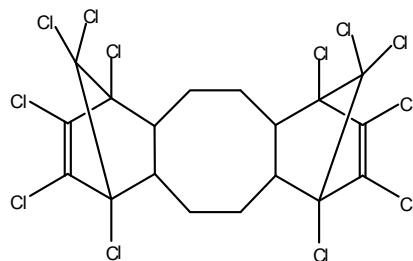


Figura 1-4. Estructura molecular del Declorane Plus.

En la formulación comercial, el DP se encuentra como dos isómeros ópticos: el *syn*-y el *anti*-DP, los cuales se producen industrialmente en una proporción aproximada de 1:2 respectivamente. Estos estereoisómeros son los productos de la reacción de Diels-Alder de 2 moles de hexaclorociclopentadieno con 1 mol de 1,5-ciclooctadieno (Tomy et al., 2007). Hay que tener en cuenta que el material a proteger puede contener hasta un 35% en peso de DP (Tomy et al., 2008a). El DP se utiliza principalmente en productos tales como recubrimientos de cables, materiales de plástico para techos, además de conectores de ordenadores y televisores. Recientemente se ha detectado la presencia de los isómeros de DP en muestras bióticas y abióticas de los Grandes Lagos

de América del Norte, que son una zona de impacto al encontrarse cerca de la industria que produce DP industrialmente (Gauthier y Letcher 2009; Hoh et al., 2006; Tomy et al., 2007).

1.3. Dinámica y distribución en el medio ambiente

El estudio intensivo de la presencia e impacto ambiental de los HFRs comenzó durante la década de los 90, con los PBDEs y se ha ido incrementando a lo largo de los años el número de investigaciones y compuestos en estudio. El objetivo de este apartado es dar ejemplos de la literatura reciente sobre niveles y tendencias de HFRs en matrices ambientales bióticas y abióticas. En la literatura es posible encontrar distintos trabajos que han revisado los niveles de PBDEs en el medio ambiente y humanos (Covaci et al., 2006; Covaci et al., 2009; 2010; Hale et al., 2006; Law et al., 2006b; Law et al., 2008), por ello se ha decidido utilizar literatura más reciente, publicada entre los años 2008 y 2010, haciendo énfasis en el estudio de HBCD, TBBPA y DP.

1.3.1. Matrices abióticas

Existen numerosos trabajos que analizan PBDEs en matrices abióticas con el objetivo de evaluar el comportamiento, distribución y destino de estos compuestos. En los últimos años la información generada respecto a otros HFRs ha ido en aumento y algunos de estos estudios se citan a continuación. En la Tabla 1-1 se resumen algunos trabajos posteriores al año 2008 donde se analizan HBCD, TBBPA y DP en distintas matrices ambientales del tipo abiótico: aire, agua, suelos, sedimentos y lodos de depuradora. Los niveles encontrados se proporcionan como promedio y, entre paréntesis las concentraciones mínimas y máximas observadas. Como antecedente adicional, se mostrarán también ejemplos de niveles de PBDEs encontrados en cada una de las matrices estudiadas.

Aire. En este apartado se trata la distribución de los HFR en el aire exterior, mientras que el comportamiento de los HFRs en el aire interior será tratado posteriormente en el apartado 1.4. de este capítulo que hace referencia a la exposición humana.

Los niveles de HFRs hallados en aire se encuentran en el intervalo de los pg/m^3 . Se han realizado diversos estudios en Norteamérica con el objetivo de estudiar el comportamiento de los PBDEs en el aire. En Point Petre, un área rural ubicada en la zona de los Grandes Lagos (Canadá) se obtuvo una concentración promedio de PBDEs de $7.0 \pm 13 \text{ pg/m}^3$ (excluyendo al BDE-209) y $1,8 \pm 1,5 \text{ pg/m}^3$ en el caso de BDE-209 (Su et al., 2009), mientras que en el aire de áreas urbanas como Chicago y Cleveland (EE.UU.) la concentración de PBDEs fue de 65 ± 4 y $87 \pm 8 \text{ pg/m}^3$, respectivamente (Venier y Hites 2008).

También se han estudiado los niveles de PBDEs en el aire de dos zonas ubicadas al oeste de Canadá: una zona remota en la Isla de Vancouver y una zona cercana a un centro urbano en el Estrecho de Georgia (Vancouver) (Noel et al., 2009). Las concentraciones detectadas fueron de 12,2 pg/m³ y 13,7 pg/m³, respectivamente. Se sugiere que el rápido movimiento de las masas de aire desde el oeste a través del Océano Pacífico, proporcionan un mecanismo que ayuda a la descarga de los PBDEs procedentes de Asia en Norteamérica. Es importante señalar que esas fuentes pueden aumentar como resultado de la producción de productos electrónicos y el reciclaje de sus residuos. Concentraciones más altas de PBDEs se encontraron en aire procedente del Delta del Río Pearl (China) (Shi et al., 2009a), con valores que van desde 240 a 9.440 pg/m³, donde el BDE-209 es el congénere dominante.

Iacovidou et al. (2009) estudiaron la presencia y variación diurna de PBDEs en la atmósfera en la zona este del mar Mediterráneo, donde la concentración promedio de PBDEs encontrada en el aire fue de 3,9±2,1 pg/m³. El perfil seguido por los PBDEs no mostró variación día/noche.

Para estudiar el HBCD en el aire, es necesario tener en cuenta las dificultades analíticas inherentes a esta clase de compuestos y las bajas concentraciones ambientales en que el HBCD se encuentra en el aire. Por esta razón los estudios existentes son escasos.

Yu et al (2008b) estudiaron los perfiles diastereoisomérico y enantioméricos de HBCD en el aire procedentes de una ciudad industrial al sur de China, encontrando niveles de HBCD entre 0,69 y 3,09 pg/m³. El análisis diastereoisomérico indica que el α -HBCD fue el isómero dominante (59-68%) y el β -HBCD fue el isómero con menos concentración en todas las muestras. La evaluación enantiomérica muestra que β - y γ -HBCD forman mezclas racémicas en las muestras de aire estudiadas, mientras que se observó un leve enriquecimiento del enantiómero (-) α -HBCD.

Recientemente el DP ha sido analizado en el aire de la capa del límite marino del mar de Groenlandia, continuando por el Atlántico norte y sur. Los niveles encontrados en la atmósfera van entre los 0,05 y 4,2 pg/m³. La presencia del DP, incluso en zonas remotas como el ártico y la antártica sugieren que el DP es susceptible al transporte atmosférico a largo alcance (Moller et al., 2010).

Tabla 1-1. Resumen de niveles de HBCD, TBBPA y DP en matrices abióticas

Tipo de muestra	País/Región	año	n	α -HBCD	promedio (min-max)		TBBPA	DP	Referencia
					γ -HBCD	Σ HBCD			
Aire (pg/m ³)	China	2004	64	(0.04-2.01)	(0.02-0.64)	(0.69-3.09)		3.5	Yu et al., 2008a
	China	2004	ni					(0.8-66)	Qiu et al., 2010
	China	2005	97					0.71 (0.04-4.26)	Ren et al., 2008
	Mar de Groenlandia	2009	ni					0.30 (0.04-1.61)	Moller et al., 2010
	Océano Atlántico	2007	ni					(5.8-87)	
Agua (pg/L)	EE.UU.	2005-2006	5						Venier y Hites, 2008
	Inglaterra	2008-2009	9	15.5 (5.0-29)	44 (15-107)	67 (28-134)	498(61-2083)		Harrad et al., 2009
	Francia	2008	5				57(50-64)		Labadie et al., 2010
	Mar de Groenlandia	2009	ni					0.050 (0.003-1.05)	Moller et al., 2010
	Océano Atlántico	2008	ni					0.058 (0.009-0.66)	
Suelos (ng/g dw)	China	2006	3			1.7-5.6			Yu et al., 2008b
	China	2009	21					5.1 (0.83-1.2x103)	Wang et al., 2010a
	Inglaterra	2008-2009	9	304 (110-570)	1666 (666-3800)	2210 (880-4000)	1700 (330-3800)		Harrad et al., 2009
	Francia	2008	5				143 (65-280)		Labadie et al., 2010
	China	2006	17				33.1 (3.8-230)		Zhang et al., 2009b
Sedimentos (ng/g dw)	Noruega	ni	ni	(28-16.000)	(130-60.000)	(190-85.000)			Haukas et al., 2010
	Corea	2005	24	1.78 (0.12-8.5)	8.22 (0.23-47)	10.9 (0.39-59)			Ramu et al., 2010
	Canadá	1997-1998	ni					(0.061-586)	Sverko et al., 2008
Lodos de depuradora (ng/g dw)	Suiza	2003-2005	16			149 (39-597)			Kupper et al., 2008
	Italia	ni	7	110 (16-358)	3.3 (0.18-9.3)	139 (20-390)	0.18 (0.016-0.54)		Mascolo et al., 2010
	EE.UU.	2002-2008	84	85000 (48-330000)	68900 (336-257500)	169240 (518-647500)		31 (11-44)	La Guardia et al., 2010

ni: no se informa
dw: dry weight, peso seco

Agua. Debido a sus características físicoquímicas los HFRs no se analizan rutinariamente en agua. Sin embargo, Harrad et al. (2009) determinaron HBCD y TBBPA en el agua de 9 lagos ingleses entre 2008 y 2009, determinando concentraciones entre 80-270 pg/L y 10-3.200 pg/L respectivamente. Por otro lado, se analizó TBBPA y PBDEs en la fase disuelta (FD) y en la fase particulada (FP) de agua del Río Prédécelle (Francia) (Labadie et al., 2010). El TBBPA fue detectado solo en la FP con concentraciones que oscilaron entre <35 y 64 pg/L, probablemente debido a que su solubilidad en agua puede variar dependiendo del pH y temperatura. Contrariamente, los PBDEs fueron detectados en las dos fases con el BDE-209 como el congéner principal, seguido del BDE-99 y del BDE-47 con concentraciones totales de 2.100-3.810 pg/L, 46-181 pg/L y 46-205 pg/L, respectivamente.

El DP ha sido analizado en la capa superficial de agua de mar procedente del mar de Groenlandia y a lo largo del océano Atlántico, encontrándose principalmente asociado a la FP, con porcentajes promedio de 97±9% y 58±33% para *syn*-DP y 80±40% y 75±26% para *anti*-DP, en el mar de Groenlandia y en el transecto estudiado del Atlántico con concentraciones inferiores a 1,3 pg/L (Moller et al., 2010).

Suelos y sedimentos. Los niveles de HFRs en suelos y sedimentos suelen ser del orden de ng/g peso seco (dw, del inglés *dry weight*), aunque en áreas contaminadas puede llegar hasta los µg/g dw.

Al analizar PBDEs en suelos y sedimentos, generalmente se encuentra una mayor proporción de BDE-209, seguido de -47, -99 y -100. Este perfil fue encontrado por Yun et al (2008) al evaluar la concentración de PBDEs en sedimentos y suelos procedentes del Río Saginaw (EE.UU.), encontrando valores promedio de 3,5 y 5,90 ng/g dw, respectivamente (Yun et al., 2008). Valores más altos tanto en suelos (63,3 ng/g dw) como en sedimentos (1.263 ng/g dw) fueron encontrados en el sur de China, donde también el BDE-209 fue el congéner principal (Shi et al., 2009a).

Debido a su carácter hidrofóbico, el HBCD se encuentra fuertemente unido a partículas sólidas como por ejemplo suelos, sedimentos y lodos de depuradora (Covaci et al., 2006). Es por ello que el HBCD se puede detectar en la mayoría de los estudios realizados.

Los PBDEs y HBCD fueron analizados en sedimentos recogidos en 24 áreas costeras de Corea (Ramu et al., 2010). Las concentraciones de PBDEs y BDE-209 varían entre 0,05-32 ng/g dw y 0,40-98 ng/g dw, respectivamente. En el caso del HBCD los valores van desde 0,39 a 59 ng/g dw. En general la distribución y frecuencia del HBCD en los sedimentos costeros de Corea se corresponden con las zonas urbanizadas e industrializadas. Respecto a la concentración individual de los diastereoisómeros del HBCD, en la mayoría de los sedimentos el patrón fue similar, siendo γ -HBCD el isómero más abundante.

En sedimentos de un fiordo contaminado de Noruega se determinaron las concentraciones de HBCD a lo largo de un transecto, partiendo de una fuente puntual conocida (Haukas et al., 2010). Así, se observa que el valor promedio de HBCD disminuye a medida que aumenta la distancia río abajo. Por otro lado, los niveles totales de HBCD encontrados en suelos de áreas urbanas del sur de China varían entre 1,7 y 5,6 ng/g dw (Yu et al., 2008a). En cuanto al patrón diastereoisomérico, se observaron diferentes distribuciones ya que en dos muestras predomina el γ -HBCD, mientras que en la otra muestra el α -HBCD es el isómero principal.

Se determinan PBDEs y TBBPA en sedimentos de río de una región fuertemente industrializada en China. Las concentraciones de TBBPA van desde 3,8 a 230 ng/g dw, mientras que los niveles de tri- a hepta-BDE y nona- a deca-BDE varían desde 0,7 a 7,6 ng/g dw y de 30 a 5.700 ng/g dw (Zhang et al., 2009b). En los sedimentos más recientes se observa un aumento en la concentración de TBBPA, reflejando el incremento en su utilización comercial.

Al analizar muestras de sedimentos del río Prédécelle (Francia), se observaron valores de PBDEs desde 3,0 a 15,1 ng/g dw siendo el BDE-209 el congénere más abundante, seguido del -47 y el -100. También se encontraron concentraciones de TBBPA que oscilan entre 70 y 280 pg/g dw. Debido a que los niveles de concentración correlacionan positivamente, Labadie et al. (2010) sugieren que tanto los PBDEs como el TBBPA tienen fuentes similares.

También se analizaron HBCD y TBBPA en sedimentos de nueve lagos procedentes de Inglaterra recogidos en 2008-2009, con niveles que van desde 0,88 a 4,8 ng/g dw y de 0,33 a 3,8 ng/g dw, respectivamente. Al mismo tiempo, se detectaron tetrabromociclododecadienos en todos los sedimentos y pentabromociclododecadienos en algunos de ellos, sugiriendo la degradación del HBCD mediante la pérdida de HBr (Harrad et al., 2009).

En cuanto a DP, en suelos de la ciudad china Huai'an, las concentraciones varían entre 0,83 y $1,2 \times 10^3$ ng/g (Wang et al., 2010a), siguiendo una distribución logarítmica. Se calculó el *fanti* para cada una de las muestras, obteniéndose valores con una gran variación espacial que va desde 0,67 a 0,85, con un valor promedio de 0,79 que se aproxima al valor observado de *fanti* para el DP comercial.

Por otro lado, los niveles de DP encontrados en sedimentos de los lagos Erie y Ontario (Canadá) fueron de 0,061-8,62 ng/g y 2,23-586 ng/g respectivamente, donde la concentración media de DP en el Lago Ontario fue más de 50 veces mayor que en el Lago Erie (Sverko et al., 2008). En este caso se calculó la *fsyn* para el DP técnico ($0,355 \pm 0,004$) y para las distintas muestras de sedimento, obteniéndose valores entre 0,069 y 0,287. Estos datos muestran un enriquecimiento estereoselectivo del isómero *anti*-DP en comparación con la mezcla técnica.

Lodos de depuradora. Los contaminantes orgánicos lipofílicos son incorporados al flujo de aguas residuales luego de su uso doméstico e industrial o vía deposición aérea en las superficies impermeables y su posterior escurrimiento en el drenaje de aguas residuales.

Kupper et al. (2008) analizaron PBDEs y HBCD en lodos de 16 estaciones depuradoras de aguas residuales (EDARs) suizas, encontrando valores entre 7,9-617 ng/g dw y 39-597 ng/g dw, respectivamente. El BDE-209 fue el congéner principal, seguido por BDE-47 y -99, ambos con concentraciones semejantes. De hecho, este es el perfil que generalmente se halla en los lodos de depuradora. Por otro lado, se determinan residuos de PBDEs, HBCD y TBBPA en los lodos procedentes de siete EDARs municipales de Italia, con valores promedios de 490, 139 y 0,18 ng/g dw, respectivamente (Mascolo et al., 2010). Los resultados muestran que el BDE-209 fue el congéner más abundante en todas las muestras analizadas, con valores entre 4,6 y 24,5 veces mayor que la suma de todos los demás BFRs analizados en este trabajo. Respecto a los otros BFRs en estudio, el α -HBCD fue el compuesto más abundante en todas las muestras analizadas y, TBBPA se determina con un valor significativo (1,8-31,1 ng/g dw) en los lodos de dos de las depuradoras estudiadas.

Los niveles de HBCD y DP en lodos de depuradora de la costa atlántica de EE.UU. (La Guardia et al., 2010) fueron elevados, con valores entre 518-647.500 ng/g dw y 11-44 ng/g dw, respectivamente. Respecto al DP, la muestra correspondiente al año 2007 fue la que presentó el valor mayor (44 ng/g dw). Además el isómero *anti*- contribuye solo con un 39-54%, aunque en la literatura se informa una contribución de entre el 65 y 80% de isómero *anti*- en la fórmula industrial.

1.3.2. Matrices bióticas

Existe gran cantidad de estudios sobre los PBDEs en la fauna silvestre, lo que se ha traducido en la generación de una importante base de datos de las concentraciones de PBDEs en todo tipo de organismos. Similares estudios se han comenzado a desarrollar involucrando FRs emergentes.

A continuación se proporcionará una visión general de los estudios más importantes en que figuran HBCD, TBBPA y DP, realizados entre los años 2008 y 2010 tanto en muestras procedentes del medio acuático como terrestre. En la Tabla 1-2 se encuentran resumidos los datos encontrados en la revisión bibliográfica.

Medio acuático. Como se observa en la Tabla 1-2, se han realizado diversos estudios con el objetivo de analizar los niveles de HBCD en biota del medio acuático. Lo contrario sucede con el TBBPA y el DP, posiblemente debido a sus propiedades fisicoquímicas en el caso del primero y a que ha sido hallado recientemente en el medio ambiente, en el caso del segundo.

Tabla 1-2. Resumen de los niveles (ng/g lw) encontrados en muestras bióticas terrestres y acuáticas.

Tipo de muestra	Región	año	Especie	α -HBCD	γ -HBCD	Σ HBCD	TBBPA	DP	Referencia
aves marinas	Noruega	1983-2003	<i>Larus argentatus</i>	52 (16-109)					Helgason et al., 2009
			<i>Rissa tridactyla</i>	76 (30-142)					
	Canadá (*) China (†)	1982-2006 2005-2008	<i>Fratercula arctica</i>	34 (12-58)					<15 Gauthier y Letcher 2009 He et al., 2010
			<i>Larus argentatus</i>	1995 (420-5058)	nd (nd-64)	1995 (460-5058)	173 (133-243)		
mamíferos marinos	China Indo-Pacífico EE.UU.	2002-2008	<i>Ardeola bacchus</i>	27.5 (nd-66.6)	27 (nd-305)	73 (nd-394)	170 (28.1-1482)		Lam et al., 2009
			<i>Amaurornis phoenicurus</i>	6.9 (nd-169)	14.1 (nd-27.1)	52 (nd-344)	54.3 (10.6-149)		
	Groenlandia (*)	1993-2004 1999-2001	<i>Gallinago gallinago</i>	nd (nd-42.5)	nd (nd-216)	nd-216	103.4 (9.0-139)		Peck et al., 2008 Gebblin et al., 2008
			<i>Soucia chinensis</i>	156 (29-511)	10.3 (<0.15-137.3)	168 (32-519)			
	Belgica Inglaterra	2000-2006 2008	<i>Neophocaena phocaenoides</i>	53 (4.0-498)	1.3 (<0.15-9.7)	55 (4.1-501)			Roosens et al., 2010 Harrad et al., 2009
			<i>Lagenorhynchus acutus</i>			130 (19-380)			
	China (†)	2005-2008	<i>Ursus maritimus</i>			41			He et al., 2010
			<i>Salvelinus namaycush</i>	22 (15-27)	3.4 (1.4-6.5)	25 (16-33)			
	Noruega	2004-2005 2004-2005	<i>Anguilla anguilla</i>	110	43	394 (16-4397)		4.6 (2.3-7.2)	Haakias et al., 2009
			<i>Oncorhynchus mykiss</i>	31 (7.8-65)	14 (3.7-30)	180	0.81 (0.32-1.3)		
aves terrestres	Grandes Lagos, EE.UU. (*) Grandes Lagos, Canadá (*)	1987-1999 2005-2008	<i>Perca flaviventris</i>	91 (43-230)	27 (11-42)	127 (66-290)	1.120.55-1.7		Venier et al., 2010 Ferne et al., 2010
			<i>Carassius carassius</i>	82 (37-160)	40 (9.2-94)	140 (35-210)	<LOQ		
	Suecia	2005-2008	<i>Rutilus rutilus</i>	86	38	150	<LOQ		Johansson et al., 2009
			<i>Cyprinus carpio</i>	37	37	95	<LOQ		
	China (†)	2001-2006	<i>Scardinius erythrophthalmus</i>	34	4.1	41	<LOQ		He et al., 2010
			<i>Tinca tinca</i>	95 (16-250)	15.9 (6.4-25)	120 (26-290)	<LOQ		
	Noruega	2005-2008	<i>Exocoetidae</i>	95.7 (35-284)	13.4 (2.2-67.8)	112.4 (21-378)	1.14 (0.23-1.74)		Haakias et al., 2009
			<i>Carassius carassius</i>	579 (12-3100)	1697 (10-9000)	2380 (20-14000)			
	Grandes Lagos, EE.UU. (*) Grandes Lagos, Canadá (*)	2005 2004-2005	<i>Arenicola marina</i> (Δ)			0.13 (0.02-0.56)		0.19 (0.02-0.68)	Venier et al., 2010 Ferne et al., 2010
			<i>Haliotis leucophaea</i>			0.02 (<LOD-0.46)			
mamíferos terrestres	China (†)	2005-2008	<i>Falco peregrinus</i>	14.2 (nd-243)	54.3 (16.6-229)	73.6 (19-492)	90.1 (39.2-503)		Johansson et al., 2009
			<i>Stercoraria pomarina</i>	27.5 (nd-66.6)	27 (nd-305)	73 (nd-394)	170 (28.1-1482)		
	Japón	2001-2006	<i>Amaurornis phoenicurus</i>	20 (10-25.6)	22 (20.2-75.2)	47.5 (30.5-99.7)	28.2 (24.2-54.8)		Kumise et al., 2008
			<i>Francolinus pintadarius</i>	0.96 (<0.005-10)	1.1 (<0.005-20)	1.9 (<0.005-29)			
	Noruega	2005-2008	<i>Nyctereutes procyonoides</i>	1.8 (0.20-4.1)	0.059 (<0.005-0.17)	1.9 (0.21-4.3)			Kumise et al., 2008
			<i>Ursus maritimus</i>						

(Δ): origen
LOQ: límite de cuantificación(*) : ww, wet weight (peso seco)
LOD: límite de detección

En aves marinas los tres compuestos han sido estudiados: el HBCD en Noruega, China y Sudáfrica, el TBBPA en China y el DP en la zona de los Grandes Lagos (Canadá). El estudio realizado en Noruega analiza los niveles y las tendencias temporales (1983-2003) del HBCD en huevos de tres especies de aves marinas del norte de Noruega: gaviota argénterea (*Larus argentarius*), gaviota de patas negras (*Rissa tridactyla*) y frailecillos atlánticos (*Fratercula arctica*) (Helgason et al., 2009). Todos los huevos estudiados presentaron niveles detectables de α -HBCD, siendo mayor la concentración en gaviota de patas negras, y luego disminuyendo en el siguiente orden: gaviota argénterea > frailecillos atlánticos. Respecto a las tendencias temporales, se observó un aumento estadísticamente significativo de α -HBCD en los huevos de las tres especies estudiadas a lo largo del período de tiempo investigado. Así, este estudio muestra que HBCD se encuentra presente en el medio ambiente de Noruega y que sus niveles en huevos de aves marinas han ido en aumento.

El HBCD y el TBBPA fueron estudiados en diferentes especies de aves originarias de una región en donde se realiza el reciclaje de desechos electrónicos en China (He et al., 2010). Las concentraciones de HBCD se encuentran entre nd y 5.058 ng/g peso lipídico (lw, del inglés *lipid weight*). Estos valores se encuentran en el extremo superior de los niveles señalados anteriormente. En cuanto al patrón isomérico, α -HBCD fue el isómero principal y los valores de la fracción enantiomérica (EF, del inglés *enantiomeric fraction*) tienen un valor promedio de 0,43, sugiriendo que estas aves pueden acumular mayor cantidad de (+) α -HBCD que de (-) α -HBCD.

El TBBPA fue detectado en todas las muestras de tejido muscular con concentraciones entre 9,0 y 243 ng/g lw (He et al., 2010). Estos valores son superiores a los encontrados en otros estudios (Morris et al., 2004; Vorkamp et al., 2005), indicando que el reciclado de residuos y las actividades de manufactura electrónica son importantes fuentes de TBBPA en el sur de China.

Gauthier y Letcher (2009) estudiaron los cambios temporales y la distribución espacial del DP entre 1982 y 2006 en huevos de gaviota argénterea (*Larus argentatus*) de la zona de los Grandes Lagos canadienses (Gauthier y Letcher 2009). Las concentraciones de DP en las siete áreas estudiadas, fueron menores a 15 ng/g peso húmedo (ww, del inglés *wet weight*), indicando que este HFR se acumuló en las gaviotas a través de la dieta y fue transferido durante la ovogénesis a sus huevos. Es importante señalar que los niveles de DP en los huevos de gaviota disminuyen a medida que aumenta la distancia del área de las Cataratas del Niágara, área ubicada muy cerca de la planta industrial de Oxychem donde se fabrica el DP. El valor de la fracción *fanti* promedio fue de $0,69 \pm 0,08$ que es muy similar al del producto comercial (0,75-0,77), indicando que no hay enriquecimiento estereoselectivo de *syn*- o *anti*- DP en los huevos de gaviota estudiados.

Solo el HBCD ha sido estudiado en mamíferos, concretamente en delfines, marsopas y osos polares, procedentes de China, EE.UU. y Groenlandia. Lam et al., (2009) estudiaron la tendencia temporal de HBCD en el tejido adiposo del delfín jorobado (*Sousa chinensis*) del Indo-pacífico y marsopas

(*Neophocaena phocaenoides*) de la región de Hong Kong entre los años 2002-2007 y 2003-2008, respectivamente.

El HBCD fue detectado en todas las muestras con niveles entre 32-519 y 4,1-501 ng/g lw para delfín y marsopas, respectivamente (Lam et al., 2009). El α -HBCD fue detectado en todas las muestras analizadas, mientras que el β - en el 54% y γ -HBCD en el 60% de los casos. La concentración de HBCD mostró una tendencia temporal positiva en las muestras de delfines, lo contrario se observó en las marsopas. Esta diferencia se puede atribuir a la distribución geográfica específica de HBCD creada por las diferencias regionales en su producción y aplicación. Los niveles de HBCD en delfines de EE.UU. (Peck et al., 2008) fueron de 19 a 380 ng/g lw, con un promedio de 130 ng/g lw. Estos valores son similares a los observados por Lam et al. (2009).

En osos polares, el α -HBCD solo fue detectado en el tejido adiposo, lo que se podría deber a que en sangre e hígado hay un menor contenido lipídico que permita retener al α -HBCD (Gebbinck et al., 2008).

En peces, el HBCD fue detectado en muestras de Canadá, Bélgica, Inglaterra, China y Japón. Al comparar las concentraciones promedio obtenidas, los niveles más altos fueron señalados en anguilas de Bélgica (394 ng/g lw), siendo en la mayoría de los peces, el α -HBCD el isómero principal (Roosens et al., 2010). Respecto al TBBPA, los niveles hallados son considerablemente más bajos, por ejemplo Harrad et al. (2009) lo detectaron en 2 de 8 grupos de peces de distintas especies (0,32-1,12 ng/g lw), mientras que en muestras de China (He et al., 2010) los niveles de TBBPA son 100 veces menos concentrados que los del HBCD.

Respecto al DP, éste fue analizado en truchas del lago Ontario (Canadá) presentando concentraciones entre $2,3 \pm 0,6$ y $7,2 \pm 1,3$ ng/g lw. Los niveles de DP disminuyeron significativamente entre 1979 y 2004, con una vida media de 14 años (Ismail et al., 2009).

Finalmente, se observaron altas concentraciones de HBCD en gusanos marinos (*Arenicola marina*) de Noruega (2.380 ng/g lw promedio), donde el isómero dominante fue el γ -HBCD (Haukas et al., 2010).

Medio terrestre. En la Tabla 1-2 también se resumen los estudios recientes sobre residuos de HFRs en muestras bióticas pertenecientes al medio terrestre. Se observa que en este medio se han realizado menos trabajos (5) en comparación al medio acuático (9) y, nuevamente el HBCD es el compuesto más analizado (86% de los trabajos).

La mayoría de los estudios están referidos a aves con dieta terrestre: águila calva (*Haliaeetus leucocephalus*), halcón peregrino (*Falco peregrinus*), paloma moteada (*Streptopelia chinensis*), gallineta pechiblanca (*Amazorhynchus phoenicurus*) y francolín chino (*Francolinus pintadeanus*), procedentes de distintos países como EE.UU., Canadá, Suecia y China. Hay que destacar la

importancia de realizar estudios con aves ubicadas en la parte superior de la cadena trófica, como es el caso del águila calva y del halcón peregrino ya que son capaces de bioacumular y bioconcentrar compuestos persistentes.

Los niveles de HBCD observados van desde 0,02 hasta 1.900 ng/g lw, siendo las concentraciones en los huevos de halcón peregrino de Suecia las más altas (Johansson et al., 2009). Solo en el caso de las muestras de tejido muscular de aves originarias de China se realizó el análisis diastereoisomérico y se encontró que el γ -HBCD es el isómero dominante en las muestras de paloma y francolín, con un porcentaje promedio de 72% y 63%, respectivamente (He et al., 2010).

En las muestras de China, también se analizó el TBBPA, donde las medianas encontradas son superiores a los valores de HBCD. Por ejemplo, la gallineta tenía una concentración promedio de TBBPA de 170 ng/g mientras que la del HBCD era de 73,4 ng/g. Hay que tener en cuenta que normalmente la concentración de HBCD es mayor que la de TBBPA (Tabla 1-2).

Venier et al. (2010) analizaron el DP en el plasma de águilas de la región de los Grandes Lagos (EE.UU.), detectándolo en el 40% de las muestras con una concentración promedio de $0,19 \pm 0,10$ ng/g ww.

Finalmente, solo fue posible encontrar un estudio realizado en mamíferos utilizando muestras de hígado (n=39) y tejido adiposo (n=8) de perro mapache (*Nyctereutes procyonoides*) de tres regiones de Japón (Kunisue et al., 2008). Se calculó la relación entre la concentración de HBCD en hígado/tejido adiposo de los perros mapaches, observándose que el α -HBCD es lípido-dependiente presentando una relación promedio de 0,9 con un rango entre 0,3 y 2,5 mientras que el γ -HBCD tiene una relación de 5,9 en un rango de 0,6 a 29. Por lo tanto, el estudio propone que el γ -HBCD se podría acumular en órganos ricos en sangre como el hígado y difícilmente puede ser acumulado en el tejido adiposo, debido a la posible unión con proteínas y/o rápida biotransformación.

1.3.3. Biodisponibilidad y bioacumulación

Biodisponibilidad y bioacumulación están intrínsecamente conectados entre sí y directamente relacionados con la interacción de las sustancias químicas con el medio ambiente abiótico y biótico. La biodisponibilidad de los contaminantes químicos en el medio ambiente depende de varios factores, incluyendo estructura y propiedades físico-químicas, presencia en el medio, capacidad de ser transportados a diferentes ambientes y la naturaleza del medio en el que estas sustancias químicas se encuentran.

Como regla general, cuanto más lipofílica es una sustancia, mayor es su capacidad de ser bioacumulada por los organismos. Las sustancias lipofílicas tienen menos probabilidades de ser diluidas o ser excretadas en la orina, por lo que se pueden acumular en los tejidos grasos.

La dehalogenación biológica por parte de los organismos, explica la razón por la cual la composición de los congéneres de PBDEs encontrada en la biota no se asemeja a la composición de las mezclas comerciales: congéneres que no han sido observados en ninguna mezcla comercial han sido encontrados en muestras biológicas (Hites 2005). Si la razón de estos cambios es debido al metabolismo o evacuación, o si son el resultado de la debromación de otros congéneres, aún esta por determinar.

Como consecuencia de la persistencia y bioacumulación de los compuestos químicos, y debido a la interacción entre los diferentes niveles tróficos en un ecosistema seleccionado, una sustancia química puede ser biomagnificada en la cadena alimentaria. Cuando un compuesto químico se bioacumula en un organismo de un nivel trófico bajo, tales como especies de invertebrados, puede dar lugar a una acumulación en el tejido adiposo de los sucesivos niveles tróficos, tales como peces, aves o mamíferos marinos. Cuando un depredador se alimenta de otro organismo, las grasas que llevan los contaminantes son absorbidos en el intestino, acumulando el compuesto en su grasa. Respecto a la evaluación de la biodisponibilidad y bioacumulación de HBCD, la información disponible es escasa. Recientemente Tomy et al. (2008b) estudiaron la bioacumulación del HBCD en una cadena alimentaria marina del ártico canadiense (Figura 1-5). En general, el orden de importancia de las concentraciones de HBCD fue: narval (*Monodon Monoceros*) > gallineta (*Sebastes mentella*) ~ almejas (*Mya truncate* y *Serripes groenlandica*) > camarones (*Pandalus borealis* y *Hymenodora glacialis*) > beluga (*Delphinapterus leucas*) > zooplancton > morsa (*Odobenus rosmarus*) > bacalao (*Boreogadus saida*), solo se detectaron α - y γ -HBCD en las muestras. No se observó ninguna tendencia clara en el perfil diastereoisomérico de HBCD en los animales analizados. α -HBCD contribuyó con más del 70% del total de HBCD en las muestras de camarones, gallineta, bacalao, narval y beluga, mientras que zooplancton, almejas y morsa, contienen más de un 60% del γ -HBCD. El patrón diastereoisomérico observado puede ser en parte explicado debido a que γ -HBCD es el isómero menos soluble en agua de los tres isómeros de HBCD. Así, la difusión pasiva del γ -HBCD desde la columna de agua hacia el zooplancton podría suceder de forma más fácil que para los otros dos isómeros. En el caso de las almejas, al ser organismos bentónicos filtradores es probable que se expongan al HBCD procedente de los sedimentos en donde se sabe que el γ -HBCD es el isómero dominante. Además, las almejas también constituyen la mayor parte de la dieta de la morsa, que se cree que tiene una baja capacidad metabólica del γ -HBCD. La predominancia de α -HBCD en los organismos superiores de la cadena trófica, como beluga y narval es consistente con la mayor capacidad metabólica de estos animales.

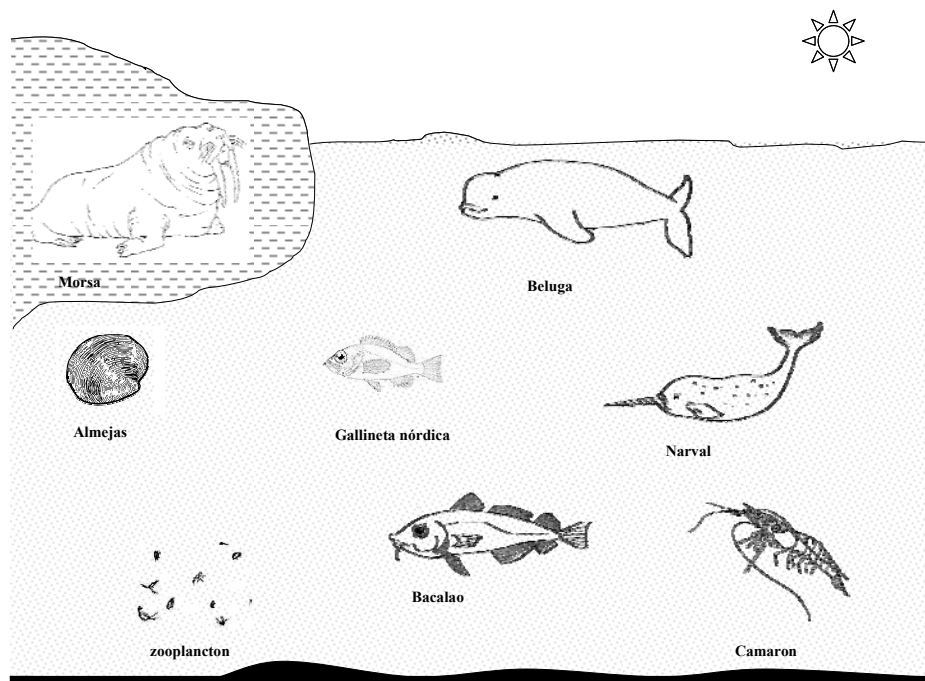


Figura 1-5. Diagrama de una cadena alimentaria del ártico canadiense (Tomy et al., 2008b).

En este trabajo también se estudiaron las EF y posible bioacumulación enantioselectiva del HBCD. Para el α -HBCD, las muestras de narval, beluga y morsa presentan valores de EF que son estadísticamente superiores a los valores del patrón analizado, sugiriendo el enriquecimiento selectivo del (-) α -HBCD por parte de estos animales. En el caso de las muestras de bacalao, almejas y camarones, todos tuvieron valores de EF para α -HBCD menores a 0,48 aunque solo las almejas tienen un valor de EF estadísticamente diferente al patrón. Finalmente narval, morsa, almejas y camarones muestran EF mayores que 0,51 para γ -HBCD pero no fueron significativamente diferentes a los valores de EF encontrado para los patrones.

Un estudio similar fue desarrollado en China (Wu et al., 2010b) evaluando una cadena trófica de agua dulce. Se incluyen dos especies de invertebrados (caracol chino (*Cipangopaludina chilensis*) y camarones (*Macrobrachium nipponense*)), tres especies de peces (carpa de barro (*Cirrhinus molitorella*), carpa dorada (*Carassius auratus*) y pez cabeza de serpiente (*Ophicephalus argus*)) y un reptil (serpiente de agua (*Enhydris chinensis*)). En las muestras de biota se obtuvieron resultados análogos, donde se observa un cambio en la composición de HBCD, con el α -HBCD como el isómero principal y aumentando su presencia a medida que se asciende en la cadena trófica. También se realizó el análisis enantiomérico, observándose residuos no racémicos de α -HBCD en

carpa de barro y dorada, y de γ -HBCD en la serpiente acuática, con preferencias para (+) α - y (+) γ -HBCD, respectivamente.

En la misma cadena trófica se evaluó la bioacumulación isómero-específica y transferencia trófica del DP, detectándolo en todas las especies acuáticas con concentraciones entre 19,1-9.630 ng/g lw (Wu et al., 2010a). Se observó una disminución de *anti*-DP en los organismos en comparación con las muestras abióticas. Además la fracción de *anti*-DP se redujo aún más al moverse a niveles tróficos superiores sugiriendo que en estos organismos sucede un metabolismo estereoselectivo del *anti*-DP y/o captación isómero-selectiva del *syn*-DP.

Finalmente hay que señalar que no se encontraron estudios respecto a la bioacumulación y/o biomagnificación del TBBPA.

1.4. Exposición humana

La exposición humana a los FR ocurre a través de diversas vías: ingestión de polvo, inhalación de aire y/o material particulado e ingestión a través de los alimentos, principalmente pescados grasos, carne, productos lácteos, además de la leche materna y la transferencia placentaria.

Sin embargo, estas vías de exposición parecen tener una contribución diferente dependiendo del tipo de compuestos considerados. En el caso de los HFRs, su presencia en el polvo doméstico y su contribución a la exposición total humana a través de la ingestión de polvo sigue siendo una cuestión crítica. Por otro lado, los HFRs pueden ser incorporados por el ser humano a través de la ingesta de alimentos, permitiendo su biomagnificación a través de la cadena alimentaria. Este es el caso del BDE-47 en los seres humanos que consumen peces del mar Báltico (Sjodin et al., 2003).

1.4.1. Ingestión de polvo

Una característica de la vida moderna es que los seres humanos pasamos mayor tiempo en el interior, tanto en el ambiente de trabajo como en casa. Como resultado, la infraestructura al interior ha cambiado significativamente, prestando más atención al ocio (por ejemplo, TV, computadores y electrodomésticos) y la seguridad. Así el desarrollo industrial ha dado lugar a una gran cantidad de productos químicos para dar cabida a este estilo de vida, donde los HFRs tienen un papel esencial.

Un punto importante sobre la ingestión de polvo y su contribución a la exposición humana a BFRs es que en el caso de los estadounidenses, es considerada como la vía de exposición principal (Lorber 2008), mientras que en el caso de los europeos la dieta parece más importante (Harrad et al., 2004). Por otra parte, hay que tener en cuenta que la importancia de la ingestión de polvo disminuye con la edad, debido a las tasas de ingestión más altas por parte de los niños pequeños.

En distintos estudios se han señalado niveles relativamente altos de PBDEs en polvo doméstico (Harrad et al., 2008; Huang et al., 2010; Toms et al., 2009) y en menor medida, en el polvo de oficinas (Huang et al., 2010) y, en microambientes como coches (Cunha et al., 2010) y aviones (Christiansson et al., 2008).

Debido al contenido de PBDEs en el polvo, algunos estudios consideran su ingestión como la principal vía de exposición a los PBDEs para la población en general (Johnson-Restrepo y Kannan 2009; Lorber 2008). La presencia de PBDEs en el polvo se ha atribuido a los productos de consumo que los contienen. Sin embargo, el mecanismo de transferencia sigue siendo poco conocido.

Se han comparado las concentraciones de PBDE en el polvo del hogar de diversos estudios realizados en distintos países, indicando que las muestras procedentes de EE.UU. y el Reino Unido contienen las cantidades más altas de PBDE totales, mientras que las muestras de Alemania y Nueva Zelanda son las que presentan una menor concentración (Harrad et al., 2008; Sjödin et al., 2008) (Figura 1-6).

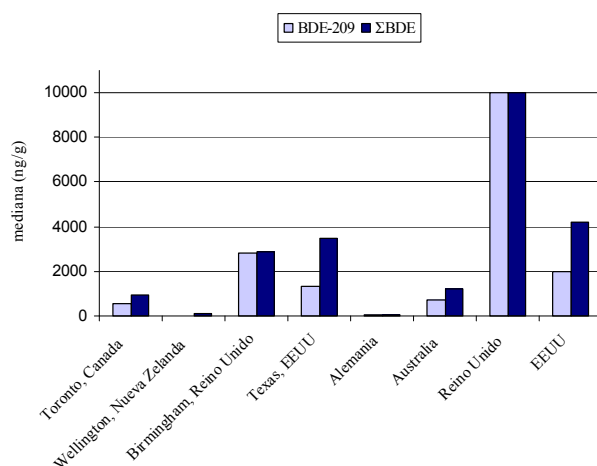


Figura 1-6. Concentración mediana de la suma de PBDEs y BDE-209 en polvo doméstico, por países.

Se han analizados distintos congéneres de PBDEs en las muestras de polvo (BDE-47, -99, -100, -153, -154, -183 y -209) y se observa una similitud con el patrón seguido por la mezcla industrial PentaBDE, donde los niveles de BDE-99 y BDE-47 son similares. En cuanto al BDE-209, se observa que fue el congénere dominante en todos los países. La mayor presencia de BDE-209 en el Reino Unido se puede deber a que en el 95% de los materiales de tapicería se utilizan FR pues éste es el único país dentro de la Unión Europea que tiene un reglamento que determina el nivel de

resistencia al fuego de la tapicería doméstica, de esta forma entre 1000-1200 toneladas/año de BDE-209 son utilizadas en aplicaciones textiles (Sjodin et al., 2008).

Varios estudios han estimado la ingesta de los PBDEs por parte de la población general y han evaluado la ingestión de polvo doméstico como una vía importante de aporte al total de la carga corporal de PBDEs (Johnson-Restrepo y Kannan 2009; Lorber 2008; Sjodin et al., 2008). Las estimaciones de la ingesta varían según la edad con diferentes cantidades de polvo ingeridos por los lactantes, niños y adultos (D'Hollander et al., 2010; Sjodin et al., 2008). Por ejemplo, D'Hollander et al. (D'Hollander et al., 2010) calcularon que la ingesta media diaria de polvo por parte de la población de Flandes es de 7 mg/día y 50 mg/día para adultos y niños, respectivamente.

Respecto a la concentración de HBCD en polvo doméstico existen pocos estudios. Recientemente los diastereoisómeros α -, β - y γ -HBCD fueron medidos en muestras de polvo doméstico del Reino Unido, EE.UU. y Canadá (Abdallah et al., 2008b). Las abundancias relativas de α -, β - y γ -HBCD varían respecto a las que se encuentran presentes en la formulación comercial, observándose una mayor presencia de α -HBCD, constituyendo más del 50% de la concentración total de HBCD en 11 de las 58 muestras analizadas. Un estudio similar se realizó en polvo interior procedente de casas y oficinas de Flandes (Bélgica) (D'Hollander et al., 2010), obteniéndose concentraciones de HBCD entre 5-42.692 ng/g dw y 256-1.153 ng/g dw, respectivamente. En este estudio, el α -HBCD predomina el perfil de HBCD (59%), seguido por γ -HBCD (29%) y β -HBCD (12%). En la Figura 1-7 se resumen las medianas de las concentraciones (ng/g dw) de HBCD obtenidas en los dos estudios.

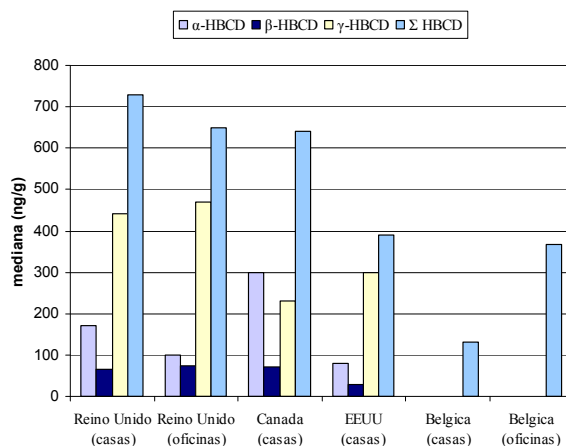
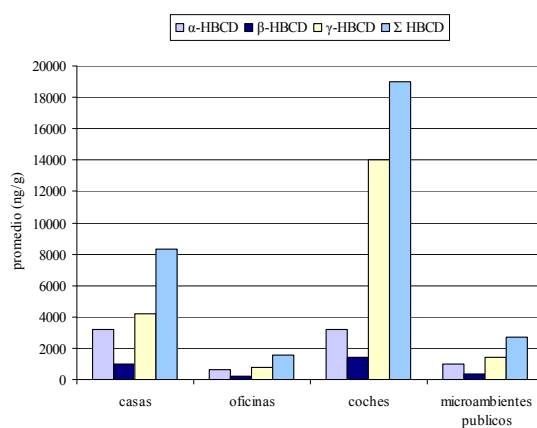


Figura 1-7. Concentración mediana de HBCD en muestras de polvo interior por países.

Stapleton et al. (2008a) realizaron un estudio sobre la presencia de HBCD en el polvo doméstico de EE.UU. La concentración máxima fue de 130.220 ng/g, siendo este dato similar al valor máximo de HBCD detectado en polvo doméstico del Reino Unido (Abdallah et al., 2008a). Las muestras fueron agrupadas según su procedencia: sala principal, dormitorio y bolsa de la aspiradora, con rangos de concentración entre <4,5 y 130.200 ng/g, <4,5 y 9.710 ng/g y 21 y 35.100 ng/g, respectivamente. El polvo del salón principal es el que presentó una mayor concentración promedio de HBCD.

El HBCD y el TBBPA fueron analizados en el polvo procedente de diferentes tipos de microambientes interiores: casas, oficinas, coches y microambientes públicos como restaurantes y bares de Birmingham (Reino Unido) (Figura 1-8).

a)



b)

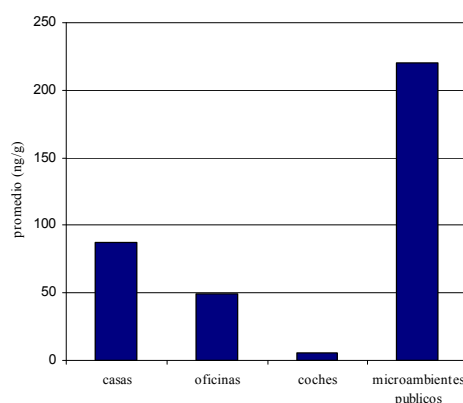


Figura 1-8. Concentración promedio de a) HBCD y b) TBBPA en distintos ambientes interiores.

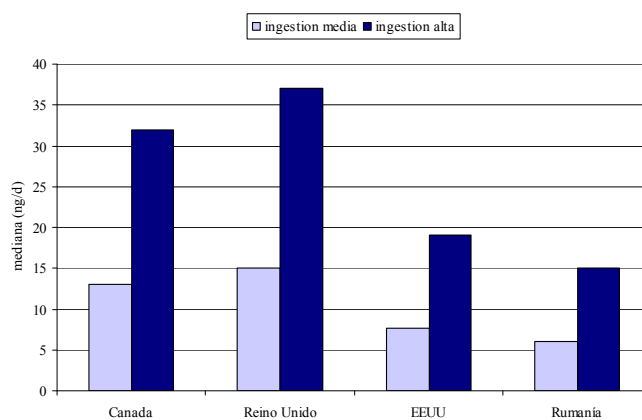
La concentración de HBCD en los coches excede la encontrada en el polvo de casas, oficinas y microambientes públicos (Figura 1-8a), mientras que para TBBPA la menor concentración fue encontrada en coches (Figura 1-8b). Respecto al TBBPA, hubo resultados similares en el polvo de 18 casas y 2 oficinas ubicadas en Bélgica, observándose un promedio de 146 ng/g y 73 ng/g, respectivamente (Geens et al., 2009). Los niveles de TBBPA encontrados en muestras de polvo interior son substancialmente menores a los niveles de HBCD. Esto es consistente con el hecho que el TBBPA se utiliza mayoritariamente como un BFR reactivo por lo tanto, es probable que su liberación desde los productos tratados sea más difícil que en el caso de los BFRs aditivos tales como el HBCD. Respecto al patrón diastereoisomérico de HBCD, en promedio la composición del polvo es de 33% de α -HBCD, 11% de β -HBCD y 56% de γ -HBCD, el cual difiere del patrón diastereoisomérico que habitualmente presenta la mezcla técnica.

En base a los resultados obtenidos se ha podido estimar la exposición humana a HBCD (Abdallah et al., 2008a; Abdallah et al., 2008b; D'Hollander et al., 2010) y a TBBPA (Abdallah et al., 2008a; D'Hollander et al., 2010) en adultos y niños pequeños. En la Figura 1-9 se compara la exposición diaria (ng/día) de a) adultos y b) niños pequeños a HBCD vía ingestión de polvo en Canadá, Reino Unido, EEUU y Rumanía. Para realizar esta estimación es necesario asumir que hay un 100% de absorción de la ingesta. Además se aplican dos valores de ingestión de polvo para adultos y niños pequeños: medio (20 y 50 mg/día, respectivamente) y alto (50 y 200 mg/día, respectivamente).

En los cuatro países estudiados se observan resultados similares de exposición a HBCD. En el caso de los valores obtenidos para Reino Unido es posible comparar con la ingesta de HBCD por medio de la dieta total en 2004 (413 ng/día de HBCD para un adulto de 70 kg y 240 ng/día HBCD para un niño de 10 kg). De esta forma es evidente que la ingestión de polvo doméstico representa una vía importante de exposición humana al HBCD en el Reino Unido.

Así, se concluye que la ingestión de polvo es una importante vía de exposición a HBCD y TBBPA para los niños del Reino Unido, incluso en un escenario de ingesta de polvo media (Abdallah et al., 2008a). Similarmente, Geen et al. (2009) evaluaron la exposición humana a TBBPA por medio de la ingestión de polvo doméstico por parte de adultos y niños pequeños. Los valores de consumo (mediana) por parte de un adulto con una ingesta media fue de 0,2 ng/día y de 0,5 para una ingesta alta de polvo. En el caso de los niños los valores fueron 14 y 34 ng/g, respectivamente.

a)



b)

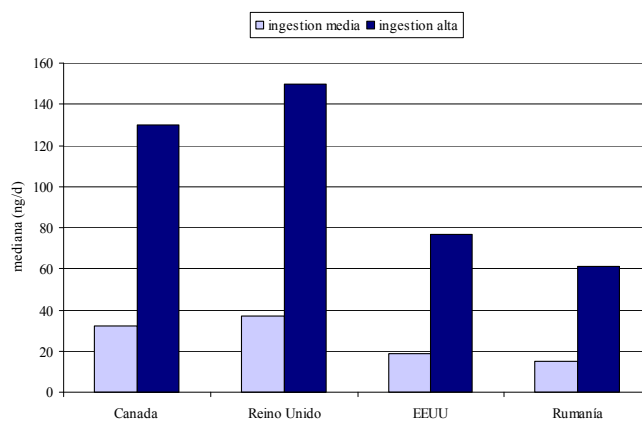


Figura 1-9. Comparación de la exposición diaria de a) adultos y b) niños a HBCD a través de la ingestión de polvo por países.

1.4.2. Inhalación de aire

Los PBDEs se distribuyen a nivel mundial a través del transporte atmosférico, por ello se encuentran por toda la atmósfera, observándose niveles en zonas remotas, incluyendo el Ártico y el Tíbet (Bossi et al., 2008; Su et al., 2009; Wang et al., 2010c). Esto pone en evidencia que la inhalación es una posible vía de exposición humana a los PBDEs.

Se han detectado diferentes gradientes de concentración de PBDEs en el aire de las zonas urbanas y rurales en distintos países (Harrad y Hunter 2006; Hoh y Hites 2005). Otros estudios han señalado de la variación del aire ambiental dentro de una misma ciudad, identificando las regiones

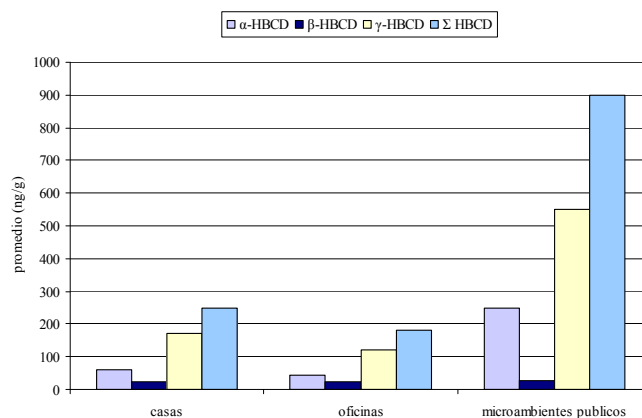
industrializadas como puntos de concentración de PBDEs (Cetin y Odabasi 2008; Chen et al., 2006a).

Los niveles de PBDEs en el aire varían entre países. En el caso del aire interior, se encontraron altas concentraciones en Canadá (Butt et al., 2004), como también en lugares de trabajo del Reino Unido (Harrad et al., 2004). Además las concentraciones de PBDE son superiores en el aire interior en comparación con el aire exterior (Butt et al., 2004; Harrad et al., 2004; Toms et al., 2009), por ello la exposición a través del aire interior ha sido objeto de varias investigaciones. La concentración relativamente alta de los PBDEs en el interior en comparación con el exterior es probable que sea relacionado con el uso y la lenta liberación de estas sustancias químicas por parte de productos de consumo y materiales de construcción que los contengan (Kemmlein et al., 2003). Como es de esperar, los congéneres con menor peso molecular son los que tienen más posibilidades de encontrarse en el aire. Esto se ve reflejado en las concentraciones encontradas en aire interior de Ottawa, donde en más del 70% de las muestras los BDE-17, -28, -47, -99 y -100 estaban presentes, con concentraciones entre nd y 1.600 pg/m³ (Wilford et al., 2004).

Se ha encontrado poca información en la literatura respecto al análisis de HBCD y TBBPA en aire interior, solo un estudio realizado en el Reino Unido analiza ambos compuestos en aire al interior de casas, oficinas y microambientes públicos (restaurantes, pubs) (Abdallah et al., 2008a). En la Figura 1-10a se muestran los valores de concentración promedio de HBCD isomérico y total, donde el γ -HBCD es el isómero que se encuentra en mayor proporción y se encontró una mayor concentración de HBCD en los microambientes públicos. Si comparamos las concentraciones de HBCD encontradas en aire con los niveles observados en polvo doméstico (Figuras 1-8a), los resultados revelan que la contribución del α -HBCD a HBCD es significativamente superior en el polvo que en el aire, con una tendencia opuesta en el caso del γ -HBCD.

Si comparamos las concentraciones de HBCD encontradas en aire con los niveles observados en polvo doméstico (Figuras 1-8a), los resultados revelan que la contribución del α -HBCD a HBCD es significativamente superior en el polvo que en el aire, con una tendencia opuesta en el caso del γ -HBCD. En promedio la composición del polvo es 33% α -HBCD, 56% γ -HBCD, mientras que en las muestras de aire es 22% α -HBCD y 65% γ -HBCD (Abdallah et al., 2008a). Al igual que ocurre con el HBCD, el TBBPA se encuentra en mayor concentración en el polvo de interior (Figura 1-10b).

a)



b)

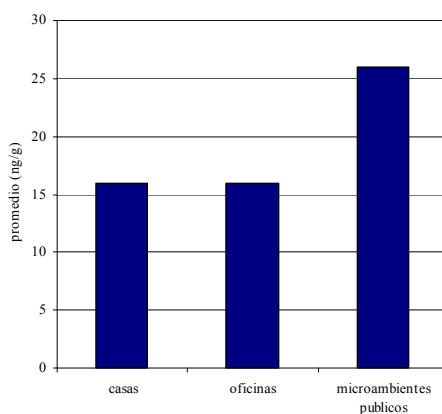


Figura 1-10. Concentración promedio de a) HBCD y b) TBBPA en aire interior del Reino Unido.

1.4.3. Ingestión de alimentos

La capacidad de los PBDEs de ser bioacumulados significa que tanto los alimentos acuáticos como los terrestres son una fuente potencial de exposición. Para los contaminantes orgánicos persistentes que presentan propiedades físico-químicas similares a los PBDEs, los alimentos también son una fuente de exposición importante.

Los PBDEs se han detectado en diversos productos alimenticios y de consumo. Las concentraciones más altas se han encontrado en aceites y grasas, productos del mar principalmente pescados grasos, carnes y derivados, huevos y productos lácteos en comparación con verduras, frutas y tubérculos

(Darnerud et al., 2006; Domingo et al., 2008; Gomara et al., 2006; Schecter et al., 2008). En la Figura 1-11 se resume el contenido de PBDEs en distintas muestras de alimentos originarios de España, Suecia y EE.UU. Como era de esperar, en los tres países las mayores concentraciones se encuentran en pescados y mariscos, donde destaca el mayor contenido de PBDEs en las muestras de EE.UU. mientras que lácteos y huevos presentan las menores concentraciones promedio. El medio acuático parece tener una mayor capacidad de concentrar y biomagnificar la mayoría de los compuestos orgánicos persistentes en biota, siendo mucho más eficaz que el compartimento terrestre (Darnerud et al., 2006).

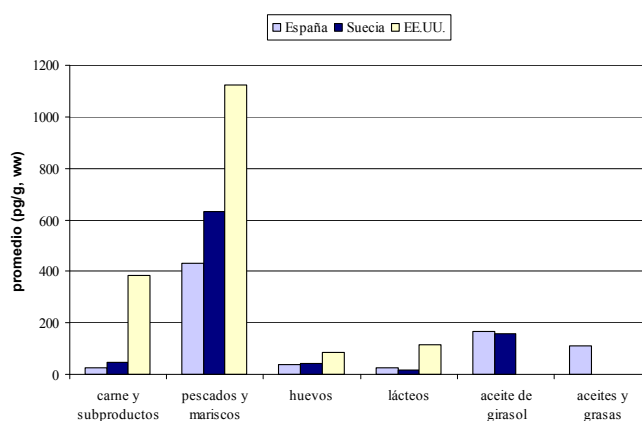


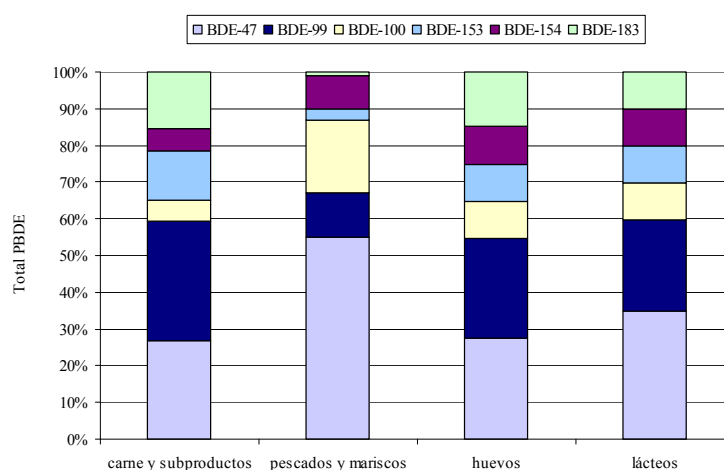
Figura 1-11. Concentración de PBDEs en alimentos de distintos países.

Respecto al patrón de comportamiento seguido por los congéneres de PBDEs (BDE-47, -99, -100, -153, -154 y -183) estos fueron estudiados en diferentes alimentos procedentes del mercado de España y EE.UU. En la Figura 1-12a podemos ver el porcentaje (%) de cada uno de los congéneres estudiados en España, donde se observa una mayor proporción de BDE-47 en todas las muestras, particularmente en las de pescados y mariscos, seguidos por el BDE-99 en las muestras de carnes y subproductos, lácteos y huevos, mientras que en el caso de las muestras de pescado hay un mayor aporte de BDE-100. Similares resultados se observan en muestras de alimentos procedentes de Rumanía (Dirtu y Covaci 2010).

El patrón de PBDEs también fue analizado en muestras de EE.UU. donde aparte de los congéneres nombrados anteriormente, se incluyó el BDE-209 (Figura 1-12b). En las muestras de pescado se observa un mayor aporte de BDE-47, mientras que en las muestras de carnes, lácteos y huevos, el BDE-99 es el que presenta un contenido mayor (Schecter et al., 2008). En los cuatro grupos de alimentos analizados hay un menor contenido de BDE-183 (0% a 3%) en comparación con las muestras de España. Al mismo tiempo se observa que el aporte de BDE-209 es de mayor

importancia en las muestras de lácteos (35%), seguido por las muestras de carne (14%), huevos (12%) y pescados (8.2%). En las muestras procedentes de Rumanía, el BDE-209 no pudo ser medido ya que en todas las muestras los valores fueron inferiores a los límites de detección (Dirtu y Covaci 2010).

a)



b)

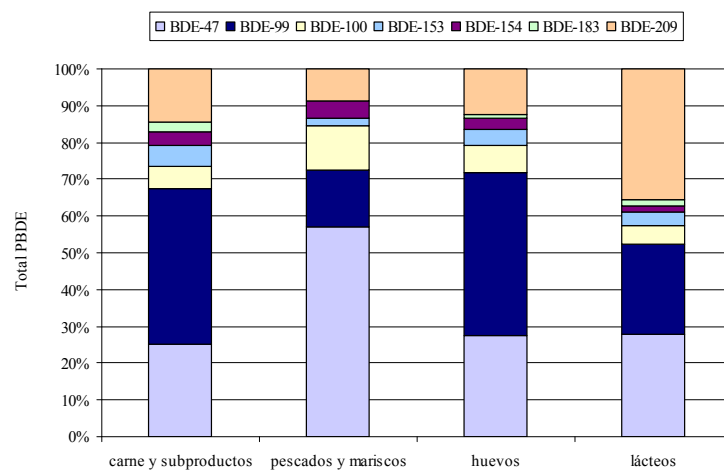


Figura 1-12. Distribución (%) de PBDEs en alimentos de a) España y b) EE.UU.

Como observamos anteriormente, el estudio de la cesta de la compra de EE.UU. (Schecter et al., 2008) mostró una alta presencia de PBDEs en pescado, luego en carnes y finalmente en lácteos,

pero se debe tener en cuenta que el estadounidense promedio tiene una ingesta dietética de PBDE procedente mayoritariamente de la carne, a continuación pescado y finalmente lácteos.

Existe poca información respecto a las concentraciones de HBCD y TBBPA en alimentos. La concentración de HBCD en alimentos ha sido evaluada en diferentes países, principalmente en pescado y sus derivados. El análisis diastereoisomérico de HBCD fue realizado en muestras de mariscos procedentes de Escocia (Fernandes et al., 2008), encontrando concentraciones totales entre 0,046 y 12,1 µg/kg ww donde el valor mayor corresponde a muestras de mejillones y el más bajo a muestras de ostión. El α -HBCD fue el isómero predominante, seguido por el γ - y el β -HBCD, tal como generalmente ocurre en muestras bióticas (Tomy et al., 2004). También se analizó la presencia de HBCD en muestras de pescado y mariscos de Holanda (Van Leeuwen y De Boer 2008), agrupándolas según su procedencia: marinas (<0,1-7,3 ng/g ww), de agua dulce (<0,1-230 ng/g ww) y cultivadas (<0,8-4,1 ng/g ww).

Dirtu y Covaci (2010) estudiaron el HBCD en muestras de carne (0,04-0,25 ng/g ww), lácteos (0,25 ng/g ww), aceite vegetal (0,25 ng/g ww) y huevos (0,04 ng/g ww) originarios de Rumanía. Con estos datos se estimó el valor de ingesta diaria (EDI, del inglés *estimated daily intake*) de HBCD por parte de un adulto (77 ng/día) y un niño pequeño (47 ng/día) a través del consumo de alimentos en Rumanía. Además, en muestras de EE.UU. las mayores concentraciones de HBCD se encontraron en muestras de sardinas enlatadas (593 pg/g), seguido por salmón fresco (352 pg/g) y mantequilla de cacahuets (300 pg/g). Por otro lado la concentración de HBCD estuvo por debajo el límite de detección (LOD, del inglés *limit of detection*) en carne picada, atún enlatado, bacalao, aceite de oliva, aceite de canola, margarina, cereales, huevos, vegetales y lácteos. Este estudio estimó el consumo total de HBCD a través de la dieta en 15,4 ng/día. La mayor contribución al consumo fue a través de la carne de vacuno (12,5 ng/día), seguido por el cerdo (4,2 ng/día) y pollo (4,2 ng/día). El consumo de HBCD por medio de pescado, productos vegetales, además de lácteos y huevos fue 0,9 ng/día, 2,0 ng/día y 0 ng/día, respectivamente (Schecter et al., 2010).

Por otro lado, Van Leeuwen y de Boer (2008) realizaron la estimación del valor de EDI de HBCD a través del consumo de pescado por parte de un holandés promedio, la cual varía entre 0,06 y 0,17 ng/kg de peso corporal (bw, del inglés *body weight*) /día para un individuo de 70 kg. En la Figura 1-13 se muestran las distintas contribuciones a la exposición por parte de diferentes especies de peces, donde se observa que un 65% se debe al consumo de arenque, seguido del bacalao y del salmón de piscifactoría.

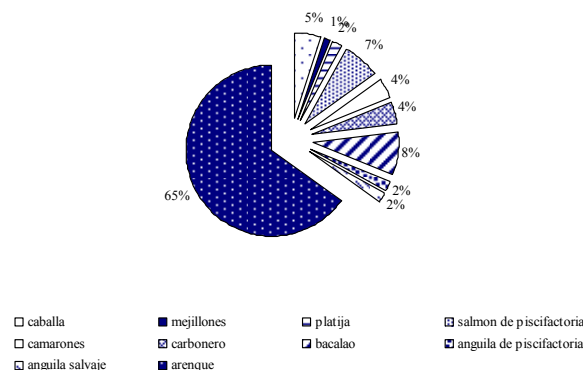


Figura 1-13. Contribución relativa (%) de varias especies marinas a la exposición de consumidores holandeses a HBCD.

También se ha calculado el valor de EDI de HBCD aportado por el consumo de mariscos en Escocia (Fernandes et al., 2008), proporcionando un valor de 5,9-7,9 ng/kg bw/día. Además se ha encontrado un estudio que evalúa el mismo parámetro en un grupo de noruegos con un amplio consumo de mariscos (entre 4-455 g/día) encontrándose un promedio de 0,3 ng/kg bw/día (Knutsen et al., 2008). Otro valor de EDI de HBCD es en mujeres de Suecia entre 17 y 74 años, con un promedio de exposición de 166 ng/día (2,5 ng/kg bw/día) (Lind et al., 2002). De esta forma se observa que el consumo de pescados y mariscos puede influir en la exposición dietética a HBCD. Además depende de la especie consumida donde los pescados con alto contenido lipídico son los que tienen una mayor contribución (Knutsen et al., 2008; Van Leeuwen y De Boer 2008), siendo un aporte importante a la ingesta total de HBCD.

Los bajos niveles encontrados de TBBPA pueden ser explicados por el valor de su log Kow (4,5-5,3), además que en la mayoría de sus aplicaciones se utiliza como BFR reactivo (Fernandes et al., 2008; Van Leeuwen y De Boer 2008). En la Figura 1-14 se muestra los resultados obtenidos para la exposición humana al TBBPA en el Reino Unido, tanto en adultos como en niños de entre 6 y 24 meses por ingestión de polvo, aire y alimentación.

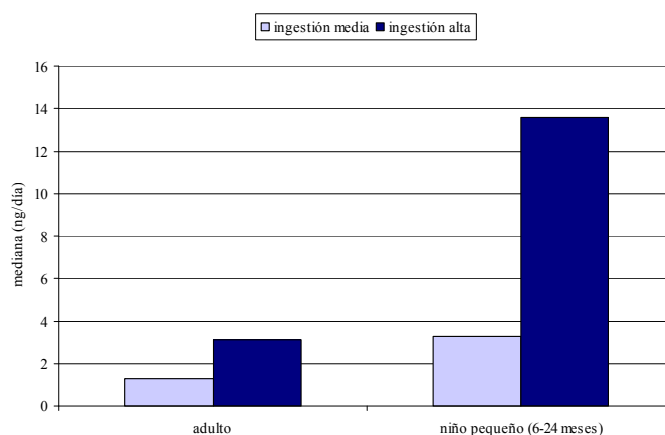


Figura 1-14. Comparación de la exposición diaria a TBBPA en el Reino Unido.

1.4.4. Grupos de Riesgo

Se debe tener en cuenta que algunos seres humanos se encuentran más expuestos a los contaminantes debido a la alimentación que reciben o por el ambiente en que residen o trabajan. Por ello tienen un mayor riesgo de incorporar estos compuestos, que pueden resultar dañinos para la salud. Este es el caso de los lactantes, que se alimentan exclusivamente de la leche materna, la cual puede tener un alto contenido de compuestos lipofílicos y, de los trabajadores expuestos a diversos contaminantes que se encuentran en su lugar de trabajo.

Lactantes. Los compuestos organohalogenados lipofílicos persistentes que se encuentran en el medio ambiente son acumulados por el cuerpo humano, siendo la eliminación de estos compuestos y sus metabolitos lipofílicos muy lenta. Debido al relativo alto contenido de grasa de la leche materna, los compuestos lipofílicos son transferidos a la leche. Así los niveles encontrados en la leche correlacionan con el contenido de grasa que tiene la matriz siendo un reflejo de la concentración acumulada en el tejido adiposo (Norén y Meirionyté 2000). De esta forma, la leche materna constituye una matriz adecuada para estudios de exposición a contaminantes organohalogenados y para estudios de la contaminación ambiental a lo largo del tiempo. Se debe tener en cuenta que los lactantes se alimentan de forma casi exclusiva de leche materna, además que su peso corporal es reducido y por ello los valores de EDI suelen ser mayores en los lactantes que en los adultos.

El análisis de la leche humana es a menudo utilizado para evaluar la carga corporal por parte de los seres humanos de contaminantes orgánicos lipofílicos persistentes, tales como los PBDEs, debido a

su contenido lipídico relativamente alto (~ 3,5 a 4%) y que los métodos de recolección no son invasivos.

Una revisión de la literatura disponible revela que las concentraciones de PBDEs en la leche materna son inferiores a 4,0 ng/g lw en países asiáticos como Indonesia (Sudaryanto et al., 2008), Japón (Eslami et al., 2006; Inoue et al., 2006; Ohta et al., 2002) y China (Li et al., 2008), mientras que en países europeos como Suecia (Lind et al., 2003; Meironyté et al., 1999), Alemania (Furst 2006), Italia (Ingelido et al., 2007) y Las Islas Faroe (Fangstrom et al., 2005) se encuentran en el mismo rango de concentración. Niveles mayores se encontraron en Australia con una concentración aproximada de 10 ng/g lw (Toms et al., 2007) y por encima de 30 ng/g lw en las muestras recogidas en EE.UU. (Johnson-Restrepo et al., 2007; Ryan et al., 2006; Schecter et al., 2003) y Canadá (Ryan y B. 2002; Ryan et al., 2006).

Hasta ahora existen pocos estudios que informen sobre la presencia de HBCD y TBBPA en leche materna. En la Figura 1-15 se señalan los intervalos de concentración encontrados en leche procedente de diferentes países. Los niveles más bajos de HBCD se obtuvieron en Suecia (0,09-0,64 ng/g lw) (Fangstrom et al., 2008) y EE.UU. (0,2-0,9 ng/g lw) (Ryan et al., 2006) mientras que los más altos se registraron en Noruega (0,58-31 ng/g lw) (Thomsen et al., 2010) y Canadá (0,4-19 ng/g lw) (Ryan et al., 2006). Lllaman la atención los niveles de HBCD en muestras de EE.UU. pues, si bien los niveles de PBDE encontrados en diferentes estudios en leche materna son bastante altos en relación a los niveles de Europa, en el caso del HBCD los niveles son similares entre ambos continentes. El α -HBCD es el isómero predominante en las muestras de leche materna de los diferentes países (Kakimoto et al., 2008; Ryan et al., 2006; Shi et al., 2009b).

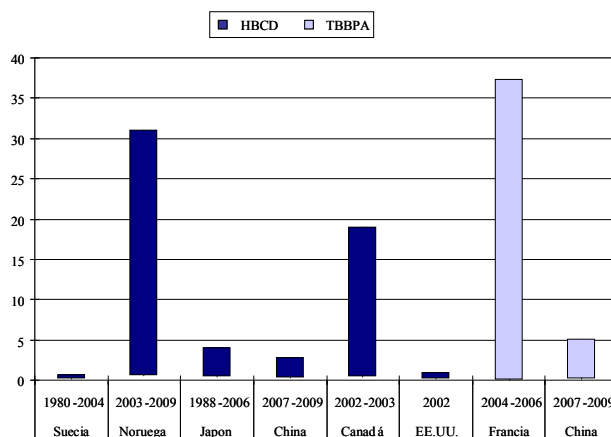


Figura 1-15. Intervalos de concentración (ng/g lw) de HBCD y TBBPA en leche materna de distintos países.

Respecto al TBBPA en leche materna, existen aún menos datos que para el HBCD. Esto se debería a que el TBBPA se une químicamente con la estructura polimérica, por lo tanto su liberación al medio ambiente es limitada. Otro punto a tener en cuenta es que el TBBPA es rápidamente metabolizado por el hígado de los mamíferos y eliminado en la bilis (Shi et al., 2009b). Se encontraron dos estudios que analizan TBBPA en leche materna procedente de Francia (Cariou et al., 2008) y China (Shi et al., 2009b) (Figura 1-15). En el primer estudio, el TBBPA fue detectado y cuantificado en 34 de las 77 muestras analizadas, con niveles que varían entre 0,062 y 37,34 ng/g lw, con un valor de mediana de 0,477 ng/g lw. En el segundo estudio, fueron analizadas 24 mezclas de leche obteniendo valores de TBBPA desde 0,11 a 5,12 ng/g lw. Además, en el 75% de las muestras la concentración de TBBPA fue inferior a 1 ng/g lw.

Exposición ocupacional. Es sabido que las personas que trabajan en ciertas industrias están predispuestas a una carga corporal potencialmente más alta de ciertos contaminantes, debido a la exposición a aire y polvo contaminado en el lugar de trabajo. Este es el caso de los PBDEs (Sjodin et al., 2003). La liberación de los PBDEs se puede acelerar en entornos industriales durante la fabricación de PBDEs, adición de PBDEs a otros productos y su posterior reciclaje, y la eliminación de productos de consumo que los contienen. Consecuentemente, en las proximidades de estas instalaciones se han observado elevados niveles de PBDEs en aire (Cahill et al., 2007; Zhao et al., 2009) y polvo (Julander et al., 2005a).

También se han encontrado elevados niveles de PBDEs en muestras de sangre de personas que trabajan en el desmantelamiento de residuos electrónicos (Sjodin et al., 1999), técnicos de una planta de reciclaje de espuma y de instalación de alfombras (Stapleton et al., 2008b), además de técnicos de informática (Jakobsson et al., 2002).

A pesar de que el HBCD y el TBBPA son FR altamente consumidos y que en general los niveles de exposición ocupacional a las sustancias químicas son mucho más altos en comparación con la exposición de fondo (Thomsen et al., 2007), el control y seguimiento de la exposición a HBCD y TBBPA en el ambiente laboral han sido escasamente estudiados.

Se ha evaluado la exposición ocupacional a HBCD por parte de los trabajadores de una planta industrial de producción de poliestireno expandible en que se utiliza HBCD como FR (Thomsen et al., 2007). Se analizó HBCD en muestras de polvo aéreo que se ha recogido cerca de la zona de respiración de 10 trabajadores, obteniendo valores de HBCD entre 0,2 y 150 $\mu\text{g}/\text{m}^3$. También se evaluó HBCD en el suero sanguíneo de los trabajadores encontrándose concentraciones que varían desde 6 a 856 ng/g lw. Por otro lado, al evaluar un grupo de referencia sin exposición laboral a HBCD, el compuesto nunca fue detectado sobre 1 ng/g. Respecto al patrón diastereoisomérico, en las muestras de polvo aéreo γ -HBCD fue el isómero con una mayor contribución promedio (81%

$\pm 6.9\%$). Este valor es similar al presentado por la mezcla comercial de HBCD ($78\% \pm 4.6\%$). En las muestras de suero sanguíneo, α - y γ -HBCD se encontraron en todas las muestras con una contribución promedio de 60 y 39% respectivamente. El valor de γ -HBCD es bastante alto en relación con otros valores observados en muestras biológicas, esto se puede deber a la exposición directa y reciente de los trabajadores a elevados niveles de la mezcla técnica de HBCD.

Finalmente, la exposición de trabajadores al TBBPA por vía respiratoria y dérmica fue evaluada en 5 entornos de trabajo: taller de muebles, instalaciones de desmantelamiento electrónico, aula de informática, oficinas y locales sociales (Makinen et al., 2009). De este estudio se desprende que el TBBPA se puede encontrar principalmente en el aire de las instalaciones de desmantelamiento electrónico ($< 4\text{--}14.600\text{ ng/m}^3$) y en el taller de muebles ($< 6\text{ ng/m}^3$), con una frecuencia de detección de 96% y 7%, respectivamente. La exposición dérmica a TBBPA se evaluó analizando parches adsorbentes que llevaron en su ropa los trabajadores. Solo en las instalaciones de desmantelamiento electrónico se obtuvieron niveles de TBBPA ($< 0.09\text{--}63\text{ ng/m}^3$) en un 92% de las muestras.

1.5. Toxicidad

La información existente acerca de la frecuencia con que son encontrados ciertos compuestos emergentes en el medio ambiente, como es el caso de los PBDEs, ha causado preocupación principalmente por el posible efecto tóxico que estos compuestos puedan tener.

En el caso de los PBDEs, la mayoría de los estudios toxicológicos se han centrado en los efectos de la exposición en los componentes del sistema endocrino (Darnerud et al., 2007; Kodavanti et al., 2010; Riu et al., 2008; Song et al., 2008; Vos et al., 2003), un sistema que juega un papel crítico en la regulación de los procesos del cuerpo humano, el desarrollo del cerebro y los tejidos nerviosos, la función y maduración sexual, el metabolismo y la homeostasis en general.

Muchos compuestos químicos sintéticos tienen la habilidad de imitar, bloquear o potenciar la respuesta de las hormonas naturales presentes en el cuerpo humano. Estos compuestos suelen ser llamados disruptores endocrinos. Uno de los órganos más importantes del sistema endocrino es la glándula tiroidea, que produce hormonas que juegan un rol esencial en el crecimiento y desarrollo (Saegusa et al., 2009). Es sabido que distintos compuestos organohalogenados pueden causar trastornos en la función normal y alteraciones en la función tiroidea, produciendo cambios en las tasas metabólicas basales, síntesis de proteínas y desarrollo celular (Palace et al., 2008; Tomy et al., 2008b).

Todas las mezclas comerciales de PBDEs, incluido el DecaBDE han mostrado tener propiedades disruptoras del sistema endocrino (Vonderheide et al., 2008). La unión competitiva de los PBDEs a los receptores de la hormona tiroidea probablemente se debe a las similitudes estructurales entre los

FR y las hormonas endógenas (Palace et al., 2008). Los PBDEs también pueden interferir alterando la función del hígado y en la homeostasis de la vitamina A, a menudo dando por resultado la eliminación de la hormona tiroidea (Zhang et al., 2009a).

Experimentos *In vivo* han demostrado que los PBDEs pueden causar diversos efectos adversos, los que dependen del tipo de producto utilizado y su dosis. Generalmente, el PentaBDE parece causar efectos con una dosis menor, mientras que son necesarias dosis mayores para obtener efectos del DecaBDE (Darnerud 2003). De esta forma, se considera que el DecaBDE tiene la menor toxicidad de las tres mezclas técnicas de PBDEs disponibles. Los efectos críticos de PentaBDE se relacionan con el desarrollo neurológico y de la hormona tiroidea en crías (Lilienthal et al., 2006; Zhou et al., 2001). Mientras que el OctaBDE principalmente origina toxicidad fetal/teratogénesis en ratas y conejos, el DecaBDE causa ciertos efectos morfológicos en la tiroides, hígado y riñones de animales adultos (Darnerud 2003).

En organismos expuestos, el metabolismo es un factor importante para determinar bioacumulación, destino, toxicocinética y toxicidad de los contaminantes. Poco se sabe acerca de la toxicocinética de PBDEs en humanos, mientras un limitado número de trabajos ha sido llevado a cabo con ratones y ratas (Chen et al., 2006b; Hakk et al., 2002; Hakk y Letcher 2003) y en peces (Stapleton et al., 2004c; Stapleton et al., 2004b; Stapleton et al., 2004a).

Los PBDEs pueden ser metabolizados parcialmente, produciendo metabolitos con características más polares para facilitar su excreción (Lupton et al., 2010), este es el caso de los PBDEs hidroxilados (OH-PBDEs), los que son estructuralmente similares a la hormona tiroxina. Debido a esta similitud, son sospechosos de causar disrupción endocrina (Chang et al., 2010; Lupton et al., 2010; Mas et al., 2007; Verreault et al., 2005). Hay que destacar que los OH-PBDEs muchas veces son más tóxicos que sus análogos PBDEs (Lupton et al., 2010), de forma similar a lo que sucede con PCBs y sus compuestos hidroxilados (OH-PCBs).

Por otro lado, algunos congéneres de PBDEs pueden tener un efecto sobre el receptor AhR del citoplasma, este es el caso de BDE-47, -77, -99, -100, -126 y -153 (Eljarrat y Barcelo 2003). Similar comportamiento tienen las dioxinas, aunque cabe destacar que la potencia tóxica de los PBDEs es muy inferior a la de las dioxinas.

Hasta la fecha, el conocimiento de los mecanismos subyacentes a la toxicidad inducida del HBCD y el TBBPA es escaso. Estructuralmente el HBCD es diferente a los PBDEs y hormonas tiroideas (Figura 1-16). Por esta razón es de esperar que el HBCD tenga un menor potencial para alterar a las enzimas de biotransformación y al eje tiroideo (Palace et al., 2008). Sin embargo, recientes estudios sugieren que al igual que algunos PBDEs, el HBCD tiene un alto potencial de bioacumulación (Law et al., 2006a) y en organismos expuestos podría afectar la biotransformación enzimática y la homeostasis tiroidea (Germer et al., 2006; Hamers et al., 2006). En estudios realizados con ratas se

ha observado que el HBCD inhibe la absorción de neurotransmisores en sinaptosomas y la absorción de la dopamina en las vesículas sinápticas. Además, en ratas expuestas a HBCD durante el desarrollo cerebral se ven afectados el comportamiento espontáneo, el aprendizaje y la memoria (Saegusa et al., 2009). Existe evidencia experimental que muestra que el α - y el γ -HBCD tienen mayor capacidad agonista frente a la hormona triyodotironina que el β -HBCD, por esto se evaluó la actividad tiroidea de truchas alimentadas con una de tres dietas enriquecidas con α -, β - o γ -HBCD, observándose que los tres diastereoisómeros tienen el potencial de alterar la homeostasis de la tiroides (Palace et al., 2008). Es importante señalar que es vital realizar estudios toxicológicos para cada uno de los isómeros de HBCD y no solo de la mezcla comercial.

Respecto a la toxicidad del TBBPA se ha sugerido que en animales de experimentación es baja, aunque se sabe que la estructura del TBBPA, al igual que los PBDEs es similar a la hormona tiroxina (Figura 1-16). En un estudio de toxicidad reproductiva en dos generaciones de ratas, el TBBPA no indujo efectos sobre la fertilidad o reproductividad y tampoco tuvo efectos convincentes sobre los puntos finales del desarrollo neurológico. Por otro lado, en estudios *in vitro* del TBBPA se han demostrado los efectos inmunológicos, las actividades antagónicas sobre los receptores de la hormona tiroidea y la inhibición de la captación de neurotransmisores sinápticos (Mariussen y Fonnum 2003). Estudios recientes han demostrado efectos neuroconductuales de TBBPA en las crías de ratas en un estudio de la reproducción de una generación (Lilienthal et al., 2006; van der Ven et al., 2009).

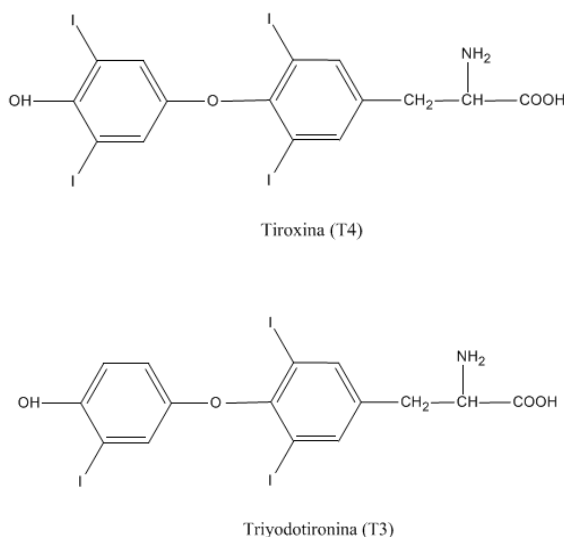


Figura 1-16. Fórmula estructural de hormonas tiroideas.

Por lo tanto, observamos que el conocimiento existente respecto a posibles efectos de los HFRs en humanos aún es muy limitado, principalmente debido a que la evaluación del riesgo se encuentra basada en modelos hechos con animales haciendo necesario mayores estudios acerca de sus posibles efectos tóxicos en humanos.

1.6. Análisis químico de retardantes de llama

El desarrollo de métodos analíticos para analizar HFRs es una tarea difícil debido a la complejidad de las matrices y las bajas concentraciones en que se suelen encontrar los compuestos de interés. Los HFRs son compuestos con una amplia variedad de propiedades físicas y químicas, y algunos de ellos, como el BDE-209, HBCD y TBBPA requieren tratamientos específicos. Básicamente, los HFRs son extraídos de la muestra, luego el extracto se purifica, fracciona y concentra antes de la separación cromatográfica y detección final mediante espectrometría de masas.

1.6.1. Preparación de muestra

La preparación de la muestra es por lo general un procedimiento en etapas independientes, por lo que es tedioso y lento, susceptible a la pérdida de analitos y a la contaminación (Mitra 2003). Por ello, aún es considerada como el punto más débil de todo el procedimiento analítico, consumiendo cerca de dos tercios del tiempo total de análisis. Además se estima que es la principal fuente de errores y discrepancias entre laboratorios. Así, la calidad de este paso es un factor clave en el éxito final del análisis (Fidalgo-Used et al., 2007; Vonderheide 2009).

Es importante señalar que en el caso del HBCD y del TBBPA, los procedimientos de tratamiento de muestras típicamente se han basado en protocolos previamente elaborados y utilizados para la determinación de los PBDEs. Por otra parte, debido a las propiedades físico-químicas particulares, la determinación de los diastereoisómeros de HBCD y TBBPA pueden requerir enfoques específicos de análisis, incluyendo un fraccionamiento adicional.

Preservación y pretratamiento. Teniendo en cuenta que suele haber una demora entre la toma de muestras y el análisis, la preservación de muestras es un paso importante. Con la preservación se asegura que la muestra mantenga sus características físicas y químicas para el análisis posterior (Mitra 2003).

El almacenamiento y transporte de muestra sólida deben ser llevados a cabo en la oscuridad, muy importante para los compuestos sensibles a la luz, como es el caso del BDE-209 y a 4°C de temperatura. El pretratamiento de la muestra debe ser realizado inmediatamente en el laboratorio incluyendo congelación, liofilización y homogenización (Eljarrat y Barcelo 2004).

Si durante la preparación de la muestra se utilizan solventes no polares, la matriz tiene que estar libre de agua para permitir la extracción de los analitos. Las muestras biológicas, tales como alimentos y tejidos, se mezclan con sulfato de sodio o liofilizan para eliminar las trazas de agua.

Las muestras de suelo, sedimentos, lodo y polvo se deben secar antes de la extracción. Además, pueden ser tamizadas para asegurar la homogeneidad del tamaño de partículas y para facilitar la manipulación posterior de las muestras (Covaci et al., 2007b).

Extracción. En aplicaciones ambientales, la desorción de los compuestos desde el sitio activo de la matriz es un paso limitante, teniendo en cuenta que las interacciones entre el soluto y la matriz son muy difíciles de superar y predecir (Camel 2001; Camel 2003). Se debe hacer énfasis en la posibilidad de mejorar la exactitud del método al utilizar técnicas de extracción, obteniendo recuperaciones cuantitativas y reproducibles de un soluto desde una matriz sólida, controlando cuidadosamente la recolección del extracto (Martens et al., 2002).

La selección del método de extracción, tanto para muestras bióticas como abióticas, depende de la naturaleza de la matriz que se desea analizar (Covaci et al., 2007b; Mitra 2003). Otro punto a tener en cuenta es la cantidad de muestra requerida para la extracción, que varía dependiendo del nivel de contaminación previsto en la muestra y de la sensibilidad de la técnica de detección.

Hay algunas particularidades que se deben tener en cuenta a la hora de extraer los HFRs desde las muestras para su posterior análisis y requieren atención adicional. En primer lugar está la amplia gama de pesos moleculares de los compuestos, es el caso de los congéneres de PBDEs (entre 250 y 960 Da), y las consecuentes diferencias en sus comportamientos físicos (Vonderheide 2009). En segundo lugar, la necesidad de obtener límites de detección más bajos requiere la adaptación de los métodos de extracción y de los procedimientos de purificación existentes.

La extracción por Soxhlet es aceptada como una técnica de extracción sólido-líquido robusta. Los solventes más utilizados para la extracción de HFRs desde muestras sólidas son: tolueno, hexano y mezclas de hexano/acetona. En general se obtienen buenos resultados pero es necesario utilizar un gran volumen de solvente orgánico (200-250 ml) además de consumir bastante tiempo (entre 12 y 24 horas) (Covaci et al., 2003; Covaci et al., 2007b; Kierkegaard et al., 2009; Vonderheide 2009). También hay que incluir el uso de extracción asistida por ultrasonidos (UAE, del inglés *ultrasonic-assisted extraction*) y agitación, aunque estas técnicas presentan también desventajas de tiempo de extracción y consumo de solventes (Bjorklund et al., 2000; Camel 2001).

Otras técnicas de extracción, como la extracción con líquidos presurizados (PLE, del inglés *pressurised liquid extraction*), la extracción asistida por microondas (MAE, del inglés *microwave assisted extraction*) y la extracción con fluido supercrítico (SFE, del inglés *supercritical fluid*

extraction), se han introducido con el objetivo de reducir el tiempo de extracción y consumo de solventes.

-Extracción por Soxhlet. Esta técnica se basa en la extracción exhaustiva de compuestos orgánicos en un sistema Soxhlet utilizando un solvente orgánico que refluye continuamente a través de la muestra contenida en un dedal poroso. Los analitos extraídos se acumulan en un matraz caliente, por lo que deben ser estables en el disolvente que se mantiene en ebullición para mantener el reflujo (Mitra 2003).

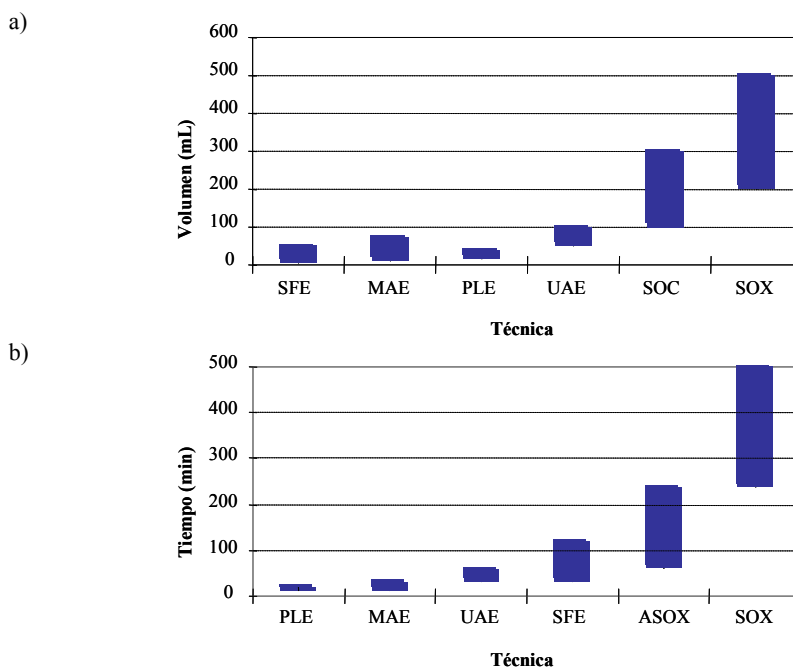
Las principales ventajas de la extracción por Soxhlet son: permite el uso de gran cantidad de muestra (1-100 g), no se necesita filtración luego de la extracción y muchos extractores Soxhlet pueden ser configurados para trabajar al mismo tiempo (Fidalgo-Used et al., 2007). Por otro lado, las desventajas se basan en las grandes cantidades de solvente que se necesitan, además que el solvente debe ser posteriormente evaporado para concentrar los analitos antes de la siguiente etapa de purificación. Por lo tanto, el proceso se prolonga por varias horas o incluso días para completar la extracción, generando extractos sucios que requieren un posterior tratamiento de limpieza.

Es necesario señalar que la extracción por Soxhlet es ampliamente utilizada como técnica estándar y de referencia para evaluar el desempeño de nuevos métodos de extracción propuestos (de Boer et al., 2001; de Boer y Cofino 2002).

- Extracción con líquidos presurizados (PLE). Este método es frecuentemente utilizado en la extracción de los PBDEs desde matrices sólidas, como sedimentos, peces o alimentos. Se obtienen altos rendimientos de extracción debido a la combinación de altas temperaturas y presiones (Camel 2003). Las principales ventajas de esta técnica son la reducción del tiempo de extracción y consumo de disolventes, en comparación con Soxhlet o UAE, y la posibilidad de llevar a cabo un gran número de extracciones sin supervisión, proporcionando una mayor eficiencia y mejor reproducibilidad. Sin embargo, el tipo de disolvente y los parámetros específicos del sistema, tales como presión, temperatura, tiempo de calentamiento, tiempo estático, volumen de descarga, tiempo de purga y los ciclos de estática, tienen que ser optimizados para una óptima recuperación del analito (Bjorklund et al., 2000).

A fin de reducir aún más el tiempo de preparación de muestra, algunos autores proponen realizar la purificación en la misma celda de extracción, relleno la celda con la muestra dispersada en un adsorbente como sílica, Florisil o alúmina para la determinación de PCBs (Gomez-Ariza et al., 2002) y PBDEs (de La Cal et al., 2003), proporcionando un método con mayor selectividad. Es importante señalar que la mayor desventaja de este tipo de técnica es el coste del aparato.

- *Extracción asistida por microondas (MAE)*. Se basa en la aplicación de la energía de microondas para calentar la extracción con solventes, mientras que se controla la temperatura, presión y energía de microondas (Fidalgo-Used et al., 2007). La ventaja principal de la MAE es la reducción del consumo de solvente. El control de temperatura y presión permiten el calentamiento del solvente a una presión por encima de su punto de ebullición, así el disolvente permanece en estado líquido promoviendo la difusión del analito (Mitra 2003; Wang et al., 2010b). El tiempo de extracción es reducido en comparación con la extracción por Soxhlet. Sin embargo es necesaria la optimización de factores como la temperatura de extracción, tamaño de partícula, tiempo de espera y el solvente empleado. La adición de solventes polares, como el metanol, es necesario para la absorción de microondas y por lo tanto para la calefacción de la extracción por solvente. Las mezclas de solventes polares con solventes no polares se emplean comúnmente en el MAE (por ejemplo, acetona: n-hexano, isopropanol: hexano, xileno: diclorometano o metanol: tolueno) (Fidalgo-Used et al., 2007; Shin et al., 2007; Wang et al., 2010b). Algunos inconvenientes de esta metodología incluyen la necesidad de filtrar los extractos tras la extracción, se utilizan solventes polares y el instrumento es moderadamente costoso (Fidalgo-Used et al., 2007).



ASOX: Soxhlet automático (del inglés, *automated soxhlet*), SOX: Soxhlet

Figura 1-17. Comparación del a) consumo de disolvente y el b) tiempo de extracción utilizando distintas técnicas de extracción.

Finalmente, en la Figura 1-17 se comparan los distintos volúmenes de solvente (1-17a) y tiempo (1-17b) que se utilizan entre las diferentes técnicas de extracción más utilizadas en el estudio de HFRs.

Purificación. La naturaleza no selectiva de los procedimientos de extracción exhaustivos y la complejidad de las matrices lleva a obtener extractos complejos (Covaci et al., 2007b) que pueden contener lípidos, carotenoides, pigmentos y resinas. Estas impurezas deben ser eliminadas para permitir una identificación y cuantificación definitiva de los niveles más bajos de analito, además de minimizar el deterioro de los resultados cromatográficos. Por lo tanto, se han desarrollado diferentes procedimientos de purificación para reducir al mínimo los efectos negativos de la matriz (Mitra 2003). Además, la etapa de purificación es necesaria para eliminar no solo la mayor parte del material co-extraído, sino también a los compuestos estrechamente relacionados con los analitos que podrían interferir en la determinación definitiva (Fidalgo-Used et al., 2007).

En el caso de matrices ambientales, como son los sedimentos y lodos de depuradora, pueden contener cantidades relativamente importantes de azufre elemental, que podría perturbar el posterior análisis, como por ejemplo la cromatografía de gases (GC, del inglés *gas chromatography*), y debe ser eliminado (Eljarrat y Barcelo 2004). Los métodos típicos para la eliminación de azufre son el tratamiento con polvo de cobre en el Soxhlet o dentro de la celda de PLE, sílice modificada con AgNO_3 en una columna de sílice de múltiples capas, o desulfuración con mercurio.

Respecto a la extracción de muestras bióticas es necesario recuperar toda la materia grasa pues es en ella donde quedan acumulados los analitos. Sin embargo, dicha materia grasa debe ser posteriormente eliminada del extracto. Existen diversos métodos en la literatura, los que se dividen en destructivos y no destructivos. Los primeros se basan en que los HFRs son estables bajo condiciones fuertemente ácidas (Covaci et al., 2007b), es por ello que uno de los métodos más utilizados es el ataque con ácido sulfúrico concentrado. La forma más simple es adicionando el ácido directamente al extracto disuelto en hexano, aunque este tratamiento requiere varias etapas de extracción líquido-líquido (LLE, del inglés *liquid-liquid extraction*) y centrifugación secuenciales lo que resulta en un procedimiento de múltiples pasos y consumidor de tiempo. Por otro lado, el uso de sílica acidificada elimina los problemas de emulsión que tiene el LLE, reduce la manipulación de la muestra y consumo de solvente, además de aumentar el rendimiento de la muestra. Sin embargo, es solo útil para pequeñas cantidades de grasa.

La cromatografía de permeación en gel (GPC, del inglés *gel permeation chromatography*) y la cromatografía de adsorción sobre diferentes sorbentes son tratamientos no destructivos aplicados para la eliminación de lípidos. GPC se realiza principalmente utilizando columnas de poliestireno divinilbenceno y diclorometano como eluyente. En el caso de muestras que contienen una cantidad limitada de lípidos, como en lodos de depuradora y peces, se utiliza solo una columna de GPC ya

que es suficiente para eliminar los lípidos del extracto. Por el contrario, en muestras más complejas es necesario utilizar dos columnas de GPC conectadas en serie o realizar una limpieza adicional por cromatografía de adsorción para la eliminación completa de los lípidos.

Sílica gel, alúmina y Florisil con diferentes grados de activación han sido ampliamente utilizados para la eliminación de lípidos por cromatografía de adsorción en condiciones atmosféricas. Debido a su limitada capacidad para retener lípidos, la sílica ha sido utilizada en combinación con alúmina y Florisil. Para PBDEs la utilización de alúmina y Florisil es recomendada, salvo en el caso de BDE-209 ya que queda retenido en el Florisil, por lo tanto es preferible el uso de alúmina.

1.6.2. Análisis instrumental

Cromatografía de gases acoplada a espectrometría de masas (GC-MS). La GC es una herramienta de análisis popular y potente, además de económica y fácil de usar. Es por ello que GC se ha convertido en una técnica de rutina para el análisis de los PBDEs. Sin embargo, debido a sus propiedades físico-químicas (como presión de vapor y polaridad) y su inestabilidad térmica, algunos congéneres específicos de PBDEs tienen necesidad de precauciones especiales durante el análisis por GC. Por lo tanto, antes de seleccionar una metodología de análisis para los HFRs, las características del sistema de GC deben ser optimizadas de acuerdo con las propiedades de estos analitos. Estas características incluyen el programa de temperaturas, el tipo de columna, la fase estacionaria como también la longitud de la columna y el diámetro interno, además de la técnica de inyección. Finalmente, la detección completa el conjunto de parámetros que deben ser optimizados para una determinación precisa de los PBDEs.

Los PBDEs pueden ser determinados de una forma relativamente fácil utilizando columnas no polares o semi-polares como las de 100% metilpolisiloxano (DB-1) y las de 5% dimetil-fenil-polisiloxanos (DB-5, CP-Sil 8 o AT-5). La longitud de columna que se utiliza más frecuentemente está entre los 25 y 60 m. Todas estas columnas presentan una buena resolución para la mayoría de los compuestos, aunque cada una de las columnas debe ser probada para evaluar posibles coeluciones de los compuestos de interés. El impacto de las posibles coeluciones depende de la limpieza de la muestra, concentración del contaminante en la muestra, sistema cromatográfico y método de detección (Covaci et al., 2003). En resumen, la columna cromatográfica óptima es aquella que combina buena resolución y discriminación de congéneres con la mayor sensibilidad en la menor cantidad de tiempo (Stapleton 2006).

Hay que prestar atención con el BDE-209 pues es sensible a las altas temperaturas, además de susceptible a la degradación en el sistema de GC. Por ello, para analizarlo la columna GC debería ser relativamente corta (10-15 m) para reducir al máximo el tiempo de residencia del BDE-209

además de cambiar el tamaño de poro de 0,25 μm a 0,1 μm , siendo muchas veces necesario analizarlo por separado del resto de PBDEs (Eljarrat et al., 2007).

Para la determinación de HFRs se ha utilizado la espectrometría de masas (MS, del inglés *mass spectrometry*), tanto en modo de baja resolución (LR, del inglés *low resolution*) como de alta resolución (HR, del inglés *high resolution*).

La determinación de los PBDEs en muestras ambientales utilizando instrumentos de LR se realiza principalmente con instrumentos que operan tanto en ionización electrónica (EI, del inglés *electronic ionization*)-MS como en ionización por captura electrónica en modo negativo (ECNI, del inglés *electron capture negative ionization*)-MS. La detección por LRMS ha sido utilizada en diversos estudios por su sensibilidad, selectividad y debido a que es un instrumento que se encuentra disponible en los laboratorios de medio ambiente, además de ser fácil de utilizar y optimizar (Van Leeuwen y De Boer 2008). En general, para el análisis de PBDEs se ha demostrado que la EI-LRMS es más selectiva que los métodos de ECNI-LRMS pues los fragmentos moleculares (típicamente $[\text{M}-\text{Br}_2]^+$) son adquiridos para cada uno de los grupos homólogos. En contraste, los métodos ECNI-LRMS adquieren los iones bromo ($[\text{Br}]^+$, m/z 79 y 81) para todos los grupos homólogos, excepto el BDE-209 que da lugar a fragmentos específicos a partir de $[\text{C}_6\text{Br}_5\text{O}]^+$ con m/z 484,6 y 486,6, además de la posibilidad de utilizar el patrón marcado ^{13}C -BDE-209 con m/z 494,6 y 496,6 para $^{13}\text{C}_6\text{Br}_5\text{O}]^+$ (Bjorklund et al., 2003). Por lo tanto, utilizando ECNI-MS no es posible cuantificar con dilución isotópica, salvo en el caso de BDE-209. Sin embargo, ECNI-LRMS es más sensible que EI-LRMS presentando un LOD con un orden de magnitud inferior (Covaci et al., 2003).

En contraste, los instrumentos HR son utilizados casi exclusivamente en modo EI (Alaee et al., 2001). La espectrometría de masas de alta resolución (HRMS, del inglés *high resolution mass spectrometry*) ofrece la mejor selectividad para medir PBDEs, con un poder de resolución ($m/\Delta m$) de aproximadamente 10.000. Sin embargo, hay que tener en cuenta que los instrumentos de HR son más caros que los instrumentos LR y se necesita personal entrenado para su mantenimiento (Stapleton 2006). En todo caso, si comparamos EI-HRMS con EI-LRMS, la primera tiene una sensibilidad mucho mayor. Recientemente se ha mostrado el uso de GC acoplada a la trampa de iones (IT, del inglés *ion trap*) en tándem como una excelente alternativa a GC-HRMS para la determinación de PBDEs en muestras ambientales y biológicas proporcionando una alta sensibilidad y excelente selectividad (Losada et al., 2010). En la Tabla 1-2 se resumen las ventajas e inconvenientes de utilizar diferentes técnicas de detección para el análisis de BFRs.

Tabla 1-3. Comparación de diferentes técnicas de detección para BFRs

Técnica	Ventajas	Inconvenientes
EI-LRMS	Se puede cuantificar por dilución isotópica, buena selectividad	Baja sensibilidad
ECNI-LRMS	Buena sensibilidad	No se puede cuantificar por dilución isotópica, baja selectividad
IT-LRMS/MS	Buena sensibilidad, buena selectividad	Poco disponible en laboratorios analíticos
EI-HRMS	Muy buena sensibilidad, muy buena selectividad	Elevado coste de adquisición y mantenimiento

Durante los últimos años, el interés científico por los FR tales como HBCD, TBBPA y DP ha aumentado, siendo necesario el desarrollo de nuevos métodos de análisis. Al principio, se utilizaron enfoques similares a los utilizados para el análisis de PBDEs para la detección de HBCD y TBBPA. Es por ello que los primeros métodos se basan en GC-MS. Así, HBCD inicialmente fue determinado mediante GC-ECNI-MS. Sin embargo, esta técnica tiene algunas limitaciones: interconversión de diastereoisómeros de HBCD por encima de 160 ° C, la descomposición del HBCD a temperaturas superiores a 240 ° C y la ruptura parcial del HBCD en los sistemas de GC sucios. Por otro lado la acidificación y los pasos de derivatización son obligatorios en el análisis por GC-MS de BFR más polares tales como el TBBPA. Respecto al DP, se han desarrollado diferentes metodologías para su análisis en muestras ambientales, todas ellas basadas en el uso de GC-MS principalmente acoplada a ECNI-LRMS (Hoh et al., 2006; Sverko et al., 2010) o EI-HRMS (Kolic et al., 2009).

Cromatografía de líquidos acoplada a espectrometría de masas (LC-MS). Las dificultades encontradas para el análisis de HBCD y TBBPA mediante GC han creado la necesidad de desarrollar métodos alternativos. A pesar del relativamente limitado poder de resolución cromatográfico de la cromatografía de líquidos (LC, del inglés *liquid chromatography*), los métodos que emplean LC-MS ofrecen una buena alternativa para analizar HBCD y TBBPA, permitiendo la determinación diastereoisomérica del HBCD. La identificación y cuantificación de los tres isómeros de HBCD es importante pues los patrones de isómeros difieren en condiciones de producción diferentes y entre las matrices (de Boer y Wells 2006).

Sin embargo, se sabe que la GC-MS tiene la ventaja de tener una mayor sensibilidad en comparación con los métodos LC-MS (Petersen et al., 2004). Para mejorar la sensibilidad y la especificidad de los métodos LC-MS en el campo de análisis ambiental se han desarrollado

enfoques basados en el acoplamiento de LC a la espectrometría de masas en tándem (MS/MS). Los primeros estudios se centraron en el uso de instrumentos triple cuadrupolo (QqQ), aunque más recientemente se han desarrollado otras configuraciones instrumentales para la determinación de BFRs. Nuevos trabajos han aplicado técnicas de LC-MS/MS a partir de instrumentos híbridos, como son el cuadrupolo-tiempo de vuelo (QqTOF, del inglés *quadrupole time of flight*) y el cuadrupolo-trampa de iones lineal (QqLIT, del inglés *quadrupole lineal ion trap*) (Petrovic y Barcelo 2006). Se han evaluado diferentes técnicas de ionización para las metodologías de LC-MS/MS, pero la ionización más utilizada es por electrospray (ESI). Es bien sabido que al utilizar ESI, la respuesta de los analitos puede verse modificada por efecto de la matriz de la muestra que pueden causar tanto el aumento como la supresión de la señal de los analitos de interés (Dodder et al., 2006; Marvin et al., 2007). Por esta razón, la posibilidad de utilizar estándares isotópicamente marcados como ^{13}C - o d_{18} - α , β y γ -HBCD y ^{13}C -TBBPA, es muy útil para compensar dichos efectos de la matriz.

Recientemente se han desarrollado metodologías LC-MS/MS multianalitos con el objetivo de estudiar una serie de HFRs con diferentes propiedades fisicoquímicas, por ejemplo HBCD, TBBPA, DP y PBDEs. Ejemplo de esto son los análisis utilizando LC-fotoionización a presión atmosférica (APPI, del inglés *atmospheric pressure photoionization*)-MS/MS (Zhou et al., 2010b) y LC-ionización química a presión atmosférica (APCI, del inglés *atmospheric pressure Chemicals ionization*)-MS/MS (Zhou et al., 2010a).

También es importante tener en cuenta que los métodos de LC pueden ser útiles en el análisis de los metabolitos de BFR y productos de transformación. Por ejemplo, el carácter no polar de la estructura del éter difenilo en los PBDEs se ve afectado por la introducción de grupos hidroxilo durante su metabolización obteniéndose una nueva molécula con propiedades diferentes como características no volátiles que no permite el análisis directo de los metabolitos de los PBDEs por GC-MS. Recientes trabajos se centran en el desarrollo de metodologías LC-MS/MS para este propósito (Lupton et al., 2010; Mas et al., 2007).

El desarrollo de métodos analíticos para BFRs es difícil debido a la complejidad de las matrices ambientales y las concentraciones generalmente bajas de los compuestos de interés. Las técnicas de preparación de las muestras también deben ser desarrolladas y optimizadas para obtener métodos sensibles y selectivos. En el artículo científico #1: “*New approaches for halogenated flame retardant determinations by means of LC-MS*” se revisan los procedimientos analíticos recientes que se basan en la utilización de la cromatografía líquida acoplada a la espectrometría de masas para el análisis de HFRs.

1.7. Validación de los métodos analíticos

Un requisito para caracterizar la eficacia de los métodos analíticos y asegurar la calidad de los resultados es disponer de métodos validados convenientemente. Diferentes organismos internacionales como la Agencia de Protección del Medio Ambiente de EE.UU. (EPA, del inglés *environmental protection agency*), el foro para la química analítica europea (EURACHEM, del inglés *european analytical chemistry*) y la unión para la química pura y aplicada (IUPAC del inglés, *union of pure and applied chemistry*), han desarrollado criterios y directrices generales para la validación de métodos, proporcionando la base para asegurar su fiabilidad.

Se pueden utilizar diferentes enfoques para validar un método de análisis. Sin embargo, es importante tener en cuenta que el objetivo de la validación es demostrar que un procedimiento es adecuado para los fines previstos. Es aconsejable que en el diseño del trabajo experimental se consideren simultáneamente las características de la validación adecuada para obtener el conocimiento general de las capacidades del procedimiento analítico desarrollado, reduciendo al mínimo el número de experimentos que se deben realizar (Chan et al., 2004).

La validación de un método de análisis debe incluir la siguiente información:

Linealidad. Es la capacidad de mantener invariable la pendiente de la función de calibración por un intervalo de concentraciones de trabajo. Esto se determina inyectando concentraciones crecientes de estándares analíticos al sistema instrumental, midiendo la correspondiente respuesta del instrumento. La linealidad del método se evalúa mediante el coeficiente de regresión (R^2) de las rectas de calibrado utilizadas para la cuantificación de los compuestos.

Exactitud. Evalúa el grado de concordancia entre el resultado y un valor de referencia certificado. La exactitud de un resultado se expresa mediante el error absoluto, el cual es la diferencia entre el valor experimental y el valor considerado como verdadero.

Precisión. Grado de concordancia entre los datos obtenidos de una serie, reflejando el efecto de los errores aleatorios producidos durante el proceso analítico. La *precisión* cuantifica la variabilidad de los resultados analíticos en función del operador, de las manipulaciones inherentes al método y al medio ambiente del laboratorio, entre otras. Dependiendo de las posibles fuentes de variabilidad, la *repetitividad* se acepta como una medida de la dispersión interna utilizando una misma muestra, el mismo laboratorio con el mismo equipo y el mismo operador en un intervalo corto de tiempo. Por otro lado, la *reproducibilidad* evalúa las posibles fuentes de variación aleatoria de los datos, registrando los resultados de experimentos independientes, realizados con el mismo método y aplicado a la muestra en diferentes condiciones. La *precisión* se expresa como la desviación estándar relativa (RSD, del inglés *relative standard deviation*).

Robustez. Capacidad de un método analítico para permanecer invariable a variaciones pequeñas pero deliberadas de los parámetros del procedimiento de análisis, indicando su fiabilidad durante el uso normal.

Especificidad. Capacidad de evaluar de forma inequívoca un analito en la presencia de componentes que se espera que estén presentes, como por ejemplo impurezas y/o productos de degradación.

El análisis de HFRs es laborioso, complejo e incluye diversos pasos, en los que se puede cometer múltiples errores, como por ejemplo durante la extracción, purificación y determinación por GC-MS o LC-MS. El análisis preciso de HFRs es importante para los responsables científicos y políticos que se basan en los datos obtenidos en los laboratorios de medio ambiente. Para reducir al mínimo las posibilidades de errores, se deben tomar medidas para mejorar el análisis, por lo tanto los sistemas de control de calidad deben establecerse y aplicarse de manera rutinaria. Esto incluye el uso de estándares de elevada calidad y uso de estándares internos, el análisis de blancos, análisis de replicados, estudios de recuperaciones, participación en ensayos interlaboratorios y el análisis de materiales de referencia certificados (CRM, del inglés *certificatted reference materials*) y materiales de laboratorio de referencia (LRM, del inglés *laboratory reference materials*) (de Boer y Cofino 2002; Van Leeuwen y De Boer 2008). La participación de ensayos interlaboratorios facilita la evaluación y valoración del desempeño del método completo. Es por ello que desde 1999 se han llevado a cabo estudios internacionales de interlaboratorios para el análisis de PBDEs en diferentes matrices (de Boer y Cofino 2002; de Boer y Wells 2006). Además existen CRMs de peces y sedimento disponibles para la evaluación de PBDEs.

Un punto importante es la falta de métodos armonizados para la determinación de HBCD y TBBPA en matrices ambientales. Los estudios regulares de intercalibración y la participación en estudios de aptitud de laboratorio (si están disponibles) son importantes para mantener la alta calidad de los datos analíticos. Hasta ahora, solo un número limitado de estudios de interlaboratorios se han llevado a cabo con un número bastante limitado de los laboratorios participantes. Por otra parte, estos estudios han proporcionado datos sobre el HBCD, pero en ninguno de ellos se realizó la determinación del TBBPA.

Con el fin de evaluar la calidad de las determinaciones de HBCD, el Instituto Noruego de Salud Pública organizó estudios de comparación en 2005 y 2007. Los objetivos de estos estudios fueron: (a) evaluar la comparabilidad de los resultados obtenidos utilizando diferentes técnicas de análisis, (b) proporcionar un instrumento que garantice la calidad de los laboratorios participantes, y (c) evaluar la disposición de los laboratorios especializados para determinar HBCD en muestras biológicas (Haug et al., 2008). Trece laboratorios analizaron la concentración total de HBCD o diastereoisomérica de HBCD en aceite de hígado de bacalao, filete de arenque, filete de salmón, mantequilla y carne de pollo. Los laboratorios fueron capaces de determinar las concentraciones

totales de HBCD en muestras marinas con una calidad satisfactoria (RSD <35%). Sin embargo, el análisis de muestras con baja contaminación de HBCD (<de 2 ng / g de peso en lípidos) debe ser mejorado. No se observaron diferencias estadísticamente significativas entre las concentraciones totales de HBCD obtenidos por LC-MS y GC-MS.

Un factor adicional a tener en cuenta, es que actualmente no existen CRM para HBCD ni TBBPA que se puedan utilizar para la validación de un método.

Artículo científico #1:

“Recent approaches for selected halogenated flame retardants determinations by means of LC-MS”

por

P. Guerra, E. Eljarrat y D. Barceló

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NEW APPROACHES FOR SELECTED HALOGENATED FLAME RETARDANT
DETERMINATIONS BY MEANS OF LC-MS

P. Guerra¹, E. Eljarrat¹, D. Barceló^{1,2}

¹Dept. Environmental Chemistry, Institute of Environmental Diagnostic and Water Studies (IDAEA), Spanish Council of Scientific Research (CSIC).

Jordi Girona 18-26, E-08034 Barcelona, Spain.

²Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona.

Pic de Peguera 15, E-17003 Girona, Spain.

**Corresponding author; tel. +34 93 400 6100, fax +34 93 204 59 04, email: eeeqam@cid.csic.es*

Abbreviations and Symbols

APCI: Atmospheric pressure chemical ionization; APPI: Atmospheric pressure photoionization; ECNI: Electron capture negative ionization; EF: Enantiomeric fraction; EI: Electron ionization; EPI: Enhanced product ion; ESI: Electrospray ionization; GC: Gas chromatography; HBCD: Hexabromocyclododecane; HRMS: High resolution mass spectrometry; ISP: Ion spray; NCI: Negative chemical ionization; QqLIT: Quadrupole linear ion trap; QqQ: Triple quadrupole; QqTOF: Quadrupole time of flight.

ABSTRACT

In this paper, an overview of current analytical methods for selected halogenated flame retardants (HFRs), focused on instrumental determination using LC-MS approaches, is presented. The strategy literature search for the preparation of this review was based on the recent articles published in peer-reviewed scientific journals or in conference proceedings. Selected HFRs includes TBBPA, HBCD, TBECH and DP, as well as some metabolites and transformation products of PBDEs and HBCD. Analytical performances of the different approaches (ESI, APCI and APPI as ionization modes) are reported, with emphasis on selectivity and sensitivity. Moreover, these parameters were compared with those obtained by gas chromatographic techniques. Conclusions and future perspectives are outlined.

Keywords: Dechlorane Plus, Hexabromocyclododecane, LC-MS-MS, Polybrominated diphenyl ethers, Tetrabromobisphenol A, Tetrabromoethylcyclohexane.

1. INTRODUCTION

Flame retardant (FR) compounds are a structurally diverse group of chemicals that are added to or reacted with polymers which are used in plastics, textiles, electronic circuitry and other materials to reduce the risk of fire. One of these groups of compounds is the brominated flame retardants (BFRs). Some of them are ubiquitous, and many have been detected in biota, sediments, air, water, marine mammals and even in human milk [1-3].

It is important to note that the bulk of research done has been focused in the study of polybrominated diphenyl ethers (PBDEs) due to their persistence and hydrophobicity, two characteristics that make them amenable to bioaccumulation and biomagnification [4]. Due to their toxicological effects, the production and use of PBDE formulations are banned in Europe and, Penta- and Octa-BDE formulations have been banned in North America. Despite these bans, PBDEs continue to leach from existing products that are in service or have been disposed of in landfills. Furthermore, industry has introduced several new halogenated flame retardants (HFRs) in response to fire safety regulations. Some of these compounds are hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), hexachlorocyclopentenyl-dibromocyclooctane (HCDBCO), 1,2-bis (2,4,6-tribromophenoxy)ethane (BTBPE), bis(2-ethyl-1-hexyl)tetrabromophthalate (BEHTBP), octabromotrimethylphenylindane (OBIND), tetrabromoethylcyclohexane (TBECH) and decabromodiphenylethane (DPDPE).

On the other hand, while policy makers consider further restrictions on these currently used HFRs, Dechlorane Plus (DP), a chlorinated flame retardant ($C_{18}H_{12}Cl_{12}$) manufactured for over 40 years, has only recently been reported in the environment and biota. DP is only produced by Oxychem (Niagara Falls, NY) however, DP is sold worldwide including Europe and Asia [5]. DP is unregulated for use even the United States Environmental Protection Agency (USEPA) categorized DP as a High Production Volume (HPV) substance. It is also listed on Canada's Domestic Substances List (DSL) but not listed high in terms of risk for bioaccumulation because of its high molecular mass (648Da) and high $\log K_{ow}$ (9.3) [6]. In commercial DP products, there are two stereoisomers, *syn*- and *anti*-DP at a ratio of approximately 1:2 respectively, and plastic formulations can contain as high as 35% (weight basis) of DP [6].

Traditionally, the analysis of HFR has been developed using gas chromatography (GC) as the principal separation technique, due to the volatility of these compounds. In the case of the analysis of PBDEs, it has been focused almost exclusively on GC - mass spectrometry (MS). The determination using low resolution instruments is typically made operating in electron capture negative ionization (ECNI) or electron ionization (EI) modes [7]. On the other hand, high resolution (HR) instruments are almost exclusively used in EI mode coupled with mass spectrometry (EI-HRMS). HRMS offers the best selectivity for PBDEs measurements but HR instruments are more expensive relative to low resolution (LR).

In the last years the analysis of other BFR compounds has been necessary, this is the case of HBCD and TBBPA. Generally, the GC-MS analysis of HBCD has been performed using ECNI-mode obtaining a

higher sensitivity. However, this technique has some limitations due to the interconversion of HBCD diastereoisomers above 160°C, decomposition of HBCDs at temperatures above 240°C and partial breakdown in dirty GC systems [8]. The analysis of more polar BFRs by GC-MS such as TBBPA would require the use of a derivatizing reagent. In the case of TBBPA, acidification and derivatization are compulsory before analysis by GC can be carried out [9].

Despite the limited chromatographic resolving power of liquid chromatography (LC), methods employing LC-MS and LC-MS-MS offer very good results for the analysis of HBCDs and TBBPA. The identification and quantification of the three HBCD isomers is important because the isomeric patterns differ under different production conditions and also differ among analyzed matrices. Besides with the use of LC-MS methods, the HBCD enantiomers can be separated and determined using LC with chiral chromatographic columns [10].

TBBPA, mainly due to its lower bioaccumulation potential, presented concentration levels lower than those of PBDEs or HBCD in the environment [11]. However, TBBPA being a phenolic compound may have a greater adverse effect on humans and wildlife, thus the determination of TBBPA by LC, together with the HBCD diastereoisomers seems to be the simplest, most attractive option [10].

The main advantage of GC-MS technique is its higher sensitivity compared with LC-MS methods. Thus, in order to improve sensitivity and specificity of LC-MS methods, in the field of environmental analysis, the main advantages are related to tandem MS (MS-MS), using triple quadrupole instruments (QqQ), and more recently, using LC-hybrid MS techniques such as quadrupole time of flight (QqTOF) and quadrupole linear ion trap (QqLIT) instruments [12].

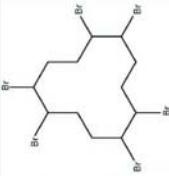
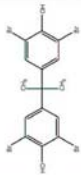
Finally, it is important to note that LC methods may be useful in the analysis of BFR metabolites and transformation products. For example, the non polar nature of the diphenyl ether structure in PBDEs and the introduction of polar hydroxyl functional groups upon their metabolization provide a new molecule with different properties such as non-volatile nature does not allows the direct analysis of PBDEs metabolites by GC-MS, the second challenge is due to these metabolites are not easily detected at low levels by LC-MS [13].

This review summarizes current analytical methods based on the use of LC-MS-MS for selected HFRs, such as TBBPA, HBCD, TBECH and DP, as well as for some metabolites and transformation products of PBDEs and HBCD. Analytical performances of the different developed approaches are reported, with emphasis on selectivity and sensitivity. Moreover, these parameters are compared with those obtained by GC techniques.

2. PHYSICO-CHEMICAL PROPERTIES

Dissimilar physicochemical properties present the different HFR studied. The main physicochemical properties of PBDEs, HBCD, TBBPA, TBECH and DP are summarized in Table 1. It is important to note that some of them are now in use, banned or in revision; this is the case of TBBPA, PBDEs and HBCD, respectively.

Table 1. Physicochemical properties of HFR analyzed by LC-MS

HFR Compound	Molecular Structure	Formula	Molecular mass	Melting Point (°C)	Solubility H ₂ O (µg/L; 25°C)	Log K _{ow}	Ref.
PBDEs		C ₁₂ H ₆ Br ₄ O	485.8	82.3	14.7	5.87-6.16	[14]
		C ₁₂ H ₅ Br ₅ O	564.7	81.0	4.4	6.64-6.97	
		C ₁₂ H ₂ Br ₈ O	801.5	200	-	8.35-8.90	
		C ₁₂ Br ₁₀ O	959.2	290-306	20-30	9.97	
HBCD		C ₁₂ H ₁₈ Br ₆	641.7	179-181	48.8	5.07	[15,16]
				170-172	14.7	5.12	
				207-209	2.1	5.47	
				175-195	6.56	5.63	
TBBPA		C ₁₃ H ₁₂ Br ₄ O ₂	543.9	178	720	4.5-5.3	[17]
TBECH		C ₈ H ₁₂ Br ₄	427.8	70-97	69.2	5.2	[6,18]
DP		C ₁₈ H ₁₂ C ₁₂	653.7	350	250	9.3	[19]

Due to its low solubility in water and high log K_{ow} [20], TBBPA is likely to be associated with suspended particulate matter once released in the water column and ultimately buried in sediment [21,22]. This compound is not frequently analyzed in environmental laboratories, may be due to its lower bioaccumulation potential, with concentrations lower than PBDEs and HBCD in the environment. However, TBBPA has been found in abiotic matrices as air, soil, sediment and sewage sludge [21,23,24]. PBDEs are highly hydrophobic compounds with log K_{ow} between 5.87 and 9.97 for mono-BDE to deca-BDE-209. The physical-chemical properties differ greatly among the BDE congeners, which leads to differences in their environmental behavior.

The physical-chemical properties of HBCD are similar to some PBDEs and other persistent organic pollutants (POPs). HBCD is not covalently bonded to the material leading to the risk of migration out of the product during use or disposal [16]. The dissimilarities in the structure of α -, β - and γ - isomer might raise differences in polarity, dipole moment and in solubility in water. As an example, the solubility of α -, β - and γ -HBCD in water was 48.8, 14.7 and 2.1 $\mu\text{g/L}$ respectively. Therefore these different properties may explain the differences observed in their environmental behavior [25]. Covaci *et al.* [26] and Morris *et al.* [27] found that in sediments, the distribution of HBCD isomers was the same of commercial mixture: γ -HBCD is the most abundant isomer. However, in biota α - isomer predominates over the γ - isomer, and little or no presence of β -isomer exists [2,26]. α -, β - and γ - HBCD diastereoisomers are chiral; thus HBCD has three pairs of enantiomers (+) α , (-) α , (+) β , (-) β , (+) γ and (-) γ . The enantiomers have identical physicochemical properties and abiotic degradation rates, but may have different biological and toxicological properties and therefore different biotransformation rates. These transformations may result in nonracemic mixtures of the enantiomers that were industrially synthesized as racemates [7,25].

TBECH is a polybrominated cycloaliphatic flame retardant and can exist as four pairs of enantiomers. At temperatures above 120°C, thermal conversion of α - and β -isomers occurs, resulting in the formation of γ - and δ -isomers [6]. Little is known about this compound although it has been detected in Canadian Arctic beluga [28], and its bioaccumulation potential is expected by the authors due to its estimated log K_{ow} .

In commercial DP products, there are two stereoisomers, *syn*-DP and *anti*-DP resulting from the Diels-Alder reaction of 2 mol of hexachloropentadiene with 1 mol of 1,5-cyclooctadiene [29]. Although DP has not been measured in the environment until recently, DP has been shown to be present in air, sediment and biota [6,19,29]. Even when its log K_{ow} (9.3) is high the detection of DP in biota suggests that the isomers are bioavailable and bioaccumulative [6].

4. INSTRUMENTAL ANALYSIS BY LIQUID CHROMATOGRAPHY

The need for increased capabilities in the environmental analysis of HFR is now driving to new strategies and instrument advances. One of these advances is the use of LC-MS instruments in order to improve the existing methodologies mainly using GC-MS. Table 2 summarized different LC-MS methods found in the literature for the analysis of different HFRs.

Table 2. LC-MS methods applied to HFR determinations

Compound	Matrix	Instrumental Technique	Equipment	LOD (pg)	mLOD	Ref
TBBPA	Standards	LC(-)APPI-MS	Q-IT (Thermo)	nr	nr	[30]
	Wastewater	LC(-)APCI-MS	Classic LCQ (Thermo)	232	nr	[31]
	Air	LC(-)ESI-MS-MS	Quattro Microass (Waters)	20	0.02 pg/g	[24]
	Sediment and sewage sludge	LC(-)ESI-MS-MS	LSQ Advantage ion-trap (Thermo)	3.1	nr	[22]
	Human serum	LC(-)ESI-MS-MS	Quattro Microass (Waters)	nr	6-18 ng/mL	[21]
α -, β - and γ -HBCD	Dust	LC(-)ESI-MS-MS	TSQ Quantum (Thermo)	nr	0.8 pg/g	[32]
	Marine sediments	LC-ESI-ITMS	Sciex API 2000 (Applied Biosystem)	nr	nr	[33]
	Suspended sediments	LC-ESI-ITMS	1100 MSD ion-trap-SL (Agilent)	nr	5-10 pg/g	[34]
	Fish sample	LC-ESI-ITMS	Quattro Microass (Waters)	nr	10 pg	[35]
	Biota	LC-ESI-MS-MS	Sciex API 2000 (Applied Biosystem)	4-6	nr	[36]
	Biota	LC-ESI-MS-MS	TSQ Quantum (Thermo)	0.5-5.0	nr	[25]
	Human biological matrices	LC-ESI-MS-MS	ABS API-4000 QTRAP (Applied Biosystem)	nr	0.3 ng/g ww	[37]
	Fish oil supplements	UPLC(-)ESI-MS-MS	Quattro Microass (Waters)	nr	30-500 ng/kg	[38]
	Sediments	UPLC(-)ESI-MS-MS	Quattro Premier XE (Waters)	nr	nr	[39]
	Bird eggs	LC-ESI-MS-MS	ABS API-4000 QTRAP (Applied Biosystem)	0.3-1.5	0.12-5.61 ng/g	[3]
(±)- α -, (±)- β - and (±)- γ -HBCD	Food	LC-ESI-MS-MS	TSQ Quantum (Thermo)	0.5-1.0	nr	[40]
	Human milk	LC-ESI-MS-MS	TSQ Quantum (Thermo)	7.5-22	nr	[41]
	Standards	LC-ESI-MS-MS	ABS API-4000 QTRAP (Applied Biosystem)	0.23-3.20	nr	[42]
	Standards	LC-ESI-MS-MS	ABS API-4000 QTRAP (Applied Biosystem)	nr	0.1-1.0 ng/g	[2]
PBDEs	Standards	LC-ESI-MS-MS	Q-IT (Thermo)	nr	nr	[30,38,43]
	House dust	LC-ESI-MS-MS	LC-AB Prominence (Shimadzu)	12.3-29.8	nr	[43]
	Sewage sludge	UPLC(-)APCI-MS-MS	3200 QTRAP (Applied Biosystem)	2.4-28	nr	[44]
α -, β - and γ -HBCD and TBBPA	Standards	UPLC(-)APCI-MS-MS	API 5000 (AB Sciex)	0.10-0.72	3-198 pg/g	[46]
	Standards	LC-ESI-MS	Quattro Microass (Waters)	0.11-6.3	4-380 pg/g	[18]
	Sewage sludge and TBBPA	LC-ESI-MS-MS	ABS API-4000 QTRAP (Applied Biosystem)	nr	nr	[3]
	Sewage sludge and TBBPA	LC-ESI-MS-MS	ABS API-4000 QTRAP (Applied Biosystem)	0.1-1.2	nr	[23]

TBBPA, α -, β - and γ - HBCD, DP, PBDEs and emerging HFR nr: not reported	sediment					
	Sewage sludge	UPLC(-)ESI-MS-MS	API 5000 (AB Sciex)	0.03-0.06	0.080-0.082 ng/g	[46]
	Landfill leachate and sediment	LC(-)ESI-MS-MS		nr	nr	[10]
	Blubber	LC(-)APCI-MS-MS	ABS API-3000 (Applied Biosystem)	nr	0.2-2.0 ng/L	[47]
	Wastewater	LC(-)ESI-MS	LSQ Advantage ion-trap (Thermo)	nr	nr	[48]
		LC(-)APCI-MS-MS		0.5-40	nr	
	Fish	LC(-)APPI-MS-MS	ABS API-4000 QTRAP (Applied Biosystem)	0.5-20	nr	[49]

4.1 LC-MS for TBBPA

LC-MS technique appears to be the method of choice for TBBPA analysis because no derivatization of the phenolic group is required [11,21,26]. TBBPA is covalently bonded with the fire protected material and is considered as the most polar molecule among major BFRs. Another advantage of the LC-MS determination of TBBPA is the possibility to use the ^{13}C -labelled TBBPA as surrogate standard. This greatly enhances the quality of the analytical data obtained by compensating for any matrix-related effects that can affect analyte ion intensity [11]. In order to obtain a good chromatographic separation of TBBPA it is important to have into account its greatly dependence with the used mobile phase. Chu *et al.* (2005) found that using methanol as mobile phase the response of target compounds was about one third greater than when using acetonitrile. Using methanol as mobile phase, better limit of quantification (LOQ) was obtained due to the more stable detector baseline obtained. In general, using methanol and water as mobile phase a more advantageous analysis was obtained [3,11].

The most suitable LC-MS interface for TBBPA analysis is electrospray ionization (ESI) operating in negative ionization mode. Tollbäck *et al.* [24] reported limits of detection (LODs) 30 to 40 times lower compared to atmospheric pressure chemical ionization (APCI). In addition, it permits the monitoring of the intact TBBPA molecule through the soft ionization of ESI resulting in improved method selectivity and accuracy. Similar results were found by Morris *et al.* [27]. In terms of sensitivity, LC-ESI-MS can be competitive with published GC-EI-MS techniques with LODs in the ppt range [32].

Another proposed method is based on the use of LC- atmospheric pressure photochemical ionization (APPI)-MS [30] in order to analyze TBBPA and its related degradation products working with a 100-C8 column. In the case of LC-APCI-MS [31], TBBPA was analyzed simultaneously with some other BPA halogenated derivatives using a SunFire C18 column with methanol/water as mobile phase. This method has been applied to the analysis of water from a recycling plant.

LC-QqLIT-MS was proposed to analyze TBBPA and related compounds (bisphenol A (BPA), monobromobisphenol A (MonoBBPA), dibromobisphenol A (DiBBPA), and tribromobisphenol A (TriBBPA)) in sewage sludge and sediment samples [23].

On the other hand, ultra performance liquid chromatography (UPLC)-ESI-MS-MS has been reported to analyze TBBPA in soil and food samples [50,51]. This technique combines all the advantages of LC-MS-MS in addition to shorter residence time of the analyte on-column, minimizing the potential for losses on-column due to adsorption and degradation. The short analysis time (4 min.) can double the efficiency of the analytical method [11].

Some derivatives of TBBPA are also used as BFRs. This is the case of tetrabromobisphenol-A-bis(2,3-dibromopropylether) (TBBP-A-dbpe), tetrabromobisphenol-A-bis(allyl ether) (TBBP-A-ae) and tetrabromobisphenol-S-bis(2,3-dibromopropyl ether) (TBBP-S-dbpe). Recently, the mass spectra characterization of TBBP-A-dbpe, TBBP-A-ae and TBBP-S-dbpe was obtained [52]. In order to obtain higher sensitivity for the quantification of these analytes, a LC-APPI-QTOF-MS method was performed

achieving LODs of the method (mLODs) of 0.07, 0.03 and 1.28 ng/g wet weight (ww) for TBBP-A-dbpe, TBBP-A-ae and TBBP-S-dbpe, respectively.

4.2. LC-MS for HBCD

4.2.1. LC-MS for HBCD diastereoisomers

To obtain HBCD diastereoisomer-specific data it is mandatory to use LC since using GC the diastereoisomers interconvert at temperatures above 160°C. Among different MS analysers, the QqQ is the most used [3,25]. However the increasing robustness and widespread use of ion trap instruments, due to their high versatility and relatively low cost, led to the development of methodologies based on this technique for HBCD detection and quantification [3,16,23,41,53,54].

There has been some concern regarding the apparent differences in the stability of HBCD diastereoisomers in different solvents. This was explained by the relatively lower solubility of γ -HBCD in acetonitrile compared to methanol. Therefore, Tomy *et al.* (2005) [16] suggested the use of methanol for final extracts to inject in LC-MS analysis.

Regarding diastereoisomers separation, using any ratio of methanol/acetonitrile as mobile phase the separation of all three diastereoisomers is obtained. However, an increase in the percentage of acetonitrile in the mobile phase resulted in a slightly better separation of HBCD diastereoisomers, mainly between β - and γ -HBCD with instrumental LOD (iLOD) between 0.5 and 50 pg on-column [25]. In order to enhance the signal or sensitivity, mobile phase modifiers have been used, commonly ammonium acetate [10,55] and acetic acid [38]. It is well documented and experimentally confirmed that HBCD tends to associate with several anions forming different adducts that can affect the sensitivity and the accuracy of the determinations [41,42,54]. The addition of different ammonium salts to the mobile phase, i.e. ammonium chloride or ammonium acetate, in order to encourage (chloride (Cl) method) or try to inhibit (ammonium acetate (Ac) method), respectively, the formation of the chlorine adducts of the molecular ion was tested. For the Cl method, the most intense ion in the adduct cluster corresponds to m/z 676.6 that was chosen as the precursor ion, while m/z 640.6 was monitored for quantification and m/z 638.6 for confirmation purposes. In the case of Ac method, as usual m/z 640.6 was the precursor ion, m/z 78.9 and m/z 80.9 being the quantification and confirmation ions, respectively. The Cl method showed higher sensitivity and LODs (0.23–0.41 pg on column) and LOQs (0.77–1.35 pg on column) were up to 14 times lower than those obtained applying the Ac method [41,42].

For the determination of HBCD diastereoisomers in sea lions a C30 analytical column [37] was coupled with LC-ESI-MS-MS although the majority of published works used C18 column. A different elution order is obtained using a C30 column: α -, γ - and β -isomer.

In order to evaluate HBCD pattern in fish and marine oil supplements a method involving the use of UPLC-ESI-MS-MS was developed [39]. The limits of quantification for α -, β - and γ -HBCD were calculated obtaining values of 20, 20 and 40 pg on-column, respectively. Using UPLC less time is required for the elution of the compounds, in this case less than 8 min were necessary.

LC-MS or LC-MS-MS using ESI or APCI are versatile tools for the isomeric specific determination of HBCDs trace levels monitoring the specific transitions m/z 640.6 $\rightarrow$ m/z 78.9 and 80.9. Budakowsky and Tomy (2003) [36] has shown that APCI have lower intensities compared with a similar experiment with ESI. Consequently, ESI mode is the preferred one for determining diastereoisomers by several studies [3,23,36]. However, Suzuki and Hasegawa [10] reported signal to noise (S/N) ratio values 2-5 times higher compared to ESI when APCI was applied to the analysis of HBCDs in a leachate.

Different methods for the analysis of diastereoisomeric HBCD using LC-ESI-MS-MS and the selective reaction monitoring (SRM) for the transition $[M-H]^+$ (m/z 640.6) $\rightarrow$ $[Br]^+$ (m/z 79 and 81) have been developed obtaining LODs between 0.5 and 6 pg on-column [25,36]. Morris *et al.* (2006) observed differences in the sensitivity of different HBCDs between two MS instruments; α -HBCD proved to be the most sensitive of the three diastereoisomers with the single quadrupole instrument, whilst γ -HBCD was the most sensitive on the ion-trap [54].

A LC-ESI-ion trap (IT)-MS method has been developed [34] to determine HBCD diastereoisomers in marine sediment samples obtaining good precision and a wide range of linearity with LOD of the method (mLOD) between 5 and 10 pg/g. The distribution of HBCD in Detroit River suspended sediments was analyzed by Marvin *et al.* (2006) [35] using PLE-LC-ESI-MS-MS and a detection limit of 10 pg on-column was estimated for individual HBCD diastereoisomers.

It is important to note that some other LC-ESI-MS-MS methods have been developed for the simultaneous analysis of diastereoisomeric HBCDs and TBBPA [3,10]. Two different LC-QqLIT-MS methods were developed by Guerra *et al.* (2008) [3] comparing a SRM experiment with an enhanced product ion (EPI) experiment to analyze α -, β - and γ -HBCD together with TBBPA and related compounds (BPA, MonoBBPA, DiBBPA and TriBBPA). The developed methods display excellent LODs in SRM mode (0.1-1.8 pg) (Figure 1), but even better results are obtained in EPI mode (0.01-0.5 pg) [3]. In order to analyze sewage sludge and sediments, the previous optimized experiment involving SRM was used [23]. The sample extraction was based on the use of sonication-SPE obtaining mLODs between 1.4 and 12 ng/g.

A comparison between the use of LC-ESI-MS-MS and LC-APCI-MS-MS was performed by Suzuki in 2006 [10] in order to analyze water and sediments, obtaining S/N ratios for HBCDs by APCI approximately two to five times higher than those for ESI [10]. In the case of TBBPA the S/N for APCI was almost half that for ESI.

Recent studies involving the use of UPLC-ESI-MS-MS for the analysis of HBCD and TBBPA through a C18-BEH column found iLODs from 0.03 to 0.06 pg on-column and mLODs between 0.080 and 0.082 ng/g for sewage sludge samples [46].

It is also important to note that matrix-related effects, either signal enhancement or more commonly signal suppression, can have a pronounced effect on quantitative measurements. Based on these observations, the use of isotopic labelled standards are helpful to achieve accurate analytical

measurement data on the diastereoisomers. Several methods found in the open literature includes the use of both ^{13}C - and d_{18} -labelled surrogates as recovery and/or instrument standards [16,25,56].

4.2.2. LC-MS for HBCD enantiomers

α -, β - and γ - HBCD diastereoisomers are chiral; thus HBCD has three pairs of enantiomers $(+)\alpha$, $(-)\alpha$, $(+)\beta$, $(-)\beta$, $(+)\gamma$ and $(-)\gamma$. The enantiomers have identical physicochemical properties and abiotic degradation rates, but may have different biological and toxicological properties and therefore different biotransformation rates. These transformations may result in nonracemic mixtures of the enantiomers that were, industrially synthesized as racemates [7,40,53]. The rates of metabolization process of the enantiomers of chiral environmental pollutants may be significantly different, for example, there are some cases where only one enantiomer is being decomposed, while the second is being accumulated in the environment, this is the case of hexachlorohexane (HCH). Because of that, an understanding of the environmental and biological fate of the HBCD enantiomers is required.

In order to separate the enantiomers $(+/-)\alpha$ -, $(+/-)\beta$ - and $(+/-)\gamma$ -HBCD, permethylated β -cyclodextrin columns have been successfully used [2,25,41,42,53,56]. Other chiral chromatographic columns such as ChiraDex and ChiraSep (LiChroCART 250-4, 5 μm) from Merck, did not resolved the enantiomeric separation [53]. On the other hand, good separation was achieved using Nucleodex β -PM (200 mm x 4.0 mm, 5 μm) in a single analysis with LODs between 10 and 20 pg injected on-column. However, it is well known that ESI is subjected to sample matrix effects that can cause enhancement or suppression of the target analytes signal and can adversely affect their quantification [7,16]. Matrix effects are caused by coeluting compounds that interfere with the ionization of the analyte. In order to avoid this effect that can affect in enantiomeric fraction (EF) calculations, Marvin *et al.* [7] introduced the corrected EF values. This correction is based on the use of isotopic labelled standards (d_{18} -HBCDs) since they behave in an identical manner to their native counterparts. Two other factors influencing the obtained EF values were observed by Marvin *et al.* (2007) [7]. The first one is the organic solvent content in the mobile phase: a decrease of EF value with increasing organic fraction content in the mobile phase was observed. The second factor is the column bleed that would compete with the enantiomers during the ion charge process in the ion spray source.

Different enantiomeric behavior studies were done by Janak *et al.* [25,40] and Guerra *et al.* [2,53]. The first author [25] studied the EF in different fish species from the Western Scheldt estuary, where a strong enrichment of $(+)\alpha$ -HBCD was observed for bib and whiting liver. In the case of sole, the EFs were similar for liver and muscle, with a slight enrichment of $(-)\alpha$ -HBCD. In 2008, the same author [40]

found clear differences about α -HBCD enantiomeric fractions among the different species analyzed, indicating species-specific stereoselective mechanism for uptake and metabolism of α -HBCD enantiomers. In Spanish sediment samples [53] EF obtained suggested a higher presence of (+)- α -HBCD and (+)- γ -HBCD as compared to technical mixtures which implies that are not racemic mixtures. Finally, EFs values in human breast milk samples from Spain have been analyzed [2]. Figure 2a shows the chromatographic separation obtained in a standard solution, and Figure 2b presents the separation of a human milk sample. As can be seen, low EF value was obtained for the human milk sample (around 0.3), indicating an enrichment of the (-)- α -HBCD enantiomer in human body.

4.3. LC-MS for PBDEs and emerging BFRs

ESI, APCI and APPI are the atmospheric pressure ionization techniques commonly used in LC-MS analyses. These techniques are suitable for the ionization of polar, less polar and slightly polar to non-polar compounds, respectively. Although ESI efficiently ionizes the HBCD isomers and TBBPA [3,16,22,57], the use of this ionization method for others HFRs has failed. APPI is the most used ionization method for the analysis of PBDEs but the limited availability of APPI in most analytical laboratories has made this mode of ionization less appealing [30,38,43,45,49]. Abdallah *et al* [44] performed a ^{13}C -labeled isotope dilution LC-APPI-MS-MS method to analyze 14 different PBDEs, between tetra- to deca-BDEs in house hold dust. Different C18-RP columns were tested and columns with 3 μm particle size were preferred, because BDE-207 and BDE-208 could not be well-separated on 5 μm particle size columns of different dimensions.

UPLC-APCI-MS-MS and UPLC-APPI-MS-MS were both tested for the analysis of eleven BDE congeners through a C18-BEH column [46]. iLOD and mLOD were reported with values between 0.10 to 0.72 pg and 3 to 198 pg/g and, 0.11 to 6.3 pg and 4-380 pg/g for APCI and APPI, respectively. Therefore, depending on the congener it seems that the APCI mode is between 2 and 8 times more sensitive than the APPI mode for the analysis of BDE congeners.

Another method was developed to analyze 36 HFRs in fish by LC-(-)APPI-MS-MS using a C18 column with ~2 μm particle size which produced narrower peaks that improved both sensitivity and resolution [49]. Some of the quantified HFR were 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EHTeBB), BEHTBP, isomeric HBCD and 14 BDE congeners (BDE-17, -47, -66, -71, -77, -100, -99, -126, -138, -154, -153, -183, -197, -205, -206 and -209) with iLOD values from 0.5 to 40 pg on-column. Moreover, LC-APCI-MS-MS method was also performed for the analysis of 38 HFRs in wastewater finding EHTeBB, DBDPE, BEHTBP and TBBPA at pg/mL levels. In this method, the optimized mobile phase consisted on a mixture of water and methanol [48]. The eluent containing only methanol generated the highest sensitivity compared to acetonitrile, acetonitrile-modified or methanol-modified mobile phases. Methanol has a lowest boiling point (64.7°C), thus consumes less desolvation energy in the APCI source. Consequently the analytes are more easily evaporated and ionized. As regards the use of APCI or APPI,

the main advantage associated with using APCI over APPI is the simplicity for the analysis of these HFRs, because APCI does not require a UV lamp and a dopant reagent to assist APPI.

The two methods above described include DP as one of the selected HFR, although this compound usually is detected using GC-HRMS. In LC-APCI-MS-MS and LC-APPI-MS-MS, displacement reactions were the most dominant pathways observed. The main precursor ion observed for DP is $[M-Cl+O]^+$ with chlorine loss and oxygen capture. Both proton elimination and combination reactions were also observed during the ionization of DP isomers obtaining $[M-H]^+$ and $[M+O_2]^+$; $[M-3Cl-2H+O]^+$ and $[M-H-4Cl]^+$ were also generated in the APCI source [48,49].

A new emerging BFR recently included in some environmental studies is the TBECH. This compound can exist as four diastereoisomers thermally sensitive, and they can interconvert at temperatures of 123°C or higher. Due to that, the development of a LC-MS method may be useful in order to obtain the separation of the four isomers. Arsenault *et al.* (2008) [18] developed a LC-ESI-MS method for the analysis of the four TBECH isomers. They could not detect the molecular ion, however analytes were detected monitoring the bromide (Br^-) ion using SIM mode. They also observed that, using APCI conditions, a very weak molecular ion was generated.

4.4. BFR metabolites and transformation products

Organic compounds can enter to the environment in different manner, mainly depending on their pattern of usage and mode of application. When they are into the environment could be widely distributed and subjected to different processes that contribute to their elimination and/or transformation [58]. Depending on the compartment in which the transformation occurs the products could be a metabolite, when the organism metabolism is involved or, a transformation product if it reacts in the environment.

In order to analyze metabolites and transformation products of BFRs, LC-MS is a useful technique due to the higher polarity of these compounds compared with the parent compound.

4.4.1. HBCD metabolites and transformation products

Abdallah *et al.* [33] found HBCD transformation products in dust samples via LC-MS-MS using a C18 analytical column and water/methanol as mobile phase in presence of ammonium acetate. These products were identified as four pentabromocyclododecene (PBCDe) isomers. Moreover, two additional peaks were observed in chromatograms, and corresponded to two congeners of tetrabromocyclododecadienes (TBCDe). The monitoring of m/z 560.8 $\rightarrow$ 79.0 and 480.4 $\rightarrow$ 79.0 was done for PBCDes and TBCDes, respectively. The elution of all compounds is obtained in less than 15 minutes. Different results were observed by Davis *et al.* [59] when analyzed wastewater sludge and freshwater sediments by LC-APPI-MS. In this study, only one TBCDe isomer as a degradation product of HBCD was detected. It was proposed that TBCDe is the result of a dihaloelimination reaction of HBCD resulting in the loss of two bromines from vicinal carbons with the further formation of a double bond between the adjacent carbon atoms.

A small number of studies have been carried out in order to determine HBCD metabolism. In dolphins, three HBCD metabolites were detected using a LC-ESI-MS instrument [60]: two metabolites were identified as monohydroxy-HBCD and, the third metabolite could not be identified. Hydroxylated metabolites of β - and γ -HBCD were found, however no significant decrease in α -HBCD concentration was observed during the incubation with rat and harbour seal microsomes. However, in vitro experiments with microsomes of dab and flounder showed that α -HBCD was also biotransformed resulting in two monohydroxy-HBCD metabolites. Huhtala *et al.* [61] confirmed this result by finding a monohydroxy-HBCD after an in vitro study in rainbow trout liver microsomes.

Brandsma *et al.* [62] studied the presence of hydroxylated metabolites of HBCD in three wildlife species (tern egg, seal and flounder) as well as Wistar rats exposed to HBCD for 28 days. A total of four different types of metabolites were found using LC-MS-MS: the monohydroxy metabolites of TBCDe, PBCDe, and HBCD, and a dihydroxy-HBCD (Figure 3). Another metabolic pathway in the rat is debromination of HBCD to PBCDe and TBCDe. In this study, all the hydroxylated metabolites found with LC-MS-MS were confirmed by GC-MS, but an additional metabolite was found with GC-MS, the dihydroxy-PBCDe [62].

4.4.2. PBDE metabolites

The nonpolar nature of the diphenyl ether structure in PBDEs and the introduction of polar hydroxyl functional groups in the molecule upon its metabolism provide a special challenge in the analysis of PBDEs metabolites. Due to the structural similarity of some hydroxylated (OH) PBDE compounds to thyroid hormones such as thyroxine (T₄), these metabolites are suspected to mimic hormones in the body making them more deleterious than the parent compounds [8].

Direct analysis of OH-PBDEs by GC-MS is not amenable due to their non-volatile nature. All analytical methods applying GC-MS for the analysis of OH-PBDE metabolites must include a derivatization step with diazomethane. This compound needs to be handled with extreme care due to its explosive characteristics. On the other hand, the efficiency of the derivatization varies from sample to sample since the reaction may give less than 100% yield. Finally, additional sample preparation/cleanup steps could introduce errors and long analysis time. For all these reasons the analysis of OH-PBDE metabolites by LC-MS seems interesting. However, these metabolites are poorly ionized by LC-ESI-MS, and consequently are not easily detected at low levels [9].

In 2007, Mas *et al.* [63] proposed the analysis of eight underivatized OH-di- to OH-tetra-BDEs by negative ion spray ionization (ISP) MS-MS in soil fish, sludge and particulate matter. This method was shown to be efficient, robust, sensitive and selective with LOQ at the high picogram per gram dry weight level. Another LC-APCI-MS-MS method was developed for the separation and detection of nine OH-PBDEs, ranging from tri- to hexa-brominated as well as MS-MS fragmentation information [8]. As mobile phase, water and acetonitrile were used and the reversed-phased separation was completed on a

C18 analytical column, resulting in a 35 min separation of the nine congeners followed by a 10 min re-equilibration.

Methoxylated (MeO) PBDEs have been reported in a few studies and the current knowledge indicates that the MeO-PBDEs found in wildlife are mostly a consequence of accumulation via natural sources in marine environments such as in sponges and green algae [64]. A study was done by Kato *et al.* [65] for the analysis of OH- and MeO-tetra-BDEs and of hydroxylated and methoxylated analogs of tetrabromobiphenyl (diOH-tetra-BB and diMeO-tetra-BB) using LC-APCI-MS-MS in marine biota. A good advantage of the proposed APCI method is that the simultaneous determination of OH- and MeO-brominated analogs could be completed within 22 min after a single cleanup GPC procedure.

A selective and sensitive method using negative LC-APCI-MS-MS [66] was also developed to enable analysis of selected natural persistent organohalogenes accumulated in marine biota. Selected analytes were three MeO-tetra-BDEs, a diMeO-tetra-BDE and two halogenated methyl bipyrroles (Cl₇-MBP and Br₄Cl₂-DBP). The method only required 10 min to allow the separation of selected analytes. In this method, the fragmentation pathways of MeO-BDEs produced characteristic SRM transitions needed to resolve isomeric compounds, [M-Br+O]⁻ and Br⁻ ions for MeO-BDE analogues and [M-Cl+O]⁻ and Br⁻ or degradation product ions for 1,1'-dimethyl-3,3',4,4'-tetrabromo-5,5'-dichloro-2,2'-bipyrrole (Br₄Cl₂-DBP) and 2,3,3',4,4',5,5'-heptachloro-1'-methyl-1,2'-bipyrrole (Cl₇-MBP), respectively.

Finally, a LC-ESI-MS-MS method with greater sensitivity and better separation efficiency [67] was established for the simultaneous analysis of four estrogens, BPA, 10 OH-PBDEs, and 15 brominated phenols (BRPs) in blood plasma sample, using dansyl chloride as derivatization agent that reacts which provides a new method to derivatize phenolic compounds prior to LC-MS analysis (Figure 4). The ionization and fragmentation of the isolated dansyl derivatives ESI-MS-MS resulted in protonated molecular ions [MH]⁺ or [MH]⁺-2 of their dansyl derivatives, producing the same major product ions at *m/z* 171 and 156. Thus, 30 analytes belonging to four classes of phenolic compounds could be analyzed within 22 min after sample treatment. However, the sample preparation is longer due to the use of dansyl chloride.

5. Conclusions and future perspectives

PBDEs have typically been analyzed using GC-MS but the high injection port temperatures required to transfer these analytes to the GC column can result in degradation of the desired compounds. These could occur in the case of highly PBDEs and other BFRs such as HBCDs. In the case of TBBPA or PBDE metabolites a derivatization step is needed, given more possibilities for errors because it is not always reproducible and quantitative.

The LC-MS and LC-MS-MS methods appear to be methodologies of choice to analyze TBBPA, HBCD diastereoisomers and HBCD enantiomers because no derivatization is needed, and the isomeric separation is obtained. The ESI, APCI and APPI are the atmospheric pressure ionization modes commonly used in LC-MS analyses. However, the use of LC-MS-MS allows the necessary sensitivity in

order to analyze environmental samples with values in the range of low ng/g. On the other hand, the use of ^{13}C -TBBPA, ^{13}C - and d_{18} -HBCD-isomer labelled standards as internal standards enhances the quality of the analytical data through compensation for matrix related effects that can affect analyte ion intensity, reproducibility and trueness.

Also important is the recent development of methods for the identification of metabolites of PBDEs and HBCD by LC-MS-MS, thereby avoiding the problems of derivatization with diazomethane.

The future challenge is to adapt these LC-MS techniques for the analysis of emerging flame retardants such as DP, TBECH, HCDBCO, BTBPE and DBDPE in different sample matrices, as well as their potential metabolites and transformation products.

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FIGURE CAPTIONS

Figure 1. SRM chromatogram of HBCD, TBBPA and related compounds in a standard solution (500 pg/ μ L) by LC-ESI-MS-MS

Figure 2. Enantiomeric HBCD analysis by LC-ESI-MS-MS for: (a) standard solution at 500 pg/ μ L, and (b) human breast milk sample.

Figure 3. (A) Selected ion LC-MS chromatogram of α -, β - and γ -HBCD standard extract. Selected ion LC-MS chromatograms in adipose tissue of a male rat sample: (B) PBCDe in fraction 6, monohydroxy-HBCD in fraction 12, (C) monohydroxy-HBCD in fraction 12, (D) monohydroxy-PBCDe in fraction 15, (E) monohydroxy-TBCDe fraction 15, (F) dihydroxy-HBCD in fraction 16. *Reproduced from reference [62].*

Figure 4. LC-MS-MS MRM chromatographic profiles of 30 analytes in a standard mixture of 10 ng/mL: E1 (1), β E2 (2), α E2 (3), EE (4), BPA (5), 2'-OH-6'-Cl-BDE-7 (6), 6'-OH-BDE-17 (7), 3-OH-BDE-47 (8), 5-OH-BDE-47 (9), 6-OH-BDE-47 (10), 4'-OH-BDE-49 (11), 2'-OH-6'-Cl-BDE-68 (12), 6-OH-BDE-90 (13), 2-OH-BDE-123 (14), 6-OH-BDE-137 (15), 3-BRP (16), 2/4-BRP (17/18), 2,6-diBRP (19), 2,3-diBRP (20), 2,5-diBRP (21), 2,4-diBRP (22), 3,4-diBRP (23), 3,5-diBRP (24), 2,3,6-triBRP (25), 2,3,4-triBRP (26), 2,4,6-triBRP (27), 2,3,5/2,4,5-triBRP (28/29), 3,4,5-triBRP (30), analyzed on a 100 mm $\times$ 2.1 mm, i.d.; Waters XBridge column. E1: estrone; β E2: 17 β -estradiol; α E2: 17 α -estradiol; EE2: Ethinyl estradiol. *Reproduced from reference [67].*

Figure 1.

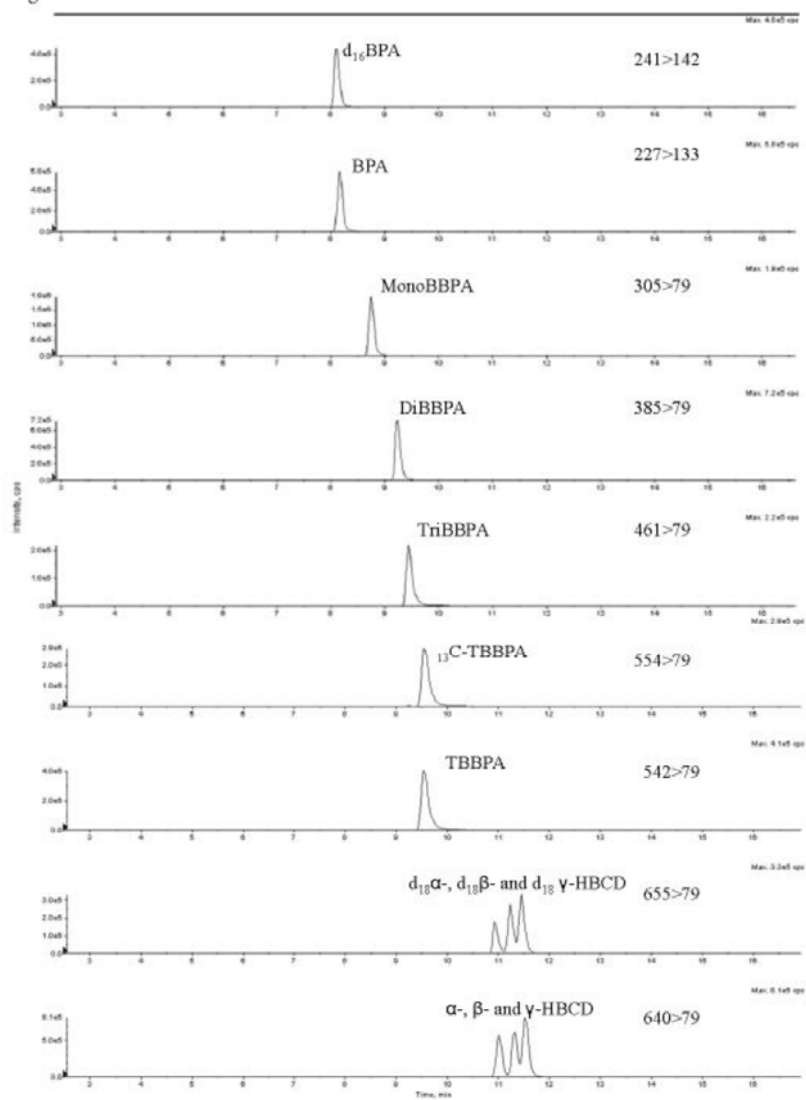
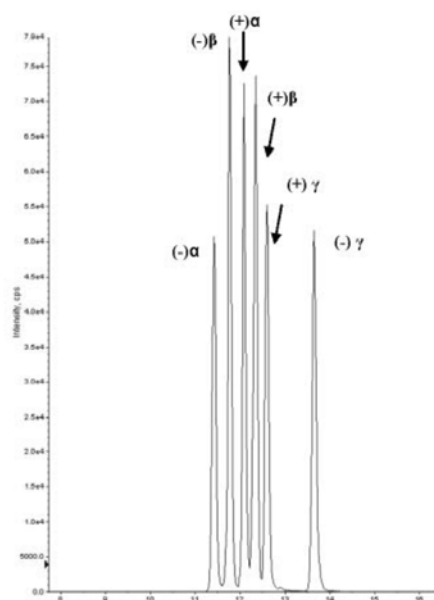


Figure 2.

a)



b)

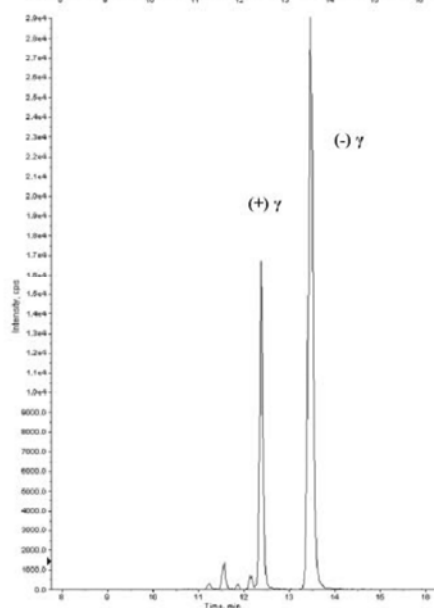


Figure 3.

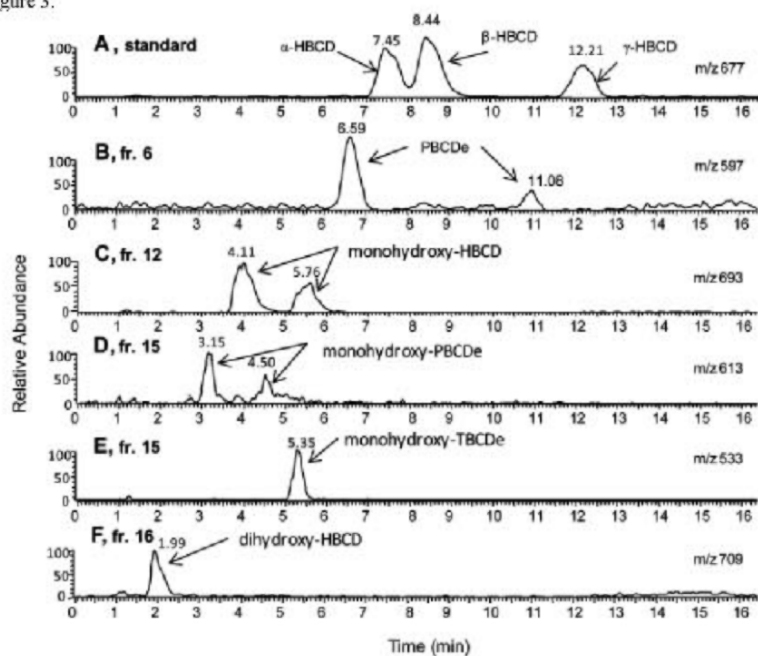
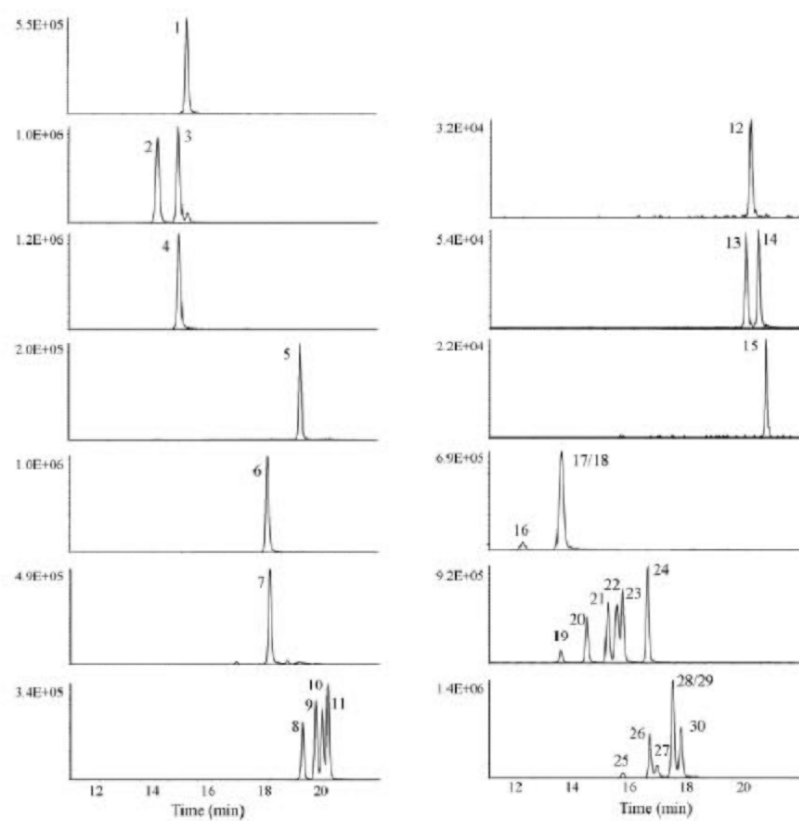


Figure 4.



1.8. Justificación de la tesis

La investigación de la contaminación química en los últimos años se ha centrado en el estudio de contaminantes que representan un riesgo para la salud humana debido a su toxicidad y a su capacidad para producir efectos cancerígenos y/o mutagénicos, así como por su elevada persistencia en el medio ambiente.

La principal problemática que presentan estas sustancias, entre ellas los HFRs, es que son introducidos continuamente al medio ambiente procedentes de su producción industrial y utilización en diversos materiales. Además se debe considerar su liberación durante el periodo de vida útil del material como también en su almacenamiento para el reciclaje y/o disposición final.

En las últimas décadas los estudios sobre FR particularmente de PBDEs, han aumentado en todo el mundo, debido principalmente a su persistencia y presencia en diversas matrices. Actualmente, dos de los productos técnicos de PBDEs (PentaBDE y OctaBDE) están siendo eliminados o han sido prohibidos en Norteamérica y Europa, mientras que el DecaBDE ha sido prohibido en Europa y, en EE.UU. su producción, importación y venta será discontinuada a fines de 2013.

Al comienzo de la presente tesis, en el año 2006, grupos de investigación involucrados en el campo de los BFRs comenzaban con la optimización de métodos que permitieran el análisis de HBCD en matrices ambientales. Por otro lado, según datos del Foro Ambiental de la Ciencia del Bromo (BSEF, del inglés *bromine science of environment forum*) del 2001 se indicaba que el uso de BFR era liderado por TBBPA (59%), además de mostrar un incremento en el uso de HBCD a nivel mundial.

Se espera que tras la disminución del consumo y producción de PBDEs, otros FR sean utilizados de forma creciente para sustituirlos. Este es el caso de HBCD y TBBPA, además de otros FR emergentes. Por ello, es de vital importancia realizar estudios de evaluación de su riesgo ambiental. Para esto es necesario obtener información sobre su presencia, destino y transformación en el medio ambiente, además de evaluar la posible exposición de los seres humanos a estos compuestos.

Para lograr obtener esta información, el primer paso imprescindible es disponer de metodologías analíticas fiables y suficientemente sensibles para lograr determinar los niveles ambientales.

Una vez la comunidad científica logre aportar datos suficientes sobre presencia, destino, comportamiento y toxicidad, se podrá evaluar si es o no aconsejable continuar utilizando estos HFRs o, si por el contrario, estos nuevos HFRs emergentes deben ser también prohibidos, al igual que sus antecesores los PBDEs.

1.9. Objetivos generales

Teniendo en cuenta todo lo expuesto anteriormente y basándonos en la necesidad de establecer nuevas metodologías de análisis y a fin de analizar el riesgo ambiental de los FR halogenados en una variedad de muestras, se establecieron los siguientes objetivos para la presente Tesis Doctoral:

1. Desarrollar y validar metodologías de análisis sensibles y selectivas utilizando la técnica de LC-MS/MS, que permitan la determinación de BFR en diversas matrices ambientales y biológicas.
2. Aplicar los métodos desarrollados para la determinación del impacto ambiental de los FR en el medio acuático. Para ello, se estudiarán los sedimentos y biota de diferentes cuencas hidrográficas españolas, así como los lodos de diferentes EDARs.
3. Estudiar también el impacto ambiental en el medio terrestre. Para ello, estudiar la biodisponibilidad de FR emergentes en huevos de aves ubicadas en la parte superior de la cadena trófica, tales como el halcón peregrino (*Falco peregrinus*).
4. Evaluar el grado de exposición humana a diversos HFRs mediante el estudio de leche materna, así como de complementos de la dieta española.

CAPÍTULO 2: DESARROLLO DE MÉTODOS ANALÍTICOS.

2. DESARROLLO DE MÉTODOS ANALÍTICOS

2.1. Introducción

En el Capítulo 1 hemos señalado que en los últimos años el consumo de HBCD ha ido en aumento, por lo tanto es de prever que cada vez tenga un mayor impacto en matrices medioambientales como sedimentos, suelos y biota, como también en muestras humanas. Los primeros métodos de análisis de HBCD se basaron en el uso de la GC, pero esta técnica tiene limitaciones, dado que solo puede proporcionar información acerca de la concentración total de HBCD. En la Figura 2-1 se observa la coelución de los diastereoisómeros de HBCD en un único pico de mayor amplitud. Es por ello que al comienzo de esta tesis el primer paso fue el desarrollo de un método sensible, selectivo y reproducible utilizando LC-MS/MS que permitiera determinar los isómeros de HBCD de una forma fiable. También se sabe que otros HFRs tienen problemas para ser analizados por GC-MS, como es el caso de TBBPA. Por lo tanto, era de nuestro interés lograr un método de análisis por LC-MS/MS capaz de llevar a cabo el análisis simultáneo de dos FR, HBCD y TBBPA.

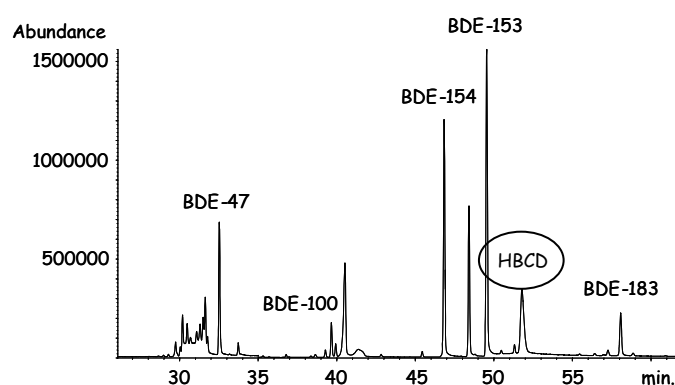


Figura 2-1. Ejemplo de la separación cromatográfica de HBCD por GC-MS utilizando una columna DB5-MS.

Para una mejor comprensión del comportamiento de HBCD en el medio ambiente es vital contar con la información adecuada respecto a la presencia de α -, β - y γ -HBCD. Consecuentemente, hemos desarrollado un método que permite no solo la determinación del HBCD, sino que también y de forma simultánea, el análisis de otro BFR, el TBBPA y sus derivados mediante el uso de LC-ESI-MS/MS el cual se encuentra detallado en el artículo #2: *Identification and trace level determination of brominated flame retardants by LC-quadrupole linear ion trap (QqLIT) mass spectrometry*. Posteriormente el método analítico lo aplicamos al análisis de dichos BFRs en muestras de lodos de depuradora y sedimentos, desarrollando un método de extracción y purificación. Este trabajo se

resume en el artículo #3: *Simultaneous determination of hexabromocyclododecane, tetrabromobisphenol A, and related compounds in sewage sludge and sediment samples from Ebro River basin (Spain)*.

Por otro lado, los isómeros del HBCD son quirales y por lo tanto pueden presentarse como parejas de enantiómeros, los cuales tienen propiedades físico-químicas idénticas, excepto en dos aspectos:

- Rotan el plano de la luz linealmente polarizada en direcciones opuestas, aunque en cantidades iguales.
- Reaccionan a diferentes velocidades con otros compuestos quirales, pero lo hacen a la misma velocidad con compuestos aquirales.

De esta forma, los enantiómeros tienen propiedades idénticas en un medio simétrico, pero sus propiedades pueden ser diferentes en un medio asimétrico. Este es el principio básico para la cromatografía enantioselectiva, donde la fase estacionaria quiral que se utiliza conforma el entorno asimétrico, según sea necesario. Por lo tanto, la formación de complejos diastereoisoméricos entre los enantiómeros respectivos y la fase estacionaria quiral dan lugar a diferentes tiempos de retención, permitiendo una separación enantioselectiva. Por el contrario, diastereoisómeros que poseen propiedades físicas diferentes, también se pueden separar en fases estacionarias aquirales (Huhnerfuss y Shah 2009).

Los procesos biológicos generalmente proceden con enantioselectividad. Por ello los resultados de investigaciones enantioselectivas de compuestos quirales pueden aportar información valiosa sobre la biodisponibilidad y la acumulación de contaminantes antropogénicos a lo largo de la cadena trófica.

En los estudios sobre enantiómeros es importante calcular su composición enantiomérica, la cual usualmente se presenta como EF y se calcula a partir de las áreas de los picos cromatográficos de los enantiómeros (A_+ y A_-), utilizando la siguiente fórmula:

$$EF = A_+ / (A_+ + A_-)$$

Los contaminantes quirales originalmente son producidos industrialmente como racematos ($EF=0,5$). Sin embargo al entrar en contacto con un sistema biológico se puede producir el agotamiento o acumulación de uno de los enantiómeros. Al comparar los EF de un patrón o del producto industrial con muestras del medio ambiente, puede observarse una desviación del valor racémico, indicando la bioactividad del compuesto quiral (Eljarrat et al., 2008).

Dada la importancia de poder determinar el comportamiento enantiomérico de HBCD, desarrollamos un método analítico para la determinación de las parejas de enantiómeros (+/-) α -, (+/-) β -y (+/-) γ -HBCD y sus respectivas EFs. Este estudio permitió la publicación del artículo #4:

Enantiomeric specific determination of hexabromocyclododecane by LC-quadrupole linear ion trap (QqLIT) mass spectrometry in sediment samples.

2.2. Desarrollo de metodología

Los tres artículos que se presentan a continuación tienen en común el desarrollo de metodologías para la detección de BFRs en muestras ambientales utilizando LC-MS/MS. En el artículo #2 se obtiene una nueva metodología para la determinación de α -, β - y γ -HBCD, además de TBBPA y los compuestos formados por su debromación. En el artículo #3 se presenta el desarrollo de un método de extracción y purificación de los compuestos en estudio en sedimentos y en lodos de depuradora que provienen de aguas residuales con diferente procedencia y tratamiento. Finalmente, en el artículo #4 encontramos el desarrollo de un método analítico que permite la determinación enantiomérica de HBCD por LC-MS/MS.

Separación cromatográfica

Para el análisis de los diastereoisómeros de HBCD se utilizó la cromatografía de líquidos en fase invertida con una columna C₁₈ Symmetry, siendo el orden de elución: α -, β - y γ -HBCD, tal como se muestra en la Figura 2 del artículo # 2. El tiempo de elución obtenido en nuestro trabajo, que incluye la separación de α -, β - y γ -HBCD, TBBPA, BPA, monobromobisfenol A (MonoBPA, del inglés *monobromobisphenol A*), dibromobisfenol A (DiBBPA, del inglés *dibromobisphenol A*), tribromobisfenol A (TriBBPA, *tribromobisphenol A*) además de 4 estándares marcados isotópicamente (d₁₆-BPA, ¹³C-TBBPA, d₁₈ α - y d₁₈ γ -HBCD) fue de menos de 17 min., utilizando una mezcla de agua y metanol como fase móvil.

Finalmente, hay que destacar que también intentamos optimizar el análisis de los mismos compuestos anteriores utilizando la cromatografía líquida de ultra eficacia (UPLC, del inglés *ultra performance liquid chromatography*)-MS/MS con una columna UPLC C₁₈, para disminuir los tiempos de análisis. Sin embargo, la separación de TBBPA y compuestos similares no fue posible. Además la separación cromatográfica de los diastereoisómeros de HBCD obtenida fue de inferior calidad, tal como se observa en la Figura 2-2.

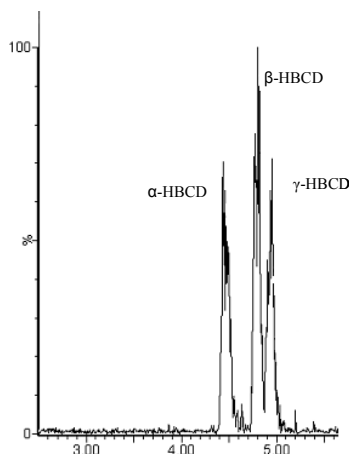


Figura 2-2. Separación cromatográfica de α -, β - y γ -HBCD (100 pg/ μ L) utilizando UPLC-QqQ-MS.

Por otro lado, en el caso de la separación cromatográfica de los enantiómeros de HBCD, utilizamos una columna quiral Nucleodex beta-PM, la cual fue seleccionada luego de probar otras dos columnas enantioméricas, tal como se comenta en el artículo #4. Al optimizar la fase móvil para la separación enantiomérica primero se utilizó agua y metanol, de forma similar al método diastereoisomérico pero la separación no fue satisfactoria, tal como se observa en la Figura 2-3a. Una vez que incorporamos acetonitrilo a la fase móvil obtuvimos la separación de los seis enantiómeros. Los tiempos de elución son en torno a los 5 min., siguiendo el siguiente orden: (-) α -, (-) β -, (+) α -, (+) β -, (+) γ - y (-) γ -HBCD, tal como en la Figura 2-3b.

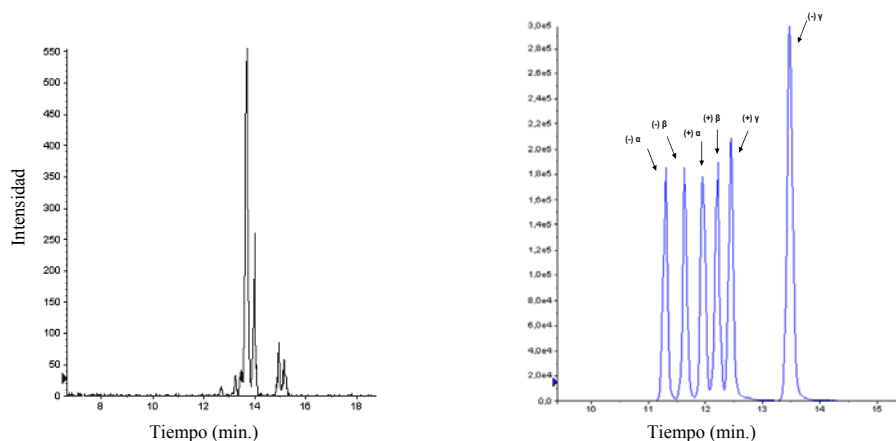


Figura 2-3. Cromatograma obtenido para una mezcla de estándares de α -, β - y γ -HBCD con a) agua y metanol, b) agua, metanol y acetonitrilo en la fase móvil.

Detección por MS

Para los tres trabajos que se revisan en este capítulo, el análisis por espectrometría de masas se basa en el uso de un instrumento híbrido del tipo Qq/trampa lineal de iones (LIT, del inglés *linear ion trap*) (MSD Sciex 4000QTrap), equipado con ESI. La detección de los isómeros de HBCD y de TBBPA se basa en la monitorización de la transición $[M-H]^+ \rightarrow [Br]^+$, donde para HBCD nativo la transición de cuantificación es $m/z\ 639 \rightarrow m/z\ 79$, mientras que la confirmación se hace con la transición $m/z\ 639 \rightarrow m/z\ 81$. En el caso de los isómeros marcados d_{18} -HBCD, se monitorizan las transiciones $m/z\ [d_{18}\text{-}M-H]^+ m/z\ 656 \rightarrow m/z\ 79$ y 81 . Mientras que para TBBPA las transiciones son $m/z\ 543 \rightarrow m/z\ 79$ y 81 .

Hay que señalar que una de las características importantes del instrumento con que hemos trabajado (QTrap) es la capacidad de trabajar con el tercer cuadrupolo (Q3) configurado como cuadrupolo o como una LIT. Por ello, nos pareció interesante poder comparar las características de funcionamiento de ambas configuraciones. Es sabido que la LIT ofrece algunas posibilidades adicionales de exploración, porque los iones pueden ser almacenados durante un tiempo determinado, dando como resultado una mayor sensibilidad. De esta forma, el QTrap fue operado en modo IDA mediante la combinación de SRM con el modo *enhanced product ion* (del inglés EPI) a tres energías de colisión diferentes (CE, del inglés *collision energy*). Esto permite maximizar la cantidad de información que puede ser aprovechada en una sola pinchada al combinar dos o más modos de análisis diferentes de una manera secuencial, además de poder obtener una confirmación adicional en la identificación de los analitos que se analizan.

Artículo científico #2:

“Identification and trace level determination of brominated flame retardants by LC-quadrupole linear ion trap (QqLIT) mass spectrometry”

por

P. Guerra, E. Eljarrat y D. Barceló

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Identification and trace level determination of brominated flame retardants by liquid chromatography/quadrupole linear ion trap mass spectrometry

Paula Guerra¹, Adrián de la Torre², M. Angeles Martínez², Ethel Eljarrat^{1*} and Damià Barceló¹

¹Department of Environmental Chemistry, IIQAB-CSIC, c/Jordi Girona 18–26, 08034 Barcelona, Spain

²Environment Department, CIEMAT, Avda. Complutense 22, 28040 Madrid, Spain

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We describe the development of instrumental methodology for the simultaneous determination of hexabromocyclododecane (HBCD) diastereoisomers and tetrabromobisphenol A (TBBPA) and its derivatives by liquid chromatography/quadrupole linear ion trap mass spectrometry (LC-QqLIT-MS). Two different experiments were developed, optimized and compared. The first is based on a selected reaction monitoring (SRM) method in which the two most abundant transitions were selected for each analyte, as well as for the internal standards. In the second, the ion trap was used for the storage and subsequent fragmentation of precursor ions, obtaining an enhanced product ion (EPI) experiment. Both methods were validated by measuring quality parameters such as linearity, sensitivity, reproducibility and repeatability. Limits of detection (LODs) were in the range of 0.1–1.8 pg and 0.01–0.5 pg for SRM and EPI experiments, respectively, being lower than those published for the LC/QqQ-MS methods. Thus, LC-QqLIT-MS, used for quantification and confirmation, proved to be a powerful and very sensitive analytical tool. Copyright © 2008 John Wiley & Sons, Ltd.

Brominated flame retardants (BFRs) are a structurally diverse group of chemicals that are used in a variety of commercial applications to prevent fires, by reducing the flammability of combustible materials such as plastics and synthetic polymers.¹ BFRs include polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA). The Bromine Science Environmental Forum (BSEF) estimated the usage of selected BFRs in different areas of the world in 2001, with TBBPA being the most used (59%) and showing an increased use of HBCD.²

Tetrabromobisphenol A is the primary flame retardant used in electronics circuits and is preferred over other BFRs because it can be covalently bound to the polymer in the manufacturing process.³ It is not, however, frequently analyzed in environmental laboratories, perhaps because of its lower bioaccumulation potential, with concentrations lower than PBDEs and HBCD in the environment. However, TBBPA, being a phenolic compound, may have a greater

adverse effect on humans and wildlife.⁴ Due to its low solubility in water (0.72 mg/L) and high log K_{ow} (4.5), TBBPA is likely to be associated with suspended particulate matter once released in the water column and ultimately buried in sediments.^{5,6} There are indications of the potential toxicity of TBBPA as an endocrine disruptor and immunotoxic compound^{7,8} and other studies showed that it is toxic to aquatic life.^{9,10} Biodegradation studies have shown that TBBPA can be partly degraded to less brominated analogues under both aerobic and anaerobic conditions in soil and river sediment.^{7,11} This degradation is dependent on type, temperature, humidity and composition of the environmental matrix;⁶ for example, Ronen and Abeliovich¹⁰ reported a half life of 5 days for TBBPA in anaerobic sediment.

Hexabromocyclododecane, a brominated cyclic hydrocarbon, is the principal flame retardant in polystyrene foams and is used as thermal insulation in the building industry.¹² Technical 1,2,5,6,9,10-HBCD is produced industrially by addition of bromine to *cis-trans-trans*-1,5,9-cyclododecatriene. This process leads theoretically to a mixture of 16 stereoisomers, six pairs of enantiomers and four mesoforms. The product is usually a mixture of the three diastereoisomers α -, β - and γ -, but some authors have also found δ - and ϵ -diastereoisomers.¹³ Normally, the γ -isomer is the most abundant in commercial mixtures (ranging between 75 and 89%), followed by the α - and then the β -isomer (10–13% and 1–12%, respectively).¹⁴ The physicochemical properties of HBCD are similar to those of PBDEs and other persistent organic pollutants; in fact the log K_{ow} of HBCD is 5.6 and that

*Correspondence to: E. Eljarrat, Department of Environmental Chemistry, IIQAB-CSIC, c/Jordi Girona 18–26, 08034 Barcelona, Spain.

E-mail: eeeqam@cid.csic.es

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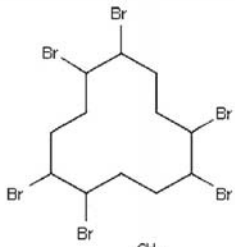
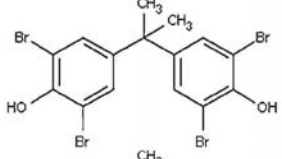
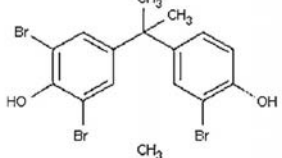
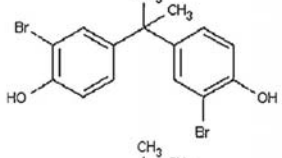
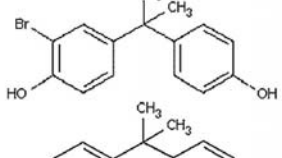
places it in the optimum range for bioaccumulation.¹² HBCD is not covalently bonded to the material into which it is impregnated, leading to the risk of migration out of the product during use or disposal.¹⁵ The dissimilarities in the structures of the α -, β - and γ -isomers might lead to differences in polarity, dipole moment and in solubility in water; for example, the solubilities of α -, β - and γ -HBCD in water are 48.8, 14.7 and 2.1 $\mu\text{g/L}$, respectively. Therefore, these different properties may explain the differences observed in their environmental behavior.¹⁶

The first methods for the analysis of HBCD were mainly based on gas chromatography (GC), but GC has limitations because it only provides information about the total HBCD

isomers due to coelution of the diastereoisomers and interconversion at temperatures over 160°C.^{15,17} Liquid chromatography/mass spectrometry (LC/MS) and tandem mass spectrometry (LC/MS/MS) are currently the best methods for measuring HBCD diastereoisomers separately in environmental samples.^{18,19} However, GC/MS has the advantage of higher sensitivity.²⁰ In the case of TBBPA, the advantage of LC/MS over GC/MS is that the derivatization step is not necessary.²¹ In order to improve sensitivity and specificity of LC/MS methods, in the field of environmental analysis, use has been made of MS/MS, with triple quadrupole instruments (QqQ), and, more recently, with LC/hybrid MS techniques such as are provided by

Determination of BFRs by LC/QqTOF-MS 917

Table 1. Structure and physicochemical properties of the selected analytes

Compound	Acronym	Chemical structure	log K_{ow}	MW
Hexabromocyclododecane	HBCD		5.6	641.7
Tetrabromobisphenol A	TBBPA		4.5	543.9
Tribromobisphenol A	Tri-BBPA		2.1*	465.0
Dibromobisphenol A	Di-BBPA		2.1*	386.1
Monobromobisphenol A	Mono-BBPA		3.7*	307.2
Bisphenol A	BPA		3.3	228.3

*Estimated, not experimental (IALOG P).

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Rapid Commun. Mass Spectrom. 2008; 22: 916–924

918 P. Guerra *et al.*

quadrupole time-of-flight (QqTOF) and quadrupole linear ion trap (QqLIT) instruments.²²

The objective of the present study was to develop a simultaneous method for the analysis of HBCD diastereoisomers (α , β and γ) and TBBPA and its derivatives (bisphenol A, monobromobisphenol A, dibromobisphenol A and tribromobisphenol A) (Table 1), based on an LC/QqLIT-MS method. The main advantage of QqLIT over QqQ is that the final quadrupole (Q3) can be operated in two different modes. The first mode is based on selected reaction monitoring (SRM), where the trap operates like a third quadrupole, similar to QqQ instruments; and the second uses the enhanced product ion (EPI) mode, where the ion trap is used for storage and subsequent fragmentation of precursor ions.^{23,24} In this work, both modes were tested and compared.

EXPERIMENTAL

Standards and reagents

α -, β - and γ -HBCD were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA) and were of minimum 97% purity; TBBPA, ¹³C-TBBPA, d₁₈- α - and d₁₈- γ -HBCD were obtained from Wellington Laboratories Inc. (Guelph, Ontario, Canada) and were of minimum 98% purity; BPA and d₁₆-BPA were from Aldrich (Milwaukee, WI, USA) and were of 99% purity. Mono-BBPA, di-BBPA and tri-BBPA were a kind gift from Dr Göran Marsh (Department of Environmental Chemistry, Stockholm University, Sweden).²⁵ Ammonium acetate was of minimum 98% purity from Aldrich. Solvents for organic trace analysis were purchased from Merck (Darmstadt, Germany). All solvents and other reagents were of analytical grade.

Individual stock standard solutions were prepared on a weight basis in methanol and stored at -20°C. A mixture of all selected analyte standards was prepared by appropriate dilution of individual stock solutions. Further dilutions of this mixture were prepared in methanol before each analytical run and were used as working standard solutions. Stock solutions of internal standards were also prepared in methanol and were stored at -20°C. A mixture of these standards, used for internal standard calibration, was also prepared by diluting the individual stock solutions in methanol.

LC/QqLIT-MS analysis

The LC system used was an Agilent HP 1100 binary pump (Agilent Technologies, Palo Alto, CA, USA) with a Symmetry C₁₈ column (2.1 × 150 mm, 5 μ m) preceded by a C₁₈ guard column (2.1 × 10 mm) supplied by Waters (Milford, MA, USA). Experiments were carried out in negative ion (NI) mode using H₂O/methanol (3:1 v/v) as eluent A and methanol as eluent B, at a flow rate of 0.25 mL/min. The injection volume was set at 4 μ L. The elution program started at an initial composition of 100% A and was ramped to 10% A in 17 min and the initial conditions were reached again in 3 min and maintained for an additional 15 min.

Mass spectrometric analysis was performed with an hybrid triple quadrupole/linear ion trap Applied Biosystem

MSD Sciex 4000QTRAP™ (Applied Biosystems, Foster City, CA, USA) instrument equipped with an electrospray ionization (ESI) Turbospray interface. All data were acquired and processed using Analyst 1.4.2. software (Applied Biosystems). For target quantitative analyses, data acquisition was performed in SRM mode, recording the transitions between the precursor ion and the two most abundant product ions. The MS/MS conditions were optimized to afford the highest relative signal intensity. Some parameters were set at default values: curtain gas pressure (CUR) at 50 psi, collision gas pressure (CAD) at 4.5×10^{-5} Torr, temperature of the turbo gas in the TurbolonSpray source (TEM) at 700°C, and the ion source gas 1 (GS1) and ion source gas 2 (GS2) pressures both at 50 psi.

Other parameters such as the declustering potential (DP), collision energy (CE), collision cell exit potential (CXP), ion spray voltage (IS) and entrance potential (EP) were optimized. The DP was modified between 55 and 130 V for TBBPA and related compounds, and between 50 and 80 eV for HBCD diastereoisomers. The CE, CXP, IS and EP optimization was carried out by modifying their values between 10 and 110 eV, 1 and 40 V, 4500 and 5500 V, and 5 and 30 V, respectively.

For the enhanced product ion scan (EPI) method the same detection conditions as used in the SRM method were applied. This experiment was performed in information-dependent acquisition (IDA) mode which combines a survey scan with an EPI carried out at three different collision energies, 10, 20 and 40 eV, at a scan rate of 4000 m/z units/s and a linear ion trap fill time of 20 ms.

Method characterization

The method characterization was carried out for the above two experiments, the first one working in SRM mode and the second in EPI mode. Quality control was carried out by repeated injections of solvent blanks (methanol) and standards. Identification of selected analytes was based on the following restrictive criteria: (i) the retention time for all monitored transitions for a given analyte should maximize simultaneously ± 1 s, with a signal-to-noise ratio ≥ 3 for each; and (ii) the ratio between the two monitored transitions should be within 15% of the theoretical value (calculated upon standards). Quantification was carried out by an isotopic dilution technique, based on the addition of labeled standards: d₁₈- α -HBCD for α - and β -HBCD quantification, d₁₈- γ -HBCD for γ -HBCD, d₁₆-BPA for BPA and ¹³C-TBBPA for TBBPA, tri-BBPA, di-BBPA and mono-BBPA. The linearity of the method was checked using calibration curves made from standard solutions at six concentration levels (5, 50, 100, 500, 1000, 2500 and 5000 pg/ μ L), and by triplicate injections. The precision of the method was determined by repeated intra-day and inter-day analysis with five successive injections of a standard solution in one day and on five successive days, respectively. The instrumental limit of detection (LOD), defined as 3 times the noise level, and the limit of quantification (LOQ), defined as 10 times the noise level, were calculated with the first SRM transition for both experiments.

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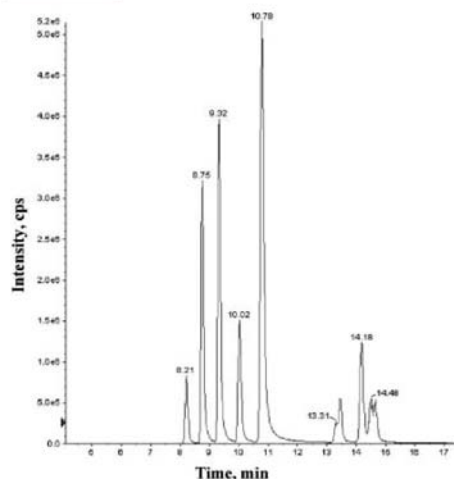


Figure 1. Total ion chromatogram (TIC) obtained for a standard mixture of 2500 pg/ μ L.

RESULTS AND DISCUSSION

Two different LC/QqLIT-MS assays were developed for the selective identification and confirmation of HBCD diastereoisomers, TBBPA and related compounds. The first is based on a SRM experiment, and the second used an EPI experiment.

Chromatographic separation

Various mobile phase compositions have been reported for BPA, TBBPA or HBCD analysis by LC/MS. A series of preliminary experiments was performed, testing different mobile phases consisting of methanol, water and acetonitrile, and using or not additives such as ammonium acetate. We

Determination of BFRs by LC/QqTOF-MS 919

found that the optimal separation of selected compounds was achieved using methanol as the mobile phase, with higher responses of target compounds than were obtained using acetonitrile. It was also observed that the use of additives gives a similar response for HBCD diastereoisomers, but that the sensitivity of TBBPA and related compounds decreased considerably. Therefore, using methanol and water as the mobile phase was concluded to be more advantageous for this quantitative analysis. A representative chromatogram of a 2500 pg/ μ L standard mixture of selected analytes and internal standards is illustrated in Fig. 1 (see also Table 2).

SRM experiment optimization

SRM transitions and other compound-dependent parameters (DP, CE and CXP) were optimized by infusing via a syringe pump a standard mixture, containing all of the compounds at 500 pg/ μ L. The optimum values are summarized in Table 2. An example of the analysis of a mixture of selected analytes using the developed SRM method is shown in Fig. 2. In all cases the $[M-H]^-$ ions were selected as the precursor ions in the NI mode. The product ions observed for each target compound were in good agreement with those previously reported. For example, the detection of HBCD isomers was based on the signal from the m/z 639 $\rightarrow$ 79 transition, with the $[M-H]^-$ to m/z 81 being used as the confirmation transition. The same transitions were selected by Budakowsky and Tomy.¹⁹ For TBBPA, Saint-Louis and Pelletier⁵ found an abundant $[M-H]^-$ ion at m/z 543 with two product ions at m/z 528 and 448 corresponding to $[M-H-methyl]^-$ and $[M-H-methyl-Br]^-$. Chu *et al.*⁶ found that the sensitivity using the m/z 543 $\rightarrow$ 81 $[Br]^-$ transition was about 10 times higher than that using m/z 543 $\rightarrow$ 528. The m/z 543 $\rightarrow$ 79 and m/z 543 $\rightarrow$ 81 transitions were selected in our study.

The MS/MS conditions were optimized to afford the highest relative intensity (Table 2). The optimized DP values were the same for the precursor and product ions, with values between 65 and 120 V for TBBPA and related compounds, and between 60 and 80 V for the HBCD diastereoisomers (Fig. 3(a)). Values between 30 and 98 eV,

Table 2. MS/MS parameters for the analysis of selected compounds by SRM in negative ion mode

Compound	Rt (min)	Precursor ion (m/z)	DP-CE-CXP (V-eV-V)	SRM1 (m/z)	DP-CE-CXP (V-eV-V)	SRM2 (m/z)	Ratio (Mean $\pm$ SD) ^a
BPA	8.3	227 $[M-H]^-$	80-30-1	227 $>$ 133	80-24-29	227 $>$ 211	0.36 $\pm$ 0.01
d_{18} -BPA	8.4	241 $[M-H]^-$	95-38-7	241 $>$ 142	95-28-19	241 $>$ 223	
Mono-BBPA	8.8	305 $[M-H]^-$	65-58-9	305 $>$ 79	65-34-5	305 $>$ 133	137 $\pm$ 85
Di-BBPA	9.4	385 $[M-H]^-$	75-44-13	385 $>$ 79	75-44-7	385 $>$ 81	1.1 $\pm$ 0.04
Tri-BBPA	10.0	461 $[M-H]^-$	120-74-1	461 $>$ 79	120-52-15	461 $>$ 338	4.1 $\pm$ 0.20
^{13}C -TBBPA	10.5	555 $[M-H]^-$	120-98-3	555 $>$ 79	120-98-5	555 $>$ 81	
TBBPA	10.9	543 $[M-H]^-$	105-84-1	543 $>$ 79	105-92-1	543 $>$ 81	0.96 $\pm$ 0.05
α -HBCD	13.3	639 $[M-H]^-$	60-46-1	639 $>$ 79	60-38-3	639 $>$ 81	2.0 $\pm$ 0.20
β -HBCD	14.4	639 $[M-H]^-$	60-46-1	639 $>$ 79	60-38-3	639 $>$ 81	2.1 $\pm$ 0.20
γ -HBCD	14.9	639 $[M-H]^-$	60-46-1	639 $>$ 79	60-38-3	639 $>$ 81	1.9 $\pm$ 0.21
d_{18} - α -HBCD	13.1	656 $[M-H]^-$	80-46-1	656 $>$ 79	80-50-1	656 $>$ 81	
d_{18} - γ -HBCD	14.7	656 $[M-H]^-$	80-46-1	656 $>$ 79	80-50-1	656 $>$ 81	

Rt: retention time; DP: declustering potential; CE: collision energy; CXP: collision cell exit potential; SRM1: first transition, used as quantitative transition; SRM2: second transition, used as confirmative transition; Ratio = SRM 1 abundance/SRM 2 abundance; SD: standard deviation; ^acalculated upon the standard calibration curves.

920 P. Guerra *et al.*

RCM

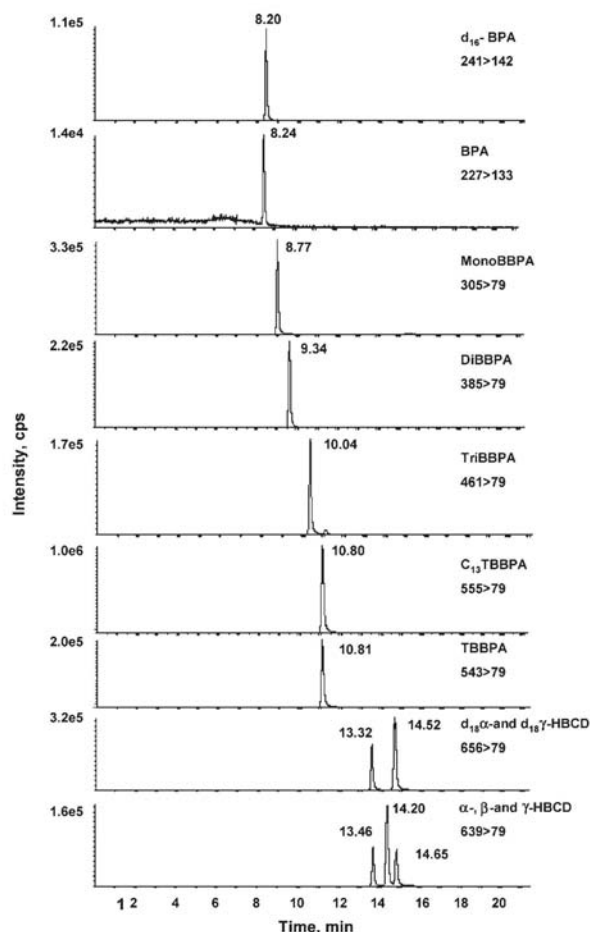


Figure 2. SRM chromatogram obtained with the LC/ESI(-)-MS/MS method for a standard solution (100 pg/ μ L).

and between 1 and 29 V, were found for CE and CXP optimization, respectively (Fig. 3(b)). Finally, the IS and EP were set at, respectively, 4500 V and 10 V for all the selected analytes.

EPI experiment optimization

In addition to all the capabilities of a triple quadrupole, the fact that the apparatus can be operated with Q3 configured as the final analysis quadrupole in a conventional tandem triple quadrupole mass spectrometer or as a linear ion trap mass spectrometer allows comparison of the performance characteristics of the two operational modes. The linear ion trap (LIT) offers some extra scan possibilities, because it can be

filled with ions during a specified time, resulting in more sensitive scan modes: enhanced MS (EMS), enhanced product ion (EPI) and MS/MS/MS. Using the QqLIT configuration and with the EPI mode, the selection of the precursor ion is performed in Q1 using radio-frequency (RF)/direct current (DC) isolation at any resolution. Collision-induced dissociation (CID) occurs in collision cell q2, and product ions are trapped in Q3 operated in the LIT mode. RF/DC isolation has a significant advantage over isolation waveform where, for the isolation of fragile ions, elimination of the precursor ion can be observed.

In addition, the QTRAPTM instrument allows us to use IDA, a procedure for maximizing the amount of information

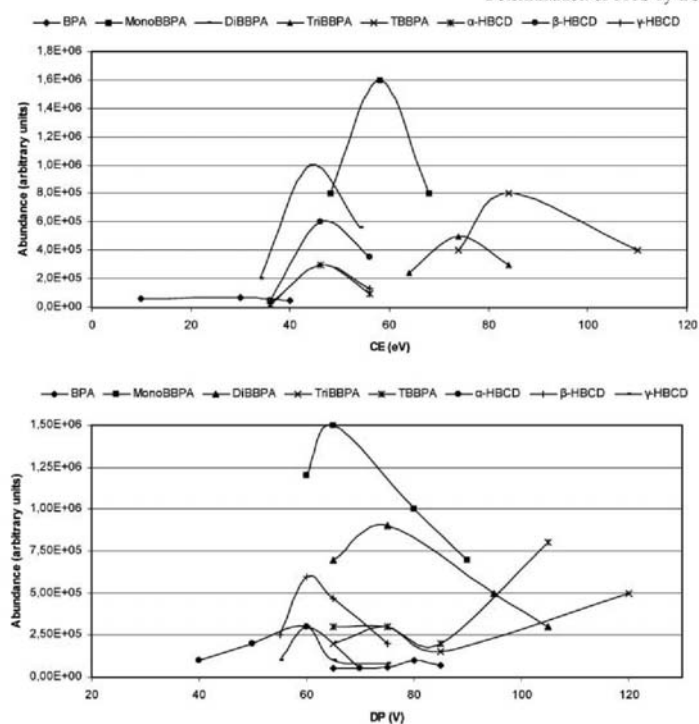


Figure 3. Variation of the abundance for selected analytes vs. (a) DP and (b) CE.

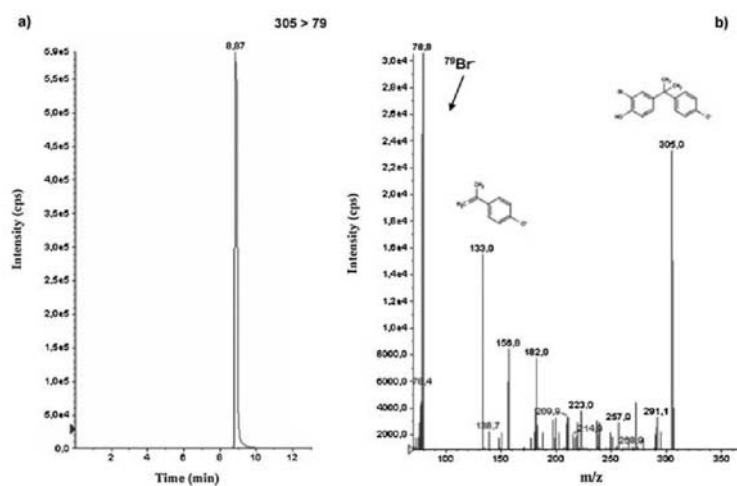


Figure 4. IDA experiment for the determination of monobromobisphenol A in a standard solution (100 pg/μL): (a) SRM transition and (b) EPI at a CE of 40 eV.

Table 3. Quality parameters for SRM and EPI methods (RSD: relative standard deviation)

Compound	SRM					EPI			
	R ²	LOD (pg)	LOQ (pg)	Repeatability (% RSD, n = 5)	Reproducibility (% RSD, n = 5)	R ²	LOD (pg)	LOQ (pg)	Repeatability (% RSD, n = 5)
Bisphenol A	0.999	1.8	6.0	5.0	1.9	0.995	0.5	1.6	2.0
Monobromobisphenol A	0.998	0.3	0.9	4.7	4.4	0.999	0.1	0.2	7.0
Dibromobisphenol A	0.999	0.2	0.7	5.3	8.9	0.998	0.03	0.1	5.6
Tribromobisphenol A	0.998	0.4	1.3	4.8	4.6	0.992	0.1	0.2	4.1
Tetrabromobisphenol A	0.998	0.1	0.2	3.1	8.1	0.996	0.01	0.03	3.5
α -Hexabromocyclododecane	0.995	0.5	1.7	5.7	12	0.992	0.2	0.6	5.8
β -Hexabromocyclododecane	0.991	0.5	1.8	5.6	11	0.995	0.1	0.3	5.5
γ -Hexabromocyclododecane	0.998	1.2	4.0	5.3	6.5	0.991	0.3	1.1	4.9

that can be exploited in a single LC/MS/MS run because it combines two or more different scans modes in a sequential fashion with an EPI.

In our QqLIT experiment, the same parameter values that were optimized for the SRM method were applied (Table 2). Moreover, the fill time of the LIT was optimized, showing the best result at 20 ms. Finally, the collision energy was set at 10, 20 and 40 eV. As an example, a representative chromatogram obtained for the determination of mono-BBPA at 100 pg/ μ L by the EPI method is shown in Fig. 4. The same precursor ion product transition selected for the SRM experiment was also used for the EPI method, corresponding to formation of the [Br]⁺ ion from the [M-H]⁺ ion.

Method characterization

Quality assurance of the developed methods was evaluated by measuring its linearity, sensitivity, reproducibility and repeatability. Characterization data for the two proposed methods are presented in Table 3. Calibration curves were generated using linear regression analysis over the established concentration range (5–5000 pg/ μ L). Both methods (SRM and EPI) gave good fits, with R² always higher than 0.99. The variability (n = 5) of the SRM experiment was acceptable, with values below 6% and below 12% for run-to-run and day-to-day, respectively. For the EPI experiment, similar values were obtained.

For the SRM experiment, the LOD and LOQ were between 0.05 and 1.8 pg and between 0.2 and 6.0 pg, respectively. For the EPI experiment, the LODs were between 0.01 and 0.5 pg. Very little published information could be found on validation data for HBCD and TBBPA determination. Morris *et al.*,¹⁷ who used a single quadrupole, found that the LOQ for HBCD was 150 pg on-column. Gómará *et al.*,²⁶ who used LC/ion trap MS/MS for HBCD determinations, obtained LODs between 30 and 86 pg. On the other hand, Budakowsky and Tomy¹⁹ obtained, using a triple quadrupole mass spectrometer, LODs of between 4 and 6 pg injected for HBCD; this was also higher than our results. For TBBPA and related compounds, there is no information about the quality control of the published methods although all the published values were higher than our results. It should be pointed out that a more realistic comparison would be with LOD values obtained using the new generation of QqQ instruments, but unfortunately these data are not available.

If we compare our LODs for the SRM and EPI modes, we observe that the EPI method gives slightly better sensitivity. The same situation was observed for other applications such as the analysis of reserpine²⁷ and the characterization of drug metabolites.²⁸

The applicability of the LC/QqLIT-MS method working in the EPI mode was tested with extracts of real samples including different matrices such as sediment and biota. Sample preparation of extracts was carried out using a selective pressurized liquid extraction (SPLE) method in which the extraction and purification steps were carried out simultaneously.²⁹ As an example, Fig. 5 shows the determination of BPA in a sediment sample working with the developed IDA experiment. We can observe the BPA transition from the [M-H]⁺ ion at *m/z* 227 to 133. Moreover, the full scans obtained at different CEs gave useful information, because the fragmentation varies with the energy applied. In the case of BPA, working at low CE (10 and 20 eV) basically only the [M-H]⁺ ion was detected. However, when the CE was increased to 40 eV, more fragmentation was observed with product ions at *m/z* 133 and 211.

CONCLUSIONS

A simultaneous method for the analysis of HBCD diastereoisomers, TBBPA and related compounds was developed and characterized. Through the use of a methanol/water LC gradient, it is possible to completely separate TBBPA, tri-BBPA, di-BBPA, mono-BBPA, BPA, α -, β - and γ -HBCD diastereoisomers in a short time (less than 15 min).

Two different MS/MS methods were developed. The first was based on a SRM experiment and the second on an EPI experiment. Both methods are more sensitive than other published studies with LODs below 1 pg. The developed methods display excellent detection limits in SRM mode (0.1–1.8 pg), but slightly better results are obtained in EPI mode (0.01–0.5 pg). Moreover, the selectivity was enhanced by working with IDA, because we can obtain the complete spectrum for the fragmentation of the [M-H]⁺ ion, opening the possibility for the identification of potential product ions of selected precursor ions. Thus, we propose for the unequivocal analysis and quantification of brominated flame retardants in environmental samples at the ultratrace level the use of the IDA experiment working in the EPI mode.

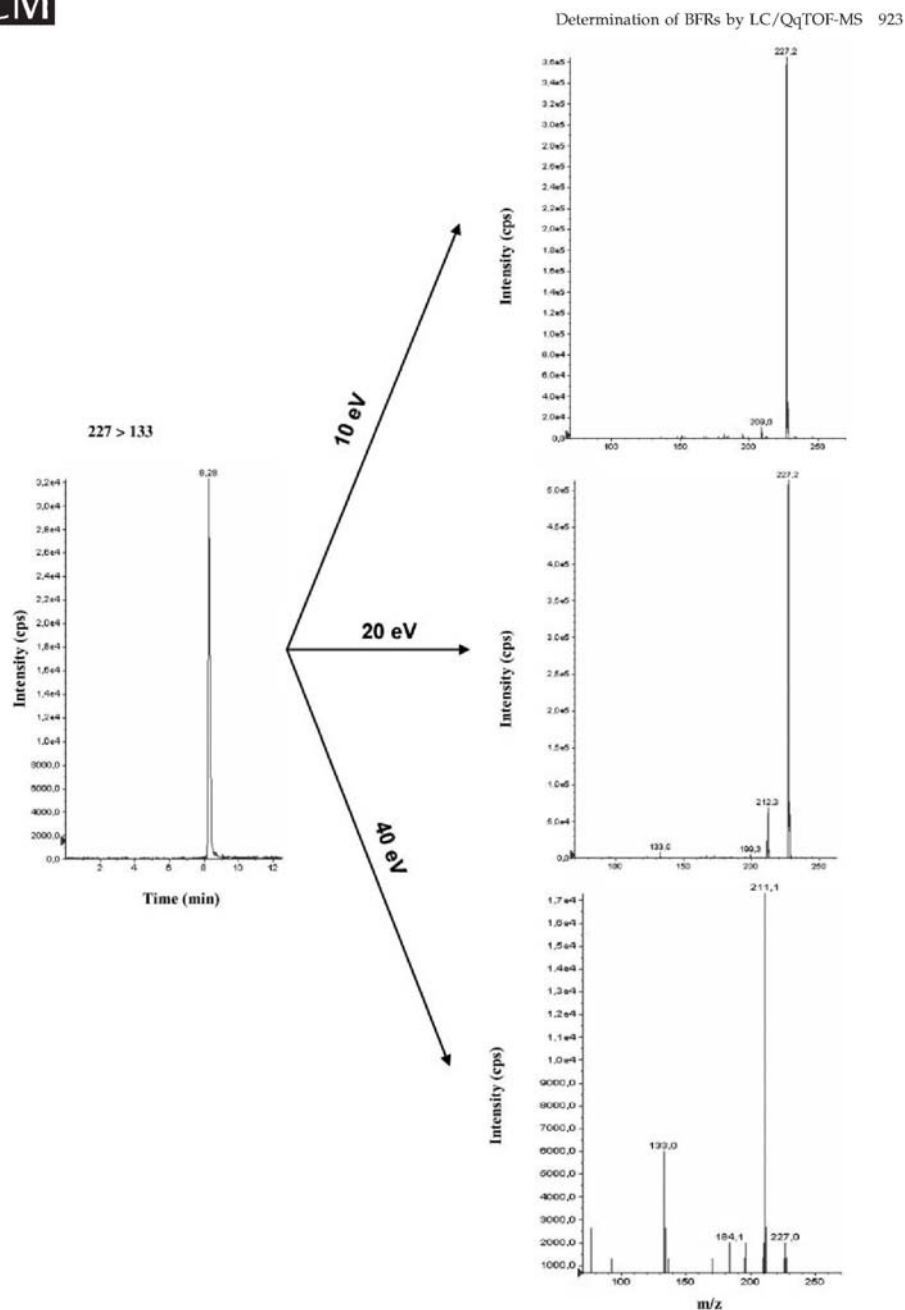


Figure 5. Determination of BPA in a sediment sample by IDA experiment, showing the selected transition and scans obtained at the different CEs applied.

924 P. Guerra *et al.*

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Artículo científico #3:

“Simultaneous determination of hexabromocyclododecane, tetrabromobisphenol A, and related compounds in sewage sludge and sediment samples from Ebro River basin (Spain)”

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ORIGINAL PAPER

Simultaneous determination of hexabromocyclododecane, tetrabromobisphenol A, and related compounds in sewage sludge and sediment samples from Ebro River basin (Spain)

Paula Guerra · Ethel Eljarrat · Damià Barceló

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Abstract This paper describes the development of a methodology for the simultaneous determination and quantification of hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), and related compounds (bisphenol A, monobromobisphenol A, dibromobisphenol A, and tribromobisphenol A) in sludge and sediment samples. The selected method is based on an extraction with dichloromethane: methanol followed by purification via SPE C₁₈ cartridges. Instrumental determination was carried out by liquid chromatography–quadrupole linear ion trap mass spectrometry (LC–QqLIT–MS), with quantification based on isotopic dilution method. Analyte recoveries were in the range of 39–120% and 88–126% for spiked sewage and sediment, respectively. Repeatability of replicate extractions was better than 13% relative standard deviation. Linearity was checked in the range of 0.05 and 25 injected nanograms. Limits of detection (LODs) and limits of quantification (LOQs) were in the range of 0.6 and 2.7 ng/g and 1.4 and 66 ng/g for sediment and sludge samples, respectively. The developed method was applied to sewage sludge and sediment samples collected along the Ebro River and Cinca River, one of its tributaries (northeast of Spain). TBBPA levels in sewage sludge ranged from not quantified to 1,329 ng/g dw, whereas levels in sediment samples were lower, between not detected and 15 ng/g dw.

As regards HBCD, concentrations were between not detected and 375 ng/g for sludge samples and 0.8 and 1850 ng/g for sediments.

Keywords Waste/sludge · HPLC · Organic compounds/trace organic compounds

Introduction

Brominated flame retardants (BFRs) are a structurally diverse group of chemicals that are used in a variety of commercial applications to prevent fires by reducing the flammability of combustible materials such as plastics and synthetic polymers [1]. BFRs include polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), hexabromocyclododecane (HBCD), and tetrabromobisphenol A (TBBPA). The Bromine Science Environmental Forum (BSEF) estimated the usage of selected brominated flame retardants in different areas of the world in 2001. TBBPA was the most used (59%) showing a usage increase for HBCD in replacement of the banned PBDEs [2].

TBBPA is employed both as a chemical bound flame retardant (~90%) in the manufacturing of epoxy, phenolic, and polycarbonate resins, and as an additive flame retardant [3]. The annual global market of this compound was over 170,000 tonnes in 2004 [4], being produced just in the USA, Israel, and Japan [5]. However, TBBPA could be imported into a country as a primary product or in finished or partially finished products.

Due to its low solubility in water (0.72 mg/l) and high log *K*_{ow} (4.5), TBBPA is likely to be associated with suspended particulate matter once released in the water column and ultimately buried in sediments [6, 7]. Although it is not frequently analyzed in environmental laboratories,

P. Guerra · E. Eljarrat (✉) · D. Barceló
Department of Environmental Chemistry, IDAEA-CSIC,
Jordi Girona 18-26,
08034 Barcelona, Spain
e-mail: ceeqam@cid.csic.es

D. Barceló
Catalan Institute for Water Research (ICRA),
Parc Científic i Tecnològic de la Universitat de Girona,
Pic de Peguera 15,
17003 Girona, Spain

may be due to its lower bioaccumulation potential, with concentrations lower than PBDEs and HBCD in the environment. Although, TBBPA has been found in abiotic matrices as air, soil, sediment, and sewage sludge [6, 8–10].

There are indications about the potentially toxicity of TBBPA as an endocrine disruptor, immunotoxic, and neurotoxic compound [3, 11, 12]. Other studies showed that it is toxic to aquatic life [13]. TBBPA could be dehalogenated under anaerobic [4] and aerobic [14] conditions to yield bisphenol A, a widely used compound with reported disrupt endocrine function [15].

HBCD is the principal flame retardant in polystyrene foams and is used as thermal insulation in the building industry [16]. The commercial product usually is a mixture of the three diastereoisomers α -, β -, and γ -. Normally, the γ -isomer is the most dominant in the commercial mixtures (ranging between 75% and 89%), followed by α - and then β -isomer (10–13% and 1–12%, respectively) [17]. The physical-chemical properties of HBCD are similar to PBDEs and other persistent organic pollutants; in fact, the log K_{ow} of HBCD is 5.6 and that valor places in the optimum range for bioaccumulation [16]. HBCD is not covalently bonded to the material leading to the risk of migration out of the product during use or disposal [18].

Some studies indicate a possible role of HBCD as endocrine disruptor [19, 20]. In other works, reproduction-related effects [21] and neurotoxic effects in mice were found [22].

First methods for the analysis of HBCD were based on gas chromatography (GC), but it has limitations because only provided information about the total of HBCD isomers due to the diastereoisomers coelution and interconversion at temperatures over 160 °C [18, 23]. Liquid chromatography mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS-MS) are currently the best methods for measuring HBCD diastereoisomers separately in environmental samples. On the other hand, TBBPA is also detected by LC-MS and LC-MS-MS [7, 24–26]. The main reason is that using gas chromatography mass spectrometry (GC-MS), a derivatization step is necessary for its detection [27].

The development of analytical methods to analyze BFRs is a hard task because of the complexity of the environmental matrices and of the usually low concentrations of target compounds [28]. Liquid-solid extraction, using Soxhlet apparatus, is a widely used standard technique. Typical solvents used for the extraction of BFRs are toluene, dichloromethane (DCM), acetone, and hexane: acetone or hexane:DCM mixtures. However, new extraction techniques such as pressurized liquid extraction (PLE) have also been evaluated [29]. Although higher costs are involved compared with Soxhlet extraction, these techniques have the advantage of lower solvent consumption, which makes the long-term cost lower and the procedures more environmentally friendly. Use of PLE has been

reported in some recent BFR studies with heptane and hexane:DCM mixture applied as solvents extractors [30].

The objective of this work was to develop a sample preparation methodology followed by LC-MS-MS determination that allows a sensitive, precise, and simultaneous analysis of HBCD, TBBPA, and related compounds (bisphenol A (BPA), monobromobisphenol A (MonoBBPA), dibromobisphenol A (DiBBPA), and tribromobisphenol A (TriBBPA)) in sewage sludge and sediments samples. The developed methodology will be then applied to study the concentration levels in sediment and sludge samples from Ebro River basin.

Materials and methods

Standards and reagents

TBBPA, ^{13}C -TBBPA, $d_{18}\text{-}\alpha$ -, $d_{18}\text{-}\beta$ -, and $d_{18}\text{-}\gamma$ -HBCD were obtained from Wellington Laboratories Inc. (Guelph, Canada) and were of minimum 98% purity; BPA and d_{16} -BPA were from Aldrich Chemical Co. (WI, USA) and were a 99% of purity. α -, β -, and γ -HBCD were obtained from Cambridge Isotope Laboratories Inc. (WI, USA) and were of minimum 97% purity. MonoBBPA, DiBBPA, and TriBBPA were a kind of gift from Dr. Göran Marsh (department of Environmental Chemistry, Stockholm University, Sweden). All solvents and other reagents were of analytical grade.

Individual stock standard solutions were prepared on a weight basis in methanol and stored at $-20\text{ }^{\circ}\text{C}$. A mixture of all selected standards was prepared by appropriate dilution of individual stock solutions. Further dilutions of this mixture were prepared in methanol, before each analytical run and were used as working standard solutions. Stock solutions of internal standards were also prepared in methanol and were stored at $-20\text{ }^{\circ}\text{C}$. A mixture of these standards, used for internal standard calibration, was also prepared by diluting the individual stock solutions in methanol.

Sample collection

The study area is located in the northeast of Spain, along the Ebro River. Previous studies showed high BFR contamination in this area [31, 32]. In 2008, seven samples of sewage sludge were obtained from different waste water treatment plants (WWTPs): Miranda de Ebro (W1), Logroño (W2), Pamplona (W3), Tudela (W4), Zaragoza (W5), Lleida (W6), and Tortosa (W7). Table 1 summarizes the characteristics of the studied WWTPs. In the case of sediments samples, three sites were selected: Logroño (S1), Tudela (S2), and Zaragoza (S3), belonging to the same sampling campaign.

Compounds in sewage sludge and sediment samples from Ebro River

2819

Table 1 Characteristics of the waste water treatment plants (WWTP) investigated

Waste water treatment plant	Sample code	Influent flow rate (m ³ /day)	Population served	Type of waste water treated	Hydraulic retention time (h)	Solids retention time (days)	Primary treatment	Secondary treatment
Miranda de Ebro	W1	10,090	42,000	Urban/industrial	32	N/A	–	Activated sludge
Logroño	W2	60,000	171,024	Urban/industrial	8	6–7	Primary settling	Activated sludge
Pamplona	W3	126,748	355,688	Urban/industrial	21	N/A	Primary settling	Activated sludge
Tudela	W4	24,680	37,300	Urban	18	–	Primary settling	Biologic filters
Zaragoza	W5	194,600	650,000	Urban	10	3–4	Primary settling	Activated sludge
Lleida	W6	54,175	137,631	Urban	7 to 10	4–6	Primary settling	Activated sludge
Tortosa	W7	6,577 ^a	40,937	Urban	46	19	Primary settling	Activated sludge

N/A not available

^a Effluent flow rate (influent data were not available)

Four other sediment samples, collected in 2006 were also selected in this study: Puente de Las Pilas (S4), 20 km upstream from Monzón (S5), a heavily industrialized town with a very important chemical industry; Alcolea de Cinca (S6) and Fraga (S7), 27 and 67 km downstream of S5, respectively. All these sampling points are located along the Cinca River, a tributary of the Ebro River.

Sample preparation optimization

Sewage sludge and sediment samples were freeze dried, and lyophilized material was ground, homogenized, and stored in sealed containers at -20°C until analysis.

A dry weight (dw) of 0.5 g of sample was spiked with 50 μL of a mixture of $d_{18}\text{-}\alpha\text{-HBCD}$ and $d_{18}\text{-}\gamma\text{-HBCD}$, and 50 μL of $^{13}\text{C-TBBPA}$, both solutions at concentrations of 5 ng/ μL . Spiked samples were kept overnight to equilibrate.

Different experiments were carried out in order to optimize the extraction procedure. First of all, and in order to reduce time of analysis, a pressurized liquid extraction (PLE) was tested [33], using a fully automated ASE 200 system (Dionex, Sunnyvale, CA, USA). Previous work was done in order to select the most appropriate extraction solvent, resulting in a selection of DCM:methanol (MeOH; 1:9, v/v; data unpublished). The aim to use non-polar DCM is to allow solvatization of target analytes whereas MeOH is dedicated to allow rupture of analyte–matrix interactions [34]. Four tests were carried out applying PLE (M1 to M4). The temperature of cell extraction was optimized, modifying its value between 50 and 100°C (M1 and M3 were carried out at 100°C , whereas M2 and M4 at 50°C).

Moreover, selective PLE was tested in M1 and M2 using aluminum oxide neutral into the extraction cell to perform an in-cell cleanup. Results were compared with the same extraction without adsorbent (M3 and M4). Moreover, ultrasound-assisted extraction (UAE) was also checked (M5 to M7). Extraction was based in the use of 10 mL of DCM:MeOH (1:9, v/v). Samples were sonicated for 60 min at 30°C and then agitated for 3 h at 25°C in a rotary shaker (Selecta, Spain). Samples were centrifuged and filtered through a 0.2 μm filter (Waters Corporation, Massachusetts, USA).

Finally, different test were performed in order to optimize the clean up procedure by solid-phase extraction (SPE). Three different cartridges were evaluated: SPE C_{18} (3 mL, Isolute, Uppsala, Sweden; test M5), SEP PAK (Waters, Massachusetts, USA; test M6) and Oasis HLB (Waters, Massachusetts, USA; test M7). The clean up was performed as follows: each cartridge was conditioned eluting 2 mL of DCM followed by 2 mL of MeOH. The extract was charged and eluted with 1 mL of MeOH. To one volume of cleaned and filtered extract two volumes of HCl (1%) and two volumes of DCM were added. After agitation and centrifugation, the organic phase was recovered and washed with deionized water. Under a gentle nitrogen stream, organic phase was reduced and re-dissolved with 50 μL of $d_{18}\text{-}\beta\text{-HBCD}$ and 50 μL of $d_{16}\text{-BPA}$ at 5 ng/ μL and 150 μL of methanol prior to analysis by LC-MS-MS. In the case of sediment samples, the acid clean up was not necessary.

For each method, three blanks and four repetitions were prepared.

Chromatographic separation

The LC system used was an Symbiosis Pico (Spark Holland, Emmen, The Netherlands) with a Symmetry C₁₈ column (2.1 mm×150 mm, 5 µm) preceded by a C₁₈ guard column (2.1×10 mm) supplied by Waters (Massachusetts, USA). Experiments were carried out in negative ionization mode using H₂O: methanol (3:1 v/v) as eluent A and methanol as eluent B, at a flow rate of 0.25 mL/min. The injection volume was set at 10 µL. The elution program started at an initial composition of 100% A and was ramped to 0% eluent A in 8 min, then eluent A increased to 10% in 17 min and initial conditions were reached again in 3 min and returned to the starting conditions in 15 min [25].

Quadrupole linear ion trap mass spectrometry analysis

Mass spectrometric analysis was performed with a hybrid triple quadrupole/linear ion trap Applied Biosystem MSD Sciex 4000QTRAP™ (Applied Biosystems, Foster City, CA, USA) instrument equipped with an electrospray (ESI) Turbo spray interface. All data were acquired and processed using Analyst 1.4.2 Software. For target quantitative analyses, data acquisition was performed in selected reaction monitoring (SRM).

The MS-MS detection conditions were optimized previously to afford the highest relative intensity: curtain gas (CUR) at 50 psi, collision gas (CAD) at 4.5×10^{-5} torr, temperature of the turbo gas in the TurbolonSpray™ source (TEM) at 350 °C, ion source gas 1 (GS1) and ion source gas 2 (GS2) at 50 psi.

Method characterization

After obtaining the optimum conditions, the whole method consisted of: 0.5 g dw of sample was spiked with 50 µL of a mixture of *d*₁₈-α-HBCD, *d*₁₈-γ-HBCD and 50 µL of ¹³C-TBBPA, both solutions at concentration of 5 ng/µL. Spiked samples were kept overnight to equilibrate and extracted by sonication with 10 mL of DCM:MeOH (1:9, v/v). The obtained extracts were cleaned with SPE C₁₈ cartridges. Finally, extracts were concentrated to incipient dryness and re-dissolved with 50 µL of *d*₁₈-β-HBCD and 50 µL of *d*₁₆-BPA at 5 ng/µL and 150 µL of methanol prior to analysis by LC-MS-MS.

Quality assurance of the developed method was evaluated by measuring parameters as linearity, recoveries, and reproducibility.

Identification of selected analytes was based on the following restrictive criteria: (1) retention time for all monitored transitions for a given analyte should maximize simultaneously ±1 s, with signal-to-noise ratio ≥3 for each; and (2) the ratio between the two monitored transitions

should be within 15% of the theoretical value (calculated upon standards).

Quantification was carried out by an isotopic dilution technique, based on the addition of labeled standards: *d*₁₈-α-HBCD and *d*₁₈-γ-HBCD were used for α- and γ-HBCD quantification, respectively. For the quantification of BPA, MonoBBPA, DiBBPA, TriBBPA, and TBBPA, ¹³C-TBBPA was utilized. The recovery evaluation was done by the addition of *d*₁₈-β-HBCD and *d*₁₆-BPA. The linearity of the method was checked using calibration curves made from standard solutions at six concentration levels (5, 50, 100, 500, 1,000, and 2500 pg/µL) and by triplicate injections.

Results and discussion

Sample preparation optimization

Sewage sludge is a challenging matrix because it is not uniform in its composition. The concentrations of pollutants present in samples vary depending on the nature of the inputs to the WWTP [28]. The complexity of sewage sludge matrices implies the use of extraction procedures followed by multi-step purification methods. Generally, for the analysis of BFRs, it is necessary to remove the high content of organic matter in sewage sludge samples.

Series of preliminary experiments with sewage sludge were performed, testing different extraction methods. First of all, different solvents were checked as solvent extraction: hexane:DCM (1:1, v/v), hexane:acetone (1:1, v/v), and DCM: MeOH (1:9, v/v). The better results were obtained using the last one, and thus, this mixture was the solvent solution used for all the extraction optimization tests.

In Table 2 are summarized the recoveries of the different extraction test that were tried. The first four methods (M1–M4) were performed using PLE with the objectives to minimize the time of analysis and to evaluate a possible degradation of the compounds with the temperature (M1–M4). The recoveries were not satisfactory; the biggest problem is the matrix effect that made difficult the quantification of the different compounds. Thus, another extraction experiment was performed using sonication extraction (M5–M7) but a different clean up was tried with another kind of cartridge. In the case of M5, using SPE C₁₈ cartridges, better quality of chromatography peaks were obtained. Therefore, M5 was selected because gives better recoveries for all compounds of interest, between 39% and 120%.

Method validation

Quality assurance of the developed method was evaluated by measuring parameters as linearity, sensitivity, recoveries, and reproducibility.

Compounds in sewage sludge and sediment samples from Ebro River

2821

Table 2 Recoveries sewage sludge

Compound	M1		M2		M3		M4		M5		M6		M7	
	%R	RSD	%R	RSD	%R	RSD	%R	RSD	%R	RSD	%R	RSD	%R	RSD
BPA	7.6	7.9	nd		26	26	182	143	115	7.5	26	32	136	6.3
MonoBBPA	37	18	nd		nd		350	98	42	8.3	35	10	46	7.6
DiBBPA	41	27	nd		nd		176	63	80	6.1	75	6.5	106	4.6
TriBBPA	43	30	90	309	nd		nd		120	6.9	160	5.2	157	5.3
TBBPA	30	17	621	127	90	14	170	58	98	9.5	108	8.6	115	8.1
α -HBCD	32	6.3	236	9.1	74	22	160	81	39	11	68	6.6	38	12
β -HBCD	39	28	417	17	200	18	69	212	57	13	29	24	56	13
γ -HBCD	24	32	92	239	86	15	336	77	48	3.1	76	2.0	52	2.9

nd not detected, RSD relative standard deviation, M extraction method

M1: selective PLE-100 °C; M2: selective PLE-50 °C; M3: PLE-100 °C-SPE C₁₈; M4: PLE-100 °C-SPE C₁₈; M5: UAE- SPE C₁₈; M6: UAE-SEP PAK; M7: UAE-OASIS HLB

Linearity Calibration curves were determined for all analytes by LC-MS-MS. The linear calibration range used was from 5 to 2,500 pg/ μ L. Good correlations were obtained within the interval studied. The correlation coefficients ranged between 0.9956 and 0.9999.

Recoveries and reproducibility The recoveries (percent of standard added to sample recovered during the whole process) and reproducibility (relative standard deviation (RSD) for quadruplicate analysis) of BFRs during the sonication-SPE and LC-MS-MS were determined by the analysis of spiked samples. The recoveries obtained with spiked experiments in sewage sludge ranged from 39% to 120%. The relative standard deviation between three values was always below 13%, indicating good reproducibility (Table 2). Recoveries in sediment were also analyzed using the same extraction method. The obtained values were 106%, 88%, 105%, 126%, and 110% for BPA, MonoBBPA, DiBBPA, TriBBPA, and TBBPA, respectively. For α -, β -, and γ -HBCD the recoveries were between 53% and 89%. The relative standard deviation between three values was always below 11%, indicating good reproducibility.

Sensitivity The sensitivity was evaluated by determining the method detection limits (LODmet). The detection limit of the method, defined as the minimum amount of analyte that can be detected with reasonable certainty for a given analytical procedure. The LODmet were calculated for each compound by a signal-to-noise ratio of 3 in spiked samples. Table 3 shows the LODmet values for the determination of the studied compounds in sewage sludge and sediment samples. The methodology applied to sediments produced LODmet in the range between 0.6 and 2.7 ng/g dw and the methodology applied to sewage sludge produced LODmet

in the range of 1.4 and 66 ng/g dw. The method quantification limits (LOQmet) were also determined. The LOQmet, were defined as the lowest concentration of a residue that can be identified and quantitatively measured in sample using a developed methodology. LOQmet were based on the peak to peak noise of the baseline near the analyte peak obtained by analysis of spiked samples and on minimal value of signal-to-noise ratio of 10. The applied methodology to sediments provided a LOQmet in the range between 1.9 and 9.1 ng/g dw and the applied methodology to sludge provides a LOQmet in the range between 4.6 and 220 ng/g dw.

Analysis of real samples

The developed method sonication-SPE and LC-MS-MS was applied to the analysis of sediment and sludge samples from the Ebro river basin. Levels of HBCD, TBBPA, and related compounds in samples are presented in Table 4.

Table 3 Limits of detection (LOD) and quantification (LOQ) for the determination of studied compounds in sewage and sediment samples

Compound	Sewage sludge (ng/g, dw)		Sediment (ng/g, dw)	
	LOD	LOQ	LOD	LOQ
BPA	34	115	3.7	9.5
MonoBBPA	1.5	5.1	0.6	1.9
DiBBPA	4.7	16	2.0	6.7
TriBBPA	66	220	—	—
TBBPA	3.0	9.9	2.7	8.9
α -HBCD	12	38	1.6	5.3
β -HBCD	1.4	4.6	—	—
γ -HBCD	2.7	9.1	2.2	8.2

Table 4 Concentration in sewage sludge and sediment samples

Sample Code	Concentration (ng/g, dw)							
	BPA	MonoBBPA	DiBBPA	TriBBPA	TBBPA	α -HBCD	β -HBCD	γ -HBCD
W1	945	103	nd	nd	411	nd	nd	nd
W2	263	nd	nd	nd	1329	nd	nd	nq
W3	834	561	660	637	597	175	6.0	375
W4	nq	4.0	nd	550	nq	nq	nq	nq
W5	nq	265	nd	nd	379	nd	nd	nd
W6	132	122	nd	nd	287	nd	nd	2.0
W7	173	145	nd	nd	1032	nd	nd	nd
S1	16	nd	nd	nq	3.1	nq	nd	4.6
S2	8.3	2.0	nd	nd	2.8	nq	nd	nq
S3	23	nq	nd	nd	nd	1.7	nd	nq
S4	26	nq	nq	nd	5.4	nd	nd	4.0
S5	24	nq	nd	nd	15	24	nd	1849
S6	6.6	nq	nd	nd	nq	nd	nd	91
S7	8.7	nq	nd	nd	nq	1.6	nd	63

nd not detected, nq not quantified

Sewage sludge The content of BFRs in sewage sludge from WWTP gives an indication of the general exposure and use of these compounds. The range of contaminants found in sewage sludge samples is a reflection of what is being used in society. TBBPA was detected in six out of seven analyzed sewage sludge samples (86%), with values between 287 and 1,329 ng/g. Only in the sample W4 was this compound not detected. The concentration in samples W2 and W7 were higher than 1,000 ng/g; both values are higher than the reviewed data by Covaci et al. with values between <0.3 and 510 ng/g dw [5]. Other values had been reported, for example in the North Sea, TBBPA concentrations from <3.9 to 600 ng/g dw were found [23]. Less concentration values were reported by Kuch et al. [35] in sediments from Germany with an average concentration of 16.2 ng/g and a maximum concentration of 62.2 ng/g.

BPA was present in five out of seven analyzed samples (71%) with values from 132 to 945 ng/g. This compound is widely used in the production of epoxy resins and polycarbonate plastics. For that reason, the presence of this compound may due to the use of BPA directly or the degradation of TBBPA. It is important to note that Ronen and Abeliovich [14] reported production of BPA through the biological reductive dehalogenation of TBBPA. This phenomenon could be a consequence of debromination of TBBPA during the wastewater treatment, or other abiotic debromination process [6]. Meesters and Schrodgers [36] found BPA with concentrations between 25–325 μ g/g in sewage sludge samples from Germany.

Mono, Di, and TriBBPA were found at concentrations between nd–561, nd–660, and nd–640 ng/g, respectively (Table 4). Chu et al. [6] reported lower values of Mono, Di, and TriBBPA from not detected to 1.0, 0.18 to 0.52, and

0.11 to 0.55, respectively, in sludge from Canada. To our knowledge, there are no more publications that report the multiple determinations of Mono, Di, and TriBBPA in environmental samples.

As regards HBCD isomers, α -, β -, and γ -HBCD were detected just in sample W3, with concentrations between 6.0 and 375 ng/g. Fifteen different WWTPs from The Netherlands, UK, and Ireland, were sampled in 2002 [23]. Total HBCD was analyzed by GC-MS and levels between not detected and 9,120 ng/g dw were found. In some selected samples, an isomeric analysis was carried out by LC-MS-MS. In these samples, γ -HBCD appeared to dominate the congener profiles, whereas α - and β -HBCD were present in almost equal quantities.

The main difference between the studied WWTPs is the hydraulic retention time (HRT; Table 1) with values between 7 h (W6) and 46 h (W7). Other factors to be in account are the population served and the type of sewage treated. For example, sample W3 contains all the studied compounds at important concentrations. This sample comes from a WWTP that treats urban and industrial sewage water and the population served is high (355,688 inhabitants).

Sediments Lower concentrations for all studied compounds were found in sediment samples from Ebro River (Table 4). DiBBPA and TriBBPA were not detected and MonoBBPA was found just in one sample (S2). However, TBBPA was found at values from not detected to 15 ng/g in four out of seven analyzed samples. Another study [6] shows concentrations lower than 0.51 and 0.34 ng/g for TBBPA and TriBBPA, respectively, in sediments from Lake Erie (Canada). Neither DiBBPA nor MonoBBPA was detectable in any of the analyzed samples.

In the case of BPA, higher values were observed, from 6.6 to 26 ng/g. These concentrations are higher than the data obtained in other studies that presented values between 0.6 and 5.0 ng/g [37, 38]. Concentrations between 1.6 and 1,849 ng/g were obtained for HBCDs. Similar concentrations were found in another study performed in the same area [39]. These values are consistent with previous reported data for river sediments. Morris et al. [23] determined HBCD in river and estuarine sediment samples. HBCD was detected in all sediments analyzed. The maximum value for total HBCD concentration was 1,700 ng/g dw in freshwater sediments from the River Skerne (England). In the River Tees, the mean concentration was 510 ng/g dw. HBCD was also detected in sediments from Scheldt (The Netherlands) basin with values between 38 and 950 ng/g dw. In the case of TBBPA, another study reports values between <0.2 and 1.6 ng/g for sediments from industrial waste landfill sites and for a marine sediment, the concentration was 5.5 ng/g [40].

Conclusions

A simple and accurate analytical method to determine simultaneously HBCD, TBBPA, and related compounds in sewage sludge and sediment samples by LC-MS/MS has been developed. This method eliminates the tedious derivatization stage that is necessary to perform using GC-MS. Ultrasound-assisted extraction followed by solid-phase extraction was used for the treatment of these environmental samples. In sewage sludge samples, recovery efficiencies were in the range of 39–120% with repeatability better than 13%. For sediment samples, the recoveries were between 88% and 126% with repeatability of 11%.

TBBPA was detected in six sewage sludge samples, with values between 287 and 1,329 ng/g. These values are higher than the published data. For related compounds, BPA was present with values from not quantified to 945 ng/g; Mono-, Di-, and TriBBPA were found at concentrations between nd-561, nd-660, and nd-637 ng/g, respectively. HBCDs were detected just in two samples with concentrations between 2.0 and 375 ng/g. However, for sediment samples, lower concentrations of all studied compounds were found. For example, Di and TriBBPA were not detected and TBBPA was observed at values from not detected to 5.4 ng/g. In the case of BPA, higher values were observed, from 8.3 to 23 ng/g.

Finally, for HBCD, the obtained values were from not quantified to 91 ng/g; just one sample (S5) from an industrialized site had higher concentration, 1,873 ng/g.

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Enantiomeric specific determination of hexabromocyclododecane by liquid chromatography–quadrupole linear ion trap mass spectrometry in sediment samples[☆]

Paula Guerra^a, Ethel Eljarrat^{a,*}, Damià Barceló^{a,b}^a Department of Environmental Chemistry, IDAEA-CSIC, c/Jordi Girona 18–26, 08034 Barcelona, Spain^b Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, Edifici Jaume Casademont, Porta A, Planta 1, Despatx 13C/ Psc de Peguera, 15 E 17003 Girona, Spain

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ABSTRACT

This paper describes the determination and quantitation of hexabromocyclododecane (HBCD) enantiomers by liquid chromatography–quadrupole linear ion trap mass spectrometry (LC–QqLT–MS). The method is based on the use of a chiral chromatographic column Nucleodex β -PM (200 mm $\times$ 4.0 mm, 5 μ m), which allows a good separation between HBCD enantiomers [($\pm$), α , ($\pm$), β and ($\pm$), γ] in less than 15 min and the detection is performed by a Q-Trap instrument. Linearity was checked between 0.05 and 25 injected ng. Limits of detection (LODs) were in the range of 0.3–1.5 pg, limits of quantification (LOQs) were between 1 and 6 pg, and both values are lower than those published in the literature applying LC–MS–MS methods. The method was applied to sediment samples collected along the Cinca River, a tributary of the Ebro River (northeast of Spain). Samples were extracted and purified following a pressurized liquid extraction method. LODs of the method were between 0.12 and 5.61 ng/g and LOQs, from 0.38 to 1.87 ng/g. Total HBCD levels in these sediments ranged from not detected to 2660 ng/g dry weight. Enantiomeric fractions (EFs) were calculated and compared with EF obtained from standard injections. It is important to note that calculated EFs were corrected using ²H₁₃-labeled HBCD standards, in order to compensate matrix effect. EFs obtained in sediment samples suggested a higher presence of (+)- α -HBCD and (+)- γ -HBCD in technical mixture, this suggests that it is not a racemic mixture.

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1. Introduction

Flame retardant chemicals are a structurally diverse group of chemicals that are used in a variety of commercial applications to reduce the risk of fire. One of this group of compounds are the brominated flame retardants (BFRs) which include polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA). The Bromine Science Environmental Forum (BSEF) estimated the usage of selected BFRs in different areas of the world in 2001, showing increase of usage for HBCD [1] mainly in polystyrene foams and as thermal insulation in the building industry.

The physical–chemical properties of HBCD are similar to some PBDEs and other persistent organic pollutants, in fact the

octanol–water partition coefficient estimation ($\log K_{ow}$) of HBCD is 5.6; this means that the compound could be bioaccumulated [2]. Thus, HBCD is not covalently bonded to the material leading to the risk of migration out of the product during use or disposal [3]. Based upon these properties, there is a high potential for this material to absorb soil and sediments.

Technical 1,2,5,6,9,10-HBCD is produced industrially by the addition of bromine to *cis-trans-trans*-1,5,9-cyclododecatene, with the resulting mixture that contains three predominant diastereoisomers α -, β - and γ -HBCD [4]. Normally, the γ -isomer is most dominant in the commercial mixtures (ranging between 75 and 89%), followed by α - and β -isomer (10–13 and 1–12%, respectively) [5–7].

The dissimilarities in the structures of the α -, β - and γ -isomers might raise differences in polarity, dipole moment and in solubility in water; for example the solubility of α -, β - and γ -HBCD in water was 48.8, 14.7 and 2.1 μ g/L, respectively. These different properties may explain the differences observed on their environmental behaviour [5]. In sediments, the stereoisomeric profile of HBCD is similar to that on commercial HBCD formulations, with γ -isomer being the most abundant stereoisomer. In contrast to sediments,

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* Corresponding author. Tel.: +34 934006172; fax: +34 932045904.

E-mail address: eeqam@cid.csic.es (E. Eljarrat).

α -isomer is the most prominent stereoisomer in the majority of aquatic invertebrate and fish samples [8,9].

Actually, LC/MS and LC/MS/MS are currently the best methods for measuring HBCD diastereoisomers separately in environmental samples [7,8,10–13]. Chromatographic columns such as C_{18} were used in the majority of HBCD-isomers studies. Moreover, electrospray ionization shows the better sensitivity.

α -, β - and γ -HBCD diastereoisomers are chiral; thus HBCD has three pairs of enantiomers (+) α , (–) α , (+) β , (–) β , (+) γ and (–) γ . The enantiomers have identical physicochemical properties and abiotic degradation rates, but may have different biological and toxicological properties and therefore different biotransformation rates. These transformations may result in nonracemic mixtures of the enantiomers that were industrially synthesized as racemates [12,14–16]. The rates of metabolism process of the enantiomers of chiral environmental pollutants may be significantly different, for example, there are some cases where only one enantiomer is being decomposed, while the second is being accumulated in the environment, this is the case of HCH [15]. Because of that, an understanding of the environmental and biological fate of the HBCD enantiomers is required.

The objective of this work was to develop an analytical method for the detection and quantitation of HBCD enantiomers and for enantiomeric fraction (EF) calculation in environmental samples. The developed method will be applied to evaluate EFs in sediment samples collected along the Cinca River, a tributary of Ebro River (northwest of Spain). Previous studies showed that there is an important HBCD contamination along this river with concentration levels up to 514 ng/g dry weight for sediment samples, and up to 1172 ng/g dry weight for fish samples [17,18].

2. Materials and methods

2.1. Standards and reagents

A solution of α -, β - and γ -HBCDs (50 μ g/mL) in toluene was obtained from Cambridge Isotope Labs. (Andover, MA, USA) and was of minimum 97% purity; [$^2H_{18}$] α - and [$^2H_{18}$] γ -HBCD (α -HBCD- d_{18} and γ -HBCD- d_{18} , respectively) were purchased from Wellington Labs. (Guelph, Canada) and were of minimum 98% purity. Solvents for organic trace analysis and neutral aluminium oxide were obtained from Merck (Darmstadt, Germany).

Individual stock standard solutions (5 μ g/mL) were prepared on a weight basis in methanol and stored at $-20^\circ C$. A mixture of all selected analyte standards was prepared by appropriate dilution of individual stock solutions. Further dilutions of this mixture were prepared in methanol before each analytical run and were used as working standard solutions. Stock solutions of internal standards were also prepared in methanol and were stored at $-20^\circ C$. A mixture of these standards, used for internal standard calibration, was also prepared by diluting the individual stock solutions in methanol. Stability of stock solutions was controlled through storage at $-20^\circ C$; moreover, the absence of potential degradation products was checked by injecting stock solutions in the MS instrument before their use.

2.2. Sample collection

The study area is located in the northeast of Spain, along the Cinca River a tributary of Ebro River (northwest of Spain). Previous studies showed high HBCD contamination in this area [17,18]. Four different sampling stations were selected: Puente de Las Pílas (site 1), 20 km upstream from Monzón (site 2), a heavily industrialized town with a very important chemical industry, Alcolea de

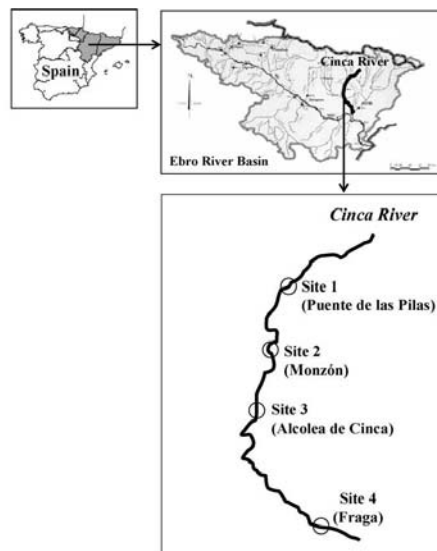


Fig. 1. Geographical situation of the Cinca River and location of sampling stations.

Cinca (site 3) and Fraga (site 4), were 27 and 67 km downstream of site 2, respectively (Fig. 1). One superficial sediment (0–2 cm) was collected at each different selected site.

Selection of sampling sites was carried out on the basis of results obtained in previous studies on this area [17,18]. In these former studies, two sites upstream Monzón (focal point of contamination) as control sites, and two other sites downstream Monzón (up to 30 km of distance from focal point) were selected. In this new monitoring, one site control was also selected, as well as the focal point of contamination (Monzón). However, in order to study the effect of the contamination along the Cinca River, different sites downstream Monzón were selected, covering a great distance from the focal point (up to 67 km).

2.3. Extraction and cleanup

Sediment samples were freeze-dried, and lyophilized material was ground, homogenized and stored in brown glass sealed containers at $-20^\circ C$ until analysis. 0.5 g of dry weight (dw) of sediment sample was spiked with 200 μ L of d_{18} - α -HBCD at 5 ng/ μ L. Spiked samples were kept overnight to equilibrate. In order to reduce time of analysis, a pressurized liquid extraction (PLE) method that automatically and rapidly achieves quantitative and selective extraction of BFRs was used [19]. PLE was carried out using a fully automated ASE 200 system (Dionex, Sunnyvale, CA, USA) using aluminium oxide neutral and Hydromatrix (Varian, Palo Alto, CA, USA). Extraction cell was heated to $100^\circ C$ and extraction was carried out using a mixture of hexane: CH_2Cl_2 (1:1). The volume of the resulting extract was about 35 mL. Extracts were finally concentrated to incipient dryness and re-dissolved with 20 μ L of γ -HBCD- d_{18} at 50 pg/ μ L and 180 μ L of methanol before analysed by LC-MS-MS.

2.4. Chromatographic separation

The LC system used was an Agilent HP 1100 binary pump (Agilent Technologies, Palo Alto, CA, USA). Three chiral chromatographic columns were tested: ChiraDex and ChiraSep (LiChroCART 250-4, 5 μ m) from Merck, and Nucleodex β -PM (200 mm $\times$ 4.0 mm, 5 μ m) supplied by Macherey-Nagel (Düren, Germany). We made different experiments for each column with different mobile phases: H₂O, methanol, acetonitrile (ACN) and mixes, as well as different gradients.

2.5. QqLIT-MS analysis

Mass spectrometric analysis was performed with a hybrid triple quadrupole/linear ion trap Applied Biosystem MSD Sciex 4000QTRAP (Applied Biosystems, Foster City, CA, USA) instrument equipped with an electrospray (ESI) Turbospray interface. All data were acquired and processed using Analyst 1.4.2 Software. For target quantitative analyses, data acquisition was performed in SRM. The $[M-H]^- \rightarrow Br^-$ transitions at m/z 640.6 $\rightarrow$ 78.9 and 640.6 $\rightarrow$ 80.9 were monitored for unlabeled HBDC. The labeled HBDCs were monitored at the 652.6 $\rightarrow$ 78.9 and 652.6 $\rightarrow$ 80.9 transitions.

The MS/MS detection conditions were optimized to obtain the highest relative intensity: curtain gas at 50 psi, collision gas at 4.5×10^{-5} Torr, temperature of the turbo gas in the TurbolonSpray source at 350 °C, ion source gas 1 at 50 psi and ion source gas 2 at 10 psi. Other parameters such as declustering potential, collision energy, collision cell exit potential, ion spray voltage and entrance potential were previously optimized [10].

2.6. Enantiomeric fraction

The enantiomeric composition was expressed as EF, which is the preferred descriptor of chiral signatures in environmental samples [20] and normally is calculated from the peak areas of the enantiomeric pairs by Eq. (1):

$$EF = \frac{A_+}{A_+ + A_-} \quad (1)$$

where A_+ and A_- are the peak area of eluting enantiomers.

It is well known that electrospray ionization is subject to sample matrix effects that can cause enhancement or suppression of the target analytes signal and can adversely affect their quantification [9,21]. Matrix effects are caused by coeluting compounds that interfere with the ionization of the analyte. In order to avoid this effect that can affect in EF calculations, Marvin et al. [12] introduced the corrected EF values, calculated by the Eq. (2). This correction is based on the use of isotopic labeled standards (HBDCs- d_{18}) since d_{18} -labeled enantiomeric analogues behave in an identical manner to their native counterparts.

$$EF_{corrected} = \frac{([A_+]/[A_{+d_{18}}]) \times pgA_{+d_{18}}}{([A_+]/[A_{+d_{18}}]) \times pgA_{+d_{18}} + ([A_-]/[A_{-d_{18}}]) \times pgA_{-d_{18}}} \quad (2)$$

Where $A_{+d_{18}}$ and $A_{-d_{18}}$ are the peak area of eluting labelled enantiomers.

For a racemic mixture, EF is 0.5. If EF value is larger than 0.5 there is an enrichment of (+) enantiomer. In contrast, if EF is smaller than 0.5 there is an enrichment of (−) enantiomer.

2.7. Method characterization

In order to characterize the method, different quality parameters such as linearity, intra- and inter-day variation and sensitivity

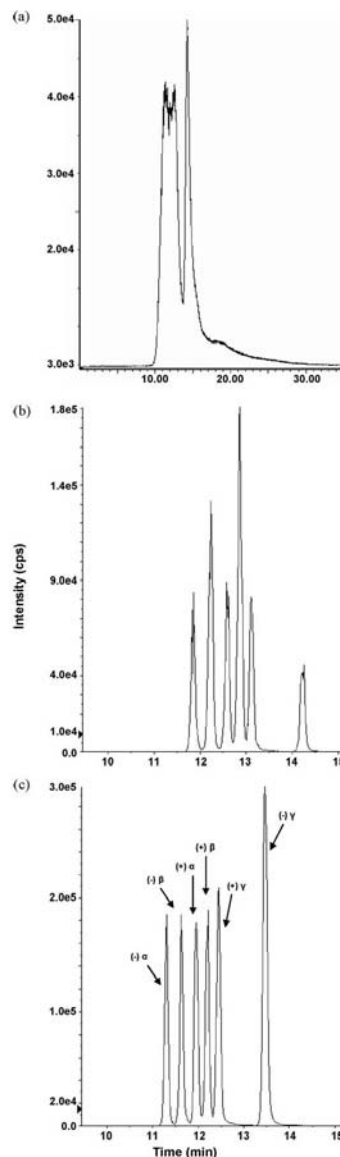


Fig. 2. Chromatographic separation of standard mixture (2500 pg/ μ L) with (a) ChiraDex column, (b) Nucleodex column using 80% H₂O:20% ACN as eluent A and 40% H₂O:60% MeOH as eluent B and (c) Nucleodex column optimized.

were studied. Identification of selected analytes was based on the following restrictive criteria: (i) retention time for all monitored transitions for a given analyte should maximize simultaneously ± 1 s, with signal-to-noise ratio ≥ 3 for each; and (ii) the ratio between the two monitored transitions should be within 15% of the theoretical value (calculated upon standards). Quantification was carried out by an isotopic dilution technique, based on the addition of labeled standards: α -HBCD- d_{18} was used for α -, β - and γ -HBCD quantification, and γ -HBCD- d_{18} for recovery evaluation. The linear-

ity of the method was checked using calibration curves made from standard solutions at six concentration levels (5, 50, 100, 500, 1000 and 2500 pg/ μ L), and by triplicate injections. The precision of the method was determined by repeated intra- and inter-day analysis with five successive injections of a standard solution in 1 day and in four successive days, respectively. The instrumental limit of detection (LOD), defined as 3 times the noise level, and the limit of quantification (LOQ), defined as 10 times the noise level, were calculated.

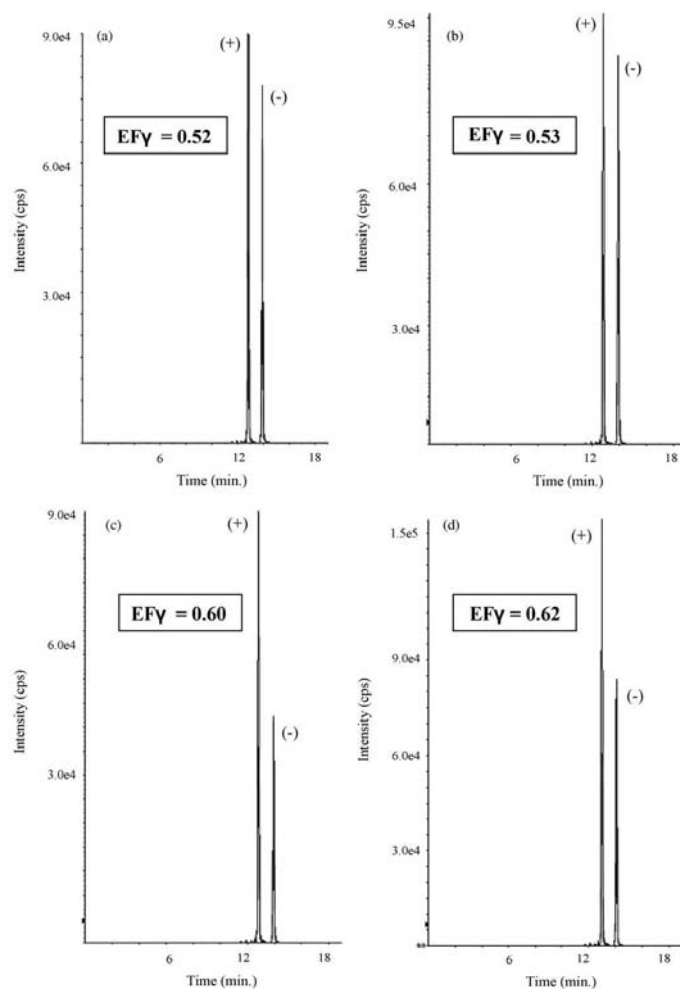


Fig. 3. Chromatographic separation of γ -HBCD standard mixture at different temperature of TurbolonSpray source: (a) 300°C, (b) 350°C, (c) 400°C and (d) 500°C.

3. Results and discussion

3.1. Chromatographic separation

A series of preliminary experiments were performed, testing different chiral chromatographic columns and mobile phases. We proved three chiral chromatographic columns. However, the use of Chiradex and Chirasep does not resolve the different HBCD enantiomers (Fig. 2a); only the use of Nucleodex β -PM (200 mm $\times$ 4.0 mm, 5 μ m) chiral column gives satisfactory results.

Once Nucleodex β -PM was selected as the optimal chiral chromatographic column, additional experiments were carried out in order to optimize the separation using different mobile phases and gradients. Fig. 2b shows the chromatographic separation using 80% H₂O:20% ACN as eluent A and 40% H₂O:60% MeOH as eluent B. On the other hand, Fig. 2c presented the optimized separation that was achieved using 70% H₂O:30% MeOH as eluent A and 70% ACN:30% MeOH as eluent B. The elution program started at an initial concentration of A at 50% decreased to 0% along the first 8 min, and was maintained for 17 min and initial conditions were reached again in 5 min and maintained for additional 12 min. The elution order of each enantiomer was assigned according to Janák et al. [7]. Good separations were obtained for (–)- α -, (–)- β - and (–)- γ -HBCD. Only in the case of (+)- α - and (+)- β -, and (+)- γ -HBCD separations there are some minor coelution (at 4.9 and 4.4% of the baseline, respectively). Moreover, this is not a problem because β -HBCD enantiomers are not usually present in environmental samples. Experiments were carried out using negative ionization mode.

The injection volume was also optimized testing different volumes. The optimum value was set at 10 μ L, for which good enantiomeric resolution was obtained, and better sensitivity than those obtained with less amount of compound injected. Values upper than 10 μ L showed worst chromatographic separation.

Finally, various flow rates have been proved. We found that the optimal separation was achieved using 0.5 mL/min. Upper values of flow rates lead to deteriorating enantiomeric separation.

3.2. MS detection

The majority of MS/MS conditions were previously optimized [10]. However, it was necessary to optimize the temperature of TurbolonSpray source to obtain the maximum sensitivity and res-

Table 1

Quality parameters for LC-QqLT-MS method

Analyte name	Analyte RT (min)	Instrumental method		Whole method	
		LOD (pg)	LOQ (pg)	LOD (ng/g)	LOQ (ng/g)
(–)- α -HBCD	11.1	0.4	1.4	0.12	0.38
(–)- β -HBCD	11.4	1.5	4.9	–	–
(+)- α -HBCD	11.7	0.9	3.0	0.24	0.79
(+)- β -HBCD	12.0	0.5	1.6	–	–
(+)- γ -HBCD	12.2	0.3	1.1	2.65	0.88
(–)- γ -HBCD	13.5	0.3	1.0	5.61	1.87

olution of both enantiomers. Fig. 3 shows different chromatograms obtained modifying the value between 300 and 500 °C for a standard solution of 500 pg/ μ L of γ -HBCD. As can be seen, the better sensitivity was obtained at 500 °C (Fig. 3d). However, the EF was different to 0.5, the expected value for a racemic solution, the same occurs at 400 °C (Fig. 3c). In contrast, at 300 or 350 °C (Fig. 3a and b), the EF was near 0.5. In this case, the selected temperature was fitted at 350 °C because it is slightly more sensitive.

3.3. Quality parameters

In order to evaluate the reliability and quality of the developed method, some analytical characteristics such as linearity, LOD, LOQ and precision were studied.

Calibration curves were obtained for all the enantiomers, in the range of 5–2500 pg/ μ L for each racemate. Good linearity was found in all cases, with correlation coefficients ranging from 0.992 to 0.996. Table 1 shows the LOD and LOQ values for HBCD enantiomers. Limits of detection were between 0.3 and 1.5 pg and limits of quantification were between 1.0 and 6.0 pg. Very little published information could be found on validation data for HBCD enantiomer determination. Gómará et al. [11], who used LC/ion trap MS/MS for HBCD enantiomers determinations, obtained LODs between 30 and 86 pg, which are values of at least one order of magnitude higher than those obtained in our study.

Precision of the method was determined by repeated intra- and inter-day analysis with five successive injections of a standard solution (100 pg/ μ L) in 1 day and in four successive days, respectively. Results for EF values, uncorrected and corrected, are gathered in Table 2. Since we have not availability of β -HBCD-*d*₁₈ standard in our laboratory, β -HBCD cannot be corrected. All RSD values

Table 2
Inter- and intra-day variation of EF values

	Inter-day						Intra-day					
	EF uncorrected			EF corrected			EF uncorrected			EF corrected		
	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)
α -HBCD	0.565	0.012	2.1	0.526	0.018	3.4	0.561	0.009	1.6	0.525	0.015	4.4
β -HBCD	0.574	0.025	4.3	–	–	–	0.574	0.016	2.8	–	–	–
γ -HBCD	0.376	0.008	2.2	0.499	0.001	0.2	0.394	0.010	2.5	0.503	0.034	6.8

Table 3
EFs variation in the function of the concentration level

	α -HBCD uncorrected	α -HBCD corrected	β -HBCD uncorrected	γ -HBCD uncorrected	γ -HBCD corrected
0.05 ng	0.580	0.510	0.600	0.355	0.480
0.5 ng	0.551	0.530	0.595	0.347	0.473
1.0 ng	0.577	0.545	0.586	0.376	0.477
5 ng	0.556	0.529	0.560	0.352	0.467
10 ng	0.555	0.501	0.538	0.377	0.481
25 ng	0.526	0.496	0.530	0.355	0.492
Mean	0.558	0.519	0.568	0.360	0.478
SD	0.020	0.019	0.029	0.013	0.008
RSD (%)	3.6	3.7	5.1	3.6	1.7

Table 4
Concentration levels and EF values for sediment samples

Sample	Concentration (ng/g) dw				EF corrected	
	α -HBCD	β -HBCD	γ -HBCD	Total	α -HBCD	γ -HBCD
Site 1	n.d.	n.d.	n.d.	n.d.	–	–
Site 2	269	n.d.	2389	2658	0.550	0.546
Site 3	12.7	n.d.	64.1	76.8	0.540	0.525
Site 4	8.0	n.d.	40.4	48.4	0.592	0.514

n.d.: not detected; dw: dry weight.

are lower than 10% and, as it was expected, RSD inter-day values are lower than the intra-day values. EF variation in the function of concentration level was also been studied. Table 3 shows the EF values calculated for a concentration range between 0.05 and 25 ng. In this case, also RSD values below 10% were obtained.

Quality parameters of the whole method, including PLE followed by LC–Q/LIT–MS, were also detected. HBCD recoveries were evaluated using γ -HBCD- d_{18} and their values were around 60%, revealing a satisfactory analysis procedure. LODs and LOQs were also calculated and values were summarized in Table 1.

3.4. Analysis of sediment samples

HBCD was detected in sediments collected downstream of Monzón (sites 2, 3 and 4), but it was not detected in site 1, site upstream of Monzón (Fig. 3). The concentration levels ranged from 50 to 2660 ng/g dw of total HBCDs, with the concentrations decreasing as long as more away from the polluted site are the sampling site (Table 4).

Only α and γ diastereoisomers were detected, whereas β was not detected in any sample. The characteristic isomeric distribution of HBCD in sediments was also found in these samples, with a major contribution of γ -HBCD (from 81 to 90% of total). Previous work on total HBCD in the Cinca River was realized [17,18] with the same extraction method but analysed by gas chromatography coupled to mass spectrometry (GC–MS), working with negative ion chemical ionization (NICI) in sediments and fish samples. In sediments it was found that the presence of HBCD near to a chemical industry and downstream was 514 and 90 ng/g dw, respectively. In the case of fish samples, in the same sites the concentration of total HBCD was between 215–1127 ng/g in muscles and 161–1172 ng/g in livers for *Barbus agnelli*. For *Alburnus alburnus* the concentration was between n.d. and 1172 ng/g in whole fish. The greater concentration of HBCD found with LC–Q/LIT–MS instrument should be due to the industrial activities that continue and produce more pollution.

EF corrected values were calculated for the sediments samples (Table 4). In the case of γ -HBCD, it was observed that EF values are between 0.55 and 0.51, with values decreasing while the site is far from the polluted area. If we compared these values with that obtained for standards (mean value of 0.48, Table 3) we can observe significant differences. EF in the contaminated site (site 2) deviates a 10.1% from the standard value, and this deviation is greater than the standard deviation with standards, previously determined (1.7%). EFs values decrease insofar the sites move further away from the industrialized area with a deviation from the standard value of 7.0% and 5.3% for sites 3 and 4, respectively. These results suggested that technical mixture of HBCD, the responsible of the contamination is not racemic, showing a higher content of (+) γ -HBCD against (–) γ -HBCD. Moreover, it seems that γ enantiomers behaviour in environment is different, with probably greater degradation rates of (+) versus (–) γ enantiomer (Fig. 4). This data has not been described before in the literature. However, further studies in different sediment samples are required to check this differential enantiomeric behaviour.

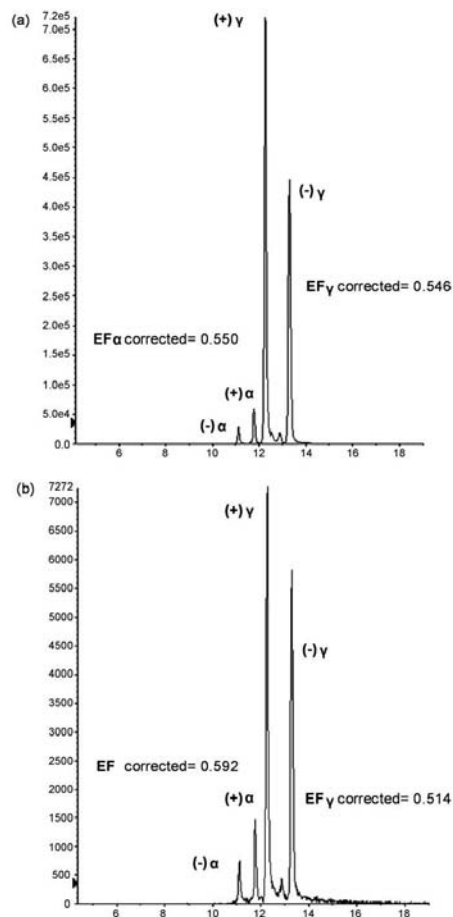


Fig. 4. Sediment samples chromatograms: (a) site 3 and (b) site 4.

As regards α -HBCD, EF values ranged between 0.54 and 0.59 which are also values significantly different to EF value established with standards (EF=0.519, Table 3). EFs in contaminated sediments divert between 2.9 and 9.9% from the standard value, being this deviation greater than that determined with standards (3.7%) for sediments at sites 2 and 4. But, in this case, no relationship between EF values and distance from the contaminated site (Monzón) was found. However, results also suggested a higher presence of (+) α -HBCD in the technical mixture, for that reason this compound should be nonracemic due to the different industrial production process. Similar findings were observed by Janák et al. [7].

Acknowledgements

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2.3. Discusión

Las metodologías de análisis desarrolladas en este capítulo, han hecho posible el análisis de BFRs de relevancia en el mercado mundial, proporcionando un mayor conocimiento sobre el comportamiento de estos compuestos en el medio ambiente.

Se han desarrollado y validado dos métodos para el análisis simultáneo de los diastereoisómeros de HBCD, además de TBBPA y compuestos relacionados utilizando LC-MS/MS. El primero se basa en modo de SRM y el segundo en un experimento IDA. Previo a su optimización se probó otro instrumento QqQ, donde se optimizó la detección de HBCD y sus isómeros pero los LOD obtenidos no permitían el análisis de HBCD a los niveles detectados en el medio ambiente (Tabla 2-1). Esta es la razón por la que se buscó un equipo alternativo que permitiera una mejor sensibilidad, coincidiendo con la adquisición de un instrumento QqLIT por parte de nuestro laboratorio. Así pues, logramos los LOD necesarios para el análisis de los BFRs en estudio. En la Tabla 2-1 se resumen los LOD obtenidos con los diferentes instrumentos utilizados. Finalmente, los métodos publicados en el artículo #2 tienen excelentes límites de detección, tanto en el modo SRM como en EPI. Además, con el uso de IDA se obtiene una mejora de la sensibilidad además del espectro completo de la fragmentación del ion molecular dando lugar a la posibilidad de identificar los posibles productos de degradación de determinados iones.

Tabla 2-1. Comparación de los LOD (pg inyectados) obtenidos en los diferentes instrumentos utilizados.

Instrumento	α -HBCD	β -HBCD	γ -HBCD	BPA	Mono BBPA	Di BBPA	Tri BBPA	TBBPA
QqQ	12	25	75	-	-	-	-	-
QqLIT SRM	0.5	0.5	1.2	1.8	0.3	0.2	0.4	0.1
QqLIT EPI	0.2	0.1	0.3	0.5	0.1	0.03	0.1	0.01

Para el caso de la optimización del método enantiomérico (artículo #4), el uso de un instrumento sensible y robusto es vital, sobre todo si tenemos en cuenta que las concentraciones de HBCD encontradas en el medio ambiente para un isómero se verán distribuidas entre los respectivos enantiómeros (+) y (-), disminuyendo aún más la señal. En la Tabla 1 del artículo #4 se presentan los LOD del método que hemos desarrollado. De hecho, al analizar muestras reales hemos visto, que para niveles bajos éramos capaces de detectar los diferentes isómeros pero al pasar al análisis enantiomérico, nos encontrábamos por debajo del LOD impidiendo así el cálculo de las EF.

Otro parámetro que puede afectar los LOD obtenidos es el efecto matriz, ya que no debemos olvidar que trabajando en ESI es probable que exista cierto aumento o supresión de la señal de los analitos

en estudio. Por ello, el uso de la dilución isotópica es primordial en los métodos optimizados, nosotros disponemos de $d_{18}\alpha$ - y $d_{18}\gamma$ -HBCD, además de ^{13}C -TBBPA y d_{16} -BPA. Debido al coste de estos estándares y al hecho que en muestras ambientales β -HBCD a menudo no se detecta, durante el desarrollo de estos tres trabajos no disponíamos del patrón $d_{18}\beta$ -HBCD aunque fue adquirido posteriormente. Por otro lado, para el cálculo de los EFs también utilizaremos los estándares marcados, tal como propone Marvin et al. (2007) para obtener EFs corregidos que nos conduzcan a resultados fiables (Ec. 2 del artículo #4).

En lo que se refiere al tratamiento de la muestra, en el artículo #3 se presenta la optimización para dos matrices complejas de importancia ambiental como son los sedimentos y los lodos de depuradora. Desde un punto de vista analítico, el análisis de lodos procedentes de EDARs es un desafío, ya que su composición no es uniforme y los extractos obtenidos son muy sucios. Además, las concentraciones de contaminantes presentes en las muestras pueden variar en función de la naturaleza del tratamiento utilizado en la EDAR.

Primero se trabajó en la extracción de los sedimentos, por ser una matriz más limpia. Fue necesario seleccionar el disolvente para realizar la extracción y se probaron tres mezclas de disolvente: hexano:diclorometano (1:1), hexano:acetona (1:1) y diclorometano:metanol (1:9). Esta última fue la mezcla seleccionada. Al comienzo se optó por realizar la optimización sin purificación, obteniendo una separación poco satisfactoria debido al efecto matriz (Figura 2-4a). Por lo tanto, decidimos hacer un paso de purificación vía SPE tanto de los sedimentos como de los lodos en estudio, obteniéndose una mejor separación cromatográfica (Figura 2-4b). En ambos cromatogramas se observa el TIC (del inglés, *total ion chromatogram*), seguido por el SRM de TBBPA y HBCD, respectivamente. Tal como se señala en el artículo #4 se probaron tres tipos de cartuchos de SPE, seleccionando el que contenía C_{18} como adsorbente. Al comparar los LOD obtenidos en sedimentos y lodos (Tabla 3 del artículo) observamos que son hasta 10 veces mejores para los sedimentos, lo cual se explica por tener un menor efecto matriz. La recuperación obtenida para cada uno de los compuestos, también es un reflejo de este efecto ya que son mejores en sedimento.

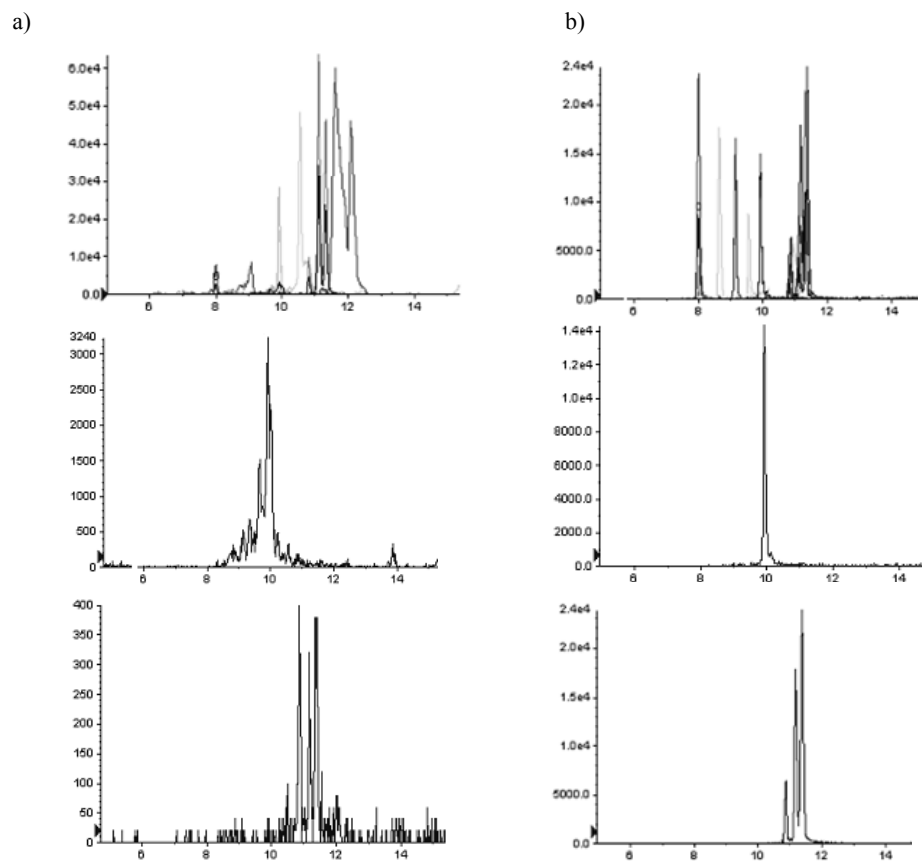


Figura 2-4. Cromatograma a) muestra de sedimento previo a la purificación y b) muestra de lodo de EDAR posterior al tratamiento de purificación por SPE.

Respecto al análisis de TBBPA, es importante destacar que en otros estudios se ha demostrado que este BFR puede degradarse hasta BPA (Ronen y Abeliovich 2000; Voordeckers et al., 2002), el cual es descrito como un disruptor endocrino en bajas concentraciones, además de ser tóxico para organismos acuáticos. La presencia de BPA en el medio ambiente se debe principalmente a que es muy utilizado en la producción de resinas epóxicas y plásticos de policarbonato (Gallart-Ayala et al., 2007), además de la degradación de TBBPA. Esto se ve reflejado en las muestras analizadas, principalmente en los lodos de depuradora donde fue posible analizar todos los compuestos formados por la debromación de TBBPA. En la Figura 2-5 se observa la distribución de los compuestos en una de las muestras (W3), de allí la importancia de realizar el análisis de los compuestos formados por la debromación de TBBPA, ya que son tanto o más abundantes que el propio TBBPA.

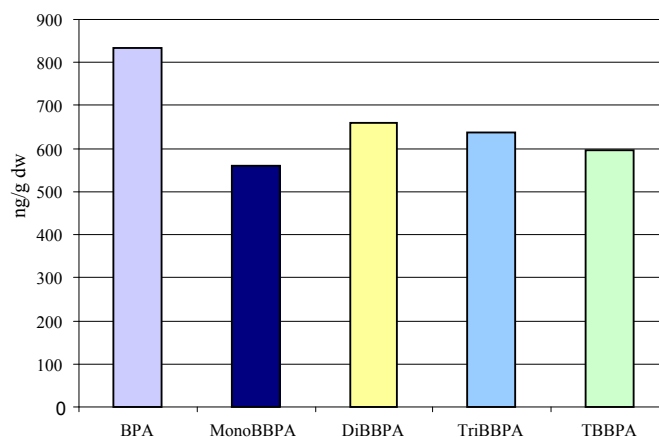


Figura 2-5. Ejemplo de la distribución de TBBPA y sus compuestos relacionados en una muestra de lodo de EDAR.

Relevancia de los resultados

El inicio de esta tesis coincidió con los primeros estudios científicos de optimización de metodologías para el análisis de HBCD diastereoisomérico y enantiomérico. Por lo tanto es un tema novedoso que ha ido evolucionando a la par que la tesis. Esto se refleja en que durante este tiempo, y posteriormente a nuestros trabajos, se han desarrollado estudios similares de otros autores en diversas matrices con hallazgos en concordancia con los nuestros. Recientemente se han publicado más trabajos que analizan el HBCD mediante LC-MS/MS con un QqQ en modo ESI, este es el caso de Harrad et al. (2009) analizaron HBCD isomérico y enantiomérico en sedimentos del Reino Unido y de un estudio realizado en muestras de sedimento de Corea (Ramu et al., 2010).

En el caso de HBCD y TBBPA la técnica de ionización por ESI es la más frecuentemente utilizada. Sin embargo recientemente se han desarrollado métodos que utilizan APPI (Mascolo et al., 2010; Zhou et al., 2010b). El interés de la APPI es la posibilidad de ionizar compuestos que no lo son utilizando ESI o APCI, particularmente compuestos que no son polares (de Hoffman y Stroobant 2007). Así, APPI se ha utilizado en la determinación de PBDEs (Debrauwer et al., 2005; Lagalante y Oswald 2008; Riu et al., 2006) y otros HFRs, incluyendo HBCD y TBBPA (Zhou et al., 2010b). Sin embargo, la disponibilidad limitada de APPI en la mayoría de los laboratorios analíticos, hacen que esta técnica de ionización sea utilizada con poca frecuencia.

CAPÍTULO 3: IMPACTO MEDIOAMBIENTAL

3. IMPACTO MEDIOAMBIENTAL

3.1. Introducción

Es importante considerar que debido a la prohibición de las mezclas PentaBDE, OctaBDE y DecaBDE, se espera que haya cada vez más FRs alternativos y que se utilicen para cumplir con las normas de consumo de productos de seguridad contra incendios. Por ello es muy importante estudiar tanto la presencia como el comportamiento de los HFR emergentes en el medio ambiente, así como su biodisponibilidad y bioacumulación.

Una vez que se han obtenido métodos de análisis sensibles, selectivos y reproducibles es posible evaluar la dinámica y distribución en el medio ambiente de los compuestos en estudio. Hay que tener en cuenta que la legislación disponible acerca de HBCD y TBBPA es mínima e inexistente en el caso de HFRs emergentes. Por lo tanto, se hace necesario el desarrollo de estudios científicos con el fin de facilitar la toma de decisiones acerca de su posterior prohibición o autorización.

En el Capítulo 2 de esta Tesis, se han desarrollado los métodos analíticos para la determinación de HBCD y TBBPA utilizando LC-MS/MS. Asimismo, contamos con metodologías de análisis previamente optimizadas que nos permitirán realizar el análisis de diversos HFRs tanto de uso habitual como emergentes mediante el uso de GC por HR-MS y LR-MS.

Medio Acuático. En este apartado se describe la vigilancia ambiental de BFRs en dos cuencas hidrográficas de España: Ebro y Llobregat, como parte de dos proyectos europeos: **Integrated modeling of the river-sediment-soil-groundwater system; advanced tools for the management of catchment areas and river basins in the context of global change** (AQUATERRA, Project number 037038) y **Models for assessing and forecasting the impact of environmental key pollutants on marine and freshwater ecosystems and biodiversity** (MODELKEY, Project N°511237) respectivamente. Ambos proyectos involucraban el análisis de una gran cantidad de contaminantes orgánicos en estas dos cuencas hidrográficas.

Respecto al río Ebro, y más concretamente al afluente Cinca, el HBCD fue el compuesto de interés y se analizó en sedimentos y biota utilizando distintas técnicas analíticas. En este caso, no solo se ha determinado la presencia y los niveles de concentración del HBCD, sino también hemos estudiado su comportamiento ambiental en lo que se refiere a biodisponibilidad y bioacumulación en organismos acuáticos. Este trabajo se resume el artículo #5: *“Transfer of hexabromocyclododecane from industrial effluents to sediments and biota: Case study in Cinca river”*.

En el caso del río Llobregat se analizaron 38 PBDEs (BDE-7, -10, -15, -17, -28, -30, -47, -49, -66, -71, -77, -85, -99, -100, -119, -126, -138, -139, -140, -153, -154, -156, -169, -171, -180, -183, -184, -191, -196, -197, -201, -203, -204, -205, -206, -207, -208 y -209) y tres BFRs emergentes:

pentabromoetilbenceno (PBEB), hexabromobenceno (HBB) y decabromodifenil etano (DBDPE, del inglés *decabromodiphenyl ethane*) en sedimentos mediante GC-ionización química negativa (NCI, del inglés *negative chemical ionization*)-MS. Este trabajo se recoge en el artículo #6 “*Analysis and occurrence of emerging brominated flame retardants in the Llobregat River basin*”. Los BFRs seleccionados corresponden a compuestos sobre los que recientemente había estudios que mostraban su presencia en el medio ambiente. Otro punto importante para elegirlos fue que se disponía de patrones comerciales para poder llevar a cabo una identificación y cuantificación fiables.

A continuación se presenta una reseña de cada uno de ellos:

- *Pentabromoetilbenceno (PBEB)*. Es un FR del tipo aditivo (Figura 3-1a) que se utiliza principalmente en resinas de poliéster termoestable, textiles, adhesivos y espumas de poliuretano (Hoh et al., 2005). Se ha detectado PBEB en muestras de aire de Chicago (Hoh et al., 2005), del sur de Inglaterra (Lee et al., 2002) y de Canadá (Gouteux et al., 2008), así como en material de desecho de una planta de reciclaje de aluminio (Sinkkonen et al., 2004). Además, es biodisponible ya que se ha detectado en truchas de lago (Ismail et al., 2009) y huevos de gaviotas (Gauthier et al., 2007; Verreault et al., 2007).

- *Hexabromobenceno (HBB)*. Pertenece a los FR aditivos (Figura 3-1b). Ha sido utilizado en plásticos, textiles y lanas, como también en papeles y productos eléctricos (Watanabe et al., 1986; Yamaguchi et al., 1988). El HBB se ha detectado en distintas matrices abióticas como en aire de Japón y Canadá (Gouteux et al., 2008; Saito et al., 2007), en material de desecho de una planta de reciclaje de aluminio en Finlandia (Sinkkonen et al., 2004) y en sedimentos de Japón (Watanabe y Sakai 2003).

Respecto a su presencia en biota, el HBB se ha encontrado en lenguados juveniles procedentes de la costa atlántica francesa (Munschy et al., 2007), en aves de Norteamérica (Gauthier et al., 2007; Gauthier et al., 2009; Park et al., 2009) y del ártico (Verreault et al., 2007). También se ha detectado en muestras humanas, como tejido adiposo (Smeds y Saukko 2003), además de leche materna y placenta de mujeres de Finlandia y Dinamarca (Shen et al., 2008).

- *Decabromodifeniletano (DBDPE)*. Es un FR aditivo (Figura 3-1c) y se utiliza como alternativa al DecaBDE. Una importante ventaja de este BFR es que no forma ni dioxinas ni furanos bromados bajo condiciones de pirólisis, al contrario de lo que ocurre con la mezcla DecaBDE. El DBDPE se ha encontrado en el aire de la zona de los grandes lagos de Norteamérica (Venier y Hites 2008), así como también en el aire, suelo y polvo del sur de China (Shi et al., 2009a). Otros estudios han evaluado la incidencia del DBDPE en aire y polvo de instalaciones industriales de Suecia (Julander

et al., 2005b; Kierkegaard et al., 2004; Pettersson-Julander et al., 2004). También se ha encontrado en muestras de lodo de depuradora de diferentes países, incluyendo Norteamérica, Asia, Europa y Oceanía, donde el DBDPE estaba presente en todas las muestras (Ricklund et al., 2008). Además ha podido ser detectado en agua y sedimentos, tanto en zonas remotas (Watanabe y Sakai 2003) como industrializadas (Zhang et al., 2009b).

Respecto a estudios de DBDPE en biota, se ha encontrado en peces del lago Winnipeg en Canadá (Law et al., 2006a). Estudios recientes han mostrado la presencia de DBDPE en diferentes especies de aves. En Norteamérica se ha analizado en huevos de gaviotas (1982-2006) de los Grandes Lagos (Gauthier et al., 2009), encontrándose en las muestras posteriores a 1996 en concentraciones que incluso superan a las del BDE-209. También ha sido analizado en hígado, riñones y músculo de gallinetas procedentes de un área de reciclaje de material electrónico en China, encontrándose la mayor concentración de DBDPE mayor en hígado y riñones (Shi et al., 2009a).

Respecto a su toxicidad, recientemente ensayos *in vivo* mostraron que DBDPE es muy tóxico para las pulgas de agua, además la presencia de DBDPE redujo las tasas de eclosión de los huevos de zebra fish expuestos y aumentó significativamente la mortalidad de las larvas (Nakari y Huhtala 2010).

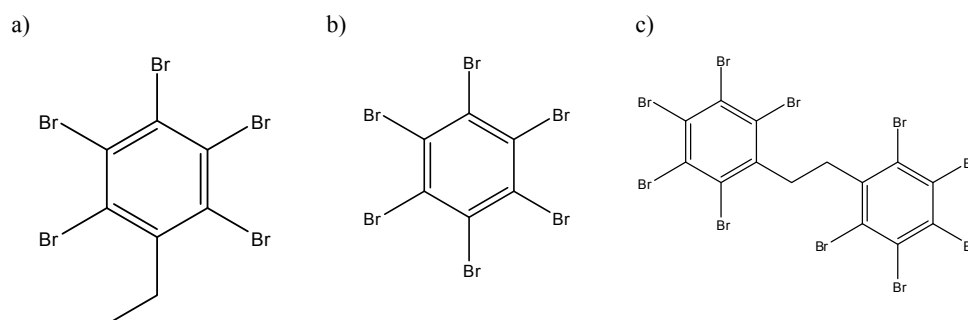


Figura 3-1. Estructura molecular del a) PBEB, b) HBB y c) DBDPE.

Medio terrestre. Durante la estancia realizada entre septiembre y diciembre de 2009, en el *Canada Centre for Inland Waters (CCIW)* en Canadá bajo la tutela del Doctor Mehran Alaei y de la Doctora Kim Fernie, se realizó un estudio de los niveles de HFRs en huevos de halcón peregrino procedentes de Canadá y de España. Se estudiaron distintos HFRs como son los PBDEs, los isómeros y enantiómeros de HBCD, el 2,2',4,4',5,5'-hexabromobifenilo (BB-153) y los HFRs emergentes HBB, hexaclorociclopentenil-dibromociclooctano (HCDBCO), bis(2-etil-1-hexil)tetrabromoftalato (BEHTBP, del inglés *bis(2-ethyl-1-hexyl)tetrabromophthalate*), octabromotrimetilfenilindano

(OBIND), DBDPE y 1,2-bis-(2,4,6-tribromofenoxi)etano (BTBPE, del inglés *1,2-bis-(2,4,6-tribromophenoxy)ethane*), además se realizó el análisis de DP y algunos compuestos relacionados: Mirex, hexaclorotriciclotridecadieno (DPMA), decacloropentaciclooctadecadieno (Cl₁₀DP), undecacloropentaciclooctadecadieno (Cl₁₁DP). Además de Declorane (Dec) 602, Dec 603 y Dec 604. Lo más interesante de este trabajo ha sido la posibilidad de comparar la biodisponibilidad y bioacumulación de una gran variedad de HFRs en una misma especie de ave.

Diferentes estudios han analizado diversas familias de contaminantes en huevos de aves, como por ejemplo pesticidas organoclorados (Jorundsdottir et al., 2010; Lavoie et al., 2010), dioxinas (Clark et al., 2009), PCBs (Clark et al., 2009; Vorkamp et al., 2009) y sus metabolitos (Jorundsdottir et al., 2010), además de FR (Chen y Hale 2010; Gao et al., 2009). Se considera que los huevos de aves son buenas herramientas para medir los niveles y posibles efectos de diferentes contaminantes en el medio ambiente, debido a que los huevos de aves no solo reflejan los niveles de contaminantes en la hembra sino también en los embriones, que es la etapa de desarrollo más susceptible a los efectos de los contaminantes (Gao et al., 2009). Las especies ubicadas en la parte superior de la cadena alimentaria, pueden acumular los contaminantes, siendo particularmente sensibles a la contaminación del medio ambiente (Chen y Hale 2010) y adecuadas para estudiar la bioacumulación y biomagnificación de los contaminantes a través de la cadena trófica (Gao et al., 2009).

Una especie depredadora importante y ampliamente estudiada es el halcón peregrino (*Falco peregrinus*). Al comienzo de la década de 1960s, esta ave parecía haber sido erradicado de la región oriental de los EE. UU. Hacia 1970 había desaparecido del este de las montañas Rocosas y, en gran parte del hemisferio occidental la población había disminuido drásticamente desde principios de 1900 (Clark et al., 2009). En junio de 1970 el halcón peregrino fue incluido en la lista federal de fauna amenazada y en peligro de los EE.UU. Los POPs, específicamente DDTs y sus productos de descomposición surgieron como los posibles causantes del adelgazamiento de la cáscara de los huevos debido a que estos compuestos interfieren con el metabolismo del calcio en las aves, lo que se asoció con la disminución de la población de halcón peregrino (Chen y Hale 2010).

Como consecuencia de la prohibición de uso de compuestos organoclorados y de la reintroducción de halcones en el medio natural, la población de estas aves se ha recuperado durante el pasado medio siglo. Sin embargo, en los últimos años se han introducido nuevos contaminantes que podrían tener un efecto en estos organismos. Un ejemplo son los PBDEs que tienen estructuras moleculares similares a algunos compuestos organoclorados como DDT. Numerosos estudios se han desarrollado con el objetivo de evaluar los niveles de estos FR en huevos de halcón peregrino, encontrándose incluso los niveles más altos de PBDEs en biota (Park et al., 2009).

Finalmente, con el trabajo realizado en Canadá se han escrito dos artículos. El primero en relación a los FRs clorados (artículo #7): *Dechlorane plus and related compounds in peregrine falcon (Falco*

peregrinus) eggs from Canada and Spain, y el segundo donde se recogen los BFRs incluyendo los clásicos y una gran lista de emergentes (artículo #8): *Concentrations of emerging halogenated flame retardants, including enantiomeric fractions of HBCD, in peregrine falcon (Falco peregrinus) eggs from Canada and Spain*.

Dentro de los FR clorados, hay que recordar que el DP ha sido tratado previamente tratado en el Capítulo 1 de esta Tesis Doctoral. Pero además, también se han estudiado:

- *Mirex*. Ha sido utilizado como plaguicida y como HFR (Figura 3-2a). Debido a su persistencia en el medio ambiente, tiene tendencia a acumularse en la cadena alimentaria, capacidad de desplazarse a larga distancia y toxicidad para seres humanos y organismos. Su producción y uso se encuentran regulados por la Convención de Estocolmo (UNEP 2001).

Debido a sus características fisicoquímicas, el Mirex ha sido muy estudiado tanto en muestras bióticas como abióticas. Es importante continuar con su análisis a fin de evaluar si las medidas de restricción tomadas en la Convención de Estocolmo han ocasionado una disminución de sus concentraciones ambientales.

- *Hexaclorotriciclotridecadieno (DPMA)*. El DPMA es el monoadducto del DP (Figura 3-2b), el cual es un diadducto formado por una reacción de Diels-Alder. Es sabido que al someterlo a un proceso de retro-Diels-Alder es posible obtener el compuesto original en su forma de monoadducto (DPMA). El DPMA (Figura 3-13a) ha sido detectado en truchas del lago Ontario (Sverko et al., 2010).

- *Decacloropentaciclooctadecadieno (Cl₁₀DP)* y *undecacloropentaciclooctadecadieno (Cl₁₁DP)*. Ambos compuestos (Figura 3-2c y d) son formados por la pérdida de cloros por parte del DP. No se han encontrado estudios de éstos compuestos en el medio ambiente.

- *Declorane 602 (Dec 602)*, *Declorane 603 (Dec 603)* y *Declorane 604 (Dec 604)*. Estos compuestos decloranes (Figura 3-2e, f y g) también han sido patentados por OxyChem en los 1970s, esta es la misma empresa que produce el DP. Son diadductos del tipo hexaclorociclopentadienos formados por reacciones de Diels-Alder.

- *2,2',4,4',5,5'-hexabromobifenilo (BB-153)*. Pertenece al grupo de los FR aditivos, del tipo bifenilos polibromados (PBB, del inglés *polybromo biphenyls*). Estos compuestos comenzaron a ser fabricados al principio de los 1970s para su uso comercial. Están formados principalmente por los congéneres hexa-, octa-, nona- y decabromodifenilo (Figura 3-2h), aunque teóricamente 209 congéneres son posibles. Se ha comprobado que estos compuestos son cancerígenos, cesando su

producción en EE. UU. en 1974 y posteriormente en Europa. Aunque ya no se utilizan en la actualidad, debido a su persistencia y toxicidad continúan siendo estudiados.

En cuanto a BFRs emergentes tenemos:

- *Hexaclorociclopentenil-dibromociclooctano (HCDBCO)*. Acerca de este FR existe muy poca información. Estructuralmente pertenece a la familia de los FR norbornenos halogenados, el que es un subgrupo de los FR halogenados alicíclicos. Como se puede observar en la Figura 3-2i, HCDBCO contiene átomos de bromo y cloro.

Recientemente ha sido detectado en aire y polvo doméstico en Ottawa, Canadá (Zhu et al., 2008). También se ha analizado en grasa de mamíferos marinos de China, encontrándose en todas las muestras a concentraciones por debajo del LOD (0,04 ng/g lw) (Lam et al., 2009).

- *Bis(2-etil-1-hexil)tetrabromoftalato (BEHTBP)*. Es un FR aditivo (Figura 3-2j), se utiliza en neopreno y PVC (Andersson et al., 2006). Ha sido detectado en el polvo de hogares de EE.UU. (Stapleton et al., 2008a) y posteriormente han sido identificados sus potenciales productos de fotodegradación (Davis y Stapleton 2009). Es importante continuar con el estudio de este FR, sobre todo después de observarse que se fotodegrada más lentamente que los congéneres con mayor peso molecular de los PBDEs (nona- y deca-BDE), lo que sugiere que al menos fotolíticamente BEHTBP podría ser mas persistente en el medio ambiente que los congéneres de mayor grado de bromación de los PBDEs.

- *Octabromotrimetilfenilindano (OBIND)*. Hay poca información disponible acerca del OBIND (Figura 3-2k). Ha sido analizado en huevos de gaviotas (*Larus argentatus*) de los Grandes Lagos Canadienses, sin ser detectado (Gauthier et al., 2009), mientras que en huevos de cigüeña (*Ciconia ciconia*) procedente de España fue detectado en una muestra a una concentración de 10,1 pg/g (Muñoz-Arnanz et al., 2010).

- *1,2-bis-(2,4,6-tribromofenoxi)etano (BTBPE)*. Es un FR del tipo aditivo, ha sido producido desde hace 25 años y actualmente se utiliza en sustitución del OctaBDE. Por lo tanto se espera un incremento en su producción durante los próximos años (Karlsson et al., 2006). BTBPE (Figura 3-2l) ha sido detectado e identificado en el aire ambiental de diversas zonas de EE.UU. observándose una mayor concentración en las muestras procedentes del sur del país (Hoh et al., 2005), también fue detectado en todas las muestras de polvo del dormitorio, del salón y de la bolsa de aspiradora de 20 casas de EE.UU. (Stapleton et al., 2008a).

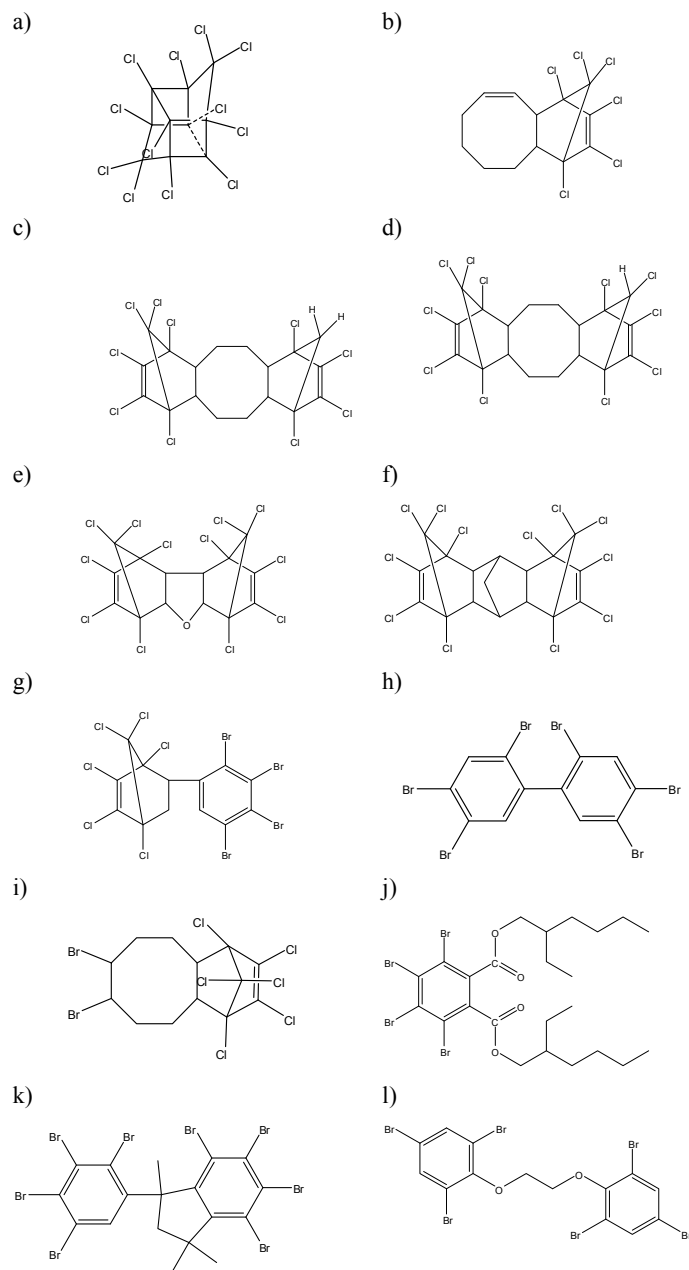


Figura 3-2. Estructura molecular del a) Mirex, b) DPMA, c) Cl_{10}DP , d) Cl_{11}DP , e) Dec 602, f) Dec 603, g) Dec 604, h) BB-153, i) HCDBCO, j) BEHTBP, k) OBIND y l) BTBPE.

Otras matrices donde se ha encontrado BTBPE son sedimentos del lago Michigan (Hoh et al., 2005) y en hielo de Noruega (Hermanson et al., 2010).

Se ha analizado BTBPE en los distintos componentes de la cadena alimentaria de un estanque natural ubicado en las cercanías de una planta de reciclaje de desechos electrónicos en China (Wu et al., 2010b), donde BTBPE fue detectado en todos los organismos analizados, además de agua y sedimento. En diferentes estudios se ha detectado el BTBPE en huevos de aves, como son fulmar boreal (*Fulmarus glacialis*) de las islas Feroe (Karlsson et al., 2006), además de gaviotas de los grandes lagos canadienses (*Larus argentarius*) (Gauthier et al., 2007; Gauthier et al., 2009) y del ártico noruego (*Larus hyperboreus*) (Verreault et al., 2007).

Artículo científico #5:

*“Transfer of hexabromocyclododecane from industrial effluents to sediments and biota: Case study
in Cinca river (Spain)”*

por

P. Guerra, E. Eljarrat y D. Barceló

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Transfer of hexabromocyclododecane from industrial effluents to sediments and biota: Case study in Cinca river (Spain)

Paula Guerra^a, Agustina De La Cal^a, Göran Marsh^a, Ethel Eljarrat^{a,*}, Damià Barceló^{a,b}

^a Environmental Chemistry Dep., IDAEA-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

^b Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, Edifici Jaume Casademont, Porta A, Planta 1, Despatx 13C/Pic de Peguera, 15 E-17003 Girona, Spain

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SUMMARY

This work is part of the research included in the European project AQUATERRA, focused on the study of different persistent organic pollutants (POPs) in different risk zones along the Ebro River basin. Within monitoring programmes, a high contaminated area was detected, located along the Cinca River, a tributary of Ebro River, downstream a heavily industrialized town (Monzón). Data showed a high hexabromocyclododecane (HBCD) contamination in this area. Our work included the analysis of sediments and biota, with special attention on aspects such as temporal trends, bioavailability and bioaccumulation of these contaminants. Moreover, an attempt of identification of source contamination was carried out, with the analysis of industrial effluents. The industry responsible of the contamination was identified.

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Introduction

Brominated flame retardants (BFRs) are a structurally diverse group of chemicals that are used in plastics, textiles, electronic circuitry and other materials to prevent fires. BFRs include polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA). The Bromine Science Environmental Forum (BSEF) estimated the usage of selected brominated flame retardants in different areas of the world in 2001, showing a usage increase for HBCD (Law et al., 2006a,b) in replace of PBDEs.

HBCD, a brominated alicyclic hydrocarbon, is the principal flame retardant in polystyrene foams and is used as thermal insulation in the building industry (Marvin et al., 2006). Technical 1, 2, 5, 6, 9, 10-HBCD is produced industrially by addition of bromine to *cis-trans-trans*-1,5,9-cyclododecatriene. This process leads theoretically to a mixture of 16 stereoisomers (six pairs of enantiomers and four mesoforms) and the product usually is a mixture of the three diastereoisomers α -, β - and γ -isomer (Petersen et al., 2004). However, some authors had found δ - and ε -isomers (Heeb et al., 2005). Normally, the γ -isomer is the most dominant in the commercial mixtures (ranging between 75% and 89%), followed by α - and then β -isomer (10–13% and 1–12%, respectively) (Barontini et al., 2001). The physical–chemical properties of HBCD are similar to some PBDEs and other persis-

tent organic pollutants (POPs). In fact the $\log K_{ow}$ of HBCD is 5.6 and that value places in the optimum range for bioaccumulation (Marvin et al., 2006). Thus, HBCD is not covalently bonded to the material leading to the risk of migration out of the product during use or disposal (Tomy et al., 2005). Based upon these properties, there is a high potential for this material to absorb to soil and sediments.

The dissimilarities in the structure of α -, β - and γ -isomer might raise differences in polarity, dipole moment and in solubility in water. For example, the solubility of α -, β - and γ -HBCD in water was 48.8, 14.7 and 2.1 $\mu\text{g/L}$, respectively. Therefore these different properties may explain the differences observed in their environmental behaviour (Janák et al., 2005). Covaci et al. (2006) and Morris et al. (2004) found that in sediments, the distribution of HBCD isomers was the same of commercial mixture: γ -HBCD is the most abundant isomer. However, in biota α -isomer predominates over the γ -isomer, and little or no presence of β -isomer has been observed.

α -, β - and γ -HBCD diastereoisomers are chiral; thus HBCD have three pairs of enantiomers (+) α -, (–) α -, (+) β -, (–) β -, (+) γ - and (–) γ -. The enantiomers have identical physicochemical properties and abiotic degradation rates, but may have different biological and toxicological properties and therefore different biotransformation rates. These transformations may result in non-racemic mixtures of the enantiomers that were industrially synthesized as racemates (Marvin et al., 2007; Janák et al., 2005; Hühnerfuss, 2000; De Geus et al., 2000). The rates of metabolism process of the enantiomers of chiral environmental pollutants may be significantly

* Corresponding author. Tel.: +34 934006172; fax: +34 932045904.
E-mail address: eeeqam@cid.csic.es (E. Eljarrat).

different. For example, there are some cases where only one enantiomer is being decomposed, while the second is being accumulated in the environment (Hühnerfuss, 2000). Because of that, an understanding of the environmental and biological fate of the HBCD enantiomers is required.

The objective of this paper is to evaluate concentration levels, time trends, bioavailability and identification of source pollution of HBCD. In order to carry out these studies, a previous work on the development of analytical methods, including diastereoisomeric- and enantiomeric-specific protocols, was done.

Development of analytical methods

Sample preparation

HBCD must be evaluated in different matrices: sediments, fish (muscle, liver and whole fish) and effluents (particulated and filtrated).

Sediment and fish samples were freeze dried, and lyophilized material was ground, homogenized and stored in sealed containers at -20°C until analysis. Five hundred milligram of dry weight (dw) of sediment and fish sample was spiked with 200 μL of $d_{18}\text{-}\alpha\text{-HBCD}$ at 5 ng/ μL . Spiked samples were kept overnight to equilibrate. In order to reduce time of analysis, a pressurized liquid extraction (PLE) method that automatically and rapidly achieves quantitative and selective extraction of BFRs was used (De la Cal et al., 2003). PLE was carried out using a fully automated ASE 200 system (Dionex, Sunnyvale, CA, USA) using aluminium oxide neutral and Hydromatrix (Varian Inc., Palo Alto, USA). Extraction cell was heated to 100°C and extraction was carried out using a mixture of hexane: CH_2Cl_2 (1:1). The volume of the resulting extract was about 35 mL. Extracts were finally concentrated to incipient dryness and re-dissolved with 20 μL of $d_{18}\text{-}\gamma\text{-HBCD}$ at 50 pg/ μL and 180 μL of methanol prior to analysis by MS.

Water samples (2 L) were filtered through 1 μm glass microfibre filters followed by 0.45 μm Whatman nylon membrane filters (Maidstone, England). Filtrate water was then mixed with methanol (30%). Extraction of the filtrate water was performed using an automated sample processor Aspec XL (Automated Sample Preparation with Extraction Columns) fitted with a 817 switching valve and an external 306 liquid chromatography (LC) pump, for selection and dispensing of samples, respectively, through the solid phase extraction (SPE) cartridges, from Gilson (Villiers-le-Bel, France). C_{18} Cartridges (200 mg), from Isolute (Madrid, Spain) were conditioned by passing 6 mL of hexane (1 mL/min) followed by 6 mL of CH_2Cl_2 (1 mL/min), 6 mL of methanol (1 mL/min) and 6 mL of LC-grade water (1 mL/min). 500 mL of the water sample were percolated through the cartridge at 5 mL/min. After sample loading, cartridges were dried by applying vacuum and subsequently eluted with 3 mL of methanol (1 mL/min) followed by 3 mL of CH_2Cl_2 (1 mL/min) and 3 mL of hexane (1 mL/min). The particulate matter retained on filters was extracted using PLE without alumina. An additional cleanup step was performed by SPE with alumina cartridges (5 g, from Isolute). Cartridges were conditioned with 20 mL hexane. The sample volume loaded was 1 mL, and the elution step was performed with 30 mL hexane: CH_2Cl_2 (1:2).

Total HBCD determination by gas chromatography coupled to mass spectrometry (GC–MS)

Separation of different HBCD stereoisomers is not possible by GC. A relatively broad, unresolved peak is obtained due to thermal rearrangement of the stereoisomers, and results reflect total HBCD concentration.

Gas chromatography working with negative ion chemical ionization mass spectrometry (GC–NCI–MS) analyses were performed on a gas chromatograph Agilent 6890 connected to a mass spectrometer Agilent 5973 Network (Agilent Technologies, Madrid, Spain). A HP-5 ms (30 m $\times$ 0.25 mm i.d., 0.25 μm film thickness) containing 5% phenyl methyl siloxane (model HP 19091S-433) capillary column was used for the determination. The temperature program was from 110°C (held for 1 min) to 180°C (held for 1 min) at $8^{\circ}\text{C}/\text{min}$, then from 180°C to 240°C (held for 5 min) at $2^{\circ}\text{C}/\text{min}$, and then from 240°C to 265°C (held for 6 min) at $2^{\circ}\text{C}/\text{min}$, using the splitless injection mode during 1 min and injecting 2 μL . The operating conditions were as follow: ion source temperature = 250°C , ammonia as chemical ionization moderating gas at an ion source pressure of 1.9×10^{-4} torr (Eljarrat et al., 2002) and the inlet temperature was 300°C . HBCD determinations were carried out monitoring the two most abundant isotope peaks from the mass spectra corresponding to m/z 79 and 81 ($[\text{Br}]^{-}$). Quantification was carried out by external standard.

HBCD isomeric determination by liquid chromatography coupled to mass spectrometry (LC–MS)

Liquid chromatography/triple quadrupole mass spectrometry (LC–QqQ–MS) method

HBCD stereoisomers can be easily separated using reversed-phase liquid chromatography, and determined by mass spectrometry (LC–MS or LC–MS–MS) (Covaci et al., 2006; Budakowsky and Tomy, 2003). An isomer-specific LC–MS–MS method was developed. Separation of α , β and γ -HBCD stereoisomers was performed on a Waters 2690 HPLC system (Milford, MA, USA) coupled to a Waters Micromass Quattro triple quadrupole mass spectrometer, equipped with a Z-spray ESI interface (Manchester, UK). A 2.1×150 mm symmetry C_{18} 5 μm column and 2.1×10 mm guard column supplied by Waters (Massachusetts, USA) was used with a 0.25 mL/min eluent flow. The injection volume was set at 20 μL . Experiments were carried out using water (A), acetonitrile (B) and methanol (C) as mobile phases. The gradient elution started at an initial composition of 60% A/10% B and 30% C and was ramped to 10% A/22.5% B and 67.5% C in 11.30 min and those conditions were maintained for 18.70 min, then initial conditions were reached again in 10 min and were maintained for additional ten min. A Quattro LC Micromass Spectrometer was operated in electrospray (ESI) ionization negative ion mode using selected reaction monitoring (SRM) for $[\text{M}-\text{H}]^{-1}$ (m/z 640.6) $\rightarrow$ Br^{-} (m/z 79.0 and 81.0). Argon of 99.999% purity was used as collision gas at a pressure of 3.54×10^{-3} mbar. All data were acquired and processed using MassLinx 4.0 software. The quantification was carried out by the isotopic dilution method, using $d_{18}\text{-}\alpha$ - and $d_{18}\text{-}\gamma$ -HBCD solutions as quantification and recovery standards, respectively. Instrumental limit of detection (LOD) was between 12 and 75 pg, whereas method LOD including PLE followed by LC–MS–MS was from 7 to 22 ng/g dw for fish and between 9 and 30 ng/g dw for sediment samples (Table 1).

Liquid chromatography/quadrupole linear ion trap mass spectrometry (LC–QqLIT–MS) method

Another methodology for the analysis of HBCD diastereoisomers (α , β and γ), based in LC–QqLIT–MS method was developed (Guerra et al., 2008a).

The LC system used was an Agilent HP 1100 binary pump (Agilent Technologies, Palo Alto, CA, USA) with a symmetry C_{18} column (2.1 mm $\times$ 150 mm, 5 μm) preceded by a C_{18} guard column (2.1 $\times$ 10 mm) supplied by Waters (Massachusetts, USA). Experiments were carried out in negative ionization mode using H_2O :methanol (3:1 v/v) as eluent A and methanol as eluent B, at

Table 1
LOD for different analytical methods for HBCD analysis.

	Information	Chromatographic column	MS detection	Quantification	LOD		
					Instrumental	Method (sediments)	Method (fish)
GC-MS	HBCD total	HP-5 ms (30 m × 0.25 mm, 0.25 μm)	SIM mode: m/z 79.0 and 81.0	External standard			
LC-MS	Isomer-specific	Symmetry C18 (2.1 mm × 150 mm, 5 μm)	SRM mode: m/z 640.6 → m/z 79.0 m/z 640.6 → m/z 81.0	Isotopic dilution technique	12–75 pg	9–30 ng/g dw	7–22 ng/g dw
LC-MS	Isomer-specific	Symmetry C18 (2.1 mm × 150 mm, 5 μm)	SRM mode: m/z 640.6 → m/z 79.0 m/z 640.6 → m/z 81.0	Isotopic dilution technique	0.5–1.2 pg		
LC-MS	Enantiomer-specific	Nucleodex β-PM (4.0 × 200 mm, 5 μm)	SRM mode: m/z 640.6 → m/z 79.0 m/z 640.6 → m/z 81.0	Isotopic dilution technique	0.3–1.5 pg	0.3–1.8 ng/g dw	1.2–3.9 ng/g dw

a flow rate of 0.25 mL/min. The injection volume was set at 4 μL. The elution program started at an initial composition of 100% A and was ramped to 10% A in 17 min and initial conditions were reached again in three min and maintained for an additional 15 min.

Mass spectrometric analysis was performed with an hybrid triple quadrupole/linear ion trap Applied Biosystem MSD Sciex 4000QTRAP (Applied Biosystems, Foster City, USA) instrument equipped with a Turbospray ESI interface. All data were acquired and processed using Analyst 1.4.1. software. For target quantitative analyses, data acquisition was performed in MRM, recording the transitions between the precursor ion and the two most abundant fragment ions.

The MS/MS detection conditions were optimized to afford the highest relative intensity. Some parameters such as curtain gas (CUR), collision gas (CAD), temperature of turbo gas in the TurbolonSpray (TEM) and ion source gas 1 and 2 were set at default value: 50 V, high, 700 °C and 50 V, respectively. Other parameters such as declustering potential (DP), collision energy (CE), collision cell exit potential (CEP), ion spray voltage (IS) and entrance potential (EP) were optimized.

The developed instrumental method display excellent LODs in SRM mode between 0.5 and 1.2 pg (Table 1).

HBCD enantiomeric separation by LC-MS-MS method

The LC system used was an Agilent HP 1100 binary pump with a Nucleodex β-PM chiral chromatographic column. The optimal separation of enantiomers was achieved using methanol, acetonitrile and water as mobile phase using 70% H₂O:30% MeOH as eluent A and 70% AcN:30% MeOH as eluent B, at a flow rate of 0.50 mL/min. The injection volume was set at 10 μL. The elution program started at an initial concentration of A at 50% decreased to 0% along the first 8 min, and was maintained for 17 min and initial conditions were reached again in 5 min and maintained for additional 12 min.

Mass spectrometric analysis was performed with a hybrid triple quadrupole/linear ion trap Applied Biosystem MSD Sciex 4000QTRAP instrument equipped with an ESI Turbospray interface. All data were acquired and processed using Analyst 1.4.2 Software. For target quantitative analyses, data acquisition was performed in SRM. The transitions for unlabelled and labelled HBCD were explained before.

The MS/MS detection conditions were optimized to obtain the highest relative intensity: curtain gas at 50 psi, collision gas at 4.5×10^{-5} Torr, temperature of the turbo gas in the TurbolonSpray source at 350 °C, ion source gas 1 at 50 psi and ion source gas 2 at 10 psi. Other parameters such as declustering potential, collision energy, collision cell exit potential, ion spray voltage and entrance potential were previously optimized (Guerra et al., 2008a). Instrumental LOD was between 0.3 and 1.5 pg, for sediments samples

from 0.3 to 1.8 ng/g dw and for fish samples the values were between 1.2 and 3.9 ng/g dw.

The enantiomeric fraction was expressed as EF, which is the preferred descriptor of chiral signatures in environmental samples (Harner et al., 2000) and normally is calculated from the peak areas of the enantiomeric pairs by

$$EF = \frac{A_+}{A_+ + A_-} \quad (1)$$

where A_+ and A_- are the peak area of eluting enantiomers.

It is well know that ESI ionization is subject to sample matrix effects that can cause enhancement or suppression of the target analytes signal and can adversely affect their quantification (Villagrana et al., 2007; Dodder et al., 2006). Matrix effects are caused by coeluting compounds that interfere with the ionization of the analytes. In order to avoid this effect that can affect in EF calculations, Marvin et al. (2007) introduced the corrected EF values, calculated by the Eq. (2). This correction is based on the use of isotopic labelled standards (d_{18} -HBCDs) since d_{18} -labelled enantiomeric analogues behave in an identical manner to their native counterparts.

$$EF_{corrected} = \frac{[A_+]/[A_{+d18}] \times PG_{+d18}}{[A_+]/[A_{+d18}] \times PG_{+d18} + [A_-]/[A_{-d18}] \times PG_{-d18}} \quad (2)$$

where A_{+d18} and A_{-d18} are the peak area of eluting labelled enantiomers.

Normally, in a racemic mixture EF is 0.5, but if the value is larger means that there are an enrichment of the (+) enantiomer and if EF is smaller than 0.5 there are an enrichment of the (–) enantiomer.

HBCD occurrence

Area of study

The study area is located in the northeast of Spain, along the Cinca river, in the Ebro River basin (Fig. 1). Different sampling stations were selected at the Cinca River: Puente de las Pílas (site C1) and La Boquera (site C2), 20 and 12 Km upstream from Monzón, respectively; just downstream from Monzón (site C3), a heavily industrialized town with a very important chemical industry, Alcolea de Cinca (site C4), Chalamera (site C5) and Fraga (site C6) were 27, 30 and 67 km downstream of site C3, respectively.

Different sampling campaigns were carried out from 2002 to 2006 (Table 2). Surficial sediments (0–2 cm) were collected at each different selected site. Attempts were made to collect several fishes from the same locations as the sediment samples. Fish specimens were collected by DC electric pulse. Fish were killed, weighed and the fork length of each fish was measured. Different fish species were sampled: barbels (*Barbus graellsii*), bleaks (*Alburnus alburnus*), southwestern nases (*Chondrostoma toxostoma*) and carps

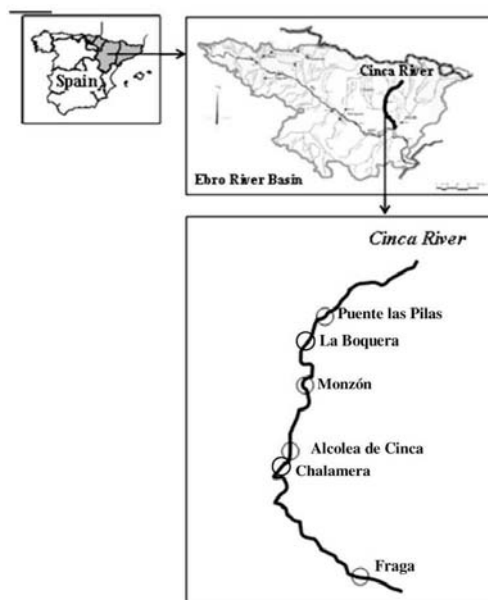


Fig. 1. Geographical situation of the Cinca River and location of sampling stations.

(*Cyprinus carpio*). Whole fish was analyzed for bleaks and southwestern nases, whereas muscle and liver tissues were analyzed for barbels. Muscle samples were collected from below the dorsal fin. The body cavity of each fish was opened and the liver was removed and preserved frozen at -20°C with the muscle sample until analysis.

Along the Cinca River and in the industrial area of Monzón, industrial effluents from two different industries were selected: the first one produced EPS (Expandable polystyrene) treated with flame retardants and ABS (acrylonitrile-butadiene-styrene), and the second one produced Pvc (polyvinyl chloride).

Sediments

Due to their hydrophobic character, HBCDs are strongly bound to solid particles such as soil and sediments. HBCD was not detected upstream Monzón; however it was detected in sediments collected downstream of Monzón. Highest concentration levels were found at site C3, with values between 514 and 2430 ng/g dw, followed by site C4 (125 ng/g dw), site C5 (90–866 ng/g dw) and finally site C6 where HBCD was detected at levels below limit of quantification (Eljarrat et al., 2004; Guerra et al., 2008b).

Table 2

Sampling campaigns carried out in the area of study between 2002 and 2006.

	Puente las Pílas (C1)	La Boquera (C2)	Monzón (C3)	Alcolea de Cinca (C4)	Chalamera (C5)	Fraga (C6)	Industrial effluent (IE)
October 2002	1 Sediment 4 Barbel ^a (M+L)	1 Sediment 8 Barbel (M+L)	1 Sediment 6 Barbel (M+L)	–	1 Sediment 5 Barbel (M+L)	–	–
	–	12 Bleak ^b (WF)	12 Bleak (WF)	–	8 Bleak (WF)	–	–
November 2004	1 Sediment	1 Sediment	1 Sediment	–	1 Sediment	–	–
	6 Southwestern nase ^c (WF)	6 Southwestern nase (WF)	–	–	6 Southwestern nase (WF)	–	–
November 2005	–	–	1 Sediment	–	–	–	–
	–	–	–	–	–	–	2 Effluent
June 2006	1 Sediment	–	1 Sediment	1 Sediment	–	1 Sediment	–
November 2006	–	–	–	–	–	–	2 Effluent

M = muscle tissue; L = liver tissue; WF = whole fish.

^a Barbel = *Barbus haasi*.

^b Bleak = *Alburnus alburnus*.

^c Southwestern nase = *Chondrostoma toxostoma*.

364

P. Guerra et al. / Journal of Hydrology 369 (2009) 360–367

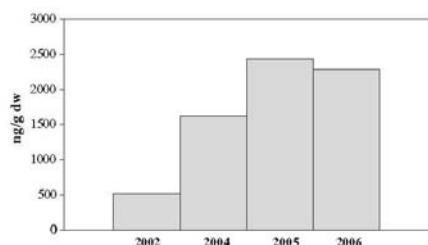


Fig. 2. Total HBCD (ng/g dw) in sediment samples collected at site C3 during the different sampling campaigns (from 2002 to 2006).

Comparing contamination values obtained at the industrial site (C3) at different sampling campaigns, we can detect an increase of the concentration levels with time (Fig. 2), reaching the maximum level in sediment sample collected in 2005 (2430 ng/g dw). Then, the concentration level remain practically constant for the following sampling (2006). It is important to note that just one sediment sample has been analyzed. But, the time trend is also reflected in fish samples, with increasing contamination with time.

Our HBCD results were consistent with reported data for river sediments. Sellström et al. (1998) reported concentration levels between nd and 1600 ng/g dw in river sediments from a Swedish river with numerous textile industries. Morris et al. (2004) determined HBCD in river and estuarine sediment samples. HBCD was detected in all sediments analyzed. The maximum value for

total HBCD concentration was 1700 µg/kg dw in freshwater sediments from the River Skerne (England). In the River Tees the mean concentration was 510 µg/kg dw. HBCD was also detected in sediments from Scheldt (The Netherlands) basin with values between 38 and 950 µg/kg dw.

The isomeric profile of HBCD found in contaminated sediment samples from Monzón was similar to that of commercial HBCD formulations, with γ -HBCD being the most abundant isomer (between 94% and 99% contribution to the total HBCD). The same findings were observed in sediments from lake Winnipeg, where HBCD contamination was dominated by γ -isomer (Law et al., 2006a,b).

EF corrected values were calculated for sediments samples from June 2006 campaign. In the case of γ -HBCD, it was observed that EF values were between 0.51 and 0.55, with values decreasing while the site is far from the polluted area. As regards α -HBCD, EF values ranged between 0.54 and 0.59. Fig. 3 shows a chromatogram for an enantiomeric separation of HBCD in a sediment sample from C4. For γ -HBCD, EF value was 0.55. Therefore, the presence of (+) γ - was greater than (–) γ -HBCD. On the other hand, α -HBCD EF value was 0.54, with a higher content (+) α - than (–) α enantiomer.

Biota

Fish often exhibit high residues of contaminants, due to their high position in the food chain and the elevated exposure in the aquatic environment. Table 3 shows the mean concentrations of HBCD in selected sites. HBCD was detected only in samples corresponding to sites downstream Monzón. In these samples, HBCD levels ranged from 90 to 4863 ng/g wet weight (ww). Similar to our findings in sediment samples, site C3 was found to be the most

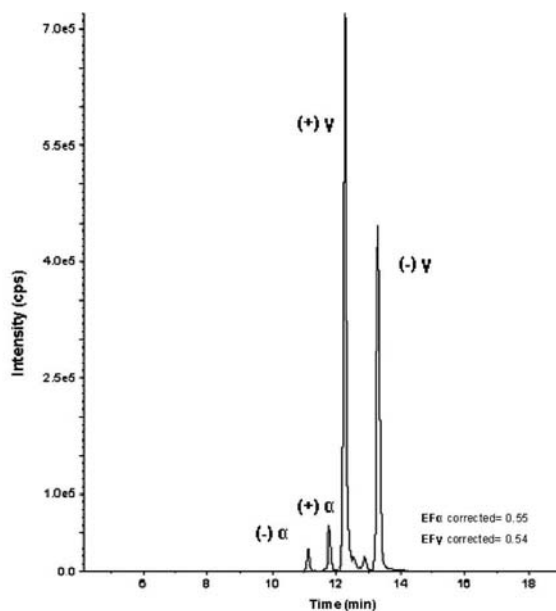


Fig. 3. HBCD enantiomeric separation sediment sample (sample collected during 2006).

Table 3
Results (ng/g ww) for fish samples.

Sampling	Matrix	Puente Las Pilas (C1)	La Boquera (C2)	Monzón (C3)	Alcolea de Cinca (C4)	Chalamera (C5)	Fraga (C6)
October 2002	Barbel*						
	Muscle	nd	nd	530 (309–750)	–	90	–
	Liver	nd	nd	554 (484–625)	–	432	–
	Whole	–	nd	1501 (1274–1643)	–	760 (460–1103)	–
November 2004	Southwestern nase*						
	Whole	nd	nd	–	–	4863 (2685–7813)	–

(–): range of concentration in samples.

* Mean values.

contaminated zone followed by site C5. In the case of C5 in 2004 sampling, the values are between 2685 and 7813 ng/g ww.

HBBCD has been detected in many biota studies (Law et al., 2006a,b; Tomy et al., 2005; 2004; Budakowsky and Tomy 2003; Janák et al., 2005; Voorspoels et al., 2003). Concentrations of α -HBBCD and γ -HBBCD diastereoisomers in fish samples from the Western Scheldt Estuary were between 28–1100 ng/g lipid weight (lw), 2–51 ng/g lw, respectively. For α -HBBCD and γ -HBBCD in muscle and liver the values were between 21–680 ng/g lw and 1–35 ng/g lw, respectively (Janák et al., 2005). In the case of fish samples from Lake Winnipeg levels of γ -HBBCD were in the range 0.15–1.11 ng/g lw. Several of samples displayed small amounts of α -isomer and β -isomer was not detected in any of the samples (Budakowsky and Tomy, 2003).

EF corrected values were calculated for the fish samples. Fig. 4 shows a chromatogram for an enantiomeric separation of HBBCD from C5 and related EFs. In the case of γ -HBBCD, EF value was 0.57. Therefore, the presence of (+) γ - was greater than (–) γ -HBBCD. On the other hand, α -HBBCD EF value was 0.45, with a higher

content of (–) α - than (+) α - enantiomer. Differences in enantiomeric distribution and behaviour were found between sediments and fish samples, as well as some deviations on the racemic value of EF in both cases.

Source of contamination

Two industries were suspected to cause the HBBCD contamination along the Cinca River. Analysis of industrial effluents of each industry in 2005 revealed that industry which produced EPS and ABS is the responsible of the HBBCD contamination, with concentration levels around 5 μ g/L. In 2006, a new sampling was done for the same industries, the results shows the same situation than in 2005: HBBCD was not detected in PVC effluent, and a concentration of 2 μ g/L was detected in the sample from EPS effluent. This sample was characterized isomerically, and the contribution of α -isomer was 14%, 11% for β -isomer and 75% for γ -HBBCD. Practically, this is the typical distribution of HBBCD commercial mix.

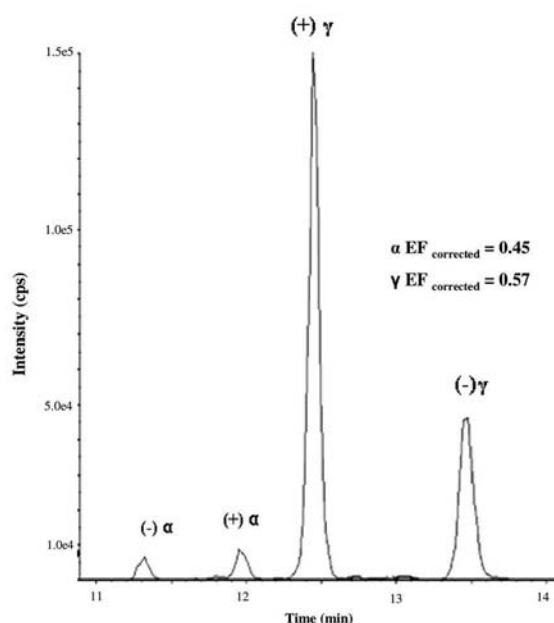


Fig. 4. HBBCD enantiomeric separation in fish sample (sample collected during 2004).

Table 4
Results (ng/g lw) for barbels obtained for samples in a bioaccumulation experiment.

	α -HBCD	β -HBCD	γ -HBCD	Total HBCD
Not exposed ^a	63 (± 9)	30 (± 8)	92 (± 2)	184 (± 15)
Sampling station C2 (upstream) ^a	15 (± 2)	2.5 (± 1.3)	12 (± 3)	28 (± 4)
Sampling station C4 (downstream) ^a	333 (± 61)	58 (± 7)	15,127 (± 2605)	15,517 (± 2673)

($\pm$): $\pm$ Standard deviation.

^a Mean values.

Studies of behaviour

Experiment in situ

An in situ experiment was performed to evaluate the HBCD bioaccumulation. In Chalamera (site C5), “clean” barbels (*Barbus graellsii*) were exposed to the environment. As a control, the same experiment was performed in La Boquera (site C2). Twenty specimens of barbel were caged into each one of two stainless steel devices that were placed on the river-bed at the two locations. Nine specimens were immediately frozen to be analyzed as blanks. After 15 days of exposure, no mortality was observed in the cages.

Table 4 shows the levels of HBCD accumulated in the caged barbels exposed in sites C2 and C4 (De la Cal et al., 2009). Accumulation at site C2 was negligible, as values measured after the exposure were even lower than those of the fishes not exposed. This could mean even some depuration during the experiment. However, at site C4, after the 15 days of exposure, concentration in caged barbel increased up to 1337 ng/g ww or 15,518 ng/g lw.

An analysis of the isomeric profile was done. For site C4, the proportion of the γ -isomer in barbel was between 96 and 98% whereas α -isomer accounted for 2–3% and β -isomer for 0.4%. On the other hand, in barbel not exposed and from site C2, the predominance of the γ -isomer dilutes. In fact, such profile is unusual in biological samples. As reviewed by Covaci et al., (2006), normally, biota shows a much higher percentage of α -HBCD than β - or γ -HBCD (Janák et al., 2005; Morris et al., 2004). The data obtained in this study show slightly lower γ -HBCD content in fish (82–98%) than in sediment (96–99%), this could be due to a recent exposition.

Depuration experiment in laboratory

The information about different isomer-HBCD bioavailability is limited, but we know that the zone under study presents impor-

tant levels of HBCD (Eljarrat et al., 2005; 2004). Because of that we made an experiment to know if the different distribution of the HBCD isomers alpha, beta and gamma found in sediments (or commercial mixture) and fish is produced by differences in the bioavailability of the isomers or by biotransformation of the isomers in the sample.

Juvenile zebrafish (*Danio rerio*) with a weight of 340 ± 33 mg and a length of 246 ± 8 mm were distributed into two groups: HBCD-treated ($0.1 \mu\text{g/L}$; $n = 60$) and effluent-treated (industrial effluent from EPS and ABS production, diluted 1:500; $n = 60$). The industry is located in C3. Zebrafish were acclimated for 2 weeks before the experiment, 40 zebrafish were sampled at time 0 to fix the basal levels of HBCD isomers in the experimental group. Fish were exposed to the HBCD/effluent in water for 12 days, changing the solution every other day. Then, pollutant was removed and fish was maintained for 16 additional days in clean water. 20 zebrafish from both groups were sampled at days, 12, 21 and 28. The extraction method was explained before.

In the Fig. 5 are presented the mean concentration and standard deviation of the HBCD in fish samples. It is observed that the contaminated effluent exposure results in an addition of HBCD in the organisms. It was detected a depuration of HBCD with the time. In the depuration data obtained is important to see that this depuration was different depending of the isomer. As an example, the depuration rate of α - or β -isomer (46% and 27% after 9 days, respectively) is lower than γ -isomer depuration rate (70% and 77% after 9 and 16 days, respectively).

Bioavailability and bioaccumulation

Sediment concentrations were compared to those in biota collected at the same sites along Cinca River. The fact that HBCD is found in fish indicates that it is also bioavailable. However, the low fish to sediment ratio (below 1) does not agree with the high ratios reported by Sellström et al. (1998) for pikes from a Swedish

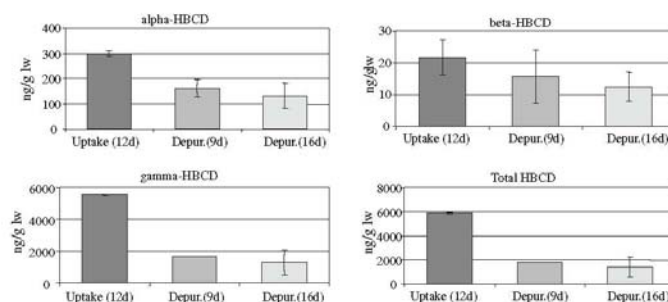


Fig. 5. Results for the zebrafish exposition to effluent treated experiment.

River. Pike is a predator at the top of the food chain and the higher fish to sediment ratio in this specie could indicate the biomagnification of this compound through the aquatic trophic chain.

Previous studies showed that PBDE levels increased with the age of the fish, indicating bioaccumulation (Haglund et al., 1997). Fish length is directly related to fish age. If the measured concentrations of HBCD are compared with the fish length at sites C3 and C4, the correlation coefficients indicate acceptable correlations ($R^2 = 0.71$). These data showed that HBCD could be bioaccumulated in barbel from Cinca River (Eljarrat et al., 2004).

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Analysis and occurrence of emerging brominated flame retardants in the Llobregat River basin

Paula Guerra^{a,*}, Ethel Eljarrat^a, Damià Barceló^{a,b}
^a Dep. of Environmental Chemistry, IDAEA, CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

^b Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, Pí de Peguera 15, 17003 Girona, Spain

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SUMMARY

In response to increasing international regulations on brominated flame retardants (BFR) formulations, alternative additive flame retardants for achieving commercial product fire safety standards are being developed and used. Some of these non-BDE (brominated diphenyl ethers) BFRs are pentabromoethylbenzene (PBEB), hexabromobenzene (hexaBBz), and decabromodiphenylethane (deBDethane).

The present study investigated the occurrence of these emerging BFRs, together with 38 BDE congeners (from di- to deca-BDE) in sediments sampled from different points along Llobregat basin (Spain) in three different sampling campaigns between 2005 and 2006. Emerging BFRs were detected in all sediment samples analyzed, at concentrations ranging from 3.1 to 9.6 ng/g for PBEB, from 0.4 to 2.4 ng/g for hexaBBz and from 4.8 to 23 ng/g for deBDethane. These levels are lower than concentrations obtained for PBDEs (from nd to 82 ng/g).

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Introduction

Flame retardant compounds are a structurally diverse group of chemicals that are added to polymers which are used in plastics, textiles, electronic circuitry and other materials to reduce the risk of fire. One of this group of compounds are the brominated flame retardants (BFRs). Some of them are ubiquitous, and many have been detected in biota, sediments, air, water, marine mammals and even in human milk (Law et al., 2006; Eljarrat et al., 2009). It is important to note that the bulk of research done has been focused in the study of polybrominated diphenyl ethers (PBDEs) due to their persistence and hydrophobicity, two characteristics that make them amenable to bioaccumulation and biomagnification (Eljarrat et al., 2004a). Due to their toxicological effect, the production and use of penta- and octa-BDE mixtures has been banned in Europe since 2004, just deca-BDE is still permitted. However, there are stocks of all PBDEs from products in service and waste.

In response to increasing international regulations on BFR formulations, alternative additive flame retardants for achieving commercial product fire safety standards are being developed and used (Verreault et al., 2007). Some of these non-BDE BFRs are pentabromoethylbenzene (PBEB), hexabromobenzene (hexaBBz) and decabromodiphenylethane (deBDethane).

PBEB, hexaBBz, and deBDethane have been detected in environmental samples from Europe and North America, including sediments (Leonards et al., 2008; Lopez et al., 2008; Hoh et al., 2005; Kierkegaard et al., 2004), sewage sludge (Eljarrat et al., 2005), air (Hoh et al., 2005), house dust (Stapleton et al., 2008), blood and eggs of gulls (Verreault et al., 2007; Gauthier et al., 2007). In addition, there is little or no toxicological data available for wildlife or humans for these additive BFRs.

The present study investigated the occurrence of emerging BFRs including PBEB, hexaBBz and deBDethane together with 38 di- to deca-BDE congeners in sediments sampled from four points along Llobregat River that belongs to internal basin of Catalonia (Spain) and three points from Anoia River, a tributary of Llobregat River. Different sampling campaigns were carried out: June and October 2005 and June 2006.

The analytical method used was based in selective pressurized liquid extraction (SPLE) without further clean up step using aluminium oxide neutral (De la Cal et al., 2003). Analysis by gas chromatography coupled to mass spectrometry (GC–MS) working with negative ion chemical ionization (NICI) was done.

Experimental

Sample collection

The study area is located in the northeast of Spain, along the Llobregat River and Anoia River, a tributary of the Llobregat River (Fig. 1). The Llobregat River is 170 km long, with 4957 km² of basin

* Corresponding author. Tel.: +34 93 400 61 00.

E-mail addresses: pggqam@cid.csic.es (P. Guerra), eeeqam@cid.csic.es (E. Eljarrat).

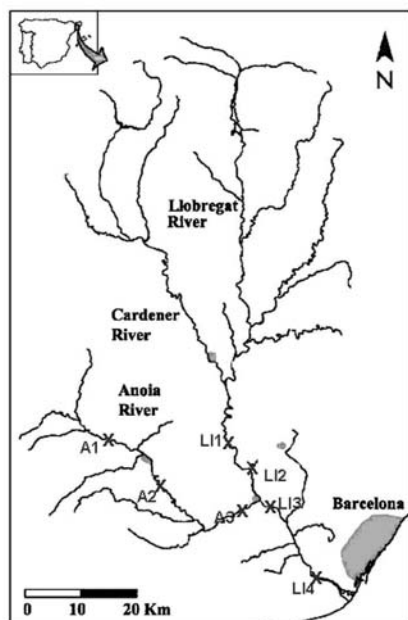


Fig. 1. Location of sampling stations in the Llobregat (LL1–LL4) and Anoia (A1–A3) rivers.

surface. It comes from the north west of Catalonia and flows into the Mediterranean Sea, close to Barcelona. It has been polluted by the effluents from different industries located along the basin (Muñoz et al., in press).

Surficial sediments (0–2 cm) were collected at each selected site (Table 1). Different sampling campaigns were carried out: June and October 2005 and June 2006. Only two samples were not collected: at site LL2 during the first campaign and at site A1 during the third campaign.

Chemicals

The polybrominated diphenyl ether native compounds stock solution BFR-PAR: 3,3',4,4'-tetrabromodiphenyl ether (BDE-77); 2,2',3,4,4',5,6-heptabromodiphenyl ether (BDE-181) and decabromo[$^{13}\text{C}_{12}$]diphenyl ether ($^{13}\text{C}_{12}$ -BDE-209) were purchased from

Wellington Laboratories (Guelph, Ontario, Canada). The components of BFR-PAR solution were: 3 di-BDEs (7, 10, 15), 3 tri-BDEs (17, 28, 30), 5 tetra-BDEs (47, 49, 66, 71, 77), 5 penta-BDEs (85, 99, 100, 119, 126), 7 hexa-BDEs (138, 139, 140, 153, 154, 156, 169), 5 hepta-BDEs (171, 180, 183, 184, 191), 6 octa-BDEs (196, 197, 201, 203, 204, 205), 3 nona-BDEs (206, 207, 208), deca-BDE-209, pentabromoethylbenzene (PBEB), hexabromobenzene (hexa-BBz) and decabromodiphenyl ether (deBDEthane). The concentrations of each compound ranged from 200 ng/mL for the di-bromo congeners to 4000 ng/mL for the decabromodiphenyl ether.

Extraction and clean up

Sediment samples were freeze dried. Lyophilized material was ground, homogenized and stored in brown glass sealed containers at -20°C until analysis. One gram dry weight (dw) of sediment sample was spiked with internal standards (10 ng BDE-77, 10 ng BDE-181 and 500 ng $^{13}\text{C}_{12}$ -BDE-209). Spiked samples were kept overnight to equilibrate. In order to reduce time of analysis, a SPLE method that automatically and rapidly achieves quantitative and selective extraction of BFRs was used (De la Cal et al., 2003). Two grams copper were added to the cell to remove sulphur interferences. SPLE was carried out using a fully automated ASE 200 system (Dionex, Sunnyvale, CA, USA) using aluminium oxide neutral and Hydromatrix (Varian Inc., Palo Alto, USA). Extraction cell was heated to 100°C and extraction was carried out using a mixture of hexane: CH_2Cl_2 (1:1). The volume of the resulting extract was about 35 mL. Extracts were finally concentrated to incipient dryness and re-dissolved with 50 μL of toluene prior to analysis by gas chromatography coupled to mass spectrometry working with negative ion chemical ionization (GC/NICI-MS).

Instrumental analysis

GC/NICI-MS analyses were performed on a trace GC ultra gas chromatograph connected to a dual stage quadrupole mass spectrometer (Thermo Electron, Texas, USA). A DB-5MS capillary column (15 m $\times$ 0.25 mm i.d., 0.1 μm film thickness) was used with ammonia as the carrier gas at an ion source pressure of 1.9×10^{-4} Torr. The temperature program was from 140°C (held for 2 min) to 325°C (held for 10 min) at $10^\circ\text{C min}^{-1}$. Injection was carried out by splitless mode for 1 min with an injector temperature of 250°C (Eljarrat et al., 2004b).

The GC/NICI-MS operating conditions were as follows: ion source temperature was 250°C and ammonia as chemical ionization moderating gas at a gas flow 3.0 mL/min. Experiments were carried out monitoring the two most abundant isotope peaks from the mass spectra corresponding to $m/z = 79$ and 81 [Br^-] for di- to nona-BDEs, PBEB, hexaBBz and deBDEthane, $m/z = 487$ and 489 for BDE-209 and $m/z = 497$ and 498 for $^{13}\text{C}_{12}$ -BDE-209.

Identification of selected analytes was based on the following restrictive criteria: (i) retention time for all monitored ions for a given analyte should maximize simultaneously $\pm 1\text{s}$, with signal to noise ratio ≥ 3 for each; and (ii) the ratio between the two monitored ions should be within 15% of the theoretical value (calculated upon standards).

Quantification of di- to penta-BDEs, PBEB and hexaBBz was carried out by internal standard procedure using BDE-77, whereas for hexa- to hepta-BDEs, BDE-181 standard was used. In the case of octa- to deca-BDEs and deBDEthane, the quantitation was carried out using $^{13}\text{C}_{12}$ -BDE-209 as internal standard.

Quality assurance

Quality parameters of the method had been evaluated. For this purpose, experiments with spiked sediment (at 5–100 ng/g) were

Table 1
Sampling point location.

Site	River	Location site	UTM X	UTM Y
LL1	Llobregat	Castellvell i el Vilar	403,818	4,607,443
LL2	Llobregat	Martorell	411,171	4,592,186
LL3	Llobregat	Abrera	410,078	4,594,291
LL4	Llobregat	Sant Joan Despi	420,247	4,577,928
A1	Anoia	Pobla de Claramunt	388,339	4,602,206
A2	Anoia	Jorba	378,856	4,606,044
A3	Anoia	Martorell	410,400	4,591,976

UTM: universal transverse mercator coordinate system, X: latitude; Y: longitude.

prepared and analyzed by PLE followed by GC/NICI-MS. Test of recoveries were carried out five times in order to evaluate reproducibility. The limits of detection (LOD) of the method, defined as 3 times the noise level, and the limit of quantification (LOQ) of the method, defined as 10 times the noise level, were calculated.

The linearity of the method was checked using calibration curves made from standard solutions at six concentration levels (ranging from 4 pg injected to 3000 pg injected) and by triplicate injections.

Results and discussion

Quality control

The sample preparation method applying SPLE procedure was previously optimized for extraction and purification of 40 different mono- through deca-BDE congeners (De la Cal et al., 2003). In the present study, the application of this procedure was tested for emerging BFRs such as PBEB, hexaBBz and deBDethane.

Quality parameters were summarized in Table 2. Recoveries of 58%, 62% and 102% were found for PBEB, hexaBBz and deBDethane, respectively. For PBDEs, similar recoveries than those found in the previous study (De la Cal et al., 2003) were obtained, ranging from 56% to 110%. Thus, recovery range of emerging BFRs is similar to that obtained for PBDEs congeners.

As regards the reproducibility of the method, a relative standard deviation (RSD) of 3%, 5% and 6% was obtained for PBEB, hexaBBz and deBDethane, respectively. These values were below 10% and in the same range than those obtained for PBDEs (from 0.6% to 8%).

The LOD of the method achieved using SPLE followed by GC-NICI-MS ranged between 1 and 41 pg/g for PBDEs, and were 18, 9 and 86 pg/g for PBEB, hexaBBz and deBDethane, respectively. Thus, similar LOD values for PBDEs and emerging BFR were found.

Little information can be found related with analytical parameters of emerging BFRs. Ricklund et al. (2008) reported values of method LOD between 0.58 and 1.6 ng/g dw for deBDethane. These values were higher than those obtained in our study (0.09 ng/g). However, no data was found for the other emerging BFRs.

PBDEs levels

PBDEs were detected in all the sediment samples at concentrations ranging from 22 to 136 ng/g dw as shown in Table 3. Site LL2 (C2 sampling campaign) was the most contaminated zone followed by site A3 (C1 sampling campaign). These sediment samples were also subjected to a monitoring study of different priority and emerging contaminants, and also for these LL2 and A3 were the

two sites presenting the highest levels of pollution (Muñoz et al., in press). High pollution at site A3 could be attributed to the application of PBDEs in the textile and tannery industries located in the surroundings of this area. As regards, LL2 is under the influence of the effluents of a waste water treatment plant (WWTP) located next to the sampling point. On the other hand, if we compare PBDEs concentration at the three different sampling sites, no significant differences were detected.

Moreover, in a previous study carried out along 2000, PBDEs were determined in Anoia and Cardener rivers, tributaries of Llobregat River. In this study, levels ranged from 2.5 to 9.8 ng/g dw (Labandeira et al., 2007), being lower than those obtained in the present study. This difference should be due to the continuous use of flame retardants and accumulation with years. It is important to note that sediment samples from cited study are from 2000 and sampling sites were different, thus the difference also should be due to an addition from different sources.

Some other data for PBDEs in Spanish sediments are available. De la Cal et al. (2003) determined seven BDE congeners (BDE-47, BDE-99, BDE-100, BDE-118, BDE-153, BDE-154 and BDE-183) in two river sediments collected in Catalonia and Σ BDE concentrations ranged from 1.8 to 2.5 ng/g dw. Eljarrat et al. (2004a) determined 40 BDE congeners, including BDE-209 in sediments from 4 sites in Ebro River. Σ BDE concentrations were from 2.4 to 42 ng/g dw.

PBDEs were also determined in samples from 32 sites along River Danube with Σ BDE concentrations between 0.06 and 84 ng/g dw (Sawal et al., 2005). In the Scheldt estuary in the Netherlands, higher levels of Σ BDE (13 congeners including BDE-209) were found, with concentrations up to 262 ng/g dw (Verslycke et al., 2005).

Of 38 PBDEs congeners included in our analytical work, 14 different PBDEs were detected, ranging from tetra- to deca-BDE: tetra-BDE-49+71, tetra-BDE-47, penta-BDE-99, penta-BDE-100, hexa-BDE-153, octa-BDE-201, octa-BDE-204+197, octa-BDE-203, octa-BDE-196, nona-BDE-208, nona-BDE-208, nona-BDE-207, nona-BDE-206 and deca-BDE-209. The dominance of BDE-209 in the total PBDEs is unquestionable such as it had been described in literature (Eljarrat et al., 2004b), which was present at concentrations varying from 3.2 to 82 ng/g dw. In these samples BDE-209 constituted between 11% and 71% of the total PBDE contamination. The presence of BDE-209 could indicate the use of commercial formulations of deca-BDE in the studied area. On the other hand, in more than 95% of samples there are also presences of BDE-206, -207 and -208, indicating a possible degradation of BDE-209 in aquatic environment (Stapleton and Dodder, 2008). When the relative contribution of octa- and nona-BDEs is com-

Table 2
Quality parameters of the SPLE followed by GC-NICI-MS method for PBDEs and emerging BFRs.

PBDEs	% R (n = 3)	RSD (%)	Instrumental (pg)		Method (pg/g)	
			LOD	LOQ	LOD	LOQ
Di-BDE	87–105	0.7–4	3.2–6.0	11–20	20–41	67–136
Tri-BDE	57–101	3–5	1.7–2.1	5.6–7.1	15–19	50–63
Tetra-BDE	64–88	0.8–8	1.2–1.5	4.1–5.1	4.7–11	15–36
Penta-BDE	96–110	0.8–7	1.0–1.7	3.5–5.7	7.7–24	25–81
Hexa-BDE	74–105	2–8	1.3–2.3	2.1–7.8	8.4–22	28–74
Hepta-BDE	82–102	0.6–8	0.3–2.6	1.1–8.7	1.0–13	29–43
Octa-BDE	66–102	2–6	1.3–6.3	4.2–21	21–36	70–120
Nona-BDE	56–71	2–8	1.2–3.1	4.0–10	13–18	43–61
Deca-BDE	83	6	2.9	9.6	25	37
<i>Emerging BFRs</i>						
PBEB	58	3	1.7	5.6	18	60
Hexabz	62	5	1.6	5.4	9.1	30
DecaBDethane	102	6	11	39	86	288

R: recovery; RSD: relative standard deviation, LOD: limit of detection, LOQ: limit of quantitation.

Table 3
BFRs concentration levels (expressed in ng/g dry mass) in Llobregat and Anoia River sediments.

	L11			L12			L13			L14			A1		A2		A3		
	C1	C2	C3	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C1	C2	C3	C1	C2	C3
BDE-49+71	nq	nq	nd	4.9	nd	nq	0.9	nq	5.3	nd	nd	nd	1.0	0.3	nq	nq	nd	4.7	nq
BDE-47	nq	nq	nq	1.4	nq	nq	nq	nq	2.6	nq	nd	nd	nq	3.2	nq	nq	nd	8.7	nq
BDE-100	nq	nq	nq	nq	nd	nq	nd	nq	1.0	nq	nd	nd	nd	nd	nq	nq	nd	nd	nq
BDE-99	nd	nq	nq	nq	nq	nq	nq	nq	10	nq	nd	nd	nq	nd	nq	nq	nd	3.3	nq
BDE-153	nd	10	17	nq	nd	4.4	nq	nd	nq	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
BDE-201	nd	nd	nd	nd	nd	nd	nd	3.3	nd	nd	nd	nd	nd	nd	nd	nd	4.2	nd	nd
BDE-204+197	nd	nd	nd	4.0	nd	nd	nd	12	nd	nd	nd	nd	nd	nd	4.5	nd	5.5	nd	nd
BDE-203	nd	nd	nd	2.7	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
BDE-196	nd	nd	nd	3.3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
BDE-208	4.6	8.0	6.3	9.5	7.6	5.8	6.4	12	9.3	7.9	5.2	6.5	6.4	6.3	7.6	6.3	8.8	6.4	4.6
BDE-207	4.5	3.8	6.1	13	8.6	6.4	7.4	12	12	9.0	5.9	7.4	6.9	6.8	8.2	7	11	6.9	4.5
BDE-206	5.0	nq	8.1	15	11	6.1	9.7	20	11	11	12	8.4	9	10	8.5	11	24	10	7.7
BDE-209	7.8	9.9	7.7	82	13	7.5	5.2	29	21	29	56	3.2	4.1	19	17	11	57	36	11
Total PBDEs*	22	32	45	136	40	30	30	89	73	57	79	26	27	46	46	35	111	76	28
<i>Emerging BFRs</i>																			
PBEB	9.0	7.7	nq	4.7	nq	6.1	8.2	nd	5.5	9.1	nd	6.4	9.0	9.6	3.1	nd	8.3	6.7	nd
HexabBz	nq	nd	nd	2.2	nd	nq	nd	nq	2.4	nd	nd	nq	nd	nd	nq	nq	nd	0.4	nd
DeBDethane	8.6	4.8	12	23	12	9.1	13	10	9.9	11	24	9.1	15	15	15	13	20	11	22

C1: first sampling campaign (June 2005); C2: second sampling campaign (October 2005); C3: third sampling campaign (June 2006); nd: below LOD; nq: below LOQ.
* Total amount calculated considering nd and nq equal zero.

pared with the relative contribution of BDE-209 it is rejected the possibility of BDE-209 degradation on column. This was proved injecting standard of BDE-209 at the same instrumental conditions. Thus, the contribution of BDE-209 plus nona- and octa-BDEs was close to 90%, this could be due to a high usage of deca-BDE mixture.

Emerging BFR levels

Table 3 shows the concentration levels found for emerging BFRs. PBEB was detected in 13 of 19 analyzed samples at concentration levels between 3.1 and 10 ng/g dw. HexaBBz was detected just in 3 out the 19 sediments, at levels ranging from 0.4 to 2.4 ng/g dw. Finally, decaBDethane was detected in all samples, at levels between 4.8 and 24 ng/g dw.

Few data are available about these compounds in sediment samples. In the case of PBEB, a previous work analyzed it in sediment samples from Michigan lake (Hoh et al., 2005), but it was not detected. Thus, our values represented the first data available of PBEB in sediment samples around the world. The current production of this compound is not available, but recent studies reported the presence of relatively high concentrations in Chicago air samples (Gauthier et al., 2007).

HexaBBz was widely used in Japan as an additive to paper, plastic and electronic goods. Watanabe et al. (1986) reported levels between 5.6 and 60 ng/g dw in Osaka sediment samples. HexaBBz is still used in Japan at lower volumes (Watanabe and Sakai, 2003). However, hexaBBz is not reported by the EU industry as a currently produced chemical (Verreault et al., 2007). Besides, it has to be taken into account that another source of hexaBBz in the environment result from thermal degradation of BDE-209 (Gauthier et al., 2007).

DeBDethane became commercially important as an alternative to the decaBDE formulation in the early 1990s (Kierkegaard et al., 2009). DeBDethane were determined in samples from Western Scheldt River (The Netherlands) at 0.65–9.8 ng/g dw (Lopez et al., 2008). A higher value was reported by Kierkegaard et al. (2004) in sediment from the same river, with a concentration of 24 ng/g dw. All these values are in accordance with our levels in the Llobregat River.

It is important to note, that a recent work analyzed PBEB, hexaBBz and DeBDethane in sediments from 6 different sites in Canada.

However, there is no report of concentration levels, just presenting a summarising graphic, rendering the comparison with our data (Kolic et al., 2009).

Comparative occurrence of selected BFRs

Fig. 2 compared the different BFR levels in the two rivers studied. As can be seen no significant difference was observed between the contamination in both rivers. Moreover, a similar distribution was observed, being higher the concentration levels of PBDEs (mainly corresponding to BDE-209), followed by deBDethane, PBEB and finally, hexaBBz.

As it was indicated before, deBDethane applications are similar as for BDE-209 with the advantage of no production of dibenzo-p-dioxins and no formation of furans under pyrolysis conditions (Kierkegaard et al., 2004; Jakab et al., 2003). Generally, the reported levels of deBDethane were lower than for BDE-209 (Kierkegaard et al., 2009). In the present study it is observed that in 43% of samples, deBDethane concentrations are higher than BDE-209, with ratios between 1.1 and 3.6. This could be due to the higher input in the use of deBDethane during last years. Although currently deBDethane is not as widely used as BDE-209 because of its higher cost, it is predicted that deBDethane will soon become one of the major flame retardants used by thermoplastics industry (Konstantinov et al., 2006).

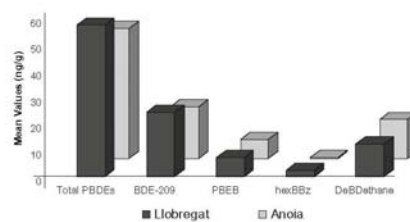


Fig. 2. Comparison of BFRs levels in Llobregat and Anoia River sediments.

As regards PBEB, its contribution represented around the 16% of that of PBDEs, even if its production was low in Europe since 2002 (Verreault et al., 2007). However, it is possible that PBEB is now being used as a synergistic co-additive in expandable graphite or inorganic flame retardants (Hoh et al., 2005).

Finally, the contribution of hexaBBz to the total PBDEs corresponded to 2% of that of PBDEs. HexaBBz is not reported by the EU industry as a currently produced chemical (Verreault et al., 2007). This could be the main reason of its low contribution.

The present findings provide evidence that currently, emerging BFRs of unrestricted usage are present in the environment. Further measurements are required to investigate the environmental sources and fate of these compounds.

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Artículo científico #7:

“Dechlorane plus and related compounds in peregrine falcon (Falco peregrinus) eggs from Canada and Spain”

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P. Guerra, K. Fernie, B. Jimenez, G. Pacepavicius, L. Shen, E. Reiner, E. Eljarrat, D. Barceló y M. Alae

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Dechlorane Plus and Related Compounds in Peregrine Falcon (*Falco peregrinus*) Eggs from Canada and SpainPaula Guerra,[†] Kim Fernie,^{*†} Begoña Jiménez,[§] Grazina Pacepavicius,^{||} Li Shen,[⊥] Eric Reiner,[⊥] Ethel Eljarrat,[†] Damià Barceló,^{†,§} and Mehran Alaei^{||}[†]Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona, 18-26, 08034 Barcelona, Spain[§]Ecotoxicology and Wildlife Disease, Science and Technology Branch, Environment Canada, 867 Lakeshore Road, Burlington, Ontario, Canada L7R 4A6[§]Department of Instrumental Analysis and Environmental Chemistry, Institute of Organic Chemistry, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain^{||}Water Science and Technology, Environment Canada, 867 Lakeshore Road, Burlington Ontario, Canada L7R 4A6[⊥]Ontario Ministry of Environment, 125 Resources Road, Toronto, Ontario, Canada M9P 3 V6^{*}Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, Pic de Peguera 15, 17003 Girona, Spain

Supporting Information

ABSTRACT: Concentrations of Mirex, Dechlorane (Dec) Plus (DP), Dec 602, Dec 603, and Dec 604 were significantly higher in peregrine falcon (*Falco peregrinus*) eggs from Canada than Spain, with the former having the only measurable concentrations of the dechlorinated DP products, decachloropentacyclooctadecadiene (aCl10DP) and undecachloropentacyclooctadecadiene (aCl11DP). Large variations also occurred in the *in ovo* concentrations of the DP monoadduct, DPMA. This is the first study to report the accumulation and metabolism of DP by peregrines, both DP and dechloranes in European biota, as well as dechloranes in a terrestrial organism and one at the top of the food web. The geographical differences in the measured *in ovo* concentrations reflect local exposure of the adult peregrines on their breeding grounds, likely differences in diet of the adults, the production of DP on the Niagara River, and the greater use of Mirex and dechloranes as flame retardants in North America than Europe.

INTRODUCTION

Halogenated flame retardants (HFRs) are a structurally diverse group of chemicals that are generally brominated or chlorinated. These compounds are added to or reacted with polymers, textiles, and electronic circuitry to reduce the risk of fire. Dechlorane Plus (DP) is a chlorinated flame retardant ($C_{18}H_{12}Cl_{12}$) manufactured in China¹ and for more than 40 years by Oxychem in North America (Niagara Falls, NY). In commercial DP products, two stereoisomers, syn- and anti-DP, occur at a ratio of approximately 1:2, respectively, and plastic formulations may contain as much as 3.5% (weight basis) of DP.² These stereoisomers are the products of the Diels–Alder reaction of 2 mol of hexachlorocyclopentadiene with 1 mol of 1,5-cyclooctadiene.³ This DP formulation is used primarily in products such as cable coatings, plastic roofing materials, and hard connectors in computers and televisions, and there are recent reports of DP isomers in biotic and abiotic samples from the Great Lakes of North America.^{3–5}

DP is a high volume production chemical (500–5000 tons/year) that has been in use since 1986 and is sold worldwide, including Europe and Asia.⁶ It replaced Dechlorane ($C_{10}Cl_{12}$), or Mirex, previously used as a pesticide and as a flame retardant until it was banned in the 1970s in North America. DP is also listed on Canada's Domestic Substances List (DSL), but in terms of bioaccumulation it is not considered to be of high risk because

of its high molecular mass (648 Da) and high log K_{ow} (9.3).⁶ DP has been measured in air,^{4,6} indoor dust,⁷ sediments,^{4,8–11} and biota.^{3–5,10–13} In the eggs of herring gulls (*Larus smithsonianus*), the sum (Σ) DP concentrations were generally <15 ng/g wet weight (ww) and the anti-DP values suggested a nonstereoselective enrichment of the DP stereoisomers.⁵ However, there was an enrichment of the syn-DP isomer relative to the commercial mixture in smelt and alewife from Lake Ontario.³

Similar to DP, Dechlorane (Dec) 602, Dec 603, and Dec 604 were developed and manufactured by the same manufacturer as DP, Oxychem, in order to improve the flame retardant property of polymers in the late 1960s and 1970s. Shen et al.¹⁰ provided the initial study of these compounds in sediments and fish from the Great Lakes, and this is the only assessment in biota to date. The concentrations of Dec 602, Dec 603, and Dec 604 in sediments were 0.97–11000, 0.61–600, and nd–8000 pg/g dry weight (dw), respectively, and in fish the concentrations ranged from 470 to 34000, 14–550, and nd–1300 pg/g lipid weight (lw), respectively.

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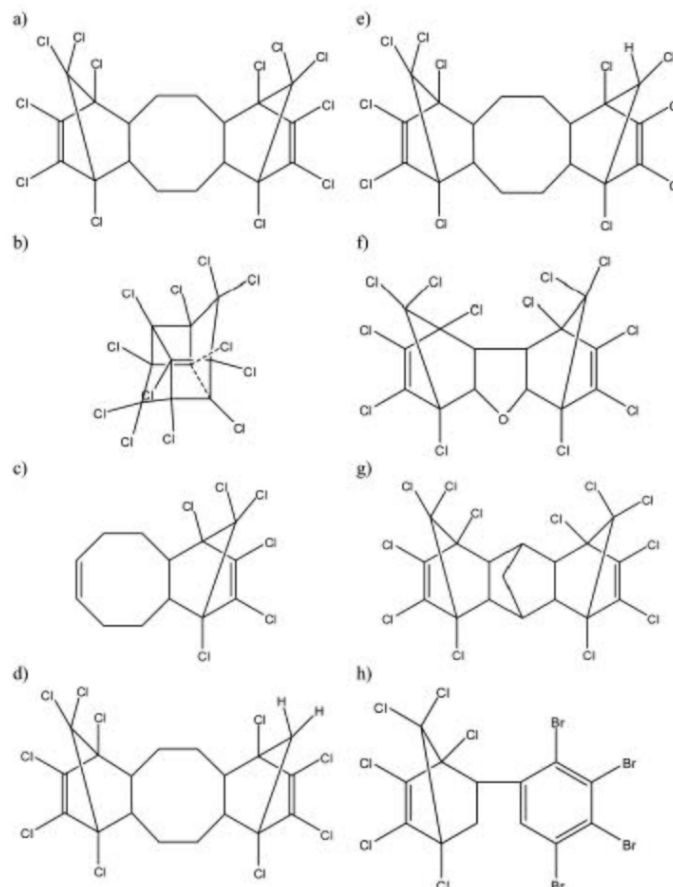


Figure 1. Molecular structures of a) DP, b) Mirex, c) DPMA, d) Cl10DP, e) Cl11DP, f) Dec 602, g) Dec 603, and h) Dec 604.

Peregrine falcons were previously endangered in the northern hemisphere partly because of the bioaccumulation of organochlorine pesticides, but their populations are now recovering. Peregrines are an excellent sentinel species for monitoring environmental organic contaminants^{14–19} because they are at the top of the food chain and consume avian prey from the aquatic and terrestrial environments. A female bird of prey gains weight immediately before egg laying, and her ability to lay eggs is related to the availability of food on her breeding territory during egg formation (ref 20 and references therein). Most peregrines that breed in the northern hemisphere, especially in Canada, overwinter in the southern hemisphere. Their exposure to organochlorine pesticides on these overwintering grounds declined between 1978 and 2004 as measured in female peregrines during migration from Central and South America.¹⁹ Peregrine eggs and nestlings have been used to assess

concentrations of new and emerging chemicals of concern, including polybrominated diphenyl ethers (PBDEs), with some of the highest PBDE concentrations ever found in biota reported in this species.^{15,17,18}

In this study, the eggs of peregrine falcons nesting in the Canadian Great Lakes and the Maritimes, as well as central and coastal Spain, were used to investigate the occurrence of halogenated norbornenes, specifically Mirex, DP, Dec 602, Dec 603, and Dec 604. Since DP is a diadduct compound, it may be affected by a retro-Diels–Alder process, leading to the possibility of a monoadduct formation. Consequently, the peregrine falcon eggs were also analyzed for the DP monoadduct, DPMA, as well as two of the dechlorinated products of DP, decachloropentacyclooctadecadiene (aCl10DP) and undecachloropentacyclooctadecadiene (aCl11DP). The molecular structures of these chemicals are illustrated in Figure 1.

■ EXPERIMENTAL SECTION

Chemicals. Dec 602 (95%), Dec 603 (98%), and Dec 604 (98%) were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). DP, DPMA, aCl10DP, and aCl11DP were obtained from Wellington Laboratories Inc. (Guelph, ON, Canada), and Mirex was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). Silica gel (100–200 mesh) was purchased from Merck (Germany). All solvents were of analytical grade (J.T. Baker, USA). Individual stock standard solutions were prepared on a weight basis in toluene and stored at -20°C . A mixture of all selected standards was prepared by appropriate dilution of individual stock solutions.

Sampling. In Canada, all collections were completed with necessary permits that prohibit the collection of eggs with live embryos. Between 2007 and 2009, eggs that had failed to hatch ($n = 12$) were collected from active nests ($n = 12$ nests) located in the Canadian Great Lakes Basin (Figure SI-1), usually from the same nests in which plasma concentrations of polychlorinated biphenyls (PCB), PBDE, and HBCD were assessed in nestlings in 2004 and 2005.¹⁵ The nests were designated as upstream (Lake Superior) of the DP manufacturing plant on the Niagara River or downstream of the plant and involved the nests on Lake Ontario and the St. Lawrence River but excluded the one nest (egg C1) in Eastern Canada (near St. John, New Brunswick) that was more than 1000 km east of the Niagara River (Table 1; Figure SI-1). From 2003 to 2006, 13 eggs were collected from 13 active nests in Central and coastal Spain, specifically Guadalajara in Central Spain and Bilbao on the North-Cantabric Coast of Spain (Figure SI-2).

Over the past 15 years, eggs that have failed to hatch, feathers, and other prey remains have been collected at Canadian nest sites when the nestlings were banded. The prey remains were identified to species and categorized according to aquatic (e.g., gull colonies) or terrestrial (e.g., passerine species) habitats. Following Fernie and Letcher,¹⁵ this information provided a preliminary classification of the diet of the adult peregrines laying eggs in this study. The classification of the Spanish eggs was based on nest location: coastal-aquatic nests and inland-terrestrial nests. Because this is a preliminary classification, the subsequent results should be interpreted with caution and subsequent stable isotope analysis is recommended.

Sample Preparation. Before extraction, sampled eggs were lyophilized, homogenized, and stored at -22°C ; 3–5 g dry weight (dw) of sample was spiked with $^{13}\text{C}_{12}$ -BDE-77 and $^{13}\text{C}_{12}$ -BDE-153 (Wellington Laboratories, Canada). Extraction was carried out by Soxhlet method using 250 mL of dichloromethane (DCM) (Caledon Laboratories, Canada). The Soxhlet extract was passed through an Allihn funnel filled with preirradiated sodium sulfate (EMD, Tracepur, GR ACS 10–60 mesh). After extraction, the crude extracts were concentrated to approximately 2 mL, and then lipids were removed by Gel Permeation Chromatography (GPC) using a column packed with BioBeads (SX-3) (Bio-Rad Laboratories, Inc.) and eluted with DCM:hexane (1:1) solution.²¹ 150 mL of DCM:hexane (1:1) was obtained with the compounds of interest. The lipid-free extracts were concentrated to 3 mL and transferred to a silica gel column (ACP Chemicals Inc., Grad 62, 60–200 mesh) and eluted with a mixture of DCM:hexane (1:1). Samples were finally concentrated to incipient dryness and redissolved, obtaining 500 μL of stable isotope labeled compound mixture containing $^{13}\text{C}_{12}$ -BDE-79 and $^{13}\text{C}_{12}$ -BDE-139 (Wellington Laboratories,

Canada) in isooctane prior to analysis by gas chromatography high resolution mass spectrometry (GC-HRMS). The percent lipid in the egg homogenates ranged from 5.2 to 8.6% as determined gravimetrically.

Chemical Analysis. The sample extracts were analyzed on a HRMS Micromass AutoSpec Ultima MS (Micromass, Manchester, UK) connected to a Hewlett-Packard 6890 GC (Hewlett-Packard, Palo Alto, CA, USA) equipped with a 15 m DB-SHT column (0.25 mm i.d., 0.10 μm film thickness) J&W Scientific, Folsom, CA). The temperature program was 100°C for 2 min, ramped to 250°C at $25^{\circ}\text{C}/\text{min}$, ramped to 260°C at $1.5^{\circ}\text{C}/\text{min}$, ramped to 325°C at $25^{\circ}\text{C}/\text{min}$, and then held for 10 min. The HRMS system was operated in EI positive mode and was tuned up to 10,000 resolving power (RP) according to 10% valley definition. Concentrations of Mirex, DP, Dec 602, Dec 603, Dec 604, aCl10DP, C111DP, and DPMA in peregrine eggs were determined by monitoring the two most abundant ions of the fragment cluster at m/z 271.81/273.80 for Mirex, syn- and anti-DP and Dec 602, 262.85/264.85 for Dec 603, 417.70/419.70 for Dec 604,¹⁰ 263.86/265.86 for DPMA, 201.89/203.88 for aCl10DP, and 237.84/239.84 for C111DP.

QA/QC. Method blank samples were run every five samples to check for interference or contamination from solvents and glassware. No presence of analytes of interest was observed. Identification and quantification were based on the following criteria: (a) sample peaks had an approximately Gaussian shape, and their GC retention times matched those of the standard compounds within ± 0.1 min; (b) all peaks had a signal-to-noise ratio of greater than 5:1; and (c) peak areas were within 15% of the corresponding theoretical isotopic ratios. The limits of detection were between 0.004 and 0.3 ng/g lipid weight (lw), and the limits of quantification ranged from 0.01 to 1.0 ng/g lw. The recovery efficiencies, based on $^{13}\text{C}_{12}$ -BDE-77 and $^{13}\text{C}_{12}$ -BDE-153 internal standards, were $85 \pm 8\%$ and $92 \pm 11\%$, respectively. All sample concentrations were recovery-corrected.

Statistical Analysis. Concentrations of DP, Dec 603, Dec 604, and DPMA were log-transformed prior to statistical analysis using one-way ANOVAs. All other data were analyzed using nonparametric Wilcoxon statistical tests. Potential differences relating to diet were not assessed in the peregrine eggs from Canada because of the very small sample size of aquatic-based eggs ($N = 2$) but were evaluated using the peregrine eggs from Spain since there was sufficient sample sizes (aquatic diet $N = 8$; terrestrial diet $N = 5$). Comparisons were made between eggs from Canada ($N = 10$) and Spain ($N = 5$) based solely on peregrines having a terrestrial diet.

■ RESULTS AND DISCUSSION

The concentrations of mirex, DP, Dec 602, Dec 603, Dec 604, DPMA, aCl10DP, C111DP, and lipids measured in the peregrine eggs (Table 1) mostly reflect contaminant exposure of the peregrines on the breeding grounds. Prior to and throughout the egg laying period, adult peregrines capture prey within their breeding territory. The occurrence of egg laying is related to the body condition of the female and the availability of food on the breeding territory (ref 20 and references therein). During egg formation, the prey of the peregrines may include migrating birds, including birds from the southern Great Lakes and those that have overwintered in Central and South America. Since 1978, concentrations of organochlorine pesticides have declined in female peregrines captured during their Spring migration from

Table 1. Concentrations of Mirex, Dieldrin (Dec 602), Dieldrin (Dec 603), Dieldrin (Dec 604), the DP Monoadduct, DPMA, DPMA:DP Ratios, and the Dechlorinated DP Products, Dieldrin, Dieldrin (Dec 602), Dieldrin (Dec 603), Dieldrin (Dec 604), and Undecachloropentacyclooctadecadiene (aC11DDP) (ng/g Lipid Weight (lw)) in Peregrine Falcon (*Falco peregrinus*) Eggs from Canada and Spain^a

country, presumed diet	N	lipid (%)	Mirex	ΣDD	f _{ant} ^a	Dec 602	Dec 603	Dec 604	DPMA	DPMA:DP	aC11DDP	CHDDP		
limit of detection			0.021	0.012		0.027	0.011	0.028	0.045		0.39	0.048		
limit of quantification			0.053	0.036		0.091	0.023	0.072	0.15		0.91	0.124		
Spain - Overall														
Spain - terrestrial	13	5.7	18.9	1.78	0.77	8.36	3.98	0.23	21.1	12.3	-	0.18		
geometric mean	5	5.5	13.5	0.6	0.8	9.78	2.33	0.33	2.19	3.75	-	-		
range	5	2.4 - 17	0.3 - 3.6	0.62 - 0.75	0.62 - 0.75	n.d. - 15	1.5 - 6.2	n.q. - 0.35	1.7 - 37	2.2 - 10	-	-		
median	5		14	0.6	0.74	4.8	2.4	0.32	2.5	4.8	-	-		
# eggs detected in	of 5		5	5		4	5	4	5	0	0	0		
geometric mean	8	5.7	29	2.81	0.77	13.3	4.91	0.18	71.2	25	-	0.18		
range	8	9.2 - 78	0.4 - 17	0.65 - 0.78	0.65 - 0.78	n.d. - 25	3.0 - 7.5	n.d. - 0.32	n.d. - 469	2.2 - 336	-	n.d. - 0.28		
median	8		29	2.3	0.69	15	5.2	0.31	51	22	-	0.13		
# eggs detected in	of 8		8	8		8	8	5	7	0	0	3		
Canada - overall														
overall GM	12	5.8	641	36.4	0.58	73.3	35.6	3.38	30.2	1.17	1.62	1.84		
geometric mean	10	5.6	733	38.4	0.58	89.2	43.9	3.7	30.5	1.2	1.58	1.81		
range	10		425 - 1353	7.5 - 209	0.41 - 0.69	44 - 211	12 - 220	1.4 - 9.8	1.2 - 1660	0.02 - 41	n.d. - 1.9	1.3 - 2.3		
median	10		744	43	0.63	71	52	3.9	62	2.1	1.7	1.9		
# eggs detected in	of 10		10	10		10	10	10	10	9	10	10		
geometric mean	2	7.1	330	27.7	0.55	27.4	12.4	2.13	28.8	1.04	1.79	1.96		
range	2		94 - 1160	6.3 - 122	0.52 - 0.60	7.3 - 104	5.3 - 29.0	1.3 - 3.5	3.8 - 218	0.6 - 1.8	1.6 - 2.0	1.6 - 2.4		
median	2		626	64	0.56	55	17	2.4	111	1.2	1.8	2		
# eggs detected in	of 2		2	2		2	2	2	2	2	2	2		
egg	year	location	presumed diet	lipid (%)	Mirex	ΣDD	f _{ant} ^a	Dec 602	Dec 603	Dec 604	DPMA	DPMA:DP	aC11DDP	CHDDP
S1	2004	Guadalupe, SP	terrestrial	6.9	2.4	3.6	0.62	0.24	6.2	0.25	37	10	n.d.	n.q.
S2	2003	Guadalupe, SP	terrestrial	6.0	14	0.81	0.75	n.d.	2.2	0.35	1.7	2.2	n.d.	n.q.
S3	2004	Guadalupe, SP	terrestrial	5.2	10	0.03	1.00	13	3.7	n.q.	2.1	6.9	n.d.	n.q.
S4	2003	Guadalupe, SP	terrestrial	5.4	14	0.9	0.75	15	2.4	0.32	2.5	2.7	n.d.	n.q.
S5	2003	Guadalupe, SP	terrestrial	5.5	17	0.62	0.73	4.8	1.5	0.31	2.6	4.8	n.d.	n.q.
S6	2006	Bilbao, SP	aquatic	5.2	9.2	1.2	0.65	15	6.5	n.d.	46	37	n.d.	n.q.
S7	2006	Bilbao, SP	aquatic	5.1	21	2.5	0.73	n.d.	5.5	n.d.	n.d.	-	n.d.	0.13
S8	2006	Bilbao, SP	aquatic	6.1	25	1.2	0.70	7.6	3.6	0.09	26	22	n.d.	n.d.
S9	2006	Bilbao, SP	aquatic	5.9	23	8.3	0.67	8.3	4.1	0.29	18	2.2	n.d.	n.d.
S10	2006	Bilbao, SP	aquatic	5.6	40	9.00	0.78	15	2.9	0.11	57	6.3	n.d.	0.16
S11	2006	Bilbao, SP	aquatic	6.4	43	17	0.68	22	4.9	0.32	123	7.2	n.d.	0.28
S12	2006	Bilbao, SP	aquatic	6.1	78	2.1	1.00	25	7.5	0.24	469	223	n.d.	n.q.
S13	2006	Bilbao, SP	aquatic	5.6	32	0.41	1.00	10	6.0	n.q.	134	336	n.d.	n.q.
C1	2008	New Brunswick, CD	terrestrial	5.6	1353	40	0.67	211	220	3.7	1656	41	1.8	1.8
C2	2008	Toronto, CD	terrestrial	5.4	471	16	0.68	143	19	2.6	72	4.6	1.7	1.7

Table 1. Continued

egg	year	location	presumed diet	lipid (%)	Mirex	Σ DP	f_{anti}^a	Dec 602	Dec 603	Dec 604	DPMA	DPMA:DP	sC10:DP	C11:DP
C3	2008	Toronto, CD	terrestrial	7.0	759	49	0.41	196	12	9.8	1.2	0.02	1.3	1.3
C5	2009	Toronto, CD	terrestrial	5.3	767	33	0.64	66	20	4.2	3.0	0.91	1.8	1.9
C6	2009	Toronto, CD	terrestrial	5.2	425	68	0.55	44	62	2.9	1.8	0.03	n.d.	2.3
C7	2009	Toronto, CD	terrestrial	5.9	1210	209	0.58	158	106	4.1	634	3.0	1.6	1.9
C11	2007	Montreal, CD	terrestrial	5.2	671	45	0.69	48	20	5.1	52	1.2	1.5	2.1
C12	2007	Montreal, CD	terrestrial	5.9	728	82	0.68	58	75	6.8	517	6.3	1.1	1.6
C4	2008	Thunder Bay, CD	terrestrial	5.3	1020	75	0.61	55	45	2.1	196	26	1.9	1.9
C10	2009	Thunder Bay, CD	terrestrial	5.6	474	17	0.43	77	60	1.4	1.5	0.088	1.7	1.8
C8	2009	North L. Superior, CD	aquatic	8.6	1160	122	0.52	104	29	3.5	218	1.8	1.8	2.4
C9	2009	North L. Superior, CD	aquatic	5.9	94	63	0.61	72	53	1.3	3.8	0.6	1.6	1.6

^aThe anti-DP fraction (f_{anti}) is calculated as the concentration of anti-DP divided by the concentration of total DP. Geometric mean DPMA concentrations exclude the outlier egg C1 collected from a nest in New Brunswick, Canada. GM: geometric mean; n.d.: not quantifiable; n.d.: not detectable. For individual eggs: S. Spain, C. Canada.

overwintering in Central and South America.¹⁹ Thus, the contaminant burden in the peregrine falcon eggs largely reflects local exposure on the breeding territory. In addition, differences in the *in ovo* contaminant concentrations in this study are not related to differences in the *in ovo* lipid concentrations, since they were comparable in the peregrine eggs from Canada and Spain, and regardless of diet for the peregrines nesting within Spain (all $P \geq 0.83$).

Prior to being banned in the 1980s in North America, Mirex was used as a flame retardant and a widely used pesticide. Although Mirex was produced and used in China as late as 2009,²⁰ the peregrine falcons in this study are unlikely to have ever been in China since they migrate to the southern hemisphere from Spain or Canada. Despite the North American ban of Mirex more than 20 years ago, it was detectable in measurable quantities in all of the peregrine eggs from the Canadian Great Lakes and Spain (Table 1). *In ovo* concentrations of Mirex were the highest of the measured compounds in the peregrine eggs, although concentrations of DPMA were higher in some of the individual eggs from Spain and the one egg from New Brunswick, Canada (Table 1). Overall, the Mirex concentrations were greatest in the eggs laid by Canadian peregrines with a terrestrial diet than the Spanish birds with a terrestrial diet ($\chi^2 = 5.5$, $df = 1$, $P = 0.02$) (Table 1), reflective of the production of Mirex in North America and not Spain. Interestingly, Mirex concentrations were significantly higher in the eggs laid by peregrines on the Spanish coast compared to the inland birds in Spain ($\chi^2 = 5.5$, $df = 1$, $P = 0.02$) (Table 1).

DP was also detected in all of the peregrine eggs, with Σ DP values ranging from 0.30 to 209 ng/g lw (Table 1). This is the first study reporting Σ DP concentrations in European biota, and there was no dietary influence on the Σ DP concentrations in these eggs from Spain ($P = 0.10$). In the peregrines with a terrestrial diet, Σ DP concentrations in the Canadian peregrine eggs were significantly and 40 $\times$ higher than in the Spanish eggs ($F_{1,14} = 55.5$, $P < 0.0001$; Table 1), suggesting localized exposure consistent with the DP manufacturing site on the Niagara River (Figure S1); this is further supported by the geographical pattern observed in the Canadian Great Lakes Basin in which Σ DP concentrations were significantly higher ($F_{1,8} = 15.26$, $P = 0.006$) in the eggs collected from nests on Lake Ontario and the St. Lawrence River that are downstream of the DP manufacturing site, than from nests which were upstream on Lake Superior (Table 1). A similar pattern was also reported in herring gull eggs nesting on the same Laurentian Great Lakes.⁵ However, the Σ DP concentrations in the peregrine eggs from Canada, both overall and laid by those birds having a terrestrial diet, are twice that of those measured in herring gull eggs (<15 ng/g ww) from the same Laurentian Great Lakes.⁵ Herring gulls can fall prey to peregrine falcons, and so may be considered at a lower trophic position than peregrine falcons. These results suggest that DP is bioaccumulating.

Commercial grade DP is synthesized by the Diels–Alder addition of 2 mol fully chlorinated cyclopentadiene to 1 mol of cyclooctadiene, and the main products of the reaction are two stereoisomers, *syn* and *anti*, along with small amounts of by-product arising from impurities within the cyclooctadiene starting material. The anti-DP fraction (f_{anti}) has been reported in fish² and herring gulls⁴ as well as sediments⁹ in the Laurentian Great Lakes, and these studies indicate that DP has been subject to stereoselective processes in the environment. In order to determine if such enrichment had occurred in the peregrines, either prior to uptake by the birds or via internal biochemical

processes by the adult prior to egg laying, f_{anti} was calculated as the concentration of anti-DP divided by the concentration of total DP (Table 1). The f_{anti} values for the peregrine eggs of Spanish birds with a terrestrial diet were significantly higher than their Canadian counterparts ($F_{1,14} = 6.7$, $P = 0.02$) (Table 1), possibly reflecting the closer proximity of the Canadian birds to the manufacturing source of DP on the Great Lakes. There was no apparent dietary influence on the f_{anti} values of the Spanish birds ($P = 0.86$), and 3 of these eggs had f_{anti} values of 1.0 with no contribution of syn-DP (S3, S12, and S13) (Table 1) perhaps suggesting differences in bioavailability. The mean f_{anti} values in the peregrine eggs (Table 1) are similar to the f_{anti} values for herring gull eggs from the same Great Lakes⁴ and in technical DP manufactured by OxyChem (0.65–0.80).^{3–6,8–10} Both peregrine falcons and herring gulls are at or near the top of their food webs, respectively, whereas fish, including rainbow smelt (*Osmerus mordax*) and alewife (*Alosa pseudoharengus*), are at a relatively lower trophic level and had lower mean f_{anti} values (0.53) in Lake Ontario.⁵ Furthermore, stereoselective enrichment of anti-DP (0.86) occurred in the sediments of Lake Ontario.⁹ Together, the results of these studies suggest that peregrine falcons are exposed to an already transformed DP prior to uptake and support the hypothesis that there are interspecies differences in bioaccumulation and biotransformation of DP.³

This is the first study to report the detection of Dec 602, Dec 603, and Dec 604 in a terrestrial organism, an organism at the top of the food web, and in European biota. Previously, only one other study reported these compounds in sediment and fish from the Laurentian Great Lakes.¹⁰ Concentrations of Dec 602, Dec 603, and Dec 604 were detected in 92%, 100%, and 84% of the peregrine egg samples, respectively (Table 1). The results of this study are consistent with the high chlorination of these compounds and indicate their persistence and bioaccumulation in biota at the top of the food web. Overall, the contaminant profile of the peregrine eggs from Canada had higher concentrations of Dec 602 than DPMA, followed by Dec 603, whereas in the Spanish peregrine eggs, concentrations of DPMA dominated the Dec compounds. For the peregrines with a terrestrial diet, eggs from Canada had significantly higher concentrations of Dec 602, 603, and 604 than the Spanish eggs (all $P \leq 0.005$), likely reflecting the production and possible greater use of these dechloranes as flame retardants in North America than Europe.¹⁰ In Spain, only the concentrations of Dec 603 were significantly higher in the eggs laid by peregrines with an aquatic diet than those with a terrestrial diet ($F_{1,12} = 5.5$, $P = 0.04$), suggesting that exposure to these compounds on the north coast of Spain may be greater than inland but further research is required.

DP is a diadduct compound that is synthesized by the Diels–Alder process, during which DPMA may be formed through partial reactions through abiotic processes.¹¹ DPMA was analyzed and detected in 24 out of 25 peregrine eggs with concentrations from 1.7 to 469 ng/g lw and 1.2 to 1660 ng/g lw in Spanish and Canadian peregrine eggs, respectively (Table 1). Furthermore, within Spain, the peregrines with an aquatic diet had significantly higher *in ovo* DPMA concentrations than the birds with a terrestrial diet ($F_{1,11} = 17.8$, $P = 0.002$). The variation in the *in ovo* DPMA concentrations likely explains the statistical similarity between the eggs laid by peregrines with a terrestrial diet from Spain and Canada, including with the removal of the outlier egg from New Brunswick, Canada ($P = 0.11$). With the removal of this outlier, the contaminant profile of the eggs from Canada and Spain is dominated by similar concentrations of

DPMA and Mirex that are much higher than Σ DP (Table 1), although the ratio of DPMA to Σ DP is similar in the eggs from both countries ($P = 0.10$). DPMA concentrations were greater than Σ DP likely because of the smaller size and lower molecular weight of DPMA resulting in DPMA having greater bioavailability.¹¹ In comparison, mean DPMA concentrations (34 ± 43 ng/g lw) in lake trout (*Salvelinus namaycush*) from Lake Ontario were comparable to those measured in the peregrine eggs, especially from Canada, but the trout had lower mean concentrations of Σ DP (0.21 ± 0.12 ng/g lw)¹¹ especially compared to levels in the peregrine eggs from Canada.

DP may be dechlorinated into two species or moieties, aCl11DP and aCl10DP, which were previously reported in sediment samples from Lake Ontario.⁹ In the current study, analysis of the elemental composition confirmed these peaks as $C_{18}H_{13}Cl_{11}$ and $C_{18}H_{14}Cl_{10}$ that correspond with aCl11DP and aCl10DP, respectively. Based on this methodology, concentrations between 1.1 to 2.4 ng/g lw were measured for aCl10DP ($N = 11$ of 12) and aCl11DP ($N = 12$ of 12) in the peregrine eggs from Canada (Table 1). This is the first study reporting quantifiable data about two dechlorinated species of DP in a terrestrial species. However, aCl11DP was detected in only three Spanish peregrine eggs at levels 10 times lower than those measured in the Canadian peregrine eggs, and aCl10DP was not detected in any of the Spanish peregrine eggs (Table 1). This pattern likely reflects the higher Σ DP concentrations measured in the Canadian peregrine eggs.

The findings of this study confirm the presence of DP and related compounds in peregrine eggs from Canada and Spain. This is the first study to document that DP is accumulated and metabolized by peregrine falcons prior to egg laying; it is also the first study to report DP and dechloranes in European biota, as well as to report dechloranes in a terrestrial organism and an organism at the top of the food web. Compared to the eggs laid by Spanish peregrines with a terrestrial diet, the peregrine eggs from Canada had significantly higher *in ovo* concentrations of Mirex, Σ DP, Dec 602, 603, and 604, the only detectable concentrations of aCl10DP and aCl11DP, and lower f_{anti} values. The geographical differences in the measured *in ovo* concentrations between Canada and Spain reflect local exposure of the adult peregrines on the breeding grounds during egg laying, likely differences in the diet of the adult birds, the production of DP on the Niagara River in Canada (Figure SI-1), and the greater use of Mirex and dechloranes as flame retardants in North America than Europe.

■ ASSOCIATED CONTENT

Supporting Information. Figure SI-1, map of the Canadian sampling area; Figure SI-2, map of the Spanish sampling area. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (905) 336-4843. Fax: (905) 336-6430. E-mail: kim.fernie@ec.gc.ca

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Artículo científico #8:

*“Concentritions of emerging halogenated flame retardants, including enantiomeric fractions of
HBCD, in peregrine falcon (Falco peregrinus) eggs from Canada and Spain”*

por

*P. Guerra, M. Alae, B. Jimenez, G. Pacepavicius, C. Marvin, G. MacInnis, E. Eljarrat, D. Barceló,
Champoux L. y K. Fernie*

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CONCENTRATIONS OF EMERGING BROMINATED FLAME RETARDANTS, INCLUDING ENANTIOMERIC FRACTIONS OF HEXABROMOCYCLODODECANE (HBCD), IN PEREGRINE FALCON (*Falco peregrinus*) EGGS FROM CANADA AND SPAIN.

Guerra Paula¹, Alaei Mehran², Jiménez Begonia³, Pacepavicius Grazina², Marvin Chris², MacInnis Gordia², Eljarrat Ethel¹, Barceló Damia^{1,4}, Champoux Louise⁵, and Fernie Kim⁶

1- Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona, 18-26. 08034 Barcelona, Spain

2- Water Science and Technology, Environment Canada, 867 Lakeshore Road, Burlington Ontario, Canada, L7R 4A6

3- Department of Instrumental Analysis and Environmental Chemistry, Institute of Organic Chemistry, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain

4- Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, Pic de Peguera 15, 17003 Girona, Spain

5-Wildlife Toxicology and Disease, Environment Canada, Sainte-Foy, Québec, Canada G1V 4H5

6-Wildlife Toxicology and Disease, Environment Canada, 867 Lakeshore Road, Burlington, Ontario, Canada, L7R 4A6

Corresponding author.

Kim Fernie

Tel.: +19053364843; fax: +19053366430

E-mail address: kim.fernie@ec.gc.ca

ABSTRACT

Overall, in peregrine falcon eggs from Canada (N = 12) and Spain (N = 13), concentrations of sum (Σ) PBDEs (16 di- deca-BDE congeners) > Σ HBCD and were an order of magnitude higher than BB-153 > hexachlorocyclopentenyl-dibromocyclooctane (HCBDCO) > 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE) > decabromodiphenylethane (DBDPE) > octabromotrimethylphenyllindane (OBIND) > hexabromobenzene (HBB) > bis(2-ethyl-1hexyl)tetrabromophthalate (BEHTBP). This is the first report of detectable HCBDCO and BEHTBP concentrations in biota, and the highest ever *in ovo* concentration of Σ HBCD (14,617 ng/g lw; Montreal, Canada). HBB, BTBPE, and OBIND were detected in eggs from both countries, but only Canadian peregrine eggs had detectable levels of HCBDCO (25%) and DBDPE (N = 1). Σ PBDE, Σ HBCD, and BB-153 levels were significantly higher in eggs laid by peregrines likely having a terrestrial diet in Canada than Spain. The *in ovo* PBDE congener profile was dominated by BDE-153 > BDE-99 > BDE-47 > BDE-183 > BDE-100 > BDE-209, with the isomeric HBCD pattern being α -HBCD > γ -HBCD (β -HBCD undetected). Four Spanish eggs were nearly racemic (0.43 – 0.45); the Canadian peregrine eggs had lower enantiomeric HBCD values consistent with their higher fractions of (-) α -HBCD, suggesting selective enantiomeric enrichment or that the (+) α -isomer is more readily metabolized and so deposited in the egg through maternal transfer.

Keywords: HBCD, HCBDCO, BTBPE, OBIND, BEHTBP, DBDPE, PBDE, HBB, eggs, peregrine falcon

1. Introduction

Brominated flame retardants (BFRs) are added to plastics, textiles and other materials to reduce the spread of fire. Many of these compounds have been detected in biota, sediment, air, water and human milk (Covaci et al., 2006; Gauthier et al., 2008; Eljarrat et al., 2009; Fernie and Letcher 2010). Most research to date has focused on polybrominated diphenyl ethers (PBDEs) because of their wide-spread commercial use, their persistence, ability to bioaccumulate and biomagnify (Chen and Hale 2010), and their toxicological effects (e.g., (Nakari and Huhtala 2009; Chen and Hale 2010)). The production and use of PBDE formulations have been banned in Europe. In the case of North America Penta- and Octa-BDE formulations are now banned and recently, bromine science environmental forum (BSEF) member companies have decided to voluntarily phase out of Deca-PBDE formulation, by the end of 2013 (Hess 2009). Despite such regulations, PBDEs continue to leach from existing products that are in service or have been disposed of in landfills.

Hexabromocyclododecane (HBCD), one of the top three BFRs in production volume (Covaci et al., 2006), is an alternative to PBDEs for some applications although it is now regulated in Canada (Gazette 2010), the USA (USEPA 2010), and by the Stockholm Convention's Persistent Organic Pollutants Review Committee (UNEP/POPS/POPRC.6/13. 2011). Technical HBCD contains three predominant diastereoisomers α -, β - and γ -HBCD, with the γ -isomer usually dominating the commercial mixture (75 - 89%), followed by the α -isomer (10 - 13%) and β -isomer (1-12%) (Tomy et al., 2005). Moreover, α -, β - and γ - HBCD diastereoisomers are chiral, leading to three pairs of enantiomers. The three diastereoisomers of HBCD have been found in the environment (Lam et al., 2009), humans (Marvin et al., 2007) and biota (Fernie and Letcher 2010).

PBDEs have been detected in peregrine falcons (*Falco peregrinus*) at the top of the food chain (Lindberg et al., 2004; Janak et al., 2008; Johansson et al., 2009), including the highest PBDE concentrations ever reported (Chen et al., 2007; Chen et al., 2008; Johansson et al., 2009) with the presence of the highly brominated BDE-153, -183 and -209 congeners (Lindberg et al., 2004; Lam et al., 2009). While Σ PBDE concentrations in the eggs of peregrines from North America are generally higher than those from Europe, BDE-153 is usually the dominant PBDE congener (Fernie and Letcher, 2010). HBCD has also been measured in peregrines in northern Europe (Lindberg et al., 2004; Helgason et al., 2009; Johansson et al., 2009) and Canada (Fernie and Letcher, 2010), although few data are available concerning the *in ovo* HBCD isomeric pattern. However, the α -HBCD isomer dominates the eggs of peregrine falcons (Janak et al., 2008; Helgason et al., 2009) and captive American kestrels (*Falco sparverius*) that were exposed to technical HBCD dominated by γ -HBCD (Fernie et al., submitted); peregrines and kestrels are part of the *Falconidae* family.

In response to flame retardant regulations, priorities of the brominated flame retardant industry has shifted to several other brominated flame retardants (BFR) including tetrabromobisphenol A (TBBPA), hexachlorocyclopentenyl-dibromocyclooctane (HCDBCO), 1,2-bis (2,4,6-tribromophenoxy)ethane (BTBPE), bis(2-ethyl-1-hexyl)tetrabromophthalate (BEHTBP), octabromotrimethylphenylindane (OBIND), decabromodiphenylethane (DPDPE), and hexabromobenzene (HBB). While some of these new HFRs (e.g., BTBPE, DBDPE, HBB) have been detected in North American and European sediments, air (Hoh et al., 2005), house dust (Stapleton et al., 2008), and birds (Gauthier et al., 2007; Verreault et al., 2007), other new HFRs have not. The objective of this study is to investigate the occurrence of and spatial differences in isomeric and enantiomeric HBCD, the emerging BFRs (HBB, HCDBCO, BEHTBP, OBIND, DBDPE and BTBPE), 16 di- to deca-BDE congeners, and 2,2',4,4',5,5'-hexabromobiphenyl (BB-153), in peregrine falcon eggs collected in coastal and central Spain, and eastern and central Canada, including the Canadian Great Lakes Basin.

2. Material and methods

2.1. Peregrine falcon eggs.

Peregrine falcon eggs that had failed to hatch ($n=12$) were collected between 2007 and 2009 from 12 active nests located in the Canadian Great Lakes Basin and the province of New Brunswick in eastern Canada. In Spain, 13 peregrine eggs were also collected from 13 active nests in Guadalajara (Central Spain) and Bilbao (North-Cantabria Coast) from 2003 to 2006. At Canadian peregrine nest sites (this study), long-term field procedures (since 1994) include banding nestlings, and collecting fail-to-hatch eggs and prey remains, the latter then identified to species and categorized according to aquatic (e.g., gull colonies) or terrestrial (e.g., passerine species) habitats and so providing a preliminary classification of the diet of the adult peregrines laying eggs (Ferne and Letcher 2010; Guerra et al., 2011). The classification of the Spanish eggs was based on nest location: coastal-aquatic nests and inland-terrestrial nests. Because this is a preliminary classification, the results should be interpreted with caution and subsequent stable isotope analysis is recommended.

2.2. Chemicals

HBB, BB-153, BTBPE, OBIND, HCDBCO, BEHTBP and DBDPE as well as α -, β -, γ -HBCD, $d_{18}\alpha$ -, $d_{18}\beta$ - and $d_{18}\gamma$ -HBCD and, $^{13}C_{12}\alpha$ -, $^{13}C_{12}\beta$ - and $^{13}C_{12}\gamma$ -HBCD were acquired from Wellington Laboratories Inc. (Guelph, ON, Canada). Internal standards, purchased from Wellington Laboratories Inc. (Guelph, ON, Canada), included BFR standards ($^{13}C_6$ -HBB, $^{13}C_{12}$ -BB-153, $^{13}C_6$ -BTBPE, $^{13}C_{14}$ -DBDPE), PBDE surrogate standards ($^{13}C_{12}$ -BDE-3, -15, -28, -47, -77, -99, -100, -126, -153, -154, -169, -183, -197, -205, -207, -209), and PBDE recovery standards ($^{13}C_{12}$ -79, -139, -180 and -206). All solvents and other reagents were of analytical grade.

2.3. Sample Preparation

Before extraction, each egg was lyophilized, homogenized and stored at -22°C . 3-5 g dry weight (dw) of sample was spiked with $^{13}\text{C}_{12}$ -labeled BDE-3, -15, -28, -47, -77, -99, -100, -126, -153, -154, -169, -183, -197, -205, -207, -209, $^{13}\text{C}_6$ -HBB, $^{13}\text{C}_{12}$ -BB153 and $^{13}\text{C}_{12}$ -DBDPE, $^{13}\text{C}_{12}$ - α -HBCD, $^{13}\text{C}_{12}$ - β -HBCD and $^{13}\text{C}_{12}$ - γ -HBCD (Wellington Laboratories, Canada). Extraction was carried out overnight using Soxhlet extraction technique with 250 mL of dichloromethane (DCM) (Caledon Laboratories, Canada). The extract was dehydrated by passing through an Allihn funnel filled with pre-rinsed anhydrous sodium sulfate (EMD, Tracepur, GR ACS 10-60 mesh). The extracts were subsequently concentrated to approximately 2 mL, followed by lipid removal using Gel Permeation Chromatography (GPC) packed with BioBeads (SX-3, Bio-Rad Laboratories, Inc.) and eluted with a DCM:Hexane (1:1) solution (Tomy et al., 2005). Further sample clean-up and fractionation was accomplished using 100% activated silica gel column (ACP Chemicals Inc., Grad 62, 60-200 mesh). Two fractions were collected using different solvents. First fraction consisted of 60 mL of hexane:DCM (1:1) which contained PBDEs and emerging HFRs (PBEB, HBB, BTBPE, BEHTBP, DBDPE and OBIND). Second fraction contained HBCD which was eluted using 60 mL of 5% methanol in DCM. Samples were concentrated to incipient dryness and re-dissolved with 500 μL of a labeled compound mixture containing either $^{13}\text{C}_{12}$ -labeled PBDE (-79, -139, -180 and -206) in isooctane for vials with PBDEs and emerging BFRs, or d_{18} - α -HBCD, d_{18} - β -HBCD and d_{18} - γ -HBCD in methanol for vials with the HBCDs. Lipid content for each egg was determined using a gravimetric technique, and ranged from 5.2 to 8.6%.

2.4. Chemical Analysis.

2.4.1. PBDEs and emerging BFRs. PBDEs (3, 15, 28, 47, 77, 99, 100, 126, 153, 154, 169, 183, 197, 205, 207 and 209) were analyzed using isotope dilution technique. The analysis of BDEs and emerging BFRs were conducted using a Micromass AutoSpec Ultima MS (Micromass, Manchester, UK) HRMS, coupled to a Hewlett-Packard 6890 GC (Hewlett Packard, Palo Alto, CA, USA); chromatographic separation was accomplished using a 15 m DB-5HT column (0.25 mm i.d., 0.10 μm film thickness J&W Scientific, Folsom, CA). The oven temperature was set to 100°C for 2 min, raised to 250°C at $25^{\circ}\text{C}/\text{min}$, then to 260°C at $1.5^{\circ}\text{C}/\text{min}$, followed by 325°C at $25^{\circ}\text{C}/\text{min}$, and held for 10 min. The HRMS was operated in EI positive mode and the resolving power was set to $\geq 10\,000$ at 10% valley.

2.4.2. HBCD Diastereoisomer analysis.

The HBCD diastereoisomer analysis was carried out using an Agilent HP 1200 binary pump SL LC system (Agilent Technologies, Palo Alto, CA, USA). Analytical separation was accomplished using a Zorbax Eclipse C_{18} column (2.1 mm x 100mm, 3.5 μm) supplied by Agilent Technologies. Experiments were carried out using $\text{H}_2\text{O}:\text{AcN}$ (1:1 v/v) as Eluent A and methanol as Eluent B, at a flow rate of 250 $\mu\text{L}/\text{min}$. The elution program started with an initial concentration of Eluent A at

50%, decreasing to 0% over 3 minutes, and then maintained for 5 minutes. Initial conditions were reached again within 3 minutes and maintained for an additional 7 min. The injection volume was set at 3 μ L. Mass spectrometric analysis was performed using a hybrid triple quadrupole/linear ion trap AB SCIEX Triple Quad™ 5500 (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray (ESI) Turbospray interface working in negative ionization mode. For target compound quantitative analyses, the instrument was operated in selected reaction monitoring (SRM). During the course of this experiment the $[M - H]^- \rightarrow Br^-$ transitions of HBCD were monitored at m/z 640.6 $\rightarrow$ 78.9 and 640.6 $\rightarrow$ 80.9; 652.6 $\rightarrow$ 78.9 and 652.6 $\rightarrow$ 80.9, 657.6 $\rightarrow$ 78.9 and 657.6 $\rightarrow$ 80.9 were monitored for unlabelled HBCD, $d_{18}\alpha$ - $d_{18}\beta$ - and $d_{18}\gamma$ -HBCD, and $^{13}C_{12}\alpha$ -, $^{13}C_{12}\beta$ - and $^{13}C_{12}\gamma$ -HBCD respectively.

2.4.3. HBCD Enantiomeric analysis.

Nucleodex β -PM (4.0 x 200 mm x 5 μ m) a chiral chromatographic column was used for enantiomer-specific separation of HBCD isomers. Experiments were carried out using 70% H_2O : 30% MeOH as Eluent A and 70% AcN: 30% MeOH as Eluent B, at a flow rate of 0.50 mL/min. Further details of the HBCD enantiomeric analysis are provided elsewhere (Guerra et al. 2008). Enantiomeric compositions were expressed as corrected enantiomeric fractions (EF). These corrections were based on the use of isotopic labeled standards since their enantiomeric analogues behave in an identical manner to their native counterparts (Marvin et al., 2007).

2.5. QA/QC.

The identification and quantification of HBB, HCDBCO, BEHTBP, OBIND, DBDPE, BTBPE, BB153, PBDEs, and α -, β - and γ -HBCD, were based on the following criteria: (a) sample peaks had an approximately Gaussian shape, and their chromatographic retention times matched those of the standard and isotope labeled compounds within ± 2 sec; (b) all peaks had a signal-to-noise ratio greater than 5:1; (c) peak areas were within 15% of the corresponding theoretical isotopic ratios. Method blank samples were run every five samples to check for interference or contamination from solvents and glassware. The presence of analytes of interest in blanks was not observed in the majority of cases and were corrected for where they were observed. The limits of detection (LOD) were between 0.09 and 2.2 ng/g lipid weight (lw), and limits of quantification (LOQ) from 0.31 to 6.9 ng/g lw were obtained. The recovery efficiencies for HBCDs, emerging BFRs and PBDEs were calculated by obtaining values from 52 to 64%, 51 to 85% and 48 to 92% of recovery, respectively.

2.6. Statistical analysis.

The limited sample size of eggs collected in Canada with birds likely having an aquatic-based diet ($N = 2$) restricted comparisons between Canada and Spain to eggs laid by peregrines likely having a terrestrial-based diet only. Similarly, potential dietary differences were restricted

to *in ovo* concentrations based on eggs from Spain only. The concentrations of all of the measured compounds were not normally distributed and could not be log-transformed successfully. Consequently, non-parametric statistical analysis was used to identify significant differences concerning *in ovo* contaminant concentrations. Statistical comparisons were not performed on the concentrations of BDE-3, -15, -28, -77, -169 and -205, or HCBCO, BEHTBP, OBI, DBDPE, or BTBPE due to the lack of quantifiable or detectable concentrations. Exceedingly high concentrations of multiple contaminants were measured in eggs C1 (from New Brunswick, Canada) and C7 (Etobicoke, Canada); as statistical outliers, they were excluded from all of the statistical analyses. Geometric means are presented in the tables but arithmetic means were used in calculating statistical significance ($P \leq 0.05$).

3. Results and discussion.

3.1. Concentration levels.

The *in ovo* concentrations of HBB, BB-153, HCDBCO, BEHTBP, OBIND, DBDPE, BTBPE, and the summed concentrations of the detected HBCD stereoisomers (Σ HBCD) and 16 PBDE congeners (Σ PBDE), in peregrines from Canada and Spain are summarized in Tables 1, 2 and 3. In general, the concentrations of these compounds were higher in the peregrine eggs from Canada than the eggs from Spain. Of the 25 peregrine eggs from both countries, Σ HBCD concentrations were detected in 80% of the samples, with the highest ever *in ovo* concentration reported to date in an egg from Montreal, Canada (15000 ng/g lw). The Σ PBDE concentrations, measured in all 25 peregrine eggs, ranged from 160 to 40 000 ng/g lw and were approximately double the Σ HBCD concentrations. Peregrine eggs from Canada had greater Σ PBDE concentrations than eggs from Spain, possibly reflecting the greater usage and release of PBDEs in North America than Europe. The Σ PBDE concentration found in one peregrine egg (C1) from eastern Canada (New Brunswick; 38000 ng/g lw) was half that of the highest Σ PBDE concentration reported in biota (72000 ng/g lw), specifically in peregrine eggs from the Northeastern U.S. (Chen et al., 2008).

3.2. Emerging, alternative BFRs currently in use

This is the first report of measurable concentrations of HCDBCO (nd - 23 ng/g lw) or BEHTBP (nd - 4.5 ng/g lw) in biota and the environment, respectively. The HCDBCO concentrations were detectable in three (25%) of the 12 peregrine eggs from Canada only, specifically from the two cities of Toronto and Thunder Bay, and the remote north shore of Lake Superior (Table 1). HCDBCO was measured at much higher concentrations in indoor air and house dust, with concentrations from nd - 3000 and nd - 93 000 ng/g lw, respectively (Zhu et al., 2008; Lam et al., 2009). The *in ovo* BEHTBP concentrations were detected in 1 egg from coastal Bilbao, Spain and 4 eggs from Toronto (N = 2) and Montreal (N = 2), Canada.

OBIND is a particularly useful flame retardant in styrenics and engineering thermoplastics because it results in high flow properties (ICL Industrial Products 2011). This study is also the first to report OBIND concentrations in peregrine eggs in North America and Spain (Table 1), although OBIND was first reported at lower concentrations in the egg of a white stork (*Ciconia ciconia*) from Spain (~0.2 ng/g lw) (Muñoz-Armanz et al., 2010). In this study, most *in ovo* OBIND concentrations, measured in 38% (5 of 13) of Spanish peregrine eggs and 33% (4 of 12) of Canadian peregrine eggs, ranged from 0.79 to 2.2 ng/g lw. However, one of the Canadian peregrine eggs (C12) had much higher OBIND concentrations (29 ng/g lw) and was collected from an active nest near Montreal, Quebec. The higher *in ovo* concentrations of OBIND in the peregrines compared to the white storks in Spain, may reflect differences in their respective trophic positions in the food chain, with storks at a lower trophic level than peregrine falcons. In Spain, white storks consume a diet of crayfish, fish, arthropods, meat scraps from refuge dumps, and earthworms (Negro et al., 2000) compared to peregrines that consume a diet consisting largely of other bird species.

DBDPE is an additive BFR and is marketed as an alternative to technical Deca-BDE (Nakari and Huhtala 2009). Previously, DBDPE was detected in air from the Great Lakes Basin (Venier and Hites 2008) and south China (Shi et al., 2009), sludge (Ricklund et al., 2008), sediment (Ricklund et al., 2010), and in fish from Lake Ontario (Ismail et al., 2009) and Lake Winnipeg. DBDPE was also detected in herring gull eggs throughout the Canadian Great Lakes (26 - 5760 ng/g lw) from 1996 onwards, and at some sites, DBDPE exceeded BDE-209 concentrations (Gauthier et al., 2009). Surprisingly, in the present study, only one peregrine egg had a measurable DBDPE concentration (8.2 ng/g lw) and this egg was from eastern Canada (New Brunswick) not from the same Great Lakes Basin as in Gauthier et al. (2009). The highest *in ovo* DBDPE levels for the herring gulls were measured in the eggs of gulls that consumed more mammalian prey than the gulls with a pure aquatic diet (Gauthier et al., 2009). The one peregrine egg with measurable DBDPE concentrations was laid by a peregrine likely having a terrestrial diet. We speculate that the detection of DBDPE concentrations in gull eggs throughout the Great Lakes in contrast to its detection in only one peregrine egg may be a function of the mammalian prey of the gulls possibly being direct consumers of larger concentrations of DBDPE in contrast to the avian prey of the peregrines.

BTBPE has been produced since the 1970s and is now used as a replacement for OctaBDE (Hoh et al., 2005). Between 1982 and 2007, the highest *in ovo* BTBPE concentrations in the Great Lakes Basin were from herring gulls nesting on the Niagara River (~ 0.94 - 14 ng/g lw) (Gauthier et al., 2007) and were primarily detected in eggs collected from the mid-1990s to 2006 (~ <1.2 - 4.0 ng/g lw) (Gauthier et al., 2009). In the marine environment, BTBPE was also found in the eggs and plasma of glaucous gulls (*Larus hyperboreus*) from the Norwegian Arctic (~ <4.0 - 19.2 ng/g lw) (Verreault et al., 2007) and in fulmar eggs (*Fulmaris glacialis*) from the

Faroe Islands ($< 0.02 - 0.17$ ng/g lw) (Karlsson et al., 2007). BTBPE (nd - 14 ng/g lw) was detected in only four of the peregrine falcon eggs (16%), specifically from Toronto (13 ng/g lw), the remote north shore of Lake Superior (14 ng/g lw), and Bilbao, Spain (3.3 and 7.4 ng/g lw). The two peregrine eggs from Canada with measureable concentrations of BTBPE (C7, C9) are 2 of the 3 eggs that had measurable concentrations of HCBCO. The *in ovo* concentrations of BTBPE are similar in the peregrine falcons, gulls (Verreault et al., 2007; Gauthier et al., 2009) and fulmars (Karlsson et al., 2007) regardless of their location in Europe or North America.

3.3. Historically-used BFRs

BB-153 was an important BFR produced and used primarily in the 1960s but due to an incident in Michigan, the sole USA manufacturer of hexabromobiphenyl ceased production in 1974. In Europe BB-153 was banned in 1984. However, despite this long-term ban, BB-153 was detected in all of the peregrine eggs from Canada and Spain. In the eggs laid by peregrines likely having a terrestrial diet, the mean *in ovo* concentrations of BB-153 were significantly and 10x higher in Canada ($12 - 1271$ ng/g lw) than Spain ($\chi^2 = 9.38$ df = 1 P = 0.002) ($3.6 - 11$ ng/g lw) (Table 1). In Spain, the peregrine eggs laid by birds with an aquatic diet ($11 - 67$ ng/g lw) were significantly higher than the birds with a terrestrial diet ($\chi^2 = 8.19$ df = 1 P = 0.004) (Table 1, 2). The BB-153 *in ovo* concentrations in the peregrines from Spain are considerably lower than levels reported in peregrines from Sweden ($0.73 - 370$ ng/g lw) (Johansson et al., 2009) or Greenland ($64 - 14\,000$ ng/g lw) (Vorkamp et al., 2005), while the *in ovo* BB-153 levels measured in the Canadian peregrines have a greater but overlapping range compared to peregrines from the mid-Atlantic states of the U.S.A. (118 ng/g lw - 1036 ng/g lw) (Potter et al., 2009).

HBB was used as an additive FR in plastics, textiles and woods (Yamaguchi et al., 1988), as well as paper, electrical and manufactured products (Watanabe et al., 1986). Historically, HBB was produced in the Great Lakes Basin by Velsicol Chemical Corporation (St Louis, MI) although production at this site stopped during the 1980s. While it is currently produced by Nippoh Chemicals (Chiba, Japan) and Dayang Chemicals (Hangzhou City, China) (Gauthier et al., 2007), the migratory patterns of peregrines in this study make it extremely unlikely that they inhabited China. Other sources of HBB in the environment result from thermal degradation of Octa- and Deca-BDE (Buser 1986). HBB concentrations were detected in 52% peregrine eggs from both Spain and Canada (Table 1). Much higher concentrations of HBB were detected in the egg yolk of glaucous gulls (*Larus hyperboreus*) from the Norwegian Arctic ($544 - 680$ ng/g lw) (Verreault et al., 2007), reflecting the higher lipid content of egg yolk compared to the whole egg. In North America, *in ovo* HBB levels of herring gulls (*Larus argentatus*) from the Great Lakes (2004: $\sim 4.8 - 10$ ng/g lw; 1982-2006: $0.2 - 78$ ng/g lw) (Gauthier et al. (2007) were similar to those in the peregrines from the same Laurentian Great Lakes as well as New Brunswick ($4.1 - 7.2$ ng/g lw).

3.4. Recently regulated BFRs

3.4.1. Hexabromocyclododecane (HBCD)

We report the highest ever *in ovo* Σ HBCD concentrations for peregrine falcons (15000 ng/g lw; Montreal, Canada, 2007), and this is the first study to measure HBCD concentrations in peregrine falcon eggs in North America. Concentrations of Σ HBCD (0.9 – 15000 ng/g lw) were detected in 80% of the peregrine eggs (Table 2). While the *in ovo* Σ HBCD levels of peregrines in Spain (0.90 – 1600 ng/g lw) were similar to concentrations (nd – 2,400 ng/g lw) for peregrines in Sweden (Lindberg et al., 2004; Johansson et al., 2009) and Greenland (Vorkamp et al., 2005), 50% of the peregrine eggs from Canada (N = 12) had higher *in ovo* concentrations (219 – 15000 ng/g lw). Compared to the peregrine eggs collected from Spain, the eggs laid by Canadian peregrines likely having a terrestrial diet had marginally higher concentrations of Σ HBCD ($\chi^2 = 3.56$ df = 1 P = 0.0593), significantly higher concentrations of γ -HBCD ($\chi^2 = 3.75$ df = 1 P = 0.0528), but comparable levels of α -HBCD (P = 0.22). Within Spain, the *in ovo* Σ HBCD concentrations were statistically similar between the inland peregrines in Guadalajara likely having a terrestrial diet (N = 5; 0.90 – 340 ng/g lw) and the coastal Bilbao birds with a likely aquatic diet (N = 8; 0.90 – 1600 ng/g lw) (P = 0.4623); these small sample sizes contributed to the large variation in Σ HBCD concentrations and likely the lack of statistical significance, suggesting the need for future investigation regarding the potential influence of dietary differences.

Currently, there is little information about isomer-specific HBCD levels in bird eggs although several studies indicate that α -HBCD is the dominant HBCD isomer measured in eggs. Braune et al. (2007) reported α -HBCD concentrations in Ivory gull (*Pagophila eburnea*) eggs from the Canadian Arctic (2.1-3.8 ng/g lw), although higher α -HBCD concentrations were reported in the eggs of Herring gulls (9-108 ng/g lw), Black kittiwakes (*Rissa tridactyla*) (26-142 ng/g lw), and Atlantic Puffins (*Fratercula arctica*) (4-58 ng/g lw) from Norway (Helgason et al., 2009). The α -HBCD concentrations reported in the eggs of these aquatic birds are lower than the *in ovo* α -HBCD concentrations laid by peregrines having an aquatic diet in Spain (N = 8; 47 – 1600 ng/g lw) or Canada (N = 2; 350 - 3400 ng/g lw), likely a reflection of the higher trophic position of the peregrines compared to these other species.

In the peregrine eggs, α -HBCD dominated γ -HBCD but β -HBCD was not quantifiable (Table 2). This distribution is consistent with reports for other wildlife (Janak et al., 2008; Brandsma et al., 2009), including the eggs of captive kestrels exposed to the technical HBCD mixture (Ferne et al., submitted). The isomer pattern in the peregrine (and kestrel) eggs strongly contrasts with the technical product formulation in which γ -HBCD is the dominant isomer, and may reflect bioisomerization of β - and γ -HBCD to α -HBCD, less efficient absorption or preferential metabolism of β - and γ -HBCD in contrast to α -HBCD (Janak et al., 2008) by peregrines.

Corrected EF values for α -HBCD were calculated for 7 of the peregrine eggs from Spain, 11 eggs from Canada, and for the commercial standards (Figure 1), using the isotopic dilution method (Marvin et al., 2007). The calculated EF values for the Spanish peregrine eggs ranged from 0.05 to 0.93 (Table 2), with four eggs being nearly racemic (0.43 - 0.45) and two eggs representing extreme EF values. Generally, the EF values for the Spanish peregrine eggs were consistently higher than the *in ovo* α -HBCD EF values (0.17 and 0.34) for peregrines in Sweden Janak et al., (2008). In the Canadian peregrine eggs, the EF values were lower, ranging from 0.164 to 0.324, consistent with the higher fractions of (-) α -HBCD in the same eggs, and similar to the EF values in Swedish peregrine eggs Janak et al., (2008). The results for the Canadian peregrine eggs are consistent with peregrine falcons more readily metabolizing the (+) α -HBCD enantiomer than the (-) α -HBCD enantiomer (Janak et al., 2008), and, may also indicate selective enantiomeric enrichment or metabolization of (+) α -isomer by the female, or preferential maternal deposition of the (+) α -isomer in the egg. Differences in EF values were observed between peregrine populations in Sweden (Janak et al., 2008), as generally they are here between the Canadian and Spanish peregrine populations (Figure 1); such differences in EF values may reflect dietary differences (Janak et al. 2008), but small sample sizes in both studies warrant further research to identify the possible role of dietary differences relative to enantiomeric enrichment.

3.4.2. PBDEs.

Of the 16 PBDE congeners analyzed in our study, all of the PBDE congeners, ranging from mono- to deca- BDE, were detected and BDE-99, -100, -153 and -183 were detected in all of the peregrine eggs (Table 3). BDE-153 was the dominant BDE congener in more than 60% of the peregrine eggs from Canada and Spain. Two of the peregrine eggs from Canada exhibited particularly high Σ 16PBDE levels of 17000 and 38000 ng/g lw (Table 3). For the peregrines with a terrestrial-based diet, the eggs from Spain had significantly lower Σ PBDE concentrations ($N = 5$; 160 – 3600 ng/g lw) than the peregrine eggs from Canada ($N = 10$; 530 – 38000 ng/g lw) ($\chi^2 = 6.0$ df = 1 $P = 0.0143$), Sweden (29 – 39000 ng/g lw), Greenland (300 – 12900 ng/g lw) or the USA (811 – 72,000 ng/g lw) (Lindberg et al., 2004; Vorkamp et al., 2005; Chen et al., 2008; Johansson et al., 2009). The significantly higher PBDE concentrations of the Canadian peregrine eggs likely reflect the greater use and release of PBDE flame retardants in North America. Similarly, the Σ PBDE levels of peregrine eggs from California (80 - 53100 ng/g lw) (Park et al., 2010) and the Northeast USA (811 - 72000 ng/g lw) (Chen et al., 2008; Park et al., 2009; Chen et al., 2010) exceeded those in peregrine falcon eggs from Sweden and Greenland (Lindberg et al., 2004; Vorkamp et al., 2005; Johansson et al., 2009).

In this study, the PBDE congener pattern in the peregrine eggs, regardless of the country of origin, was BDE-153 > BDE-99 > BDE-47 > BDE-100 > BDE-183 (Figure 2), constituting $65.8 \pm 24\%$ and $85.0 \pm 7.8\%$ of the Σ PBDE for Spain and Canada, respectively. The

concentrations of each of these individual congeners were significantly greater in the peregrine eggs laid by birds with a terrestrial diet from Canada than from Spain (all P-values ≤ 0.0492), and in the eggs laid by Spanish peregrines with an aquatic diet than those with a terrestrial diet (all P-values ≤ 0.0190) although concentrations of BDE-47 were similar. Similar congener profiles, including the dominance of BDE-153, were also found in wild peregrine eggs from Sweden (Johansson et al., 2009) and in the U.S.A. (Chen et al., 2008). In the plasma of peregrine falcon nestlings in the Canadian Great Lakes Basin, the same geographical region as that from which eggs were collected in this study, the profile of major PBDE congeners consisted of BDE-99 > BDE-153 > BDE-47 > BDE-100 > BDE-183 (Femie and Letcher 2010), generally consistent with the results in this study. The dominance of BDE-153 in peregrine eggs may reflect the longer half-life of BDE-153 compared to other PBDE congeners in kestrels (Johansson et al., 2009), or the significant biomagnification but low biotransformation of BDE-153 compared to BDE-47 in terrestrial birds of prey (Chen and Hale 2010; Chen et al., 2010).

4. Conclusions

The results of this study show the presence of emerging HFRs in peregrine falcon eggs from Canada and Spain, with Σ PBDEs > Σ HBCD > BB-153 > HCBDCO > BTBPE > DBDPE > OBIND > HBB > BEHTBP. This is the first study to document the presence of HCBDCO, BEHTBP and OBIND in peregrine falcon eggs, and reports the highest *in ovo* Σ HBCD concentration in peregrine eggs to date (an egg collected in Montreal, Canada). The *in ovo* HBCD isomer pattern was dominated by α -HBCD > γ -HBCD (β -HBCD undetected). The peregrine eggs from Canada had lower enantiomeric HBCD values consistent with their increased (-) α -HBCD proportions, possibly reflecting selective enantiomeric enrichment or that the (+) α -isomer is more readily metabolized by peregrines.

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FIGURE CAPTIONS

Figure 1. α -HBCD EF values in standards (mean $\pm$ standard deviation) and peregrine eggs.

Figure 2. Mean relative contribution of some individual PBDE congeners to the Σ PBDE concentration detected in peregrine eggs from Canada and Spain with terrestrial (T) or aquatic (A) feed.

Table 1. Overall *in ovo* concentrations of HCDBCO, BEHTBP, OBIND, DBDPE, BTBPE BB-153 and HBB in peregrine falcon eggs from Canada (Great Lakes Basin, New Brunswick) and coastal and central Spain, 2007 - 2009. LOD: limit of detection; LOQ: limit of quantification; nd: not detected; T: terrestrial; A: aquatic; HBB: hexabromobenzene, BB-153: 2,2',4,4',5,5'-hexabromobiphenyl, HCDBCO: hexachlorocyclopenthenyl-dibromocyclooctane, BEHTBP: Bis (2-ethyl-1-hexyltetra bromophenyl ether, OBIND: octabromotrimethylphenylindane, DBDPE: decabromodiphenylethane, BTBPE: 1,2-Bis(2,4,6-Tribromophenoxy)ethane.

		HCDBCO	BEHTBP	OBIND	DBDPE	BTBPE	BB-153	HBB
		ng/g lw	ng/g lw	ng/g lw	ng/g lw	ng/g lw	ng/g lw	ng/g lw
	LOD	2.2	0.6	0.21	1.1	0.67	0.49	0.09
	LOQ	6.9	1.0	0.53	5.2	2.2	1.7	0.31
Canada Aquatic	N							
	Geometric mean	17		N.A.	N.A.	14	57	N.A.
	Median	13				8	230	
	Maximum	19				14	450	
	Minimum	nd				nd	nd	
	Detected in	1	0	0	0	1	2	0
Canada Terrestrial	N							
	Geometric mean	21	1.8	4.2	8.2	13	192	6.1
	Median	3.5	0.5	0.27	6.7		154	4.8
	Maximum	23	4.5	29	8.2		1300	7.2
	Minimum	nd	nd	nd	nd		12	nd
	Detected in	2	4	4	1	0	10	6
Spain Aquatic	N							
	Geometric mean	N.A.	1.7	0.04	N.A.	3.0	7.4	0.81
	Median		1.1	0.27		5.1	23	0.35
	Maximum		1.2	1.0		7.4	67	0.99
	Minimum		nd	nd		nd	11	nd
	Detected in	0	1	3	0	1	8	4
Spain Terrestrial	N							
	Geometric mean	N.A.	N.A.	0.81	N.A.	N.A.	7.1	1.8
	Median			0.53			8.0	1.5
	Maximum			0.84			11	3.1
	Minimum			nd			3.6	nd
	Detected in	0	0	2	0	0	5	3

Table 2. Overall *in ovo* concentrations of α -HBCD, γ -HBCD and Σ HBCDs in peregrine falcon eggs from Canada (Great Lakes Basin, New Brunswick) and coastal and central Spain, 2007 - 2009. LOD: limit of detection; LOQ: limit of quantification; nd: not detected; T: terrestrial; A: aquatic. Σ HBCDs: total HBCD.

		Σ HBCD	α -HBCD	γ -HBCD	EF α -HBCD
		ng/g lw	ng/g lw	ng/g lw	ng/g lw
LOD			0.08	0.06	
LOQ			0.14	0.1	
Canada Aquatic	N	2	2	2	2
	Geometric mean	1100	1100	6.3	0.20
	Arithmetic Mean	1900	1800	6.4	0.21
	SEM	1500	1500	1.3	0.04
	Median	1900	1800	6.4	0.21
	Maximum	3300	3300	7.7	0.25
	Minimum	350	350	5.1	0.16
	Detected in	2	2	2	
Canada Terrestrial	N	9	9	5	9
	Geometric mean	2300	2200	18	0.24
	Arithmetic Mean	4100	4000	26	0.24
	SEM	1500	1500	6.5	0.02
	Median	2800	2800	29	0
	Maximum	15000	15000	39	0.32
	Minimum	220	170	1.9	0.16
	Detected in	9	9	5	
Spain Aquatic	N	7	6	2	6

18

Geometric mean		170	400	3.2	0.26
Arithmetic Mean		570	660	6.0	0.44
SEM		220	240	5.1	0.12
Median		300	560	6.0	0
Maximum		1600	1600	11	0.93
Minimum		0.91	47	0.91	0.01
Detected in		7	6	2	
Spain Terrestrial	N	2	1	2	1
	Geometric mean	18	340	1.2	0.45
	Arithmetic Mean	170	340	1.2	0.45
	SEM	170	.	0.30	.
	Median	170	340	1.20	
	Maximum	340	340	1.5	0.45
	Minimum	0.91	340	0.91	0.45
	Detected in	2	1	2	

Table 3. Overall *in ovo* concentrations of BDE-47, BDE-99, BDE-100, BDE-153, BDE-183, BDE-197, BDE-207 and BDE-209 in peregrine falcon eggs from Canada (Great Lakes Basin, New Brunswick) and coastal and central Spain, 2007 - 2009. LOD: limit of detection; LOQ: limit of quantification; nd: not detected; T: terrestrial; A: aquatic, Σ PBDEs: total PBDEs.

		Σ PBDE ng/g lw	BDE47 ng/g lw	BDE99 ng/g lw	BDE100 ng/g lw	BDE153 ng/g lw	BDE154 ng/g lw	BDE183 ng/g lw	BDE197 ng/g lw	BDE207 ng/g lw	BDE209 ng/g lw
	LOD		0.041	0.03	0.02	0.05	0.08	0.06	0.06	0.05	0.07
	LOQ		0.12	0.09	0.05	0.09	0.12	0.58	0.11	0.09	0.13
Canada Aquatic	N	2	2	2	2	2	0	2	2	2	2
	Geometric mean	3300	20	180	68	1600	N.A.	950	280	92	77
	Arithmetic Mean	4400	10	330	160	2000	.	1200	520	140	91
	SEM	3000	10	270	150	1200	.	720	440	100	49
	Median	4400	20	330	160	2000	.	1200	520	140	91
	Maximum	7300	20	600	310	3200	.	1900	950	240	140
	Minimum	1500	nd	56	15	780	.	470	79	36	42
	Detected in		1	2	2	2	0	2	2	2	2
Canada Terrestrial	N	10	10	10	10	10	6	10	9	8	10
	Geometric mean	4200	680	650	380	1400	240	110	160	140	140
	Arithmetic Mean	8300	2600	1400	940	2400	340	210	250	170	220
	SEM	3600	1600	600	440	840	120	61	58	34	48
	Median	4300	640	370	230	1800	280	160	290	180	270
	Maximum	38000	16000	6000	4500	9200	850	540	490	280	400
	Minimum	530	0	190	64	160	48	7.0	10	33	15
	Detected in		8	10	10	10	6	10	9	10	10
Spain Aquatic	N	8	8	8	8	8	8	8	7	8	6
	Geometric mean	1600	N.A.	200	68	480	100	120	44	41	85
	Arithmetic Mean	2200	39	200	70	560	110	250	110	200	620
	SEM	680	13	9.6	7.1	130	9.4	120	55	120	380
	Median	1200	63	190	69	380	110	65	32	20	57
	Maximum	5300	100	250	110	1200	140	910	360	750	2000
	Minimum	710	0	170	47	250	65	35	8.6	7.0	2.0
	Detected in		5	8	8	8	8	8	7	6	6
Spain Terrestrial	N	5	5	5	5	5	5	5	5	5	5
	Geometric mean	430	17	33	10	87	16	21	6.6	11	13
	Arithmetic Mean	950	15	34	10	89	17	28	9.5	640	26
	SEM	670	5.1	3.4	1.2	11	2.2	12	4.9	630	18
	Median										
	Maximum	3595	27	41	14	120	25	78	29	3200	100
	Minimum	162	0	22	6.3	68	13	14	3.9	1.5	7.5
	Detected in		4	5	5	5	5	5	5	5	5

Figure 1

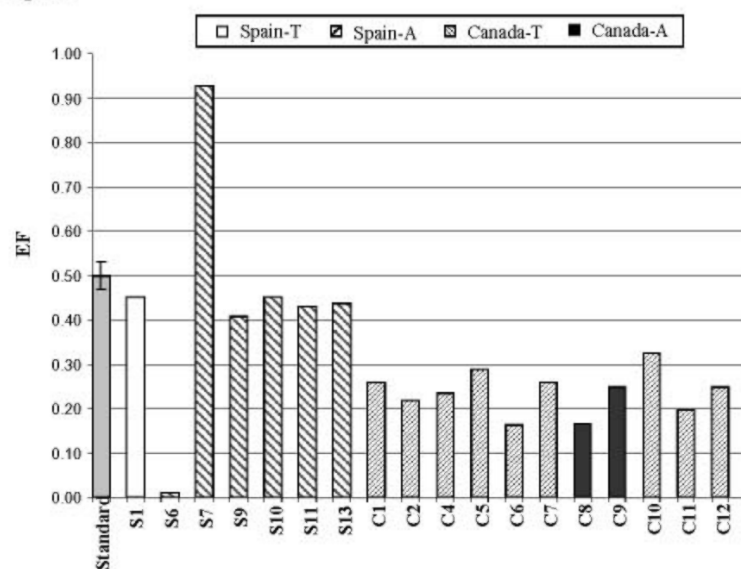
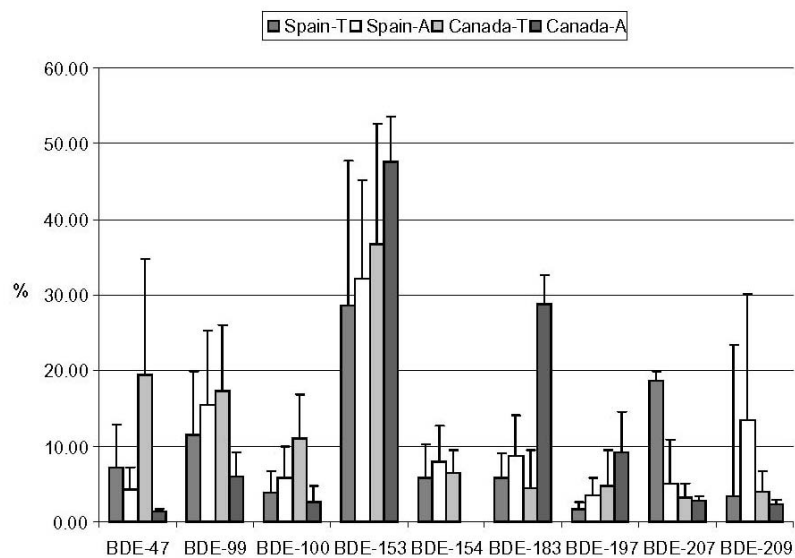


Figure 2.



3.2. Discusión

En el desarrollo del presente Capítulo hemos evaluado la presencia de HFRs emergentes en el medio acuático y terrestre sabiendo que su consumo aumentará con el tiempo, por lo tanto será necesario un mayor conocimiento sobre su comportamiento y destino.

Teníamos antecedentes de la contaminación del río Cinca por FR a partir de un estudio realizado en 2002, en el cual se detectó la presencia de dos familias de compuestos, los PBDEs y el HBCD en sedimentos y peces, mediante el uso de GC-MS (Eljarrat et al., 2004; Eljarrat et al., 2005). Los resultados obtenidos en el muestreo de Octubre de 2002 mostraban niveles de FR bajos en la zona de aguas arriba de Monzón, mientras que los niveles aguas abajo de Monzón eran considerablemente elevados y, especialmente los correspondientes al HBCD.

Una vez que desarrollamos las metodologías de análisis de HBCD utilizando LC-MS/MS nos ha parecido interesante realizar un estudio en muestras de sedimentos y biota procedentes del río Cinca, dentro del proyecto AQUATERRA que se resume en el artículo #5.

Los sedimentos fueron muestreados a lo largo del río Cinca en junio de 2006. Analizamos el HBCD y encontramos una distribución similar al estudio anterior, donde la mayor concentración de HBCD está en la zona industrial (Monzón), y va disminuyendo aguas abajo y, a unos 70 Kms (Fraga) la contaminación ha desaparecido (Tabla 3-1). Es interesante observar el patrón isomérico de HBCD en estas muestras donde predomina γ -HBCD, tal como sucede en la mezcla industrial.

Tabla 3-1. HBCD (ng/g dw) en sedimentos del río Cinca

	Km*	α -HBCD	β -HBCD	γ -HBCD	HBCD total
Puente las Pilas	-20	nd	nd	nd	nd
Monzón	0	30	nd	2251	2281
Alcolea de Cinca	27	nq	nd	37	125
Fraga	67	nq	nd	nq	nq

nd: bajo el límite de detección, nq: bajo el límite de cuantificación, * distancia desde punto de contaminación

En el artículo #5 nos interesaba identificar las posibles fuentes de contaminación de la cuenca en estudio por HBCD. Dado que existían dos industrias sospechosas, en colaboración con la confederación hidrográfica del Ebro (CHE) obtuvimos muestras de los efluentes industriales. Así, hemos comprobado el uso de HBCD en España al encontrarlo en el efluente de una industria productora de poliestireno expandible (EPS, del inglés *expandable polystyrene*) con un patrón isomérico similar al de la mezcla comercial. Además, el muestreo del efluente de la industria sospechosa de causar la contaminación, se volvió a recoger unos meses después a fin de evaluar si la

primera analítica correspondía a un vertido puntual o por el contrario, se trataba de un vertido continuo. El análisis de esta segunda muestra de efluente industrial confirmó el uso continuado de HBCD por parte de la industria.

Por otro lado, se analizaron muestras de peces procedentes de los diferentes puntos de muestreo. En 6 muestras de madrillas (*Southwestern nase*) procedentes de aguas abajo de la zona de Monzón se encontró la siguiente distribución de HBCD (Figura 3-3). Como puede observarse, γ -HBCD predomina en todas las muestras, pero hay una diferencia en la distribución de los isómeros de HBCD entre sedimentos y biota. Mientras que la distribución en el sedimento es similar a la mezcla comercial de HBCD, en los peces se observa un cambio al incrementarse el contenido relativo de los isómeros α - y β -HBCD, disminuyendo el aporte de γ -HBCD. Este fenómeno ha sido observado anteriormente en muestras de biota procedentes de otras zonas geográficas y podría deberse a las diferentes propiedades fisicoquímicas de los isómeros. Así, podría ser consecuencia de:

- α -HBCD es más biodisponible que γ -HBCD
- γ -HBCD se transforma en α -HBCD debido a reacciones metabólicas
- γ -HBCD tiene una depuración más rápida que α -HBCD

Cabe destacar que normalmente en muestras biológicas se observa que la proporción de α -HBCD aumenta, superando incluso a la de γ -HBCD. En nuestro caso, considerando que los peces están expuestos a una aportación constante de la mezcla industrial de HBCD, hay una variación menor de las proporciones isoméricas.

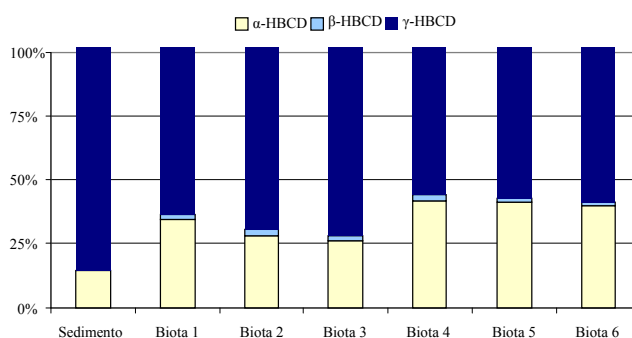


Figura 3-3. Distribución de los diastereoisómeros de HBCD en peces y sedimento de Monzón.

También se realizó un experimento en el campo para el que se cogieron muestras de barbos (*Barbus graellsii*) de los canales del delta del Ebro (teóricamente libres de contaminación por HBCD) y se repartieron en dos jaulas, cada una con aproximadamente 20 peces. Una jaula fue colocada aguas arriba del foco de contaminación (La Boquera), mientras que la otra se colocó aguas debajo de

Monzón (Alcolea de Cinca). La exposición duró 14 días y luego del análisis de las muestras (Tabla 4 del artículo) vimos que en las muestras ubicadas aguas abajo llegaron a acumular 15.500 ± 2.700 ng/g lw de HBCD, demostrándose así la biodisponibilidad del HBCD.

Por otro lado, llevamos a cabo un experimento en el laboratorio con el que se pretendía obtener información acerca de la depuración de cada uno de los isómeros del HBCD. Para ello se expusieron zebra fish al efluente industrial diluido durante 12 días. Posteriormente, los zebra fish se depuraron por 12, 21 y 28 días. Así se observó que la exposición al efluente contaminado provoca la incorporación de HBCD en los organismos. También se detectó que HBCD se depura con el tiempo y, en el caso de γ -HBCD esta depuración resultó más rápida que para el resto de isómeros (Figura 5 del artículo #5). Este fenómeno podría afectar la variación en la proporción de isómeros que se observa entre muestras ambientales (sedimento) y biota (peces), tal como explicamos anteriormente en el artículo #4.

Por otro lado, en sedimentos de la cuenca del río Llobregat (artículo #6), se han analizado los PBDEs así como tres BFRs emergentes que se utilizan sin restricciones, ya que no han sido legislados. Hemos analizado los niveles de PBDEs mediante GC-NCI-MS, encontrando niveles similares a los de un trabajo que realizamos en sedimentos costeros procedentes de Cantabria y el País Vasco (Tabla 3-2).

En lo que se refiere a BFRs emergentes, podemos destacar la presencia del DBDPE que se encontró con mayor frecuencia en las muestras analizadas de Cataluña. Las concentraciones encontradas en el río Llobregat son inferiores a las de BDE-209, al mismo tiempo que su ubicuidad es inferior a la de los PBDEs (Tabla 3-2).

Tabla 3-2. Comparación de concentraciones de BFRs en sedimentos de España.

	Llobregat	Anoia	Cantabria	P. Vasco
Total PBDEs	22-136	26-111	67-90	59-150
BDE-209	5.2-82	3.2-57	21-30	20-28
PBEB	5.5-9.1	nd-9.6	nd	nd
HBB	nd-2.4	nd-0.4	nd	nd
DBDPE	4.8-23	9.1-24	nd	nd

De esta forma hemos comprobado la utilización de DBDPE en Cataluña, ya que es sabido que DBDPE sustituye al DecaBDE, por lo tanto es de esperar que su concentración en el medio ambiente pueda aumentar luego de la prohibición del uso de esta mezcla de PBDEs. Respecto a HBB y PBEB, se encontraron en los sedimentos del Llobregat y Anoia, mientras que en las muestras del norte de España no fueron detectados.

Una vez observada la presencia de DBDPE, HBB y PBEB en el medio ambiente fue necesario evaluar su biodisponibilidad. Es por ello que se analizó la presencia de estos 3 BFRs emergentes en huevos de halcón peregrino (artículo #8). Así, pudimos observar que en las muestras de España no se detectaron DBDPE y PBEB, mientras que HBB fue detectado en el 54% de las muestras con una concentración máxima de 3,1 ng/g lw.

Para poder llegar a conclusiones respecto a la biodisponibilidad de estos BFRs emergentes sería necesario realizar muestreos más amplios. Aunque por lo que se refiere a la estructura molecular, es de esperar que el DBDPE, de forma similar a lo que ocurre con BDE-209, sea menos biodisponible por su gran tamaño molecular.

Por otro lado, en las muestras de huevos de halcón procedentes de Canadá no se encontró PBEB, aunque se observa que las concentraciones de DBDPE y HBB son mayores a las halladas en España. En el artículo #8 también fue posible analizar otros HFRs que la industria ha introducido recientemente en respuesta a las regulaciones de los BFRs más utilizados, estos son: BTBPE, BEHTBP, HCDBCO y OBIND.

También analizamos HBCD isomérico y enantiomérico, además de los PBDEs. En la Tabla 3-3 se indican los niveles encontrados para HCBCO, BEHTBP, OBIND y BTBPE. Nuestro trabajo ha sido el primero en informar la cuantificación de HCDBCO en biota y de BEHTBP en el medio ambiente. HCDBCO solo fue detectado en los huevos de halcón peregrino procedente de Canadá, tanto con dieta terrestre como acuática.

Tabla 3-3. Resumen de niveles de HFRs encontrados en huevos de halcón peregrino

		ng/g (lw)			
		HCBCO	BEHTBP	OBIND	BTBPE
España-T (n=5)	Muestras cuantificadas	0	0	2	0
	Rango (ng/g, lw)			nd-0.84	
	Mediana (ng/g, lw)			0.53	
España-A (n=8)	Muestras cuantificadas	0	1	3	1
	Rango (ng/g, lw)		nd-1.2	nd-1.0	nd-7.4
	Mediana (ng/g, lw)		1.1	0.27	5
Canadá-T (n=10)	Muestras cuantificadas	2	4	4	0
	Rango (ng/g, lw)	17-23	nd-4.5	nd-29	
	Mediana (ng/g, lw)	3.5	0.5	0.27	
Canadá-A (n=2)	Muestras cuantificadas	1	0	0	1
	Rango (ng/g, lw)	nd-19			nd-14
	Mediana (ng/g, lw)	13			8

Respecto a BEHTBP, se detectó en una muestra de España y en 4 huevos de halcón con dieta terrestre de Canadá. Nuestro estudio también ha sido el primero en informar el hallazgo de OBIND en huevos de halcón peregrino, tanto de Norteamérica como de Europa y se ha encontrado en el 38% de las muestras de España y en el 33% de las de Canadá. BTBPE fue encontrado en solo 2 huevos, uno de España y el otro de Canadá, siendo mayor la concentración en este último. Tal como podemos ver en la Tabla 3-3, en general los niveles de FRs encontrados en las muestras de Canadá son superiores a las de España.

En cuanto al análisis de HBCD en los huevos de halcón peregrino, los niveles encontrados se resumen en la Tabla 3-4. Se encontró HBCD en el 70% de los huevos de España y en el 92% de los huevos de Canadá. Incluso en una muestra de Canadá hemos hallado la mayor concentración de HBCD hasta ahora encontrada en huevos de halcón peregrino (14.617 ng/g lw).

Tal como ocurre en muestras de biota, β -HBCD se encontró por debajo de los LOD. Al comparar los niveles de HBCD encontrados en los huevos de ambos países vimos que los huevos de Canadá tienen concentraciones significativamente superiores de HBCD que los de España, lo que reflejaría un mayor uso de HBCD en Norteamérica.

En cuanto al patrón isomérico de HBCD en los huevos de halcón peregrino (Tabla 3-4), observamos que α -HBCD es el isómero principal lo que contrasta fuertemente con el patrón de la mezcla comercial, donde γ -HBCD es el isómero dominante. Este hecho ha sido observado anteriormente en muestras biológicas, pero se debe tener en cuenta que la exposición constante a un efluente industrial que contenga la mezcla industrial de HBCD puede causar que haya un aumento en la proporción de α -HBCD pero que no sea tan importante, como fue el caso de las jaulas de madrillas en nuestro artículo #5.

En el artículo #8 también se calcularon los EF corregidos de α -HBCD en las muestras donde estaba suficientemente concentrado para ser detectado enantioméricamente (Tabla 3-4). Hemos encontrado que en los huevos de España los valores son variables, mientras que en los huevos de Canadá los EF tienen valores entre 0,164 y 0,324. Estos resultados sugieren que hay una mayor concentración de (–) α -HBCD en los huevos, por lo tanto hay una acumulación enantiómero- selectiva.

Tabla 3-4. Niveles de HBCD diastereoisomérico y EF de α -HBCD en huevos de halcón peregrino

	Muestra	ng/g, lw		Σ HBCD	EF α -HBCD
		α -HBCD	γ -HBCD		
España-T	S1	338	1.5	342	0.451
	S2	nq	nd	nq	
	S3	nq	0.9	0.9	
	S4	nq	nd	nq	
	S5	nq	nd	nq	
España-A	S6	47	nd	47	0.05
	S7	812	nd	812	0.920
	S8	nq	nd	nq	
	S9	299	nd	299	0.408
	S10	1598	nd	1598	0.450
	S11	242	nq	242	0.430
	S12	nq	0.9	0.9	
	S13	941	11	952	0.436
Canadá-T	C1	4379	29	4408	0.259
	C2	2199	1.9	2200	0.220
	C3	nq	nd	nq	
	C4	572	35	607	0.235
	C5	6913	nd	6913	0.288
	C6	3180	nq	3180	0.164
	C7	2748	24	2772	0.260
	C10	170	39	219	0.324
	C11	14617	nd	14617	0.196
	C12	1454	nd	1454	0.249
Canadá-A	C8	3335	5.1	3340	0.164
	C9	345	7.7	353	0.247

nq: inferior al LOQ, nd: inferior al LOD

En los huevos de halcón peregrino también analizamos otros HFRs (artículo #7) del tipo norbornenos clorados. Este ha sido el primer trabajo que ha encontrado DP en muestras de biota de

Europa, además que ha sido la primera vez que se detectan Dec 602, Dec 603 y Dec 604 en un organismo terrestre ubicado en la parte superior de la cadena trófica (Tabla 3-5).

Tabla 3-5. Niveles de norbornenos clorados en huevos de halcón peregrino

		Σ DP		Dec 602	Dec 603	Dec 604
		<i>fanti</i> *				
España-T (n=5)	Muestras cuantificadas	5		4	5	4
	Rango (ng/g, lw)	0.3-3.6	0.62-0.75	nd-15	1.5-6.2	nq-0.35
	Mediana (ng/g, lw)	0.6	0.74	4.8	2.4	0.32
España-A (n=8)	Muestras cuantificadas	8		8	8	5
	Rango (ng/g, lw)	0.4-17	0.65-0.78	nd-25	3.0-7.5	nd-0.32
	Mediana (ng/g, lw)	2.3	0.69	15	5.2	0.31
Canadá-T (n=10)	Muestras cuantificadas	10		10	10	10
	Rango (ng/g, lw)	7.5-209	0.41-0.69	44-211	12-220	1.4-9.8
	Mediana (ng/g, lw)	43	0.63	71	52	4
Canadá-A (n=2)	Muestras cuantificadas	2		2	2	2
	Rango (ng/g, lw)	6.3-122	0.52-0.6	7.2-104	5.3-29	1.3-3.5
	Mediana (ng/g, lw)	64	0.56	55	17	2

**fanti* se calcula dividiendo la concentración de *anti*-DP por la concentración total de DP.

Al comparar los huevos de halcones con dieta terrestre de ambos países, los niveles de DP en las muestras de Canadá son 40 veces mayores que en los huevos de España. Esto se debe a que en el río Niágara se encuentra la industria que produce el DP desde hace más de 40 años. También hemos calculado los *fanti* en todas las muestra y se observa que los valores son mayores en los huevos españoles, lo cual podría deberse a que los halcones de Canadá se encuentran en contacto con el efluente industrial del DP.

Hemos cuantificado Dec 602, Dec 603 y Dec 604 en más del 84% de muestras analizadas, en concentraciones superiores a 1,5 ng/g lw (Tabla 3-5). Hay que destacar que en las muestras de Canadá se hallaron los niveles mayores, reflejando la cercanía a la industria que los produce (Oxychem), además del posible mayor uso de estos FR en Norteamérica que en Europa.

Así pues, el análisis de esta amplia gama de HFRs en un organismo ubicado en la parte alta de la cadena trófica, nos ha permitido estudiar su biodisponibilidad y bioacumulación. Así, hemos hecho un aporte importante para los estudios a nivel global acerca de la presencia, distribución y comportamiento de estos compuestos.

CAPÍTULO 4: EXPOSICIÓN HUMANA

4. EXPOSICIÓN HUMANA

4.1. Introducción

Tal y como se trató en el Capítulo 1 de esta Tesis Doctoral, existen diversas vías por las cuales un BFR puede ser incorporado al cuerpo humano. En diferentes estudios se han detectado residuos de BFRs en diversas muestras humanas (Schechter et al., 2007; Thomsen et al., 2008), lo cual demuestra que los seres humanos estamos expuestos a estos contaminantes.

Es sabido que una importante ruta de exposición a los PBDEs para la población es a través de la dieta. Esto ha sido confirmado en un estudio que se llevó a cabo en el Reino Unido, donde se estimaba la exposición a PBDEs por parte de adultos, mostrando que un 98,7% provenía de la alimentación, mientras que un 0,9% del aire y el resto del polvo doméstico (Harrad et al., 2006). Se debe tener en cuenta que la estabilidad y las características lipofílicas de los PBDEs permiten su biomagnificación en la cadena alimentaria, aumentando la concentración sucesivamente a medida que aumenta el nivel trófico.

Por otro lado, la autoridad europea de seguridad alimentaria (EFSA, del inglés *european food safety authority*) ha reconocido la preocupación por la contaminación de los alimentos y piensos con BFRs (EFSA 2006) y recomienda la vigilancia de BDE-28, -47, -99, -100, -153, -154, -183 y -209 y BB-153, además del HBCD total e isómero-específico si los niveles son muy elevados. Opcionalmente aconseja incluir también el DBDPE, el HBB y el BTBPE en los análisis de control.

Un punto a considerar en la exposición humana por dieta incluye no solo la concentración en que los contaminantes se encuentran en los alimentos, sino también los hábitos alimenticios de los consumidores, esto es la frecuencia de su consumo en la dieta de cada grupo de alimentos. En este sentido hay que destacar que existen diferentes hábitos dietéticos en función de la región geográfica en estudio. Así, por ejemplo, la dieta japonesa se caracteriza por un mayor consumo de pescados, la dieta americana por un mayor consumo de cárnicos o la holandesa por una mayor proporción de lácteos.

En el Capítulo 1 se observó que los mayores niveles de BFRs se encuentran en alimentos con alto contenido graso, siendo el consumo de pescado una forma importante de exposición a los PBDEs (Van Leeuwen y De Boer 2008). Se debe tener en cuenta que la exposición a BFRs no solo es a través del consumo directo de pescado, sino que también puede ser por medio de productos derivados tales como el aceite de pescado. Este subproducto se consume con fines dietéticos, debido a la presencia de ácidos grasos *n*-3 poliinsaturados de cadena larga que tienen beneficios para la salud y son esenciales en la dieta humana.

El aceite de pescado también es utilizado como ingrediente en los piensos para el crecimiento de los animales, desempeñando un papel importante en las dietas preparadas para las especies carnívoras y omnívoras. Es una fuente de energía ideal, imparte propiedades saludables que aportan los ácidos grasos al producto final. Sin embargo, la principal preocupación para la seguridad alimentaria es la posible transferencia de contaminantes desde los piensos a los alimentos, lo que podría provocar a largo plazo efectos sobre la salud de los animales de granja y de los seres humanos que los consumen (Mantovani et al., 2009).

Tanto en aceites de pescado para consumo humano como animal existen estudios con el objetivo de estudiar los niveles de PBDEs y su comportamiento (Covaci et al., 2007a; Domingo et al., 2008). Sin embargo respecto a HBCD la información disponible es mínima. Es por ello que hemos analizado 25 muestras de aceite de pescado utilizadas en España tanto para el consumo humano como animal. Posteriormente en las muestras con una mayor concentración de HBCD calculamos las fracciones enantioméricas para investigar posibles procesos de enriquecimiento enantiómero-selectivos. Además estimamos la dosis de ingesta diaria por el consumo de estos complementos y lo que representaría en la dosis total de ingesta por dieta alimenticia. Este trabajo queda reflejado en el artículo #9 titulado: *“Diastereoisomer- and enantiomer-specific determination of hexabromocyclododecane in fish oil for food and feed”*.

Dentro de la vía de exposición humana a través de la dieta, merece una atención especial el estudio de la exposición a un grupo de riesgo como son los lactantes. Dicho grupo solo se alimenta de leche materna. Además su masa corporal es pequeña por lo que el impacto de los contaminantes puede resultar superior, sin olvidar que dicho impacto se produce en una etapa de formación y desarrollo del individuo. El primer estudio de PBDEs en leche materna fue realizado por Norén y Meironyté (2000), con muestras procedentes de Suecia, donde se observaba un aumento continuo y exponencial de la concentración de PBDEs entre 1972 y 1997. Tras la publicación de este trabajo, se vio la importancia de estudiar el comportamiento y destino de los PBDEs en el medio ambiente. Se han desarrollado otros estudios posteriores de estos compuestos en leche materna, por ejemplo en Suecia (Lignell et al., 2009), Islas Faroe (Fangstrom et al., 2005), Australia (Toms et al., 2007), China (Li et al., 2008), República Checa (Kazda et al., 2004), Estados Unidos (Schecter et al., 2003), Noruega (Thomsen et al., 2010) y Francia (Antignac et al., 2008). La mayor concentración ha sido encontrada en leche materna de EE.UU. (72 ng/g lw) seguido de la procedente de Australia, con una concentración 7 veces menor.

El estudio de otros FR en leche materna como HBCD no es habitual, sobre todo si deseamos obtener información de su distribución diastereoisomérica. En la Figura 4-1 se señalan los niveles de HBCD total encontrados en estudios anteriores en leche materna de distintos países. Los mayores niveles fueron encontrados en Canadá (3,8 ng/g lw), seguido de México (2,1 ng/g lw), Japón (1,7 ng/g lw) y Noruega (1,7 ng/g lw). Así se puede comprobar la distribución de HBCD alrededor del mundo y su biodisponibilidad al ser humano y posterior bioacumulación, aunque generalmente las concentraciones observadas de HBCD son menores que las de PBDEs, debido al mayor uso de los PBDEs y por un mayor periodo de tiempo. De esta forma, vemos que los niveles de HBCD son inferiores a los observados para PBDEs.

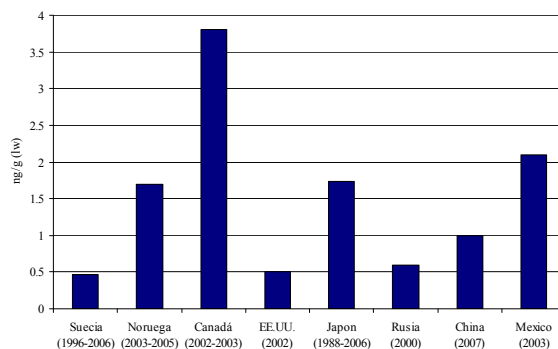


Figura 4-1. Concentración promedio de HBCD (ng/g lw) en diferentes países

Tanto en Japón (Kakimoto et al., 2008) como en Suecia (Fangstrom et al., 2008) se ha realizado un estudio de la tendencia temporal de HBCD, el cual se encuentra resumido en la Figura 4-2. La leche humana de Suecia muestra un aumento de las fluctuaciones en el tiempo: desde 1988 la concentración de HBCD ha aumentado hasta concentraciones similares a la del BDE-99, -100 y -153, congéneres de PBDEs que fueron analizados conjuntamente. Estos resultados muestran que el HBCD es transferido a la leche, por lo tanto es persistente y se bioacumula. Respecto al trabajo de Kakimoto et al. (2008), las muestras analizadas entre los años 1973 y 1983 tienen concentraciones de HBCD menores al LOD y los valores aumentan a partir de las muestras del año 1988. A partir del año 2000 las concentraciones se encuentran entre 1,0 y 4,0 ng/g lw. Los niveles de HBCD en Japón son mayores que los observados en Suecia, esto muestra su mayor utilización y/o producción industrial. Solo en el caso de las muestras procedentes de Japón se realizó el análisis diastereoisomérico de HBCD, encontrándose que generalmente α -HBCD predomina (62-100%), mientras que en los años 1993 y 2006 γ -HBCD fue el isómero principal (56 y 65%, respectivamente) y el β -HBCD no fue detectado en ninguna de las muestras.

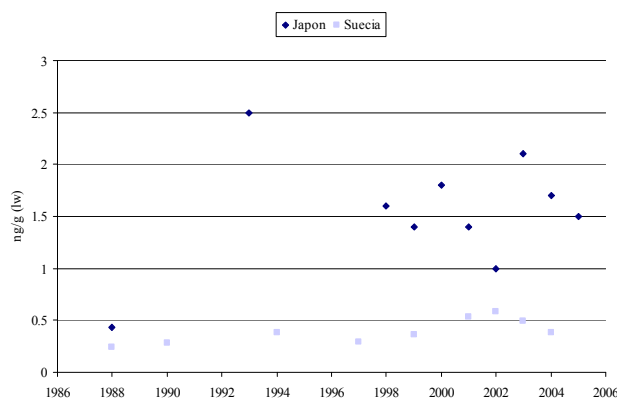


Figura 4-2. Tendencia temporal de HBCD en Japón y Suecia.

Luego de observar que existen diversos trabajos que analizan PBDEs en leche materna, nos pareció interesante poder realizar un aporte al conocimiento científico sobre los niveles de HBCD en esta matriz. Por lo tanto, durante el desarrollo de esta tesis hemos determinado los niveles de concentración de HBCD en muestras de leche materna de España, evaluando la distribución de isómeros y, por primera vez se ha estudiado la distribución enantiómero-específica de HBCD a fin de determinar si existe o no acumulación enantiómero-selectiva en el cuerpo humano. Finalmente, se evaluó la dosis diaria de ingesta del lactante. Este trabajo se sintetiza en el artículo #10 *“Hexabromocyclododecane in human breast milk: levels and enantiomeric patterns”*.

Por otro lado, se realizó un estudio similar con muestras de leche materna procedentes de Australia a fin de comparar los resultados con el estudio previo realizado en España. Además y dada la disponibilidad de muestras de leche materna desde los años 1993 a 2009, el análisis de HBCD en estas muestras nos puede dar una idea de la evolución de los niveles de HBCD con el tiempo en Australia y compararlo con los niveles y las tendencias encontrados de PBDEs. Con los datos de este estudio se ha escrito el artículo #11: *“Temporal trends of brominated flame retardants in Australia: 1993-2009”*

.

Artículo científico #9:

“Diastereoisomer- and enantiomer-specific determination of hexabromocyclododecane in fish oil for food and feed”

por

*X. Ortiz, P. Guerra, J. Díaz-Ferrero, E. Eljarrat y D. Barceló
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Diastereoisomer- and enantiomer-specific determination of hexabromocyclododecane in fish oil for food and feed

Xavier Ortiz^{a,1}, Paula Guerra^b, Jordi Díaz-Ferrero^{a,1}, Ethel Eljarrat^{b,*}, Damià Barceló^{b,c}^a Environmental Laboratory, Institut Químic de Sarrià, Ramon Llull University, Via Augusta 390, 08017 Barcelona, Spain^b Department of Environmental Chemistry, IDAEA, CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain^c Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, Pic de Peguera 15, 17003 Girona, Spain

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ABSTRACT

Fish oils are one of the main sources of ω -3 fatty acids. However, they can present elevated levels of some lipophilic pollutants, such as hexabromocyclododecanes (HBCDs). Since data about HBCDs in fish oil samples are very limited, in this study, 25 samples of fish oil for feed and food have been analyzed. Total HBCDs, as well as, α -, β - and γ -diastereoisomers have been determined. Total HBCDs ranged from 0.09 to 26.8 ng g⁻¹, with higher concentrations in fish oil for feed (average value of 9.69 ng g⁻¹) than those for food (1.14 ng g⁻¹). Concentrations of α -HBCD (average value of 4.12 ng g⁻¹ in feed samples and 0.48 ng g⁻¹ in food samples) and γ -HBCD (5.05 and 0.43 ng g⁻¹ respectively) were higher than that of β -HBCD (0.52 and 0.19 ng g⁻¹ respectively) in most of the samples. However, none of them was predominant in the samples. Concentrations of HBCDs were compared to concentrations of other pollutants and correlation between dioxin and dioxin-like PCBs levels and HBCDs levels were observed. Intake of HBCDs was calculated for fish oil with human consumption purposes and it ranged from 0.08 to 5.38 ng HBCDs d⁻¹, which could contribute significantly to HBCDs total intake. Enantiomeric fractions were also determined. No clear enrichment was observed for γ -HBCD, while ()- α -HBCD enrichment was detected in some samples.

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1. Introduction

Fish oils are one of the main sources of ω -3 fatty acids (mainly cis-5,8,11,14,17-cicosapentaenoic acid – EPA – and cis-4,7,10,13,16,19-docosahexaenoic acid – DHA), well-known for being blood vessel dilators and preventing heart attacks by blood pressure reduction (Shidu, 2003). For this reason, these oils have been introduced steadily in human and animal diets. However, they can present elevated levels of lipophilic pollutants (Zennegg and Schmid, 2006), because they bioaccumulate in fatty tissues and fish have very low metabolism capability for these compounds (Voorspoels et al., 2004). According to this, one of the pollutants that could be easily found in fish oil are hexabromocyclododecanes (HBCDs). These compounds have been used as a brominated flame-retardant in polystyrene foams, which are mainly used for thermal insulation in buildings (Law et al., 2006). Their production have increased steadily during the last years due to the ban in the EU of other similar flame retardants such as polybrominated diphenyl ethers (PBDEs) or polybrominated biphenyls (2006/11/EC Directive).

Considering all elements of symmetry, 16 different HBCD diastereoisomers including six pairs of enantiomers as well as four meso forms are possible theoretically (Heeb et al., 2007). Technical HBCDs are produced industrially by addition of bromine to cis-trans-trans-1,5,9-cyclododecatriene, with the resulting mixture that contains three predominant diastereoisomers α -, β - and γ -HBCDs. Normally, the γ -isomer is the most dominant in the commercial mixtures (ranging between 75% and 89%), followed by α - and β -isomer (10–13% and 1–12%, respectively) (Covaci et al., 2006). Moreover, α -, β - and γ -HBCDs diastereoisomers are chiral, leading to three pairs of enantiomers.

Although there are some publications reporting the levels of other persistent organic pollutants in fish oil (Eljarrat et al., 2002; Zennegg and Schmid, 2006; Martí et al., 2010), very few studies have reported the occurrence of HBCDs in this kind of samples. To the knowledge of the authors, there is only one study reporting the diastereomeric HBCDs distribution in fish oil samples so far (Kakimoto et al., 2007), although there is more information of this compound in fish (Eljarrat et al., 2004; Sørmo et al., 2009; Zhang et al., 2009). Moreover, this paper reports for the first time HBCDs levels in fish oils from the Spanish market.

The objective of this study was to determine HBCDs diastereoisomer levels in fish oil samples used in Spain for animal and human consumption, since this pollutant can bioaccumulate in

^{*} Corresponding author. Tel.: +34 93 4006100; fax: +34 93 2045904.E-mail addresses: jordi.diaz@iqs.edu (J. Díaz-Ferrero), eeeqam@cid.csic.es (E. Eljarrat).¹ Tel.: +34 93 2672000; fax: +34 93 2056266.

fat tissues and biomagnify through the food chain. Moreover, enantiomeric fractions (EFs) were calculated in order to investigate potential selective enantiomeric enrichment processes.

2. Experimental

2.1. Fish oil samples

For this study, 25 fish oil samples were collected. Ten samples were raw fish oils for animal consumption with different origin, mainly from Northern Atlantic and Southern Pacific. The other twelve collected samples were for human consumption, and were acquired from drug and dietetic stores. These oils had been previously refined according to the usual industrial processes applied to oils for human consumption (Hilbert et al., 1998). They are commercialized as health supplements for nutritive purposes and for this reason some of these oils contained other compounds, such as vitamins and minerals. Moreover, three out of these twelve oils contained vegetable oil as well, for their high content in ω -6 fatty acids. Finally, in order to compare the obtained results, three health supplements based only in vegetable oil were included in the study. More detailed information of all the analyzed samples can be found in Table 1.

2.2. Chemicals

Hexane was supplied by Baker (Deventer, Holland), dichloromethane was acquired from Carlo Erba (Val de Reuil, France) and concentrated sulphuric acid was supplied by Scharlau (Barcelona, Spain). Alumina solid phase extraction cartridges were acquired from IST (Uppsala, Sweden). D_{18} -labeled α -, β - and γ -HBCDs standards were acquired from Wellington Laboratories Inc. (Guelph, Canada) and were of minimum 98% purity. α -, β - and γ -HBCDs standards were obtained from Cambridge Isotope Laboratories Inc. (WI, USA) and were of minimum 97% purity.

2.3. Sample preparation

One gram of fish oil samples were spiked with d_{18} -labeled α - and γ -HBCDs as internal standards. Samples were dissolved in

10 mL of hexane and matrix was degraded by the addition of 10 mL of concentrated sulphuric acid. Organic fraction (containing HBCDs) was evaporated to 3 mL and the whole clean-up procedure was repeated until the acid did not turn yellow. Final purification of the extract was carried out in 5 g alumina SPE cartridges. Cartridges were conditioned with 20 mL of hexane and eluted with 30 mL of hexane:dichloromethane (1:2). Purified extracts were concentrated with a gentle nitrogen flow and spiked with d_{18} labeled β -HBCD as injection standard. Since elevated temperatures can isomerize stereoselectively these compounds, no heating of the samples was performed during their preparation.

2.4. Diastereoisomer analysis

The LC system used was an Symbiosis Pico (Spark Holland, Emmen, The Netherlands) with a Symmetry C_{18} column (2.1 mm $\times$ 150 mm, 5 μ m) preceded by a C_{18} guard column (2.1 $\times$ 10 mm) supplied by Waters (Massachusetts, USA). Experiments were carried out in negative ionization mode using H_2O : methanol (3:1 v/v) as eluent A and methanol as eluent B, at a flow rate of 0.25 mL min⁻¹. The injection volume was set at 10 μ L. The elution program started at an initial composition of 100% A and was ramped to 0% A in the first 8 min, then eluent A increased to 10% in 17 min and initial conditions were reached again in three min and returned to the starting conditions in 15 min.

Mass spectrometric analysis was performed with a hybrid triple quadrupole/linear ion trap MSD Sciex 4000QTRAPTM (Applied Biosystems, Foster City, CA, USA) instrument equipped with an electrospray (ESI) Turbospray interface, and working in negative ionization mode. For target quantitative analyses, data acquisition was performed in selected reaction monitoring (SRM). The $[M - H]^- \rightarrow Br^-$ transitions at m/z 638.7 $\rightarrow$ 78.9 and 638.7 $\rightarrow$ 80.9 were monitored for unlabelled HBCDs. The labeled HBCDs were monitored at the 655.8 $\rightarrow$ 78.9 and 655.8 $\rightarrow$ 80.9 transitions. The MS/MS detection conditions were optimized to obtain the highest relative intensity: curtain gas at 50 psi, collision gas at 4.5×10^{-5} Torr, temperature of the turbo gas in the TurboIonSprayTM source at 350 $^{\circ}$ C, ion source gas 1 at 50 psi and ion source gas 2 at 10 psi (Guerra et al., 2008a).

Table 1
Specifications of the analyzed fish oil samples.

Sample code	Consumption	Main component	Other components	Origin	Format	Recommended dose
FE1	Feed	Fish oil	–	North Atlantic	Raw oil	–
FE2	Feed	Salmon oil	–	–	Raw oil	–
FE3	Feed	Fish oil	–	North Atlantic	Raw oil	–
FE4	Feed	Fish oil	–	South Pacific	Raw oil	–
FE5	Feed	Fish oil	–	North Atlantic	Raw oil	–
FE6	Feed	Fish oil	–	North Atlantic	Raw oil	–
FE7	Feed	Fish oil	–	South Pacific	Raw oil	–
FE8	Feed	Fish oil	–	–	Raw oil	–
FE9	Feed	Fish oil	–	South Pacific	Raw oil	–
FE10	Feed	Fish oil	–	North Atlantic	Raw oil	–
FO1	Food	Salmon oil	Protein, carbohydrates, vitamin E	–	Pearls	2–3 pearls d ⁻¹
FO2	Food	Fish oil	Protein, carbohydrates, vitamin E	–	Pearls	2 pearls d ⁻¹
FO3	Food	Fish oil	–	–	Bottle	1 tablespoon (3.3 g) d ⁻¹
FO4	Food	Fish oil	Vitamins and minerals	–	Pearls	1 pearl d ⁻¹
FO5	Food	Fish oil	Vitamins and minerals	–	Pearls	5–10 pearls d ⁻¹
FO6	Food	Fish oil	Vitamins	–	Pearls	2–3 pearls d ⁻¹
FO7	Food	Fish oil	–	–	Pearls	4 pearls d ⁻¹
FO8	Food	Cod liver oil	Vitamins	–	Pearls	1 pearl d ⁻¹
FO9	Food	Fish oil	Vitamin E	–	Pearls	4 pearls d ⁻¹
FO10	Food	Fish oil + evening primrose oil	Isoflavones, calcium and magnesium	–	Pearls	2 pearls d ⁻¹
FO11	Food	Fish oil + evening primrose oil	Vitamins and minerals	–	Pearls	1 pearl d ⁻¹
FO12	Food	Salmon oil + boraje oil	Vitamin E	–	Pearls	3–5 pearls d ⁻¹
FO13	Food	Pumpkin seed oil	Tocopherols	–	Pearls	6 pearls d ⁻¹
FO14	Food	Vegetable oil rich in omega-6	–	–	Pearls	2–3 pearls d ⁻¹
FO15	Food	Evening primrose oil	α -Tocopherol	–	Pearls	1–2 pearls d ⁻¹

2.5. Quality assurance/quality control

Method blank samples were performed to check for interferences or contamination from solvents and glassware. No presence of analytes of interest (α -, β - and γ -HBCDs diastereoisomers) was observed.

Spiked samples with native compounds (α -, β - and γ -HBCDs) were analyzed using the described methodology to check recoveries. These samples were concentrated to incipient dryness and redissolved with 50 μ L of labeled compounds mixture, containing d_{18} - α -HBCD and d_{18} - γ -HBCD at 125 $\text{pg } \mu\text{L}^{-1}$, prior to the instrumental analysis by LC-MS-MS. Recovery values of labeled compounds ranged from 78% to 98%, with relative standard deviation ($n=4$) values between 7% and 10%. Detection limits, defined as the minimum amount of analyte which produces a peak with a signal-to-noise ratio equal to 3, were 0.03 ng g^{-1} for α -HBCD, 0.05 ng g^{-1} for β -HBCD and 0.06 ng g^{-1} for γ -HBCD. Limits of quantification, defined as the minimum amount of analyte which produces a peak with a signal-to-noise ratio equal to 10, were 0.11, 0.18 and 0.20 ng g^{-1} for α -, γ - and β -HBCD, respectively.

2.6. Enantiomeric analysis

A chiral chromatographic column, Nucleodex β -PM (4.0 mm $\times$ 200 mm $\times$ 5 μm), was used to afford the enantiomer-specific determination. The optimal separation of enantiomers was achieved using methanol, acetonitrile and water as mobile phase. Experiments were carried out using 70% water:30% methanol as eluent A and 70% AcN: 30% methanol as eluent B, at a flow rate of 0.50 mL min^{-1} . The injection volume was set at 10 μL . The elution program started at an initial concentration of A at 50% decreased to 0% along the first 8 min, and was maintained for 17 min and initial conditions were reached again in 5 min and maintained for additional 12 min (Eljarrat et al., 2008; Guerra et al., 2008b). Mass spectrometric analysis was performed using the same conditions as for diastereoisomer analysis.

Enantiomeric composition was expressed as enantiomeric fraction (EF), which is normally calculated from the peak areas of the enantiomeric pairs by:

$$\text{EF} = \frac{A_+}{(A_+ + A_-)} \quad (1)$$

where A_+ and A_- are the peak area of eluting enantiomers.

It is well known that ESI is subject to sample matrix effects that can cause enhancement or suppression of the target analytes signal and can adversely affect their quantification. In order to avoid this effect that can affect in EF calculations, these values are calculated according to Eq. (2) (Marvin et al., 2007). This correction is based on the use of isotopic labeled standards (d_{18} -HBCDs) since d_{18} -labeled enantiomeric analogues behave in an identical manner to their native counterparts:

$$\text{EF}_{\text{corrected}} = \frac{([A_+]/A_{+d_{18}}) \times \text{pg } A_{+d_{18}}}{([A_+]/A_{+d_{18}}) \times \text{pg } A_{+d_{18}} + ([A_-]/A_{-d_{18}}) \times \text{pg } A_{-d_{18}}} \quad (2)$$

where $[A_+]$ and $[A_-]$ are the peak areas of each (+) and (−) HBCDs enantiomers, $[A_{+d_{18}}]$ and $[A_{-d_{18}}]$ are the peak areas of each (+) and (−) HBCDs d_{18} labeled standards, while $\text{pg } A_{+d_{18}}$ and $\text{pg } A_{-d_{18}}$ stand for the non-corrected determined HBCD concentration.

2.7. Comparison with PCDD/F, dl-PCB and PBDE levels

In order to compare the determined HBCDs levels with other persistent organic pollutants, concentrations of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs), dioxin-like

polychlorinated biphenyls (dl-PCBs) and polybrominated diphenylethers (PBDEs) were analyzed for some of the samples. For this purpose, 6 g of fish oil samples were spiked with ^{13}C -labeled standards. The purification was carried out with multilayer silica columns, containing H_2SO_4 , NaOH and AgNO_3 modified silica. Analytes were fractionated by high performance liquid chromatography equipped with a 2-(1-pyrenyl)ethyl column (HPLC-PYE). Final instrumental determination was carried out by high resolution gas chromatography/high resolution mass spectroscopy. More detailed information of these analyses can be found in elsewhere (Ortiz et al., 2010; Martí et al., 2010).

3. Results and discussion

3.1. Levels of HBCD in fish oil

Levels of α -, β - and γ -diastereoisomers and total HBCDs are summarized in Table 2, determined according to the previously described method. Twenty-two of the 25 analyzed samples presented quantifiable HBCDs levels within the range of 0.09 to 26.8 ng g^{-1} . Samples for feed purposes showed higher total levels (average value of 9.69 ng g^{-1}) than those for human consumption (average value of 1.14 ng g^{-1}). No clear correlation was observed between the predominant diastereoisomer and the origin or purpose of the fish oil, although γ -HBCD had slightly higher average values in feed samples whereas α -HBCD was slightly higher in food samples. Nine of the analyzed samples had α -HBCD as the predominant diastereoisomer, whereas in eight samples γ -HBCD was the predominant one. α/γ ratios (isomers with higher values) were within the range of 0.09 and 2.00, which differs from the composition of the technical mixture (Tomy et al., 2004) and shows metabolic activity. In most of the analyzed fish oils, β -HBCD presented the lowest values among the three diastereoisomers, usually below the quantification limit. Average concentrations for this diastereoisomer were clearly the lowest in both food and feed samples. Despite this, β -HBCD showed similar concentrations to the other two diastereoisomers in six of the twelve studied oils for human consumption, being the predominant diastereoisomer in four of the samples.

Most of the samples for feed purposes had known origin. Thus, FE1, FE3, FE5, FE6 and FE10 were from the Northern Atlantic while FE4, FE7 and FE9 were from Southern Pacific. In general, fish oils from Northern Atlantic presented higher levels (4.78–15.49 ng g^{-1}) than those from the Southern Pacific (3.34–5.49 ng g^{-1}).

Regarding food samples, those nutritive supplements based on fish oil had slightly higher values than those based on mixtures of fish and vegetable oil or even vegetable oils only (Fig. 1). This observation suggests that fish oils present higher levels of HBCDs than vegetable oils, that would be related to the bioaccumulation phenomenon in the fish trophic chain. Nevertheless, all these oils had remarkably lower HBCDs levels than those raw oils for animal consumption, which suggests that the oil refining process (including neutralization, bleaching and deodorization steps (Hilbert et al., 1998)) could lower the concentration of these pollutants.

Determined HBCDs concentrations were within the same range already reported in literature (Kakimoto et al., 2007), with the exception of some values detected by that author in sardine and shark liver oils, which presented 10-times higher levels (between 44 and 67 ng g^{-1} lw) than the concentrations determined in our study. Due to the lack of existing literature, fish oil concentrations were also compared with the levels of HBCDs in fish, expressed on a lipid base. The concentrations of these pollutants in our fish oil samples were remarkably lower than those reported for crucian carp (*Carassius carassius*) and loach (*Misgurnus anguillicaudatus*) in previous publications (Zhang et al., 2009), with average values of 377 and 1791 ng g^{-1} lw. For these two cases, α -HBCD was

Table 2
Concentrations of HBCD in the analyzed fish oil samples.

Sample code	Concentration (ng g ⁻¹)				HBCD intake (ng HBCD d ⁻¹)	α/γ ratio	Enantiomeric Fractions		Σ PBDES (ng g ⁻¹)	Σ PCDD/Fs (pg TEQ g ⁻¹)	Σ dI-PCBs (pg TEQ g ⁻¹)	Σ PCDD/Fs + PCBs (pg TEQ g ⁻¹)
	α -HBCD	β -HBCD	γ -HBCD	Total			α -HBCD	γ -HBCD				
FE1	5.79 (52)	0.23 (2)	5.09 (46)	11.1	–	1.14	0.58	0.55	4.88	2.98	9.59	12.6
FE2	3.71 (38)	0.89 (9)	5.28 (53)	9.88	–	0.70	0.45	0.53	–	0.37	1.59	1.96
FE3	3.14 (66)	NQ (0)	1.64 (34)	4.78	–	1.91	–	–	–	1.22	5.11	6.33
FE4	2.13 (64)	NQ (0)	1.21 (36)	3.34	–	1.76	–	–	–	0.5	1.85	2.35
FE5	3.82 (57)	NQ (0)	2.84 (43)	6.66	–	1.35	0.02	0.48	–	1.12	6.24	7.36
FE6	6.58 (42)	NQ (0)	8.91 (58)	15.5	–	0.74	0.61	0.49	8.64	2.68	19.2	21.8
FE7	2.61 (50)	NQ (0)	2.65 (50)	5.26	–	0.98	0.56	0.52	–	0.39	1.97	2.36
FE8	6.13 (23)	3.95 (15)	16.7 (62)	26.8	–	0.37	0.02	0.51	1.82	0.39	0.95	1.34
FE9	2.62 (48)	NQ (0)	2.87 (52)	5.49	–	0.91	0.70	0.45	–	0.4	1.88	2.28
FE10	4.65 (59)	0.11 (1)	3.29 (41)	8.05	–	1.41	0.17	0.57	4.81	1.54	7.71	9.25
Average value in feed	4.12 (43)	0.52 (5)	5.05 (52)	9.69	–	1.13	0.39	0.51	5.04	1.16	5.61	6.76
Standard deviation	1.59	1.24	4.67	7.01	–	0.49	0.28	0.04	2.79	0.93	5.33	6.16
FO1	2.42 (58)	NQ (0)	1.77 (42)	4.19	5.15	1.37	–	–	–	0.14	0.46	0.6
FO2	0.13 (48)	0.14 (52)	NQ (0)	0.27	0.27	–	–	–	0.21	0.63	1.39	2.02
FO3	NQ	ND	NQ	NQ	–	–	–	–	–	0.29	0.88	1.17
FO4	NQ	NQ	NQ	NQ	–	–	–	–	–	1.26	0.29	1.55
FO5	0.48 (45)	0.59 (55)	NQ (0)	1.07	4.01	–	–	–	–	0.45	0.32	0.77
FO6	0.24 (21)	0.91 (79)	NQ (0)	1.15	1.44	–	–	–	–	0.31	0.15	0.46
FO7	0.23 (22)	0.24 (23)	0.59 (56)	1.06	5.38	0.39	–	–	–	–	–	–
FO8	1.99 (65)	NQ (0)	1.09 (35)	3.08	1.26	1.83	–	–	3.18	–	–	–
FO9	0.40 (43)	NQ (0)	0.52 (57)	0.92	1.84	0.77	–	–	–	–	–	–
FO10	0.79 (100)	ND (0)	NQ (0)	0.79	0.55	–	–	–	2.63	0.33	0.39	0.72
FO11	NQ	ND	NQ	ND	–	–	–	–	–	–	–	–
FO12	0.32 (52)	0.29 (48)	0.16 (0)	0.77	2.16	2.00	–	–	–	–	–	–
FO13	0.04 (21)	0.15 (79)	NQ (0)	0.19	0.46	–	–	–	0.21	0.02	0.23	–
FO14	NQ (0)	NQ (0)	0.09 (100)	0.09	0.08	–	–	–	0.18	0.02	0.20	–
FO15	0.20 (8)	NQ (0)	2.19 (92)	2.39	3.59	0.09	–	–	34.1	0.20	0.05	0.25
Average value in food	0.48 (46)	0.19 (15)	0.43 (39)	1.14	2.51	0.43	–	–	0.45	0.40	0.40	0.80
Standard deviation	0.74	0.27	0.72	1.27	1.88	0.71	–	–	0.42	0.34	0.44	0.61

Contribution of each diastereomer in the total HBCD is included in brackets (%).

ND: Below limit of detection.

NQ: Below limit of quantification.

clearly the predominant diastereoisomer. This fact, which disagrees with our results (no predominant diastereoisomer was observed in the samples), could be because most fish oils are obtained from a mixture of different fish. Concentrations for total HBCDs in Black goby (*Gobius niger*), Sand goby (*Pomatoschistus microps*) and Whiting (*Merlangius merlangus*) have been reported (Sørmo et al., 2009), with average values of 6.04, 14.3 and 31.9 ng g⁻¹ lw. These concentrations are within the same range of our results.

Total HBCDs daily intake was calculated for fish oils with human consumption purpose (Table 2). Intake estimates were calculated by multiplying the concentration of the pollutant by the average recommended daily dose, according to the manufacturer's label instructions. Daily intakes ranged between 0.08 and 5.38 ng HBCDs d⁻¹. In general, our results showed that the consumption of fish oil-based health supplements involved a higher intake of HBCDs (average value of 2.45 HBCDs d⁻¹) as that derived from vegetable oil supplements (average value of 1.38 ng HBCDs d⁻¹). These intakes are within the same range than those reported by Knutsen et al. for oily fish (Knutsen et al., 2008) but they are much lower (ca. 10 times) than those reported by UK COT for fish (Committee on Toxicity, 2006). Although there is no data about the total daily intake of HBCDs in Spain, the comparison of the values obtained in our study with the total daily intake in other countries (Knutsen et al., 2008; Roossens et al., 2009) suggests that the ingestion of fish oil supplements could contribute to increase significantly the HBCDs intake. Although the dose of fish oil in feed is variable, the intake of HBCDs by animals could be

higher than the intake for humans because of the higher levels of HBCDs in fish oil for feed.

3.2. Enantiomeric fractions

EF are deduced from the chiral column LC data and the values are corrected according to Eq. (2) (Table 2). For β -HBCD, it was not possible to calculate EF values due to the low isomeric concentration of this diastereoisomer, and taking into account that the signal is further reduced when the enantiomeric separation was performed. In the case of γ -HBCD, it was observed that EF values are between 0.45 and 0.57, whereas EF for α -HBCD ranged between 0.02 and 0.70. If we compare these EF values with those obtained with standard solutions (Fig. 2), we can observe that no significant differences were detected for γ -HBCD. The EF mean value obtained for standard solutions was 0.49 (with 7% of relative standard deviation (RSD)). In general, samples were between the EF value $\pm$ RSD range established with standard solutions. Two samples (FE1 and FE10) presented a slight increase of EF value compared to standard value, indicating an enrichment of the (+)- γ -HBCD enantiomer in fish oil samples. On the other hand, one sample (FE9) showed a slight decrease for γ -HBCD EF value compared to standard value, indicating an enrichment of the (–)- γ -HBCD enantiomer in this fish oil sample.

In the case of α -HBCD, and for three samples (FE8, FE10 and FE5), results showed an important decrease of EF values (ranging from 0.02 to 0.17) with respect to the standards (mean value of 0.49 with 14% of RSD). Thus, we can assume that an enrichment

X. Ortiz et al./Chemosphere 82 (2011) 739–744

743

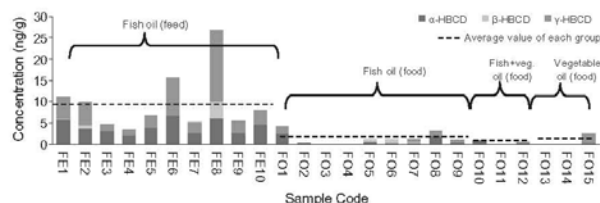
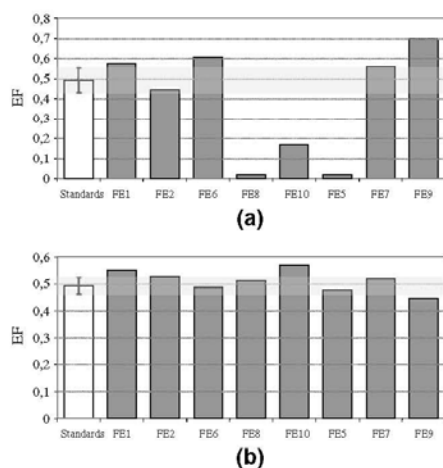


Fig. 1. Concentrations of total HBCD of the analyzed fish oil samples.

Fig. 2. Enantiomeric fraction (EF) values (mean $\pm$ standard deviation) in standards and fish oil samples for (a) alpha-HBCD, and (b) gamma-HBCD.

of the (–)- α -HBCD enantiomer was observed for some of the fish oil samples. Moreover, one sample (FE9) also showed an important increase of EF value, indicating an (+)- α -HBCD enrichment. Janák et al., 2005a reported for the first time the selective accumulation of different HBCDs enantiomers in marine fish from the Western Scheldt estuary. In two further studies, the database of EFs for α -HBCD has been extended to herring and several predatory birds (Janák et al., 2005b) and dolphins (Peck et al., 2005). In all cases, a deviation from the racemic mixture (EF = 0.5) has been observed, suggesting that an enantioselective uptake and/or metabolism of (+)- α -HBCD and (–)- α -HBCD must occur. Interestingly, no clear preference for one or the other α -HBCD enantiomer was found. Herring muscle and falcon eggs were clearly enriched in (–)- α -HBCD, whereas in whiting liver and sea eagle eggs, (+)- α -HBCD dominated. Similar behavior, with no clear enrichments was observed for γ -HBCD, but significant enrichments for α -HBCD, was also observed in human breast milk samples study (Eljarrat et al., 2009).

3.3. Comparison with PCDD/F, dl-PCB and PBDE results

Levels of HBCDs in fish oil determined in this study were compared with their respective levels of PCDD/Fs, PCBs and PBDEs, in

order to determine if any relationship between concentrations of the different families of pollutants is observed. Some of these values were already published (Martí et al., 2010) and some of them were analyzed for this study.

HBCDs levels were compared with the sum of PCDD/Fs and dl-PCBs (expressed as pg WHO-TEQ/g, which ponderates the toxicological effect for each of the individual congener) as it is shown in Fig. 3. As it can be observed, there is a positive correlation between the levels of different classes of pollutants: while the concentration of HBCDs increases, the concentration of PCDD/Fs+PCBs increases as well. Without taking into account the values for FE8 (it clearly differs from the observed trend, with a much higher value), HBCDs and PCDD/Fs+PCBs concentrations have a correlation (r^2) of 0.73, which tells us that there is a relationship between the levels of these different families of pollutants in fish oil. Nevertheless, correlation observed between these contaminants in feed samples ($r^2 = 0.79$) was much higher than in food samples ($r^2 = 0.11$).

HBCDs concentrations found in the analyzed fish oil samples were also compared with their respective levels of PBDEs (Fig. 4).

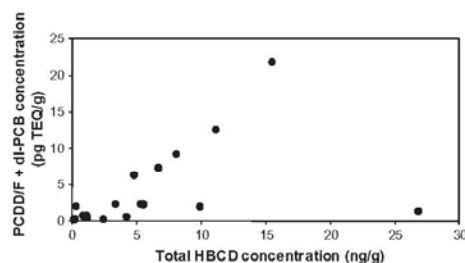


Fig. 3. Correlation between PCDD/F + dl-PCB and total HBCD in fish oil samples.

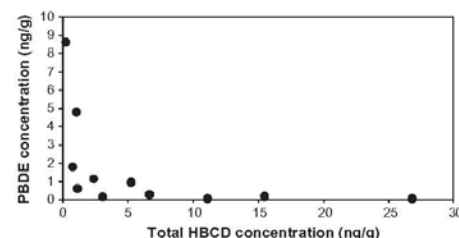


Fig. 4. Correlation between PBDE and total HBCD in fish oil samples.

Both products were used as brominated flame retardants. Despite this, there was not a clear relationship between both pollutants, with a correlation coefficient (r^2) of 0.33. This lack of correlation was confirmed by statistical hypothesis testing (Braumoeller, 2004). This test rejected the null hypothesis, which considered that both series have a significant correlation (p -value = 0.05). Probably, the alternative use of PBDEs or HBCDs as flame retardants could have influence on this low correlation. Additionally, it has to be mentioned that in this study, only the dominant PBDE congeners only were monitored: PBDE 28, PBDE 47, PBDE 99, PBDE 100, PBDE 153, PBDE 154 and PBDE 183. This fact could have some influence on the comparison.

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Hexabromocyclododecane in Human Breast Milk: Levels and Enantiomeric Patterns

ETHEL ELJARRAT,^{*,†} PAULA GUERRA,[†]
ELENA MARTÍNEZ,[†] MARINELLA FARRÉ,[†]
JUAN G. ALVAREZ,[†]
MARISA LÓPEZ-TEIJÓN,[†] AND
DAMIA BARCELÓ^{*,‡}

Department of Environmental Chemistry, IDAEA, Consejo Superior de Investigaciones Científicas, Jordi Girona 18-26, 08034 Barcelona, Spain, Servicio de Reproducción, Instituto Marqués, Fundación Leonardo Marqués, Manuel Girona 33, 08034 Barcelona, Spain, and Catalan Institute for Water Research, Parc Científic i Tecnològic de la Universitat de Girona, Pí de Peguera 15, 17003 Girona, Spain

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Samples of human breast milk ($n = 33$) from A Coruña (NW of Spain) collected in 2006 and 2007 were analyzed for HBCD diastereoisomers and enantiomers. HBCD was detected in 30 out of 33 human milk samples, at concentration levels ranging between 3 and 188 ng/g lw, with a median value of 27 ng/g lw, showing higher levels than those published for other countries. Diastereoisomeric pattern shows the predominance of the γ -isomer, with low contribution of the α -isomer and the β -isomer being below the limit of quantification. However, in six samples, there is a dominance of the α -isomer versus the γ -isomer. For the first time, HBCD enantiomeric analysis was carried out in human breast milk samples, showing a selective enantiomeric enrichment in human body. As regards α -HBCD, an enrichment of (–)-enantiomer was observed. However, in the case of γ -HBCD, no clear preference for one or the other enantiomer was found. Finally, and based on the calculated HBCD concentrations in human breast milk from Spain, the daily ingestion rate of HBCD was estimated. The nursing infant dietary intake for HBCD was set at 175 ng (kg of body weight)^{–1} day^{–1}.

Introduction

Hexabromocyclododecane (HBCD) is a brominated aliphatic cyclic hydrocarbon used as a flame retardant in thermal insulation building materials, upholstery textiles, and electronics. In 2001, the world market demand for HBCD was 16 700 tins, from which 9500 tons was sold in the European Union (1). These figures make HBCD the second highest-volume brominated flame retardant (BFR) used in Europe, after tetrabromobisphenol-A and before decabromodiphenyl ether (2). The production and use of penta- and octabromodiphenyl ethers has been restricted in Europe (3). HBCD may be used as an alternative for polybrominated diphenyl ethers (PBDEs) in some applications (4). To date, there are

no restrictions on the production or use of HBCD. As a result of their widespread use and their physical and chemical properties, HBCD are now ubiquitous contaminants in the environment and humans (5).

When all elements of symmetry are considered, 16 different HBCD diastereoisomers, including six pairs of enantiomers as well as four meso forms, are possible theoretically. Technical HBCD is produced industrially by addition of bromine to *cis-trans-trans*-1,5,9-cyclododecatene, with the resulting mixture that contains three predominant diastereoisomers known as α -, β -, and γ -HBCD. Normally, the γ -isomer is the most dominant in the commercial mixtures (ranging between 75% and 89%), followed by α - and β -isomers (10–13% and 1–12%, respectively) (6). Moreover, α -, β -, and γ -HBCD diastereoisomers are chiral, leading to three pairs of enantiomers.

Biomonitoring studies on persistent organic pollutants (POPs) in human tissues and fluids provide a direct measure of the internal dose of these substances. At present, human biomonitoring data represent the most reliable estimate of human exposure resulting from multiple sources to be used in risk assessment. The chemico-physical stability of POPs is responsible for their long persistence in humans, whereas their high lipophilicity determines their accumulation and storage in the lipid fraction of tissues. The relatively rich lipid content of human milk (compared to blood or urine) makes it an ideal matrix for measurement of POPs. Moreover, breast milk is a noninvasive way of taking samples. In addition, levels in human milk reflect accumulation in the mother but also indirect exposure of the fetus and direct exposure of the breast-fed infant at a sensitive stage at the beginning of life. Breast milk monitoring studies of POPs are of primary importance for carrying out an adequate risk assessment at the actual levels of human exposure and represent a major source of information on infant perinatal exposure. During the past few decades, numerous investigations on pollution of POPs in human breast milk have been conducted in various countries (7, 8). As regards BFRs, a considerable number of studies of PBDEs in human breast milk have been undertaken (9, 10). Moreover, PBDEs were also included on the Breast Milk Monitoring Program (BMMP) managed by the WHO, in which many countries are participating (11). However, very few studies have reported the occurrence of HBCD in human samples, and little information is available regarding HBCD concentrations in human breast milk. Moreover, HBCD consists of several diastereoisomers, but the majority of published works did not give information on isomeric HBCD content. To our knowledge, there are only two studies reporting the isomeric HBCD distribution in human breast milk samples (12, 13).

The objective of this study was to determine HBCD diastereoisomer levels in human breast milk from Spain and to provide valuable information to evaluate the body burden of mothers and the exposure to this flame retardant in infants via breast-feeding. Moreover, enantiomeric fractions (EFs) were calculated in order to investigate potential selective enantiomeric enrichment in human bodies. To our knowledge, this is the first time that enantiomeric HBCD patterns have been investigated in human breast milk.

Experimental Procedures

Sample Collection. The experimental protocol was approved by a local ethical committee in accordance with Spanish regulation, and the informed consent of all participating subjects was obtained. The mothers were asked to complete a questionnaire for information about residence, maternal

* Corresponding author phone: 34-93-4006100; fax: 34-93-2045904; e-mail: eeeqam@cid.csic.es.

[†] Consejo Superior de Investigaciones Científicas.

[‡] Fundación Leonardo Marqués.

[§] Parc Científic i Tecnològic de la Universitat de Girona.

age, number of infants previously breast-fed, newborn weight, and newborn sex.

The breast milk (about 10 mL) was collected either by use of a breast pump or by hand-expressing the milk into prewashed glass containers 40 days postpartum at the hospital. After milk collection, samples were stored in the freezer at -22°C until analysis in the laboratory.

Sample Preparation. Before extraction, samples were lyophilized, homogenized, and stored at -22°C . Samples of 0.5 g dry weight (dw) of milk were spiked with 50 μL of the labeled standard γ -HBCD- d_{18} at 125 pg/ μL . Spiked samples were kept overnight to equilibrate. Extraction was carried out by the pressurized liquid extraction (PLE) method on a fully automated ASE 200 system (Dionex, Sunnyvale, CA). An 11 mL extraction cell was loaded by inserting two cellulose followed by the human milk sample. The dead volume was filled with anhydrous sodium sulfate for residue analysis previously activated at 150°C for 4 h (Panreac Química S.A., Montcada i Reixac, Barcelona, Spain). Extraction conditions were as follow: dichloromethane/hexane (2:1) mixture as solvent extractor, temperature of 100°C , pressure of 14 MPa, two static extractions of 5 min and 100% flush. After extraction, the crude extracts were concentrated to 3 mL and then subjected to a purification step via acid attack with concentrated H_2SO_4 (95–97% purity) (3×2 mL). Samples were finally concentrated to incipient dryness and redissolved with 50 μL of the labeled standard α -HBCD- d_{18} at 125 pg/ μL , prior to instrumental analysis by LC-MS.

All milk material was used for HBCD analyses, and no extra sample was available for the determination of lipid content on additional subsample. That is because calculations were carried out through the concentration to dryness of crude extracts and final determination of lipid content gravimetrically.

Diastereoisomer Analysis. The LC system used was an Agilent HP 1100 binary pump (Agilent Technologies, Palo Alto, CA) with a Symmetry C_{18} column (2.1 mm $\times$ 150 mm, 5 μm) preceded by a C_{18} guard column (2.1 $\times$ 10 mm) supplied by Waters. Experiments were carried out with water/methanol (3:1 v/v) as eluent A and methanol as eluent B, at a flow rate of 0.25 mL/min. The injection volume was set at 4 μL . The elution program started at an initial composition of 100% A and was ramped to 0% A in the first minute, then eluent A was increased to 10% in 17 min, and initial conditions were reached again in 3 min and returned to the starting conditions in 15 min. Mass spectrometric analysis was performed with a hybrid triple-quadrupole/linear ion trap MSD Sciex 4000 QTRAP (Applied Biosystems, Foster City, CA) instrument equipped with an electrospray (ESI) Turbospray interface, working in negative ionization mode. For target quantitative analyses, data acquisition was performed in selected reaction monitoring (SRM). The $[\text{M} - \text{H}]^{-} \rightarrow \text{Br}^{-}$ transitions at m/z 638.7 $\rightarrow$ 78.9 and 638.7 $\rightarrow$ 80.9 were monitored for unlabeled HBCD. The labeled HBCD were monitored at the 655.8 $\rightarrow$ 78.9 and 655.8 $\rightarrow$ 80.9 transitions. The MS/MS detection conditions were optimized to obtain the highest relative intensity: curtain gas at 50 psi, collision gas at 4.5×10^{-6} Torr, temperature of the turbo gas in the TurbolonSpray source at 350°C , ion source gas 1 at 50 psi, and ion source gas 2 at 10 psi (14).

Quality Assurance/Quality Control. Method blank samples were performed to check for interference or contamination from solvents and glassware. No presence of analytes of interest (α -, β -, and γ -HBCD diastereoisomers) was observed.

Spiked samples with native compounds (α -, β -, and γ -HBCD) were analyzed by the described methodology to check recoveries. These samples were concentrated to incipient dryness and redissolved with 50 μL of labeled compounds mixture, containing α -HBCD- d_{18} and γ -HBCD-

d_{18} at 125 pg/ μL , prior to instrumental analysis by LC-MS. Recovery values ranged from 79.9% to 119%, with a relative standard deviation ($n = 5$) values between 7% and 10%. Detection limits, defined as the minimum amount of analyte that produces a peak with a signal-to-noise ratio equal to 3, were 0.1 ng/g lw for α - and γ -isomers and 1.0 ng/g lw for β -HBCD. Limits of quantification, defined as the minimum amount of analyte that produces a peak with a signal-to-noise ratio equal to 10, were 0.2, 0.3, and 3.3 ng/g lw for α -, γ -, and β -HBCD, respectively.

Enantiomeric Analysis. A chiral chromatographic column, Nucleodex β -PM (4.0 mm $\times$ 200 mm $\times$ 5 μm), was used to afford the enantiomer-specific determination. The optimal separation of enantiomers was achieved with methanol, acetonitrile, and water as mobile phase. Experiments were carried out with 70% water/30% methanol as eluent A and 70% acetonitrile/30% methanol as eluent B, at a flow rate of 0.50 mL/min. The injection volume was set at 10 μL . The elution program started at an initial concentration of eluent A at 50%, decreased to 0% along the first 8 min, and was maintained for 17 min, and initial conditions were reached again in 5 min and maintained for additional 12 min (15, 16). Mass spectrometric analysis was performed under the same conditions as diastereoisomer analysis.

Enantiomeric composition was expressed as enantiomeric fraction (EF), which is normally calculated from the peak areas of the enantiomeric pairs by

$$\text{EF} = \frac{A_+}{(A_+ + A_-)} \quad (1)$$

where A_+ and A_- are the peak areas of eluting (+)- and (–)-enantiomers, respectively.

It is well-known that ESI is subject to sample matrix effects that can cause enhancement or suppression of the target analytes signal and can adversely affect their quantification. In order to avoid this effect, which can affect EF calculations, Marvin et al. (17) introduced the corrected EF values, calculated by eq 2. This correction is based on the use of isotopic labeled standards (HBCD- d_{18}) since d_{18} -labeled enantiomeric analogues behave in an identical manner to their native counterparts.

$$\text{EF}_{\text{corrected}} = \frac{(A_+/A_{+d_{18}})[\text{picograms of (+)-HBCD-}d_{18}]}{(A_+/A_{+d_{18}})[\text{picograms of (+)-HBCD-}d_{18}] + (A_-/A_{-d_{18}})[\text{picograms of (–)-HBCD-}d_{18}]} \quad (2)$$

Results and Discussion

Diastereoisomer Levels. HBCD was detected in 30 out 33 human milk samples, at concentration levels ranging between 3 and 188 ng/g lw (Table 1), with the mean and median concentrations being 47 and 27 ng/g lw, respectively. The lipid content ranged from 0.6% to 7.1%.

In comparison to PBDEs, much less information is available on the HBCD concentrations in human tissues such as human breast milk. Thus, a comparison of our results with other available data is hardly achievable. The number of published studies related to HBCD concentration levels in human breast milk is limited (Table 2). HBCD was detected in human milk from primiparous Swedish women collected in 2001, with the mean and maximum concentrations being 0.5 and 2.4 ng/g lw, respectively (18). Similar results were obtained in subsequent monitoring carried out in 2002 and 2003 (19). HBCD was also found in Norwegian human milk at concentrations between 0.3 and 20 ng/g lw (20, 21). HBCD levels have been reported in breast milk samples from Mexican mothers at concentrations ranging from 0.8 to 5.4 ng/g lw, with a mean concentration of 1.2 ng/g lw (22). Another breast milk study in North America reported the HBCD contamination in samples collected in Ontario

TABLE 1. Concentration Levels and EF Values for Human Breast Milk Samples^a

sample code	% lw ^b	concentration level (ng/g of lipid weight)				EF _{corrected}	
		α-HBCD	β-HBCD	γ-HBCD	total HBCDs	α-HBCD	γ-HBCD
L-1	4.0	12.0	NQ	176	188		0.602
L-2	2.6	1.59	NQ	141	143		0.494
L-3	5.8	NQ	NQ	66.5	66.5		0.507
L-4	4.2	NQ	NQ	68.7	68.7		0.554
L-5	6.4	0.25	NQ	7.80	8.05		0.595
L-6	7.1	5.35	NQ	22.6	28.0		0.490
L-7	3.7	NQ	NQ	27.0	27.0		0.553
L-8	5.7	2.24	NQ	16.4	18.6		0.508
L-9	4.1	2.82	NQ	13.7	16.5		0.507
L-10	5.3	NQ	NQ	7.90	7.90		0.344
L-11	3.1	2.21	NQ	23.1	25.3		nc
L-12	4.3	NQ	NQ	21.7	21.7		nc
L-13	1.7	ND	ND	ND	ND		
L-14	5.0	ND	ND	ND	ND		
L-15	2.8	71.5	NQ	NQ	71.5	0.102	
L-16	2.1	7.51	ND	28.9	36.4		
L-17	3.2	17.5	ND	53.9	71.4		
L-18	4.1	10.4	ND	25.2	35.6		
L-19	3.0	19.1	ND	22.8	41.9		
L-20	5.1	3.94	ND	9.49	13.4		
L-21	2.4	1.10	ND	61.9	63.0		
L-22	1.7	13.8	ND	9.13	22.9		
L-23	2.0	1.77	ND	0.95	2.72		
L-24	0.6	4.95	ND	60.5	65.5		
L-25	6.0	14.7	ND	134	148		0.268
L-26	1.5	3.56	ND	NQ	3.56		
L-27	2.5	9.49	ND	17.5	27.0		
L-28	2.8	2.11	ND	23.4	25.5		
L-29	5.5	NQ	ND	12.9	12.9		
L-30	5.4	0.76	ND	5.43	6.19		
L-31	5.3	NQ	ND	NQ	NQ		
L-32	2.6	2.76	ND	NQ	2.80		
L-33	0.8	122	ND	14.2	136	0.204	

^a ND, below limit of detection; NQ, below limit of quantification; nc, not calculated. ^b Lipid weight percentage referred to wet weight

TABLE 2. HBCD Concentrations in Human Breast Milk from Different Countries^a

country	year	α-HBCD	β-HBCD	γ-HBCD	total HBCD	positive	ref
Sweden	2001	nr	nr	nr	ND–2.4	12 (<i>n</i> = 33)	18
	2002–2003	nr	nr	nr	ND–1.5	24 (<i>n</i> = 30)	19
Norway	2001	nr	nr	nr	0.25–2.0	nr (<i>n</i> = 9)	20
	1993–2001	nr	nr	nr	0.4–20	49 (<i>n</i> = 85)	21
Mexico	nr	nr	nr	nr	0.8–5.4	7 (<i>n</i> = 7)	22
Canada	2002–2003	3.8	nr	nr	0.4–19	nr (<i>n</i> = 8)	12
United States	2002	0.5	nr	nr	0.2–0.9	nr (<i>n</i> = 9)	12
Japan	1973–1988	ND	ND	ND	ND	nr	13
	1988–2006	0.43–1.9	ND	ND–2.6	0.43–4.0	11 (<i>n</i> = 11)	13
Russia	2000–2002	nr	nr	nr	ND–1.67	11 (<i>n</i> = 37)	23
France	2005	ND–5	ND	ND	ND–5	7 (<i>n</i> = 23)	24
Spain	2006–2007	ND–122	ND	ND–176	ND–188	30 (<i>n</i> = 33)	this study

^a Results are expressed in nanograms per gram of lipid weight. ND, below limit of detection; nr, not reported.

(2002–2003) and Texas (2002), with mean concentration values of 3.8 and 0.5 ng/g lw, respectively (12). HBCD have been also detected in human samples from Japan (1988–2006), with levels ranging from 0.4 to 4 ng/g lw (13). Finally, two recent studies reported the first values for HBCD concentrations in human breast milk from Russia (23) and France (24), with values up to 1.7 and 5 ng/g lw, respectively. It should be pointed that our values are higher than those available for other countries (Sweden, Norway, Mexico, Canada, United States, Japan, Russia, or France), which are always below 20 ng/g lw. However, it is important to notice that the majority of published data are based on sampling campaigns carried out some years ago. There is a lack of information related to

recent HBCD levels in human breast milk. Since usage of HBCD is increasing, it is expected that HBCD levels in human samples also increased. This could be one explanation of our higher results.

As regards the diastereoisomeric pattern, there is a predominance of the γ-isomer (from 54% to 100% of total contribution), with a low contribution of the α-isomer and the β-isomer being below the limit of quantification. However, in six samples, this diastereoisomeric pattern was different, showing dominance of the α-isomer (from 60% to 100% of the total contribution) versus the γ-isomer. This inconsistency in the diastereoisomeric pattern may be due to individual

variability in metabolizing capacity or other unknown factors, such as the frequency of HBCD exposure.

Some HBCD diastereoisomeric pattern studies have been carried out in human samples. Some reports have suggested that β - and γ -HBCD diastereoisomers can be more extensively metabolized in organisms than can α -HBCD diastereoisomer. Weiss et al. (25) found that the most abundant stereoisomer in serum samples from Sweden was α -HBCD. In contrast, and similar to our findings, Johnson-Restrepo et al. (26) found that γ -HBCD was the dominant isomer present in human adipose tissue samples from the United States, accounting for 83% of the total HBCD concentrations, and followed by α -HBCD (17%). β -HBCD stereoisomer was not detected in any of the human adipose tissue samples analyzed. However, 35% of the human tissue samples contained α -HBCD concentrations higher than the concentrations of γ -HBCD, suggesting that some individuals are exposed to elevated concentrations of α -HBCD. Thomsen et al. (27) also found higher concentrations of γ -HBCD than the other HBCD diastereoisomers in serum of workers from an industrial polystyrene plant in Scandinavia.

As regards information related to HBCD diastereoisomeric patterns in human breast milk, there are only two published works reporting data. The first study was carried out analyzing breast milk samples from North America (12). In these samples, the α -diastereoisomer predominated, with concentration levels ranging between 0.2 and 19 ng/g lw. The second study reported the diastereoisomeric pattern in human breast milk from Japanese women (13). In the majority of these samples, the α -diastereoisomer also predominated, but some exceptions were observed in which the contribution of the γ -diastereoisomer was higher than that of α .

As the literature regarding diastereoisomer distribution of HBCD in human milk is very scarce, further studies are needed to elucidate the relationship between HBCD diastereoisomers found in humans and those found in source materials.

Enantiomeric Fractions. For the first time, HBCD enantiomeric analysis was carried out in human breast milk samples, showing the presence of the two pairs of enantiomers, (-)- α - and (+)- α -HBCD and (-)- γ - and (+)- γ -HBCD (Figure 1). EF_{corrected} values were calculated for different samples (Table 1). In the case of γ -HBCD, it was observed that EF values are between 0.268 and 0.602, whereas EF for α -HBCD was calculated only for two samples, with values of 0.102 and 0.204. If we compare these EF values with those obtained with standard solutions (Figure 2a), we can observe that no significant differences were detected between EF mean values for γ -HBCD. The EF mean value obtained for standard solutions was 0.487 [1.14% of relative standard deviation (RSD)] and 0.493 for human breast milk samples. However, the RSD for milk samples was set at 20.6%, indicating that we can sort all the samples into three different groups (Figure 2b). The first one corresponded to samples with no significant differences from standards. This group was formed by five different samples, with an EF mean value of 0.501 and RSD of 1.70%. The second one, formed by four milk samples, showed an increase of EF value compared to standard value: mean value of 0.576 and 4.54% of RSD. Thus, samples included in this second group showed an enrichment of the (+)- γ -HBCD enantiomer in the human body. Finally, the third group consisted of samples ($n=2$) where EF values are lower than those obtained for standard solutions: 0.268 and 0.344. In this case, the lowering of EF value indicates an enrichment of the (-)- γ -HBCD enantiomer in human body.

In the case of α -HBCD, it was possible to calculate the EF value only for two human breast milk samples. Results showed an important decrease of EF values (0.102 and 0.204) in these samples with respect to the standards (mean value of 0.531). Thus, we can assume that an (-)- α -HBCD

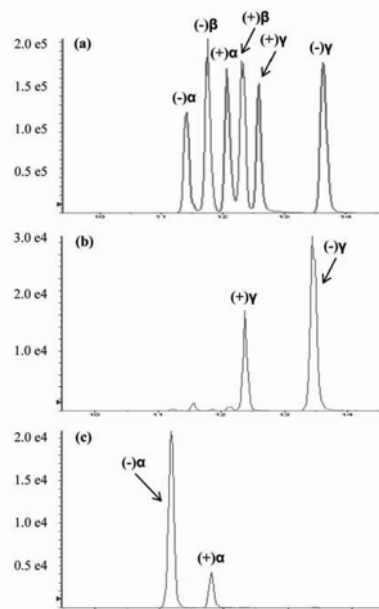


FIGURE 1. Enantiomeric analysis for (a) standard solution and (b, c) human breast milk samples.

enrichment occurs, showing a selective enantiomeric enrichment in human body.

In a published work on marine species from the Western Scheldt estuary (28), the authors have reported for the first time selective accumulation of different HBCD enantiomers in marine fish. In two further studies, the database of EFs for α -HBCD has been extended to herring and several predatory birds (29) and dolphins (30). In all cases, a deviation from the racemic mixture (EF = 0.5) has been observed, suggesting that an enantioselective uptake and/or metabolism of (+)- and (-)- α -HBCD must occur. Interestingly, no clear preference for one or the other α -HBCD enantiomer was found. Herring muscle and falcon eggs were clearly enriched in (-)- α -HBCD, whereas in whiting liver and sea eagle eggs (+)- α -HBCD dominated.

Our data in human samples have not been described before in the literature. Further studies in a great number of human breast milk samples are required in order to check this differential enantiomeric behavior.

Nursing Infant Dietary Intake Estimation. POPs may accumulate for a long period in the body of the mother and then be partially transferred to the nursing infant via mother's milk. It is realized that the presence of POPs in human breast milk has been of great concern because these lipophilic chemicals are readily transferred and absorbed to infants. In the case of dioxins, it is reported that 1- to 3-month-old infants absorb more than 90% of most dioxin congeners containing in their mothers' milk (31). Hence they may be exposed to relatively high levels of POPs during this period.

To understand the magnitude of exposure to HBCD by infants, we estimated the daily intake (EDI). EDI of contaminants by infants via breast milk can be calculated as

$$EDI_i = C_i F M b \quad (3)$$

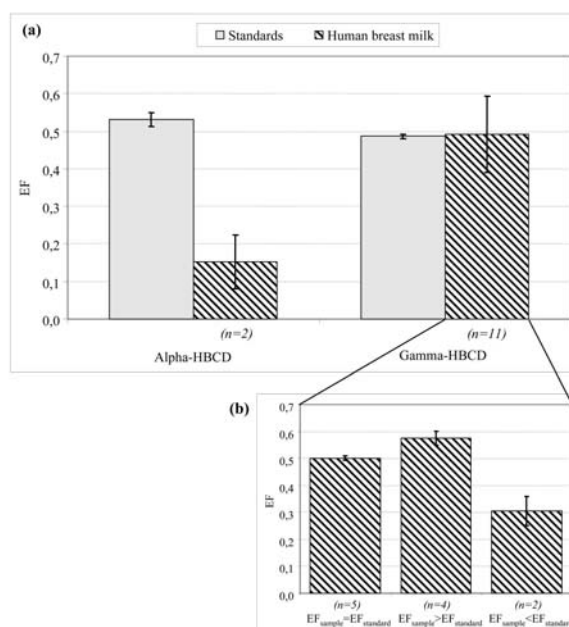


FIGURE 2. Enantiomeric fraction (EF) values (mean $\pm$ standard deviation) in standards and human breast milk samples: (a) mean values of all samples analyzed; (b) breakdown into samples with EF equal to, lower than, or higher than standard EF.

where EDI_i is the estimated daily intake [micrograms per kilogram of body weight (bw) per day]; C_i is the median concentration of HBCD in milk samples (micrograms per gram of lipid weight); F is the lipid content in milk samples (grams of lipid per 100 g of milk); and Mb is the daily consumption of milk (grams per kilogram of body weight per day). For this calculation, guidelines suggested by the U.S. EPA (32), such as the daily intake rate of breast milk and body weight for a 1-month-old infant were on average 702 mL of milk per day (723 g of milk per day) and 4.14 kg, respectively, were used to calculate an infant's average milk consumption, which was (Mb) 175 g of milk (kg bw)⁻¹ day⁻¹. The mean value of lipid content in analyzed samples was used for F estimation, with a value of 3.71 g of lipid per 100 g of milk; and the median concentration of HBCD in analyzed samples was applied for C estimation, with a value of 0.027 μ g/g lw. The infant's daily intake rate estimated for HBCD was 175 ng (kg bw)⁻¹ day⁻¹. The possible toxicological impact of such an EDI should certainly be investigated.

Our daily dietary intake estimation through breast-feeding was compared with published data on human adult dietary intake through the diet (Figure 3). There are few data available on the dietary HBCD intake. A median intake of 141 ng of HBCD per day [2.15 ng (kg bw)⁻¹ day⁻¹] was estimated for the Swedish population, with a maximum of 1076 ng per day [21.5 (kg bw)⁻¹ day⁻¹] (33). In another study (34), an estimation of the fish-related dietary exposure of HBCD for the average Dutch population was done. The median bound intake in that study was estimated at 8.3 ng/day for a 70-kg person [0.12 ng (kg bw)⁻¹ day⁻¹]. The estimated adult dietary intake of HBCD arising from the United Kingdom diet was in the range of 5.9–7.9 ng (kg bw)⁻¹ day⁻¹ (35). Finally, dietary exposure to HBCD in the Norwegian population was established at 0.3 ng (kg bw)⁻¹ day⁻¹ (36). As can be seen, all

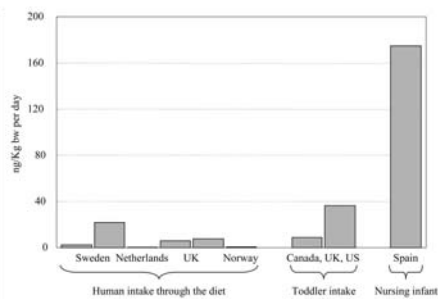


FIGURE 3. HBCD dietary intake evaluations (expressed as nanograms per kilogram of body weight per day) through different routes of exposition and for different groups of population.

these studies presented estimated HBCD dietary intake very much lower than those obtained in our study for nursing infants. Our daily intake of HBCD by Spanish infants via breast-feeding value is 25–1458 times greater than the estimated adult dietary intakes reported from Sweden, Netherlands, United Kingdom, and Norway. However, it should be pointed that EDI for infants changes when body weight increases (POP diluting effect), whereas estimation for adults is based on a lifelong exposure.

Human exposure to HBCD occurs through multiple routes. For nonoccupationally exposed persons, the major intake of HBCD is probably from food and indoor air or dust. However, the relevance of human HBCD exposure originating

from house dust versus food-based HBCD exposure is still unknown. Recently, Abdallah et al. (37) estimated the exposure of toddlers (6–24 months) to HBCD via dust ingestion in three different countries (Canada, United Kingdom, and United States) if 100% absorption of intake was assumed. The median exposure for mean dust ingestion (50 mg/day) was established between 19 and 37 ng/day. However, for high dust ingestion (200 mg/day), the values reached up to 150 ng/day. By comparison of orders of magnitude obtained from dust ingestion intake with those obtained with our daily dietary intake estimation through breast-feeding, it seems that, in Spain, food intake represents the main HBCD exposure for a nursing infant (Figure 3). However, it should be pointed that a correct comparison between different routes of exposure must be done with data coming from the same place. But actually, there is a lack of data in the same location.

Although discussions on the safety of breast-feeding have come to the firm conclusion that exclusive breast-feeding should continue to be strongly supported and promoted, the HBCD and POP contamination problem should not be ignored. Continued breast milk monitoring programs are important exercises as surveillance and early warning systems for abnormal exposure of the population to HBCD and other POPs.

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“Temporal trends of brominated flame retardants in Australia: 1993-2009”

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L. Toms, P. Guerra, E. Eljarrat, F. A. Harden, P. Hobson, A. Sjodin y J. F. Mueller

E. Eljarrat, P. Guerra y D. Barceló

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TEMPORAL TRENDS OF BROMINATED FLAME RETARDANTS IN AUSTRALIA: 1993-2009

Leisa-Maree L. Toms^{a,b,c}, Paula Guerra^c, Ethel Eljarrat^c, Dania Barcelo^{c,d}, Fiona A. Harden^e, Peter Hobson^f, Andreas Sjödin^f and Jochen F. Mueller^a

- 1 The University of Queensland, National Research Centre for Environmental Toxicology (Entox), Brisbane, Australia
- 2 Medical Radiation Sciences, Queensland University of Technology, Brisbane, Australia
- 3 Department of Environmental Chemistry, IDAEA, CSIC, Barcelona, Spain.
- 4 Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, Pic de Peguera 15, 17003 Girona, Spain
- 5 Sullivan Nicolaides Pathology, Brisbane, Australia
- 6 Centers for Disease Control and Prevention, Atlanta, USA

Corresponding author: Leisa-Maree Leontjew Toms, Medical Radiation Sciences, Queensland University of Technology, Brisbane, 4001, Australia

Email: leisamaree.toms@qut.edu.au

Abstract

Brominated flame retardants, including polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) are used to reduce the flammability of a multitude of electrical and electronic products. While the use of selected PBDEs has ceased, use of decaBDE and HBCD continues. Limited data show PBDE concentrations have increased over time to peak around the late 1990s. Despite elevated human concentrations, no temporal trend data is available on PBDEs or HBCD in Australia.

This study aimed to assess temporal trends of PBDEs and HBCD from 1993 to 2009 and in so doing provide for the first time, background HBCD concentrations from a representative sample of the Australian population.

Samples of human milk collected in Australia from 1993 to 2009, primarily from primiparae mothers were combined into 12 pools from 1993 (2 pools); 2001; 2002/2003 (4 pools); 2003/2004, 2006; 2007/2008 (2 pools); and 2009. Blood serum was collected in 2002/03, 2004/05, 2006/07 and 2008/09 from 9694 samples in 204 pools.

In human milk, concentrations of total HBCD ranged from not quantified (n.q.) to 19 ng/g lipid while α -HBCD and γ -HBCD ranged from n.q. to 10 ng/g lipid and n.q. to 9.2 ng/g lipid. β -HBCD was detected in only one sample at 3.6 ng/g lipid while Σ PBDE ranged from 2.5 -

15.8 ng/g lipid. In blood, Σ_4 PBDE concentrations for adults ranged from 5.6 – 30.3 ng/g lipid in 2002/03; 4.0 – 12.2 ng/g lipid in 2004/05; 5.5 – 14.5 ng/g lipid in 2006/07 and 6.6 – 13.7 ng/g lipid in 2008/09.

No temporal trend was apparent in HBCD concentrations in human milk collected in Australia from 1993 to 2009 while PBDE concentrations show a peak around 2002/03 (mean Σ_4 PBDEs = 9.6 ng/g lipid) and 2003/04 (12.4 ng/g lipid) followed by a decrease in 2007/08 (2.7 ng/g lipid) and 2009 (2.6 ng/g lipid). While no trend is apparent for PBDE concentrations in the adult population, there appears to be a trend of decreasing concentrations in children. This may indicate that youth body burden may better reflect actual exposure. Continuing monitoring including a larger time span may be required to observe a definitive decrease in PBDE body burden following legislative changes to usage.

Introduction

Usage of brominated flame retardants (BFRs) including polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) has changed over recent years. Commercial production of PBDEs commenced in Germany in the 1970s (Siddiqi *et al.* 2003) and by 2001, around 50% of the global production of penta-, octa- and deca-BDE commercial products were used in the Americas, followed by 37% in Asia and 12% in Europe (BSEF 2005).

In Australia, in 2003/04, usage of raw chemical PBDEs was reported to account for less than 1% of global production (NICNAS 2005) with importation of these products ceasing in 2005 (NICNAS 2007). The quantity of PBDEs imported into Australia in manufactured products, however, remains unknown. HBCD has been used “for many years” (BSEF 2009) with continued usage in products such as upholstered textiles, rigid form plastics and polystyrene foam used in baby car seats (NICNAS 2005). Importation of HBCD raw product into Australia increased 78% between 1998/99 and 2003/04 (NICNAS 2005). The commercial HBCD product usually contains three stereoisomers alpha, beta and gamma at approximately 6, 8 and 80% (Environment Canada 2011). HBCD is subject to Risk Phrases R50-R53 under EU Directive 67/548: “Dangerous for the environment – very toxic to aquatic organisms – may cause long-term adverse effects in the aquatic environment”. The commercial product HBCD has a high bioaccumulative potential, and there are indications of biomagnification comparable with or even exceeding that of BDE-47 (Vorkamp *et al.* 2011).

PBDEs and HBCD have been described as cost effective and highly efficient ways to reduce flammability and therefore potentially reduce the harm to humans caused by fires. Human exposure to these BFRs is suggested to occur via food and dust ingestion and inhalation (Harrad *et al.* 2004; Wilford *et al.* 2005; Thomsen *et al.* 2008; Roosens *et al.* 2009), however, investigations to elucidate these pathways are continuing.

Increased worldwide interest in PBDEs lead to the cross-sectional assessment of human body burden and exposure pathways of PBDEs in Australia in the early 2000's. Despite the low levels of importation, the concentrations in the Australian population were around twice that found in Europe and Asia but 10 times lower than in North America (Toms *et al.* 2007; 2008; 2009; 2009). With the cessation of PBDE use, it is hypothesized that exposure and in turn human body burden will decrease. Studies from Sweden, Norway and Japan show PBDE concentrations in humans peaked around the late 1990s (Meironyte Guvenius and Noren 2001; Darnerud *et al.* 2002; Akutsu *et al.* 2003; Thomsen *et al.* 2005) while a recent study from Sweden showed no consistent pattern of temporal trends in human milk from 1996 to 2006 with HBCD below the limit of quantification in most samples (Lignell *et al.* 2009). In the US, concentrations have increased significantly between 1985 and 2002 (Sjodin *et al.* 2004). Reports of BFR temporal trends in environmental samples over the last 30 years are varied. HBCD concentrations determined in peregrine falcon eggs from 1973 – 2001 in the

UK showed no clear temporal trends (Leslie *et al.* 2011) while in herring gull eggs from Norway, PBDE concentrations increased from 1983 to 1993 but leveled out until 2003 and alpha-HBCD increased over the time period (Helgason *et al.* 2009). Results from Asia show levels of PBDEs and HBCDs have increased over the last 30 years with concentrations of HBCD appearing to exceed those of PBDEs which the authors attribute to increased usage of HBCD compared to PBDEs (Tanabe *et al.* 2008).

Due to the longevity of products containing PBDEs e.g. televisions, mattresses, these products will remain in use for some time and therefore provide both primary and secondary sources of PBDEs to the environment and humans. HBCD use continues unrestricted in Australia and is not limited by EU regulations (European Chemical Industry Council accessed 20/9/09) thus human body burden would be expected to reflect current usage over time.

The main objective of this study is to present human biomonitoring data for BFRs in Australia including reporting the first data on HBCD in pooled human milk samples collected from 1993 – 2009. Furthermore we evaluate whether the available data from Australia indicate temporal trends of human body burden in the Australian population. Existing data on BFRs in Australia, archived samples and newly collected samples will be used to make an assessment of whether BFR usage is reflected in human body burden. The data are important for future monitoring of PBDE and HBCD body burden trends in Australia and to assess whether or not regulatory changes have affected human concentrations of BFRs.

Methods and Materials:

Sample Collection

Individual human milk samples and human blood sera have been collected throughout Australia from 2001 to 2009 and 2002 to 2009, respectively. In addition, human milk samples collected in 1993 (Quinsey *et al.* 1995) were also available for analysis. The 2001 – 2009 human milk was collected from volunteering mothers who were: primiparous; baby aged two to eight weeks; exclusively breastfeeding; healthy pregnancy; and a resident of the area for the past five years, see Toms *et al.* (2007) for full details. Participants were recruited through maternal and child health clinics and by word-of-mouth. Individual samples were pooled based on year of collection with 4 – 10 samples per pool. Where possible more than one pool was used for a specific time period, as follows: 1993 (2 pools); 2001; 2002/2003 (4 pools); 2003/2004; 2006; 2007/2008 (2 pools); and 2009. Human blood serum samples (n=9694 in 204 pools) were collected from surplus de-identified pathology samples through Sullivan Nicolaides Pathology primarily from South East Queensland, Australia. Samples were stratified by age (<16 (2002/03); 0-4 and 5-15 (2004/05 and 2008/09); cord blood; 0–0.5; 0.6–1; 1.1–1.5; 1.6 – 2; 2.1–2.5; 2.6–3; 3.1–3.5; 3.6–4; 4.1–6; 6.1–9; 9.1–12; 12.1–15 (2006/07); 16–30; 31–45; 46–60 and > 60 years); gender; and year of collection 2002/03

(n=2474), 2004/05 (n=2400), 2006/07 (n=2420) and 2008/09 (n=2400). Up to 30 samples per pool were used in the 2006/07 pools and 100 samples per pool in 2002/03, 2004/05 and 2008/09. The University of Queensland Medical Research Ethics Committee approved the study of human milk and human blood serum.

Analysis

Concentrations of HBCD and PBDEs in human milk were determined at the Department of Environmental Chemistry, IDAEA, CSIC, Barcelona, Spain by Liquid chromatography-mass spectrometry (LC-MS-MS) and gas chromatography coupled to mass spectrometry working with negative ion chemical ionization (GC-NCI-MS), respectively with full details of the methodologies listed in the Supporting Information. The pools of human blood were analysed using gas chromatography high resolution mass spectrometry. The 2002/03 and 2004/05 pools were analysed at Eurofins, Hamburg, Germany as described previously (Toms *et al.* 2008) and the 2006/07 and 2008/09 pools were analysed at the Center for Disease Control (CDC), Atlanta, Georgia, USA as described previously (Sjodin *et al.* 2004).

Results and Discussion

HBCD in human milk

In this first report of HBCD in the Australian population, this BFR was detected in 9 out of 13 human milk pools with the mean ($\pm$ standard deviation) concentration of sum HBCD (sum of α -, γ - and β -HBCD) in all samples of 10.2 ± 5.2 ng/g lipid (Table 1).

Table 1. Concentration (ng/g lipid) of PBDEs and HBCD in human milk by year of collection

Code	Origin	Fat content**	BDE-28	BDE-47	BDE-100	BDE-99	BDE-154	BDE-153	BDE-183	Σ ₄ PBDEs	α-HBCD	β-HBCD	γ-HBCD	Total HBCD
1	Blank ^a	2.7	na	na	na	na	na	na	na	na	nq**	<1	nq	nq
2	1993	3.8	<0.3	5.6	1.2	3.5	1.9	1.4	nq	8.7	nq	<1	nq	nq
3	1993	3.3	<0.3	4.4	1.1	5.1	1.2	1.4	1	6.7	10	<1	9.2	19
4	2001	3.2	<0.3	6.4	nq	2.7	1.1	1.6	<0.2	7.5	nq	nq	nq	nq
5	2002/03 ^b	3.2	<0.3	4.9	0.5	1.8	<0.2	1.2	nq	5.4	4.5	<1	6.8	11
6	2002/03 ^c	3.1	<0.3	4.0	nq	1.5	nq	1.0	<0.2	4.0	5.9	<1	4.8	11
7	2002/03 ^d	2.7	<0.3	11.5	2.3	4.5	2.0	3.2	2.1	15.8	3.1	nq	2.6	5.7
8	2002/03 ^e	2.2	<0.3	6.8	1.8	2.7	nq	3.3	3.0	8.6	nq	<1	nq	nq
9	2002/03 ^f	4.1	<0.3	7.2	1.3	3.7	nq	2.4	2.0	8.5	4	nq	6.6	11
10	2003/04	4.5	<0.3	9.3	1.4	2.8	1.7	4.5	nq	12.4	nq	nq	nq	nq
11	2006	3.7	<0.3	8.5	1.4	1.7	nq	1.5	nq	9.9	2.8	nd	2.2	5
12	2007/08 ^g	2.9	<0.3	3.0	<0.1	0.6	<0.2	2.0	nq	3.0	7.8	nq	4.5	12
13	2007/08 ^h	3.1	<0.3	2.5	nq	0.9	nq	1.6	<0.2	2.5	6.8	3.6	4.4	15
14	2009	3.3	<0.3	1.4	1.2	0.6	nq	1.3	nq	2.6	2.1	<1	0.4	2.5

a – cows' milk; b – Brisbane; c – duplicate of 2002/03 Brisbane; d – Wollongong; e – Melbourne; f – Hunter Valley; g – Brisbane pool 1; h – Brisbane pool 2; * Expressed as % relative to dry weight, ** - Above detection limit (0.1), but below limit of quantification (0.2); na – not assessed, nq – not quantified, nd – not detected, value following < is the limit of detection

Generally, the HBCD concentrations observed in human milk were lower than total PBDE concentrations which were detected in all pools of human milk. The determination of the diastereoisomeric pattern showed a predominance of the α - and γ -isomers, with the β -isomer being below the limit of quantification (with the exception of one unique sample). In samples from 1993 to 2006, the contribution of the α - and γ -isomers was similar. However, samples from 2007 to 2009 showed a dominance of the α -isomer (from 54 to 84% of the total contribution) versus the γ -isomer. This inconsistency in the diastereoisomeric pattern may be due to individual variability in metabolizing capacity or other unknown factors such as the frequency of HBCD exposure. HBCD enantiomeric analysis was carried out showing the presence of the two pairs of enantiomers, (-)- α - and (+)- α -, and (-)- γ - and (+)- γ -HBCD, see Supporting Information for further details. The concentrations of HBCD are below those found in studies from Spain but are in accordance with those obtained for other countries (Table 2).

Table 2. HBCD concentrations in human breast milk from different countries. (Results expressed in ng/g of lipid weight)

Country	Year	α -HBCD	β -HBCD	γ -HBCD	Total HBCD	Positive (n)
Sweden	2001	nr	nr	nr	ND-2.4	12 (33)
	2002-2003	nr	nr	nr	ND-1.5	24 (30)
Norway	2001	nr	nr	nr	0.25-2.0	nr (9)
	1993-2001	nr	nr	nr	0.4-20	49 (85)
Mexico	nr	nr	nr	nr	0.8-5.4	7(7)
Canada	2002-2003	3.8	nr	nr	0.4-19	nr (8)
USA	2002	0.5	nr	nr	0.2-0.9	nr (9)
Japan	1973-1988	ND	ND	ND	ND	nr
	1988-2006	0.43-1.9	ND	ND-2.6	0.43-4.0	11 (11)
Russia	2000-2002	nr	nr	nr	ND-1.67	11 (37)
Philippines	2004	0.13-2.0	ND-0.46	ND-1.9	0.13-3.2	nr (33)
France	2005	ND-5	ND	ND	ND-5	7 (23)
Spain	2006-2007	ND-122	ND	ND-176	ND-188	30 (33)
China	2007	ND-2.8	ND	ND-0.46	nr	nr (24)

ND: Below limit of detection; nr: not reported

Overall and in recognition of the fact that the presented data is based on pooled human milk samples it suggests that HBCD concentrations may be relatively variable with concentrations between pools from a given year varying by more than an order of magnitude.

PBDEs in human milk and blood serum

In human milk, the Σ_4 PBDE (sum of BDEs – 47, -99, -100 and -153) concentrations ranged from 2.5 to 15.8 ng g⁻¹ lipid across all collection periods (Table 1). Concentrations across all pools for BDEs-47, -99, -100 and -153 ranged from 1.4 to 11.5, 0.6 to 5.1, not quantified to 2.3 and 1.0 to 4.5 ng/g lipid, respectively.

In human blood, PBDEs were detected in all pools with the Σ_4 PBDEs for adults (> 16 years) ranging from 5.6 – 30.3 ng/g lipid in 2002/03; 4.0 – 12.2 ng/g lipid in 2004/05; 5.5 – 14.5 ng/g lipid in 2006/07 and 6.6 – 13.7 ng/g lipid in 2008/09. Across all collection periods, PBDE concentrations in human blood have been found to be highest in the youngest age groups and decrease until around middle age (Figure 2). The observed age trend is likely related to the history of PBDE exposure, the half-lives of the chemicals and the exposure pathways.

In human milk and human blood, BDE-47 dominated the congener profile contributing around 60 and 50 % to the Σ_4 PBDE concentration, respectively, followed in descending order by BDEs-99, -153, and -100. BDE-209, where determined in human blood (2002/03; 2004/05; and 2008/09 samples), was reported as not detected or close to the detection limit. A comparison with studies on PBDEs in human milk and blood samples from other countries shows that the concentrations of these chemicals in milk from mothers in Australia are equal to or higher than those in Europe and Asia (e.g. Polder *et al.* 2008; Sjödin *et al.* 2008; Haraguchi *et al.* 2009; Lignell *et al.* 2009; Lunder *et al.* 2010; Schecter *et al.* 2010; Windham *et al.* 2010)

Temporal trends

HBCD concentrations by year of collection are depicted in Figure 1. While the concentrations were highest in one of the two (pooled) sample collected in 1993 at 19 ng/g lipid, HBCD was not detected in the other sample from this period. The result for 2009 was similar to that from 2001 with varying levels detected in between. This may be due to the small number of samples in each pool where a highly contaminated sample may increase the result of the pool. Statistical analysis of the results is not possible due to the small number of samples in each year of collection, however, from the Figure there is no visual trend in HBCD concentrations by year of collection.

In comparison, PBDE concentrations in human milk appear to show a temporal trend with a peak around 2002/03 and 2003/04 with Σ_4 PBDEs of 9.6 (mean) and 12.4 ng/g lipid, respectively, followed by decrease to a mean of 2.7 ng.g lipid in 2007/08 and 2.6 in 2009 (Figure 1).

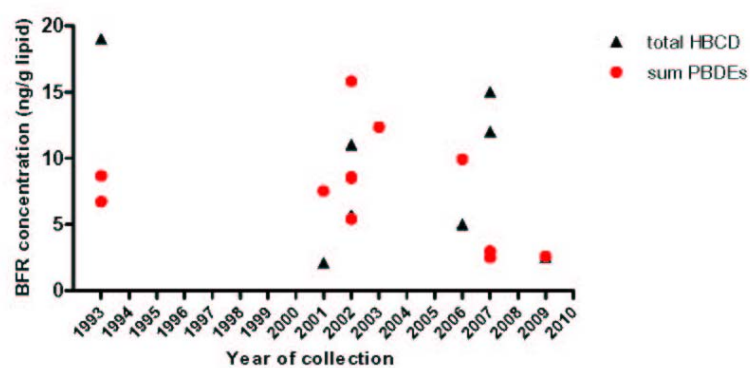


Figure 1. Concentration of HBCD and Σ_4 PBDEs (ng/g lipid) in human milk by year of collection

The use of human blood serum pools allowed an assessment of temporal trends using a larger sample size for PBDEs. To date, the serum samples have not been analysed for HBCD. Using the example of BDE-47 in human blood, the results suggest the possibility of a temporal trend. A comparison of concentrations in the 2002/03 and 2008/09 collection data shows that the mean concentration of BDE-47 in the 2008/09 was lower for all age groups by about 10 – 60 % (Figure 2). A plot of the mean sample age versus concentration of BDE-47 for all sampling periods (2002/03, 2004/05, 2006/07 and 2008/09) shows the consistent age trends but also shows that despite the relatively large pools (100 – all years except 2006/07 where 30 samples per pool were used) the variability between sample replicates, that is, samples obtained from the same strata, appears to overshadow temporal trends.

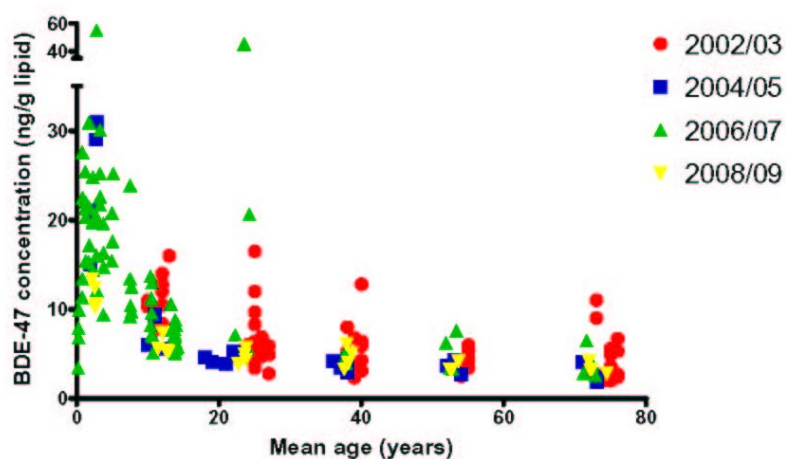


Figure 2 Concentrations of BDE-47 (ng/g lipid) by mean age (years) for males and females combined by collection date.

In order to investigate temporal trends, BDE-47 concentrations in adults (Figure 3 A) and children (Figure 3 B) have been separated and compared. The data indicate no obvious changes in the BDE-47 concentration in adults, however a decrease in the concentration is observable in children. For relatively persistent chemicals such as BDE-47 with a half-life in humans of approx. 3 - 4 years one could expect that decreases in adults are slower than in children and infants. This is likely due to the ratio of newly accumulated PBDEs in the period when exposure decreased, relative to the already 'stored' mass of PBDEs in a given body (that is, children have small bodies and high intake in comparison to adults hence the chemical profile in a child may be changed more rapidly).

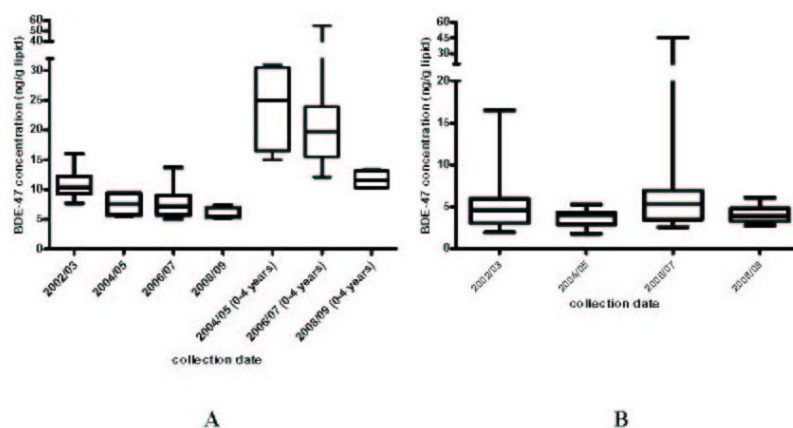


Figure 3 Box and whisker plot with median, minimum and maximum data for BDE-47 combined by gender and age - A < 16 years and 0-4 years and B > 16 years by collection date.

Continued monitoring over time with the use of large pools of human blood serum for PBDEs and HBCD will be required to further assess the body burden of BFRs in the Australian population.

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Supporting Information**Analytical methodology**HBCD

Before extraction, samples were lyophilized, homogenized, and stored at -22 °C. Samples of 0.5 g dry weight (dw) of milk were spiked with 50 µL of the labeled standard α - and γ -HBCD- d_{18} at 125 pg/µL. Spiked samples were kept overnight to equilibrate. Extraction was carried out by the pressurized liquid extraction (PLE) method on a fully automated ASE 200 system (Dionex, Sunnyvale, CA). An 11mL extraction cell was loaded by inserting two celluloses followed by the human milk sample. The dead volume was filled with anhydrous sodium sulfate for residue analysis previously activated at 150 °C for 4 h (Panreac Quimica S.A., Montcada i Reixac, Barcelona, Spain). Extraction conditions were as follow: dichloromethane/hexane (2:1) mixture as solvent extractor, temperature of 100 °C, pressure of 14 MPa, two static extractions of 5 min and 100% flush. After extraction, the crude extracts were concentrated to 3 mL and then subjected to a purification step via acid attack with concentrated H_2SO_4 (95-97% purity) (3×2 mL). Samples were finally concentrated to incipient dryness and redissolved with 50 µL of the labeled standard β -HBCD- d_{18} at 125 pg/µL, prior to instrumental analysis by LC-MS-MS.

Diastereoisomer Analysis. The LC system used was an Agilent HP 1100 binary pump (Agilent Technologies, Palo Alto, CA) with a Symmetry C18 column (2.1 mm $\times$ 150 mm, 5 µm) preceded by a C18 guard column (2.1 $\times$ 10mm) supplied by Waters. Experiments were carried out with water/methanol (3:1 v/v) as eluent A and methanol as eluent B, at a flow rate of 0.25 mL/min. The injection volume was set at 4 µL. The elution program started at an initial composition of 100% A and was ramped to 0% A in the first minute, then eluent A was increased to 10% in 17 min, and initial conditions were reached again in 3 min and returned to the starting conditions in 15 min. Mass spectrometric analysis was performed with a hybrid triple-quadrupole/linear ion trap MSDSciex 4000QTRAP (Applied Biosystems, Foster City, CA) instrument equipped with an electrospray (ESI) Turbospray interface, working in negative ionization mode. For target quantitative analyses, data acquisition was performed in selected reaction monitoring (SRM). The $[M - H]^-$ - Br^- transitions at m/z 638.7 - 78.9 and 638.7 - 80.9 were monitored for unlabeled HBCD. The labeled HBCD were monitored at the 655.8 - 78.9 and 655.8 - 80.9 transitions. The MS-MS detection conditions were: curtain gas at 50 psi, collision gas at 4.5×10^{-5} Torr, temperature of the turbo gas in the TurboIonSpray source at 350 °C, ion source gas 1 at 50 psi, and ion source gas 2 at 10 psi [7].

Enantiomeric analysis. A chiral chromatographic column, Nucleodex β -PM (4.0 mm $\times$ 200 mm $\times$ 5 µm), was used to afford the enantiomer-specific determination. The optimal separation of enantiomers

was achieved using methanol, acetonitrile and water as mobile phase. Experiments were carried out using 70% water: 30% methanol as eluent A and 70% AcN: 30% methanol as eluent B, at a flow rate of 0.50 mL/min. The injection volume was set at 10 μ L. The elution program started at an initial concentration of A at 50% decreased to 0% along the first 8 minutes, and was maintained for 17 minutes and initial conditions were reached again in 5 min and maintained for additional 12 min [ii]. Mass spectrometric analysis was performed using the same conditions as for diastereoisomer analysis.

Enantiomeric composition was expressed as enantiomeric fraction (EF), which is normally calculated from the peak areas of the enantiomeric pairs by Eqn. 1:

$$EF = \frac{A_+}{(A_+ + A_-)} \quad (\text{Eqn. 1})$$

where A_+ and A_- are the peak area of eluting enantiomers.

It is well known that ESI is subject to sample matrix effects that can cause enhancement or suppression of the target analytes signal and can adversely affect their quantification. In order to avoid this effect that can affect in EF calculations, these values are calculated according to equation 2 [iii]. This correction is based on the use of isotopic labeled standards (d_{18} -HBCDs) since d_{18} -labeled enantiomeric analogues behave in an identical manner to their native counterparts:

$$EF_{\text{corrected}} = \frac{([A_-] / [A_{+d_{18}}]) \times pg A_{+d_{18}}}{([A_+] / [A_{+d_{18}}]) \times pg A_{+d_{18}} + ([A_-] / [A_{-d_{18}}]) \times pg A_{-d_{18}}} \quad (\text{Eqn. 2})$$

where $[A_+]$ and $[A_-]$ are the peak areas of each (+) and (-) HBCDs enantiomers, $[A_{+d_{18}}]$ and $[A_{-d_{18}}]$ are the peak areas of each (+) and (-) HBCDs d_{18} labeled standards.

PBDEs

0.5 gram dry weight (dw) of milk was spiked with internal standards (10 ng BDE-77, 10 ng BDE-181 and 200 ng [$^{13}\text{C}_{12}$]-BDE-209). Spiked samples were kept overnight to equilibrate. A PLE method that automatically and rapidly achieves quantitative extraction of BFRs was used. PLE was carried out using a fully automated ASE 200 system (Dionex, Sunnyvale, CA, USA) using aluminium oxide neutral and Hydromatrix (Varian Inc., Palo Alto, USA). Extraction cell was heated to 100°C and extraction was carried out using a mixture of hexane:CH₂Cl₂ (1:1) [iv]. After extraction, the crude extracts were concentrated to 3 mL and then subjected to a purification step via acid attack with concentrated H₂SO₄ (95-97% purity) (3 $\times$ 2 mL). Final purification of the extract was carried out in 5g

alumina SPE cartridges. Cartridges were conditioned with 20 ml of hexane and eluted with 30 ml of hexane:dichloromethane (1:2). Purified extracts were finally concentrated to incipient dryness and re-dissolved with 50 μ L of toluene prior to analysis by gas chromatography coupled to mass spectrometry working with negative ion chemical ionization (GC-NCI-MS).

GC-NCI-MS analyses were performed on a trace GC ultra gas chromatograph connected to a dual stage quadrupole mass spectrometer (Thermo Electron, Texas, USA). A DB – 5MS capillary column (15 m x 0.25 mm i.d., 0.1 μ m film thickness) was used with ammonia as the carrier gas at an ion source pressure of 1.9×10^{-4} Torr. The temperature program was from 140 °C (held for 2 min) to 325 °C (held for 10 min) at 10 °C min⁻¹. Injection was carried out by splitless mode for 1 min with an injector temperature of 250 °C [°]. Experiments were carried out monitoring the two most abundant isotope peaks from the mass spectra corresponding to $m/z = 79$ and 81 [Br⁻] and $m/z = 487$ and 489 for BDE-209 and $m/z = 497$ and 498 for ¹³C₁₂-BDE-209. Identification of selected analytes was based on the following restrictive criteria: (i) retention time for all monitored ions for a given analyte should maximize simultaneously ± 1 s, with signal to noise ratio >3 for each; and (ii) the ratio between the two monitored ions should be within 15% of the theoretical value (calculated upon standards). Quantification was carried out by internal standard procedure using BDE-77 and BDE-181 standards. In the case of deca-BDE, the quantification was carried out using ¹³C₁₂-BDE-209 as internal standard.

ⁱ P. Guerra, A. de la Torre, M.A. Martínez, E. Eljarrat, D.Barceló. Identification and trace level determination of brominated flame retardants by LC-quadrupole linear ion trap (Qq-LIT) mass spectrometry. *Rapid Communication in Mass Spectrometry*, 2008, 22, 916-924.

ⁱⁱ P. Guerra, E. Eljarrat, D.Barceló. Enantiomeric specific determination of hexabromocyclododecane by LC-quadrupole linear ion trap (Qq-LIT) mass spectrometry in sediment samples. *J. of Chromatogr.*, 2008, 1203, 81-87.

ⁱⁱⁱ Marvin, C., MacInnis, G., Alahee, M., Arsenault, G., Tomy, G., 2007. Factors influencing enantiomeric fractions of hexabromocyclododecane measured using liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 21, 1925-1930.

^{iv} A. de la Cal, E.Eljarrat, D. Barceló. Determination of 39 PBDE congeners in sediment samples using fast selective pressurized liquid extraction and purification. *Journal of Chromatography A*, 2003, 1021, 165-173.

^v Eljarrat, E., De la Cal, A., Barceló, D., 2004b. Determination of decabromodiphenyl ether in sediments using selective pressurized liquid extraction followed by GC-NCI-MS. *Anal. Bioanal. Chem.* 378, 610–614.

Diastereoisomeric and enantiomeric analysis

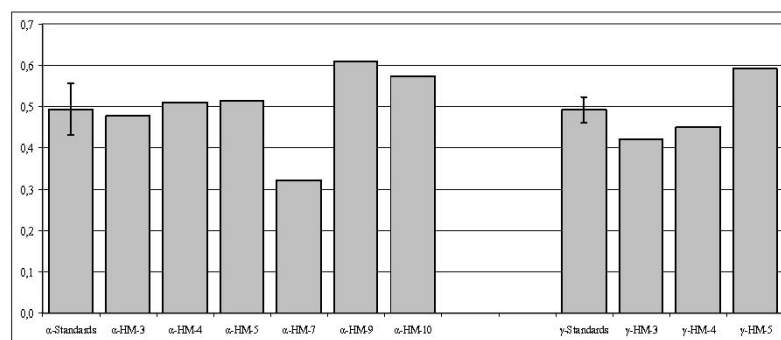
Enantiomeric fractions (EF) corrected values were calculated for different samples (Figure S1). In the case of γ -HBCD, it was observed that EF values were between 0.21 and 0.59, whereas EF for α -HBCD was between 0.32 and 0.61. If these EF values are compared with those obtained with standard solutions (EF = 0.49 for both α - and γ -HBCD) a different behavior is observed depending on the selected sample. In the case of α -HBCD, there are three samples with no significant differences with standards. However, two other samples showed an increase of EF value compared to standard value, indicating an enrichment of the (+)- α -HBCD enantiomer in human body. Finally, there is a sample with EF value of 0.32, which is lower than those obtained for standard solutions. In this case, the lowering of EF value indicates an enrichment of the (-)- α -HBCD enantiomer in human body. This (-)- α -HBCD enrichment in human milk samples was previously observed by Eljarat et al. (2008) in samples collected from Spain. A deviation from the racemic mixture (EF = 0.5) suggests that an enantioselective uptake and/or metabolism of (+)- α -HBCD and (-)- α -HBCD must occur.

Some HBCD diastereoisomeric pattern studies have been carried out in human samples. Some reports have suggested that β - and γ -HBCD diastereoisomers can be more extensively metabolized in organisms that can α -HBCD diastereoisomer. Weiss et al. (7) found that the most abundant stereoisomer in serum samples from Sweden was α -HBCD. In contrast, Johnson-Restrepo et al. (8) found that γ -HBCD was the dominant isomer present in human adipose tissue samples from United States, accounting for 83% of the total HBCD concentrations, and followed by α -HBCD (17%). β -HBCD stereoisomer was not detected in any of the human adipose tissue samples analyzed. However, 35% of the human tissue samples contained α -HBCD concentrations higher than the concentrations of γ -HBCD, suggesting that some individuals are exposed to elevated concentrations of α -HBCD. Thomsen et al. (9) also found high concentrations of γ -HBCD than the other HBCD diastereoisomers in serum of workers from an industrial polystyrene plant in Scandinavia. As regards information related with HBCD diastereoisomeric pattern in human breast milk, Ryan et al. (2010) observed that alpha diastereoisomer predominated, with concentration levels ranging between 0.2 and 19 ng/g lw, in samples from North America. In another study dealing with HBCD levels in Japanese women (2010), in the majority of the samples, the alpha diastereoisomer also predominated; but some exceptions were observed in which the contribution of the gamma diastereoisomer was higher than that of alpha.

In a published work in marine species from the Western Scheldt estuary (10), authors have reported for the first time the selective accumulation of different HBCD enantiomers in marine fish. In two further studies, the database of EFs for α -HBCD has been extended to herring and several predatory birds (11) and dolphins (12). In all cases, a deviation from the racemic mixture (EF = 0.5) has been observed, suggesting that an enantioselective uptake and/or metabolism of (+)- α -HBCD and (-)- α -HBCD must occur. Interestingly, no clear preference for one or the other α -HBCD enantiomer was found. Herring muscle and falcon eggs were clearly enriched in (-)- α -HBCD, whereas in whiting liver and sea eagle eggs, (+)- α -HBCD dominated.

In the case of γ -HBCD, one sample with no significant differences with standards was found, and one sample with an (+)- γ -HBCD enrichment. However, the rest of the samples showed a decrease of EF value compared to standard value, indicating an enrichment of the (-)- γ -HBCD enantiomer in human body.

Figure S1. Enantiomeric fraction (EF) values in standards (mean $\pm$ standard deviation) and human breast milk samples.



4.2. Discusión

En el artículo #9 analizamos los niveles de HBCD en muestras de aceite de pescado para consumo humano (FO) y animal (FE), donde hallamos niveles importantes de HBCD tal como se resume en la Tabla 4-1. La concentración de HBCD va desde nd a 4,19 ng/g y desde 3,34 a 26,8 ng/g para FO y FE, respectivamente. Tal como vemos, las muestras que se utilizan para la alimentación animal tienen niveles mayores de HBCD. Respecto a la distribución isomérica, se observó que en las muestras de aceite para consumo animal predomina levemente γ -HBCD, mientras que en los aceites para consumo humano el isómero principal es α -HBCD.

Tabla 4-1. Concentración de HBCD (ng/g) en aceites de pescado.

Muestra	α -HBCD	β -HBCD	γ -HBCD	Total
FE1	5.79	0.23	5.09	11.11
FE2	3.71	0.89	5.28	9.88
FE3	3.14	nq	1.64	4.78
FE4	2.13	nq	1.21	3.34
FE5	3.82	nq	2.84	6.66
FE6	6.58	nq	8.91	15.49
FE7	2.61	nq	2.65	5.26
FE8	6.13	3.95	16.73	26.81
FE9	2.62	nq	2.87	5.49
FE10	4.65	0.11	3.29	8.05
FO1	2.42	nq	1.77	4.19
FO2	0.13	0.14	nq	0.27
FO3	nq	nd	nq	nq
FO4	nq	nq	nq	nq
FO5	0.48	0.59	nq	1.07
FO6	0.24	0.91	nq	1.15
FO7	0.23	0.24	0.59	1.06
FO8	1.99	nq	1.09	3.08
FO9	0.4	nq	0.52	0.92
FO10	0.79	nd	nq	0.79
FO11	nq	nd	nq	nq
FO12	0.32	0.29	0.16	0.77
FO13	0.04	0.15	nq	0.19
FO14	nq	nq	0.09	0.09
FO15	0.2	nq	2.19	2.39

En el caso de los aceites para consumo humano, hemos calculado el EDI debido al uso de estos complementos alimenticios utilizando la siguiente ecuación:

$$EDI_{\text{HBCD}} = [\text{HBCD}] \times \text{DDR}$$

Donde [HBCD] es la concentración de HBCD y DDR es la dosis diaria del complemento promedio recomendada.

En la Tabla 4-2 vemos las diferentes EDI que obtuvimos para cada una de las muestras, cuyos valores van desde 0,08 a 5,38 ng HBCD/día. Es importante señalar que en los suplementos en base a aceite vegetal y/o mezclados los valores de EDI son inferiores. Así, hemos calculado el consumo diario de HBCD debido al uso de suplementos alimenticios, por lo tanto habría que sumarle el HBCD incorporado por la dieta. En España no existen estudios sobre la cantidad total de HBCD que ingresa al cuerpo humano diariamente mediante la dieta, por lo tanto hemos buscado estudios en otros países. Se han publicado diferentes valores de EDI para Suecia (141 ng HBCD/ día) (Lind et al., 2002), Holanda (9 ng HBCD/día) (Van Leeuwen y De Boer 2008), Noruega (20 ng HBCD/día) (Knutsen et al., 2008) y Reino Unido (500 ng HBCD/día) (Fernandes et al., 2008). Así, observamos que la ingesta de suplementos alimenticios que contengan aceite de pescado pueden contribuir significativamente a la ingesta de HBCD. Por ejemplo, si tomamos como base el EDI de Holanda, el aporte del consumo de los suplementos alimenticios analizados sería de entre un 0.9 y 57%.

Tabla 4-2. Valores de EDI de HBCD debido al consumo de suplementos alimenticios

Muestra	Componente principal	ng HBCD/día
FO1	Aceite de pescado	5.15
FO2	Aceite de pescado	0.27
FO3	Aceite de pescado	-
FO4	Aceite de pescado	-
FO5	Aceite de pescado	4.01
FO6	Aceite de pescado	1.44
FO7	Aceite de pescado	5.38
FO8	Aceite de pescado	1.26
FO9	Aceite de pescado	1.84
FO10	Aceite de pescado+ aceite vegetal	0.55
FO11	Aceite de pescado+ aceite vegetal	-
FO12	Aceite de pescado +aceite vegetal	2.16
FO13	Aceite vegetal	0.46
FO14	aceite vegetal rico en omega-6	0.08
FO15	Aceite vegetal	3.59

En aquellas muestras donde la concentración del contaminante lo permitía hemos calculado los EFs de α -HBCD y γ -HBCD. Ambos se resumen en la Figura 2 del artículo #9 y se observa que en el

caso de α -HBCD existen variaciones de los EFs que indicarían un comportamiento enantiómero-específico.

En los artículos #10 y #11 realizamos el análisis de HBCD isomérico y enantiomérico en leche materna de madres españolas y australianas, respectivamente. El interés de estos trabajos es debido a que los lactantes se alimentan exclusivamente de leche materna por lo tanto, los BFRs que han sido acumulados durante un largo período de tiempo en el cuerpo de la madre, les pueden ser transferidos durante la lactancia y ser absorbidos por los bebés.

En el artículo #10 se analizaron 33 muestras de leche, obteniéndose concentraciones de HBCD que varían entre 2,72 y 188 ng/g lw, siendo estos niveles muy superiores a los existentes en bibliografía (ver Figura 4.1). En lo que respecta al patrón isomérico, β -HBCD estaba por debajo de los LOD, mientras que γ -HBCD fue el isómero principal, salvo en dos leches donde α -HBCD fue el predominante.

Para comprender la magnitud de la exposición a HBCD por los lactantes, hemos calculado el valor de EDI utilizando la siguiente ecuación:

$$EDI_{HBCD} = [HBCD]FMb$$

Donde [HBCD] es la concentración media de HBCD en la leche, F es el contenido lipídico en la leche y Mb es el consumo diario de leche, sabiendo que un bebé de 1 mes consume un promedio de 702 mL de leche/día y pesa unos 4,14 kg. Así, la EDI para un lactante español fue de hasta 175 ng/día. Posteriormente a nuestro trabajo, Shi et al. (2009b) calculó el EDI para un lactante en China (45,5 ng/día). De esta forma vemos que el valor promedio de EDI de España es cerca de 4 veces mayor que el de los lactantes de China.

En este estudio se calculan por primera vez los EFs de HBCD en leche materna. En el caso de γ -HBCD, se observó que los valores de EF se encuentran entre 0,268 y 0,602, mientras que el EF de α -HBCD sólo se pudo calcular en dos muestras, con valores de 0,102 y 0,204. En la Figura 4-3 se comparan los valores de EF obtenidos tanto para las muestras como para los estándares. Para α -HBCD, observamos una importante disminución en el valor de EF. Podemos decir que se produce un enriquecimiento de (-) α -HBCD, mostrando un enriquecimiento enantiomérico-selectivo en el cuerpo humano. Respecto a γ -HBCD para un total de once muestras, hay cinco muestras sin diferencias significativas con las soluciones estándares. Mientras que la segunda y tercera columna muestran que para 2 muestras disminuye el valor de EF, y en cuatro muestras se incrementa el valor de EF. Por lo tanto, para γ -HBCD el patrón enantiomérico no está claro.

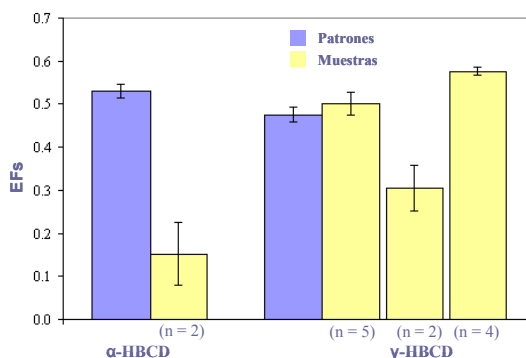


Figura 4-3. Gráfico de EFs de α -HBCD y γ -HBCD en leche materna.

Así pues, se observó enantioselectividad de α -HBCD, con enriquecimiento de (-) α -HBCD. Sin embargo, debido a que el número de muestras es pequeño ya que solo se pudo ver en dos muestras, no se pudo llegar a una conclusión clara. También analizamos los EFs de α -HBCD en las leches de Australia y obtuvimos valores que varían desde 0,32 a 0,61, respectivamente. Dada la variabilidad de los resultados, no podemos concluir nada concreto acerca de su comportamiento enantiómero-específico. Sin embargo, posteriores estudios en leche materna han corroborado nuestro hallazgo (Abdallah y Harrad 2011), indicando la presencia de posibles procesos enantioselectivos que pueden involucrar la absorción, el metabolismo y/o la excreción del HBCD y concretamente de un enriquecimiento de (-) α -HBCD.

En el caso del artículo #11 nos interesaba estudiar la tendencia temporal de HBCD y PBDEs en leche materna de Australia analizando muestras compuestas entre 1993 y 2009 ya que es sabido que en muestras de leche materna de Australia los niveles de PBDEs son superiores a los encontrados en Europa y Asia, aunque inferiores a los de EE.UU. (Toms et al., 2007) y se quería evaluar si sucedía o no lo mismo con el HBCD. Así pues, analizamos 13 muestras compuestas de leche y encontramos niveles entre nq y 19 ng/g lw de HBCD total (Tabla 1 del artículo #11). En general, no observamos una tendencia clara para HBCD en la leche materna de Australia entre los años 1993 y 2009. Esto se debería principalmente al número pequeño de muestras de leche individuales que conforman la muestra compuesta analizada, donde una muestra altamente contaminada podría aumentar la concentración total. Además el pequeño número de muestras analizadas no permite realizar análisis estadístico. Por el contrario, en el caso de los PBDEs se observó una tendencia temporal, con una concentración máxima alrededor de 2002/03 y 2003/04 para luego disminuir en las muestras pertenecientes a 2007/08 y 2009. Estos resultados cuadrarían con el mayor uso y posterior cese de los PBDEs.

CONCLUSIONES GENERALES

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Los estudios llevados a cabo durante la presente tesis permiten llegar a las siguientes conclusiones generales:

1. Se ha logrado el desarrollo y validación de dos métodos sensibles, selectivos, precisos y reproducibles para el análisis de HBCD, tanto diastereoisomérico como enantiomérico, basados en el uso de un instrumento de última generación, como es la LC-QqLIT-MS. El método diastereoisomérico permite el análisis simultáneo de TBBPA y compuestos procedentes de su debromación hasta llegar al BPA.
2. En muestras ambientales, y especialmente en las bióticas, se ha podido ver el interés de analizar el HBCD por vía diastereoisomérica y enantiomérica, en lugar del HBCD total tal como se hacía inicialmente por GC-MS.
3. Los métodos de análisis previamente desarrollados fueron utilizados para realizar la vigilancia ambiental de BFRs en sedimentos y biota procedentes de dos cuencas hidrográficas de España. Estos estudios han demostrado la presencia los BFRs clásicos como de los emergentes, aunque a niveles más bajos debido a su menor uso actual. En el caso de HBCD, el estudio en una zona altamente contaminada (el río Cinca a su paso por Monzón) ha servido para evaluar su presencia así como su comportamiento en lo que a su biodisponibilidad y bioacumulación se refiere.
4. El estudio de los huevos de halcón peregrino ha sido de gran utilidad para corroborar la presencia de distintos BFRs así como de una nueva familia de FRs, los Decloranes, en muestras correspondientes a un nivel trófico superior. Por consiguiente, estos resultados confirman la biodisponibilidad y bioacumulación a lo largo de la cadena trófica de estos nuevos contaminantes.
5. El análisis enantiomérico de HBCD ha sido de gran utilidad para detectar casos de enantioselectividad como por ejemplo, la acumulación de (-) α -HBCD observada en huevos de halcón peregrino como también en leche materna. El hallazgo de una acumulación enantiomero-específica del HBCD, sugiere la necesidad de disponer datos toxicológicos para los enantiómeros.
6. Se ha evaluado el grado de exposición humana a HBCD por diferentes vías, una por la ingesta de complementos alimenticios basados en pescado, y otra para el grupo de riesgo de los lactantes a través del análisis de leche materna. En el primer caso, esta exposición puede contribuir entre un 1 y un 50% del total por ingesta de alimentos. En el caso de los lactantes españoles, el EDI se sitúa en torno a 175 ng/kg peso día, siendo un valor muy por encima de los datos disponibles sobre ingesta en adultos, así como los de ingesta de polvo.

7. Se necesita más investigación para comprender la naturaleza y el alcance de los procesos enantioselectivos de HBCD en la naturaleza y en muestras humanas, además de los factores que influyen en la retención enantioselectiva de HBCD en las diferentes especies.
8. Se necesita una mayor disponibilidad de información acerca de los otros FRs emergentes incluidos en esta tesis, especialmente en muestras humanas.

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ANEXOS

A1. Lista de publicaciones presentadas en esta tesis

1. **P. Guerra**, K. Fernie, B. Jiménez, G. Pacepavicius, C. Marvin, G. MacInnis, E. Eljarrat, D. Barceló. M. Alae. Concentrations of emerging halogenated flame retardants, including enantiomeric fractions of HBCD, in peregrine falcon (*Falco peregrinus*) eggs from Canada and Spain. *Enviado a Environmental International*.
2. L. Toms, **P. Guerra**, E. Eljarrat, D. Barceló, F. A. Harden, P. Hobson, A. Sjödin, J. F. Mueller. Temporal trends of brominated flame retardants in Australia: 1993-2009. *Enviado a Environmental International*.
3. **P. Guerra**, E. Eljarrat, D. Barceló. Recent approaches for selected halogenated flame retardants determinations by means of LC-MS. *Trends in Analytical Chemistry*, 2011. *En prensa*
4. **P. Guerra**, K. Fernie, G. Pacepavicius, B. Jimenez, E. Eljarrat, D. Barceló, M. Alae. Dechlorane Plus and related compounds in peregrine falcon (*Falco peregrinus*) eggs from Canada and Spain. *Environ. Sci. Technol.*, 2011, 45, 1284-1290.
5. X. Ortiz, **P. Guerra**, J. Díaz-Ferrero, E. Eljarrat, D. Barceló. Diastereoisomer- and enantiomer-specific determination of hexabromocyclododecane in fish oil for food and feed. *Chemosphere*, 2011, 82: 739-744.
6. **P. Guerra**, E. Eljarrat, D. Barceló. Simultaneous determination of Hexabromocyclododecane, Tetrabromobisphenol A and related compounds in sewage sludge and sediment samples from Ebro River basin (Spain). *Anal. Bioanal. Chem.* 2010, 397: 2817-2824.
7. **P. Guerra**, E. Eljarrat, D. Barceló. Analysis and occurrence of emerging brominated flame retardants in the Llobregat river basin. *J. of Hydrology*, 2010, 383: 39-43.
8. E. Eljarrat, **P. Guerra**, E. Martínez, M. Farré, J.G. Alvarez, M. López-Teijón, D. Barceló. Hexabromocyclododecane in human breast milk: levels and enantiomeric patterns. *Environ. Sci. Technol.*, 2009, 43: 1940-1946.
9. **P. Guerra**, A. de la Cal, G. Marsh, E. Eljarrat, D. Barceló. Transfer of hexabromocyclododecane from industrial effluents to sediments and biota: case study in Cinca River (Spain). *J. of Hydrology*, 2009, 369: 360-367.
10. **P. Guerra**, E. Eljarrat, D. Barceló. Enantiomeric specific determination of hexabromocyclododecane by LC-quadrupole linear ion trap (Qq-LIT) mass spectrometry in sediment samples. *J. of Chromatogr.*, 2008, 1023, 81-87.
11. **P. Guerra**, A. de la Torre, M.A. Martínez, E. Eljarrat, D. Barceló. Identification and trace level determination of brominated flame retardants by LC-quadrupole linear ion trap (Qq-LIT) mass spectrometry. *Rapid Comm. Mass Spectrom.* 2008, 22, 916-924.

A.2. Lista de publicaciones no presentadas en esta tesis

1. E. Eljarrat, **P. Guerra**, D. Barceló. Enantiomeric determination of chiral persistent organic pollutants and their metabolites. Trends in Analytical Chemistry, 2008, 27, 847-861.
2. M.S. Díaz-Cruz¹, M.J. García-Galán, **P. Guerra**, A. Jelic, C. Postigo, E. Eljarrat, M. Farré, M.J. López de Alda, M. Petrovic, D. Barceló. Analysis and occurrence of selected emerging contaminants in sewage sludge. Trends in Analytical Chemistry, 2009, 28, 1263-1275.

A.3. Lista de publicaciones en revistas no incluídas en SCI realizadas durante la tesis.

1. **P. Guerra**, M. Alaei, E. Eljarrat, D. Barceló. Recent methodologies for BFR determinations by means of liquid chromatography-mass spectrometry, 2011. Series: The Handbook of Environmental Chemistry, volume: Brominated flame retardants (BFRs). *En prensa*
2. **P. Guerra**, A. Covaci, E. Eljarrat, D. Barceló. Recent methodologies for halogenated flame retardants determinations by LC-MS, 2011. Series: The Handbook of Environmental Chemistry, volume: Brominated flame retardants (BFRs). *En prensa*
3. **P. Guerra**, G. Pacepavicius, B. Jimenez, E. Blázquez, K. Fernie, L. Champoux, C. Marvin, G. Macinnis, E. Eljarrat, D. Barceló, M. Alaei Hexabromocyclododecane in peregrine falcon (*Falco peregrinus*) eggs from Canada and Spain: levels and enantiomeric patterns. Organohalogen Comp., 2010, 72, 710-713 (Presentación oral, Conferencia internacional).
4. **P. Guerra**, G. Pacepavicius, B. Jimenez, J. Muñoz-Arnanz, E. Blázquez, K. Fernie, L. Champoux, C. Marvin, G. Macinnis, E. Eljarrat, D. Barceló, M. Alaei. Emerging halogenated flame retardants in peregrine falcon (*Falco peregrinus*) eggs from Canada and Spain. Organohalogen Comp., 2010, 72, 718-722 (Poster, Conferencia internacional).
5. **P. Guerra**, E. Martínez, M. Farré, E. Eljarrat, D. Barceló. Hexabromocyclododecane in human breast milk: levels and enantiomeric patterns. Organohalog. Comp., 2008, 70, 309-312 (Presentación oral, Conferencia internacional).

A.4. Contribuciones a congresos

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