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POTENCIAL DEL ACOPLAMIENTO GC-MS PARA LA DETERMINACIÓN DE CONTAMINANTES ORGÁNICOS EN MUESTRAS DE MATRIZ COMPLEJA

Tesis Doctoral

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Certifican: que la Tesis Doctoral "Potencial del acoplamiento GC-MS para la determinación de contaminantes orgánicos en muestras de matriz compleja" ha sido desarrollada bajo su dirección, en el Instituto Universitario de Plaguicidas y Aguas, Departamento de Química Física y Analítica de la Universitad Jaume I de Castellón, por **Jaime Nácher Mestre**.

Lo que certificamos para los efectos oportunos en Castellón de la Plana, a 11 de Enero de 2011.

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Esta Tesis ha sido realizada, y consecuentemente será defendida, para la obtención del título de Doctorado en Química Analítica de la Universidad Jaume I.

Previamente a la defensa de la Tesis Doctoral, este trabajo ha sido evaluado por tres censores independientes directamente relacionados con el área de investigación, Dra. Maria Teresa Galceran Huguet (Catedrática del Departamento de Química Analítica de la Univesidad de Barcelona), Dra. Rosa Maria Marcé Recasens (Catedrática del Departamento de Química Analítica de la Univesitad Rovira i Virgili de Tarragona) y Dra. Inmaculada Varó Vaello (Científica Titular del Instituto de Acuicultura de Torre de la Sal del Consejo Superior de Investigaciones Científicas).

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Resumen

A lo largo de esta Tesis Doctoral se investiga el potencial analítico y la aplicabilidad del acoplamiento instrumental GC-MS mediante diferentes analizadores de masa.

La Tesis Doctoral consta de tres partes diferenciadas. En la primera se desarrolla, valida y aplica metodología analítica basada en el acoplamiento GC-MS para la determinación de contaminantes orgánicos persistentes en muestras procedentes de la acuicultura marina. Entre las muestras se incluyen materias primas de origen vegetal y animal, piensos con diferente composición y filetes de dorada cultivada. Concretamente se desarrollan metodologías para la determinación de PAHs y PBDEs. Para ello se utilizaran analizadores de triple cuadrupolo y tiempo de vuelo. La utilización de un analizador de triple cuadrupolo como herramienta de análisis ofreció una elevada sensibilidad y selectividad para el análisis de compuestos *target* a niveles traza. La utilización del analizador de tiempo de vuelo permitió una confirmación adicional de los positivos de PAHs determinados mediante el triple cuadrupolo.

En la segunda parte de la Tesis se han explorado las posibilidades que ofrece el analizador de tiempo de vuelo desde el punto de vista cualitativo y confirmativo de contaminantes orgánicos mediante técnicas de *screening*. Concretamente se realizaron estudios sobre muestras de sales marinas, salmueras y aguas medioambientales. La utilización del analizador de tiempo de vuelo ha permitido la realización de diferentes enfoques de análisis *screening*, bien con analitos preseleccionados (análisis *target*), analitos no seleccionados previamente (análisis *non-target*) y analitos seleccionados post adquisición con equipos de MS (análisis post-target).

Por último, las metodologías analíticas disponibles se aplican al estudio de la posible bioacumulación de PAHs, OCs y PBDEs en los productos de la acuicultura marina, mediante un estudio experimental que incluye un ciclo completo de engorde de doradas (*Sparus aurata L.*) en el marco del Proyecto AQUAMAX (Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers. www.aquamaxip.eu). Las muestras

consideradas en este estudio fueron materias primas de origen vegetal y animal, piensos con diferente composición y filetes de dorada cultivada.

El trabajo desarrollado en esta Tesis Doctoral ha permitido concluir que la estrategia a seguir depende principalmente del objetivo que se desea alcanzar, considerando el uso de una instrumentación u otra en función de las necesidades requeridas.

En esta Tesis Doctoral se ha desarrollado y aplicado metodología analítica altamente especializada con instrumentación avanzada basada en el acoplamiento GC-MS. La metodología desarrollada presenta excelentes características analíticas y resulta de interés tanto en análisis de rutina de estos contaminantes como para la investigación y búsqueda de contaminantes de creciente interés, tanto en el mundo alimentario como en el campo medioambiental.

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ASE	Extracción acelerada con disolventes
BaPE	Equivalentes tóxicos del Benzo(a)Pireno
BMF	Biomagnificación
CI	Ionización química
DDD	Diclorodifenildicloroetano
DDE	Diclorodifenildicloroetileno
DDT	Diclorodifeniltricloroetano
ELL	Extracción líquido-líquido
EI	Ionización electrónica
EtOH	Etanol
EU	Unión europea
GC	Cromatografía de gases
GC-MS	Cromatografía de gases acoplada a espectrometría de masas
GPC	Cromatografía por permeación en gel
H_2SO_4	Ácido sulfúrico
HPLC	Cromatografía líquida de alta resolución
ICP	Fuente de plasma de acoplamiento inductivo
IT	Analizador de trampa de iones
K_{ow}	Constante de reparto octanol/agua
KOH	Hidróxido potásico
LC	Cromatografía líquida
LC-MS	Cromatografía líquida acoplada a espectrometría de masas
LOD	Límite de detección
LOI	Límite de identificación
LOQ	Límite de cuantificación
m/z	Relación masa/carga
MAE	Extracción asistida por microondas
MeOH	Metanol

MRM	Monitorización de transiciones seleccionadas (multiple reaction monitoring)
MS	Espectrometría de masas
MS/MS	Espectrometría de masas en tándem
NaCl	Cloruro sódico
NaOH	Hidróxido sódico
NCI	Ionización química negativa
NL	Pérdidas neutras
NPLC	Cromatografía líquida en fase normal
OCs	Organoclorados
OCPs	Pesticidas organoclorados
OPs	Organofosforados
OPE	Éster organofosforado
PAHs	Hidrocarburos policíclicos aromáticos
PBDEs	Difenil éter polibromados
PCBs	Bifenilos policlorados
PCDDs	Dibenzodioxinas
PCDFs	Dibenzofuranos
PCI	Ionización química positiva
PLE	Extracción líquida presurizada
POPs	Contaminantes orgánicos persistentes
Q	Analizador de masas cuadrupolar
QqQ	Analizador de masas triple cuadrupolo
RSD	Desviación estándar relativa
SIM	Monitorización de iones seleccionados (selected ion monitoring)
SIR	Monitorización de iones seleccionados (selected ion recording)
SPE	Extracción en fase sólida
SPME	Microextracción en fase sólida
SRM	Monitorización de transiciones seleccionadas (selected reaction monitoring)
TEF	Factor de equivalencia tóxica

TIC	Cromatograma de iones totales (total ion chromatogram)
TOF	Analizador de masas de tiempo de vuelo
USEPA	Agencia americana de protección del medioambiente

CAPÍTULO 1

INTRODUCCIÓN GENERAL

1.1. Introducción

En las últimas décadas, el aumento progresivo de la presencia de algunos contaminantes orgánicos persistentes (POPs) y la aparición de nuevos contaminantes, producidos por la actividad humana en el medio ambiente, ha supuesto una creciente preocupación acerca del efecto que estos pueden tener sobre los ecosistemas y los organismos vivos.

La presencia de estos contaminantes en organismos vivos hace que sea viable su ingesta y posterior asimilación por organismos de niveles tróficos superiores. Estos contaminantes suponen una amenaza importante para la industria alimentaria. Los organismos de control oficial deben asegurar la inocuidad de los mismos, proporcionando las máximas garantías de calidad y seguridad alimentaria.

Se conocen como contaminantes orgánicos aquellas sustancias orgánicas con características definidas de toxicidad, carcinogeneidad y mutagenicidad presentes en cualquiera de los compartimentos medioambientales (Clement et al., 1999). Entre los diferentes tipos de contaminantes orgánicos existentes en la actualidad, posiblemente son los plaguicidas y los POPs los que han recibido mayor atención (Eljarrat et al., 2003). Su demostrada toxicidad y elevado uso en todo el planeta, así como las posibilidades de contaminación de distintos tipos de muestras (suelos, aguas, aire, productos alimenticios, etc.) son, sin duda, las causas de su carácter prioritario como contaminantes a controlar. Además, presentan un importante riesgo de bioacumulación a lo largo de la cadena trófica, llegando a alcanzar, en ocasiones, concentraciones alarmantes en los últimos eslabones de la misma (Hites et al., 2004). Por otro lado, algunas de estas sustancias muestran una

elevada persistencia en el medio ambiente. Esto se debe a su lenta degradabilidad, por lo que pueden llegar a detectarse residuos hasta varios años después de haber aplicado el producto.

La preocupación por los efectos nocivos derivados de estos contaminantes es una constante de nuestra sociedad actual. Asimismo, el número de compuestos orgánicos de toxicidad reconocida usados en la actualidad es muy elevado y su composición química muy variada. Por ello, los efectos tóxicos y los problemas ambientales derivados de su utilización son complejos y muy variados. Así, resulta necesaria una prevención adecuada, con el objeto de proteger la salud de toda la población en general.

La problemática de estos contaminantes en el mundo sigue siendo en la actualidad uno de los temas más desconocidos por diversos motivos. La dificultad de analizar estos compuestos a los bajos niveles de concentración exigidos por la legislación y la carencia de listas prioritarias a controlar en distintas áreas de interés, son quizás las razones que, en principio, pueden indicarse como más importantes. La contaminación orgánica conlleva una problemática que, a menudo, sobrepasa fronteras, lo que implica necesariamente la adopción de medidas legales a nivel internacional como las que se citan a continuación:

- CONVENIO DE BASILEA (22 de Marzo de 1989, entró en vigor en 1992).

  Sobre el control de los movimientos transfronterizos de los desechos peligrosos y su eliminación.

- CONVENIO DE ROTTERDAM (11 de Septiembre de 1998).

  Para la aplicación del procedimiento relativo a ciertos plaguicidas y químicos peligrosos objeto de comercio internacional.

- CONVENIO DE ESTOCOLMO (23 de Mayo de 2001, ratificado 17 de mayo de 2004).

  El objetivo del Convenio es proteger la salud humana y el medio ambiente frente a los POPs.

- REACH – 'The New EU Chemicals Legislation' (29 de Octubre de 2003).

  Los objetivos son mejorar la protección de la salud humana y del medio ambiente, manteniendo la competitividad y promoviendo la innovación de la industria química en la Unión Europea (EU).

Un ejemplo claro de la problemática de la contaminación es el caso de la acuicultura. El estudio de contaminantes orgánicos tales como los OCs, pesticidas organoclorados (OCPs), los bifenilos policlorados (PCBs), hidrocarburos policíclicos aromáticos (PAHs), retardantes de llama como los difenil éter polibromados (PBDEs), dioxinas y compuestos similares, muestran valores divergentes entre animales salvajes y los que se crían en granjas, y a su vez, entre los que se crían en América y Europa (Hites et al., 2004; Serrano et al., 2008). Atribuir este fenómeno a problemas de tipo medioambiental sería difícil de asumir. Entre otras razones, porque significaría que las condiciones ambientales de los animales criados en cautividad son mucho peores que las de las aguas donde crecen en libertad. Aun admitiendo que hubiera contaminación ambiental, que ciertamente existe en las piscifactorías de dorada, lubina o salmón por ejemplo, y por supuesto en los ambientes marinos libres, hay que considerar en paralelo el fenómeno de la bioacumulación. En los animales salvajes, probablemente debido a que su dieta en los primeros estadios se basa en insectos y otros animales que no han llegado a acumular niveles importantes de estos contaminantes, la presencia de compuestos OCs en sus tejidos grasos no es alta a no ser que se dé una exposición aguda. Los mayores niveles sólo suelen presentarse en los animales de mayor edad.

Consecuentemente, si en las piscifactorías se detectan contaminantes OCs, que típicamente se acumulan en hígado y partes grasas del animal, y no hay exposición aguda o mecanismos que propicien la bioacumulación, la única vía que explicaría su presencia son los piensos. La vía de contaminación mediante piensos en producción animal es bien conocida. Piensos en mal estado, tratados inadecuadamente o enriquecidos con materiales que se han demostrado poco apropiados, están en el origen de más de una crisis alimentaria. Ejemplos recurrentes de ello es el uso irregular e indiscriminado de antibióticos

u otros medicamentos a través de los piensos, la todavía candente crisis de las vacas locas y la pérdida de confianza en los productos cárnicos alemanes de principios de año como consecuencia del escándalo generado por la presencia de contaminantes en piensos animales.

A diferencia de la alimentación natural, los animales de piscifactoría se alimentan con harinas que se obtienen a partir de restos de pescado, entre otros. Entre los componentes importantes se encuentra el aceite de pescado, ya que de otra manera, difícilmente se podría conseguir una composición de calidad de la grasa del pescado cultivado, y no encontraríamos los efectos beneficiosos del consumo reiterado de su grasa (aceites esenciales omega-3 y omega-6) sobre las enfermedades cardiovasculares. Es en esta fracción grasa donde nos encontraríamos el principal problema. La mayor parte de los POPs analizados, tales como los PAHs, PBDEs, PCBs y algunos OCs, son acumulables en la fracción grasa de las muestras analizadas; harinas y aceites de pescado, piensos y filetes de pescado entre otros. Si se utilizan especies menores para la elaboración de harinas de pescado, o incluso restos de otras especies grasas de mayor tamaño, se aprecia, como han puesto de manifiesto numerosos estudios, un incremento de la concentración de los niveles de la contaminación química (Dórea et al., 2006).

Estos hechos ponen de manifiesto una verificación de este principio ecológico. Esta situación, que si bien no parece de un gran peligro, si que presenta un problema que requiere solución, siendo ésta un adecuado y riguroso control de las materias primas empleadas en la elaboración de los piensos para la alimentación de los peces (Newsletters from Aquamax Project, http://www.aquamaxip.eu).

Como ejemplo, la Organización Mundial de la Salud considera como máximos aceptables concentraciones de dioxinas comprendidas entre 1-4 pg/Kg peso vivo del consumidor al día. Por tanto, un individuo adulto de 70 Kg de peso podría ingerir 280 pg/día. Si vemos que los máximos detectados son de 3 pg/g de pescado, se podrían ingerir hasta 90 g de salmón al día (Organización mundial de la salud, 2001, http://www.who.int/inf-pr-2001/en/state2001-01.html).

Efectivamente, las cifras no pueden ser consideradas tan alarmantes desde este punto de vista. Sin embargo, si en el futuro no se consideran medidas de control que consigan reducir las concentraciones actuales, sí que podríamos vernos abocados a una situación de peligro difícilmente controlable.

Con el fin de evitar la contaminación en alimentos derivados de origen animal y vegetal se han de vigilar y controlar rigurosamente las etapas de producción, almacenamiento y distribución de los piensos hasta el momento de su consumo. Para ello se requieren técnicas de análisis avanzadas para la identificación de estos agentes nocivos. Estas técnicas han de ser capaces de proporcionar resultados fiables para la determinación de estos contaminantes en cortos periodos de tiempo y de manera fiable. La elevada toxicidad de los contaminantes presentes en el medio ambiente obliga además a disponer de metodología analítica fiable, rápida y suficientemente sensible que permita el control de todo tipo de contaminantes en el medio ambiente.

Otra problemática sobre contaminación medioambiental lo constituye la contaminación de los recursos hídricos. Mares, ríos y aguas superficiales han sido y siguen siendo en la actualidad el destino de gran cantidad de residuos, deshechos y vertido de compuestos químicos que en muchas ocasiones presentan toxicidad. El origen de esta posible contaminación procede de la industria, de las zonas urbanizadas, de actividades de la agricultura y ganadería, entre otros. Las aguas superficiales, subterráneas e incluso los mares contienen una elevada carga de contaminantes de origen antropogénico, muchos de los cuales tienen una alta persistencia en el medio ambiente y la capacidad de bioacumularse en las redes tróficas acuáticas. Como ejemplo de compuestos antropogénicos podemos citar a los OCs, PCBs, PBDEs, PAHs, entre otros. Estos contaminantes han sido ampliamente estudiados debido a la gran preocupación que han provocado por su peligrosidad para el medio ambiente y la salud pública. En este contexto, la aparición de nuevos contaminantes en muestras ambientales, como es el caso de los compuestos farmacéuticos y de cuidado personal, ha despertado el interés hasta el punto de ser considerados como un problema medioambiental aún por determinar (Daughton et al 1999; Ternes et al., 2004; Muñoz et al., 2008).

La contaminación de las aguas medioambientales, y en concreto de las aguas marinas, tiene como consecuencia la aparición de contaminantes en productos de consumo humano. En este sentido las salmueras y sales marinas de uso alimentario, proceden de la evaporación del agua de agua de mar y la cristalización de la sal presente en ella. Muchos de los contaminantes presentes en el agua de mar que llegan a las salinas, enclaves muy expuestos y sensibles a la contaminación, pueden permanecer tanto en la sal como en las salmueras después del proceso de evaporación. Este hecho se convierte en una amenaza para la seguridad alimentaria, ya que gran cantidad de alimentos de consumo humano contienen sal. También las salmueras constituyen cierto riesgo por ser ampliamente utilizadas en alimentación, pesquerías y productos de la acuicultura.

Resulta de gran interés el análisis y control de sales y salmueras ya que, hasta hoy, y bajo nuestro conocimiento, existen pocos trabajos o estudios publicados acerca de la presencia de contaminantes en este tipo de muestras. Su estudio contribuye a un mayor conocimiento y percepción en materia de salud pública y seguridad alimentaria.

El análisis de contaminantes orgánicos en muestras medioambientales sufrió un excelente impulso con la aparición de la cromatografía de gases (GC). El uso correcto de esta técnica permitió la separación de un gran número de compuestos. Posteriormente, la introducción de la GC capilar a finales de los años 70 y la posibilidad de disponer de sistemas de detección de espectrometría de masas (MS) selectivos y fiables, contribuyó a la emergente popularidad obtenida por los procedimientos basados en GC. A principios de los años 80, los primeros sistemas de cromatografía líquida acoplada a espectrometría de masas (LC-MS) empezaron a comercializarse. Gracias a la técnica LC-MS se pudo ampliar el análisis de contaminantes orgánicos ya que además de ofrecer una excelente separación, fue posible llevar a cabo el análisis de analitos que por características físico-químicas (termolabilidad, volatilidad, polaridad) eran más problemáticos mediante GC-MS.

En la actualidad, la determinación de contaminantes orgánicos se lleva a cabo mayoritariamente mediante técnicas cromatográficas. El análisis de compuestos orgánicos a niveles de traza, no se puede llevar a cabo con un análisis directo de la muestra, sino que

es necesario realizar un pretratamiento de esta que permita su introducción en el equipo de medida.

1.2. Determinación analítica de contaminantes orgánicos en muestras complejas.

La determinación de contaminantes orgánicos en muestras complejas con alto contenido graso, en aguas medioambientales, muestras de sales o salmueras, es una tarea complicada. Resulta ser un procedimiento especialmente delicado por tratarse de muestras con gran cantidad de interferentes que complican la determinación instrumental, tanto cualitativa como cuantitativa. Por ello, es imprescindible la aplicación de técnicas eficaces que permitan la obtención de extractos limpios compatibles con los sistemas de medida, para proporcionar resultados fiables y representativos. Otro factor que complica la determinación es la baja concentración de estos contaminantes en las muestras de análisis, lo que hace necesario la aplicación de técnicas sensibles y selectivas.

Atendiendo a los compuestos a determinar y la tipología de las muestras, se deberá seguir un procedimiento específico para cada caso con el fin de obtener resultados satisfactorios. Cabe destacar, que en todas las experiencias de esta Tesis se prestó especial atención a las guías SANCO (SANCO/10684/2009; SANCO/3131/2007) para la validación y control de calidad de la metodología analítica desarrollada.

1.2.1. Determinación de POPs en muestras con alto contenido graso

El primer paso en la determinación de POPs es la extracción de los compuestos de interés de la matriz. Posteriormente, dependiendo del origen de la muestra y de su complejidad, serán necesarias una o varias etapas de purificación y eliminación de interferentes con el fin de obtener resultados satisfactorios y fiables.

En nuestro caso, en muestras de matriz compleja como los filetes de pescado, piensos o materias primas animales o vegetales, la extracción sólido-líquido mediante un

sistema de reflujo resulta ser una excelente técnica para extraer adecuadamente los compuestos orgánicos de la matriz sólida y transferirlos a la disolución orgánica (Serrano et al., 2003). El contacto directo del disolvente orgánico extractante con la matriz, generalmente n-hexano o diclorometano, hace que los analitos afines al disolvente sean atraídos por éste consiguiéndose una extracción más efectiva. El efecto de la temperatura en la etapa de reflujo facilita la disgregación de la muestra mejorando el contacto del disolvente con la matriz. De esta manera también se obtiene una extracción más óptima. Se debe tomar especial precaución con aquellos compuestos de elevada volatilidad, teniendo en cuenta la temperatura en la que se realiza el reflujo para evitar pérdidas. Los principales inconvenientes a destacar en esta técnica de reflujo son, un elevado consumo de disolventes orgánicos y tiempo de extracción, que alcanza en muchos casos las cuatro horas. Asimismo, presenta dificultad de automatización.

Otro sistema de extracción ampliamente conocido es el Soxhlet. Esta técnica tiene un fundamento similar al reflujo, siendo muy efectiva y ampliamente utilizada para la extracción de compuestos orgánicos en matrices complejas. El uso de disolventes con elevada afinidad por los analitos permite una óptima extracción de estos de matrices complejas, lo que hace que estas técnicas sean frecuentemente utilizadas en numerosas aplicaciones (De Koning et al., 2009).

Metodologías de extracción como la extracción líquida presurizada (PLE), extracción acelerada con disolventes (ASE), extracción asistida por microondas (MAE), microextracción en fase sólida (SPME) y extracción con fluidos supercríticos (Schantz et al., 2006; Fidalgo-Used et al., 2007), aceleran el tiempo de extracción y facilitan la manipulación de la muestra después de la extracción (van Leeuwen et al., 2008; Björklund et al., 2006).

Después de la etapa de extracción, los analitos objeto de estudio se encuentran asociados a la matriz y conviene realizar alguna etapa de purificación lo más específica posible que garantice la eliminación de los interferentes. De esta forma, será viable la concentración de los analitos en el extracto y así poder conseguir una metodología más sensible y selectiva. Todos aquellos compuestos, contenidos en la muestra, que no son

objetivo de análisis, pueden llegar a enmascarar nuestros analitos y disminuir la sensibilidad, selectividad y especificidad de nuestra metodología. Los interferentes presentes en este tipo de muestras son generalmente compuestos de elevado peso molecular, principalmente lípidos, que dificultan el análisis.

La purificación o "*clean-up*" de una muestra es una de las etapas más importantes en el análisis de muestras complejas. Para la determinación de compuestos ácido-resistentes como los PBDEs, los PCBs y algunos OCs, el tratamiento de la muestra con ácido sulfúrico garantiza la eliminación de más del 90% del contenido lipídico de la muestra (Serrano et al., 2003). En el caso de los compuestos ácido-lábiles como los PAHs, es necesario proponer rutas de purificación alternativas (Fernández-González et al., 2008 a, b). Una buena solución para la eliminación de interferentes sería la realización de una purificación mediante una etapa de saponificación. En esta etapa, la fase lipídica de las muestras se transforma en jabón y los analitos orgánicos, PAHs en este caso, pasan a la fase apolar (Perugini et al., 2007).

Otra técnica de purificación ampliamente utilizada en los laboratorios de rutina para la determinación de contaminantes orgánicos ha sido la extracción en fase sólida (SPE) (Picó et al., 2007). Esta técnica supone una alternativa rápida y eficaz comparada con la etapa tradicional de extracción líquido-líquido (ELL). Comparando ambas técnicas, la SPE es una técnica que precisa una menor utilización de disolventes orgánicos, un volumen menor de muestra, evita además la formación de emulsiones y, lo más importante, permite automatizar el proceso y así realizar la purificación de más de una muestra al mismo tiempo.

El procedimiento de SPE se debe optimizar con el fin de obtener resultados satisfactorios para la eliminación de interferentes y concentración de nuestros analitos. La selección de la fase estacionaria, el disolvente de elución, el estudio del patrón de elución y volumen de ruptura (*breakthrough*) son variables críticas a optimizar.

Para purificar muestras con contenido graso mediante SPE se suele trabajar en fase normal. En esta modalidad, se pretende eluir los analitos mientras que los interferentes

(generalmente lípidos) son retenidos en la fase estacionaria y eluidos posteriormente en otra fracción diferente a la de los analitos de interés (Meloan et al., 1999). Una técnica de purificación muy similar a la anterior es la cromatografía líquida en fase normal (NPLC), también empleada para la purificación de muestras con contenido lipídico (Serrano et al., 2003). Las fases estacionarias comúnmente empleadas son de silica y Florisil.

Por último, cabe destacar también la cromatografía por permeación en gel (GPC). La GPC es una técnica ampliamente utilizada en laboratorios de análisis de pesticidas en muestras con contenido graso. Esta técnica permite la separación casi completa entre los lípidos y los pesticidas (Tindle et al., 1972; Griffitt et al., 1974). La GPC permite la purificación de extractos grasos o la separación de fracciones lipídicas. La separación se basa en el tamaño molecular de los compuestos. El sistema GPC consiste básicamente en una columna rellena de un polímero poroso en forma de bolas. Al introducir el extracto lipídico, las moléculas más pequeñas se retienen más en la columna puesto que deben recorrer mayor camino a través de los poros del relleno polimérico mientras que las moléculas grandes eluirán primero. Una buena elección del relleno de la columna de GPC y del disolvente empleado para la elución permite generalmente una separación de los pesticidas de la fracción lipídica en la mayoría de los casos.

A pesar de disponer de técnicas excelentes para la purificación de muestras grasas, la correcta determinación de POPs a niveles de traza resulta en muchas ocasiones insuficiente mediante una única etapa de purificación. Generalmente, después del primer *clean-up* todavía quedan interferentes por lo que es necesario realizar un segundo tratamiento de la muestra que nos permita cuantificar los analitos a niveles bajos de concentración con una buena sensibilidad (Serrano et al., 2003).

En nuestro caso, para la determinación de PAHs, el tratamiento de las muestras consistió en una saponificación y posterior SPE con Florisil mientras que para la determinación de PBDEs, se realizó una extracción con reflujo seguida de tratamiento con ácido y posterior SPE con Florisil obteniendo resultados satisfactorios en ambos casos. Los procedimientos desarrollados permiten llevar a cabo la purificación de muestras de la

acuicultura con elevado contenido graso, pudiendo inyectar los extractos resultantes directamente en el sistema GC-MS.

1.2.2. Determinación de contaminantes orgánicos en muestras acuosas y muestras salinas.

Como se ha comentado anteriormente, la SPE es una de las técnicas más populares y ampliamente aceptadas para el análisis de contaminantes orgánicos. Hoy en día, el tratamiento de muestra mediante SPE junto al potencial del acoplamiento GC-MS ha sido ampliamente utilizado para la determinación de contaminantes orgánicos en muestras ambientales (Rubio y Pérez-Bendito, 2009; Pitarch et al., 2010).

Para el análisis de contaminantes orgánicos en muestras de disoluciones acuosas y salinas, se emplean preferentemente cartuchos de SPE en los que se trabaja en fase inversa. La purificación de las muestras se realizará con cartuchos de SPE de fase estacionaria apolar, entre las que destacan las fases C_{18}, $C_{8,}$ fenilo, entre otras. Los analitos de interés son retenidos al pasar la muestra por la fase estacionaria y posteriormente estos analitos se eluyen mediante un disolvente apolar.

En estos casos, la SPE es una técnica económica, rápida, de fácil automatización y que no emplea gran cantidad de disolventes, además de permitir una buena concentración del extracto de la muestra. Únicamente pasarán al extracto concentrado final aquellas sustancias apolares afines a la fase estacionaria y al disolvente orgánico escogido para la elución. De esta manera se obtiene un tratamiento suficientemente selectivo de los analitos objeto de estudio permitiendo alcanzar excelente sensibilidad y niveles de cuantificación bajos.

En la presente investigación se realizó la técnica de SPE mediante cartuchos C_{18} consiguiendo una eficiente purificación y concentración de las muestras acuosas y salinas.

1.3. Cromatografía de gases

La GC es una técnica de separación ampliamente utilizada en análisis de contaminantes orgánicos en muestras ambientales (Poster et al., 2006; Van Leeuwen et al., 2008). Los objetivos de una técnica cromatográfica en columna son la separación de las diferentes especies químicas en zonas o bandas que se mueven a diferente velocidad, las cuales se visualizan en un cromatograma mediante un sistema de detección a la salida de la columna. La diferente distribución de los componentes de la muestra entre una fase estacionaria y una fase móvil junto a las diferentes velocidades de desplazamiento de los componentes al ser arrastrados por la fase móvil a través de la fase estacionaria serán factores que determinaran la cromatografía.

Pese a la importancia de los factores que intervienen en una buena separación en GC, su discusión teórica escapa de los objetivos de la presente Tesis, pudiéndose consultar bibliografía sobre los aspectos teóricos de la GC (Meloan et al., 1999; Cela et al., 2002; Van Leeuwen et al., 2008) así como parámetros básicos indispensables a optimizar a la hora de obtener unos resultados analíticos satisfactorios (Kitson et al., 1996; McMaster et al., 1998).

Cabe destacar que todos los trabajos que se presentan en los siguientes capítulos están basados en GC debido a la volatilidad y carácter hidrófobo que presentan los analitos de estudio.

1.4. Espectrometría de masas

La técnica de MS ha supuesto una mejora en la medida de la masa molecular de compuestos y átomos mediante la conversión de estos en iones cargados. La popularidad de esta técnica radica fundamentalmente en su especificidad, sensibilidad, información estructural, entre otras (Barker et al., 1998). Esta técnica se basa principalmente en diferentes procesos de ionización, separación y detección (Chhabil et al., 2007). Estos procesos transcurren en condiciones de alto vacío lo que permite que los iones se muevan

libremente sobre un espacio sin interacciones con otras especies. Las posibles colisiones producidas en un analizador de masas, podrán dar lugar a fragmentaciones de los iones moleculares pudiendo producir, mediante reacciones moleculares, diferentes especies iónicas.

Los componentes de un MS se muestran en la **Figura 1.1** y estos son los siguientes:

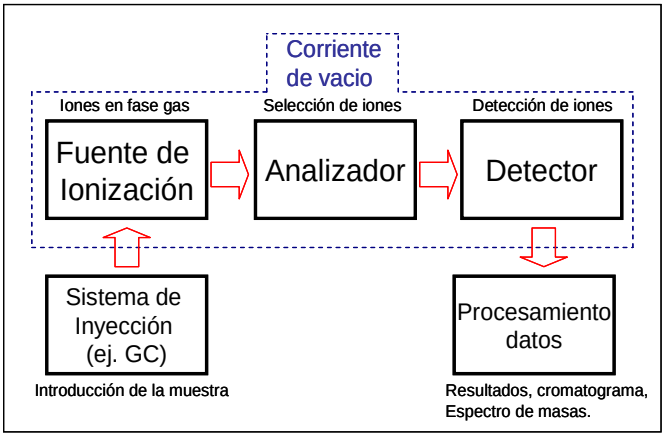

Figura 1.1. Componentes de un espectrómetro de masas.

1. Sistema de Inyección. Espacio reservado para la entrada de la muestra y su transformación previa entrada a la fuente de ionización. En este proceso la muestra entra al sistema a presión atmosférica y entra en el vacío generado por el sistema.

2. Fuente de ionización: Espacio en el cual las moléculas neutras procedentes de la muestra se ionizan y se mezclan con el gas de iones. La fuente de ionización empleada cuando se realiza un acoplamiento GC-MS puede ser de dos tipos:

   2.1. Ionización electrónica (EI): ionización mediante la cual un electrón interacciona con la molécula arrancando un electrón de la misma. En el proceso, la molécula queda con un exceso de energía que se disipa por vibración, rotación, reordenación molecular o mediante fragmentación.

   2.2. Ionización química (CI). Ionización más débil que la anterior. El fundamento consiste en transferir la carga desde un gas reactivo (ej. Metano) a las

moléculas de analito. El exceso de energía sería mínimo y se produce una escasa fragmentación a diferencia de la fuente EI. Su principal uso está dirigido a potenciar la abundancia del ión molecular en aquellas sustancias en las que su presencia en el espectro de EI es escasa o no aparece. Esta ionización puede dar lugar a especies positivas (ionización química positiva, PCI) o especies negativas (ionización química negativa, NCI).

3. Analizador de masas: El analizador es el responsable de separar los distintos fragmentos en función de su relación *m/z*. Las características principales de un analizador de masas son la resolución, sensibilidad, rango de masas y capacidad de medida exacta de masas. Existen diferentes tipos de analizadores entre los que podemos destacar el sector magnético, cuadrupolo (Q), triple cuadrupolo (QqQ), trampa de iones (IT) y tiempo de vuelo (TOF). Como los trabajos realizados en esta Tesis se basan en el desarrollo de metodologías analíticas mediante analizadores QqQ y TOF, y en el último capítulo se presenta una aplicación con analizador IT, este apartado se centra exclusivamente en estos analizadores (funcionamiento, ventajas, inconvenientes…) ya que el resto se encuentra ampliamente descrito en bibliografía (Barker et al., 1998; Chhabil et al., 2007).

   3.1. Analizadores QqQ e IT: En los últimos años, la espectrometría de masas en tándem (MS/MS) ha sido cada vez más utilizada para el análisis de contaminantes orgánicos en matrices complejas. En esta Tesis se han utilizado analizadores QqQ e IT para aplicaciones MS/MS. Gracias al acoplamiento MS/MS se obtiene gran sensibilidad y selectividad en la determinación de contaminantes orgánicos, dado que se minimizan en gran medida las interferencias debidas a la matriz. MS/MS se refiere a la combinación de dos puntos de análisis de masas que pueden darse tanto en el tiempo como en el espacio. Hasta ahora, las aplicaciones y la popularidad de esta técnica han ido creciendo paulatinamente (Santos et al., 2003; Chhabil et al., 2007; Fernández-González et al., 2008b). Se le pueden atribuir contribuciones en el campo de la elucidación de estructuras moleculares de

compuestos desconocidos, identificación de compuestos en matrices complejas, elucidación de rutas de fragmentación y cuantificación de compuestos en muestras reales. Las características de un MS/MS podrían resumirse en los siguientes puntos:

- Proporciona información estructural.
- Eliminación de picos interferentes. Excelente selectividad.
- Mejora de la señal/ruido.
- Mejora de la sensibilidad.
- Aplicación a muestras de matriz compleja.

Por otra parte, los inconvenientes de esta técnica serían:

- Instrumentación más cara y compleja.
- Búsqueda en librerías no disponible.

En un analizador QqQ existen diferentes modalidades de trabajo puesto que disponemos de dos analizadores (**Figura 1.2**). El primer analizador permite

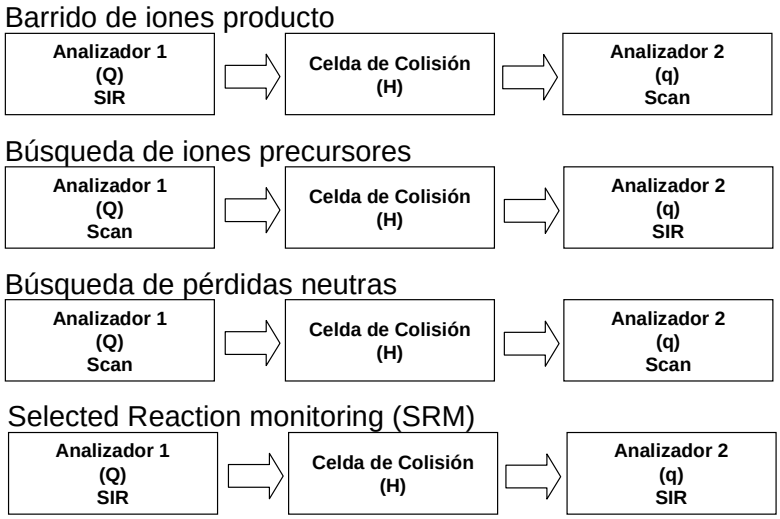

Figura 1.2. Modos de trabajo en MS/MS.

trabajar en modo SIR (*Selected ion Recording*) o en modo "*full scan*". En modo SIR, se selecciona un ión producto específico, y sólo se mide éste. En "*full scan*", se adquiere un espectro de masas continuo en un rango definido, a una velocidad de barrido adecuada. El segundo analizador permite múltiples posibilidades: barrido de iones producto, búsqueda de iones precursores, búsqueda de pérdidas neutras (NL), *selected reaction monitoring* (SRM). En la modalidad SRM, se disminuye en gran medida el ruido químico aumentando significativamente la relación S/N y observando un notable aumento de la sensibilidad. Trabajando con transiciones específicas en modo SRM se obtienen metodologías altamente selectivas incrementando la confirmación de la identidad de los analitos.

En el analizador IT, la ruptura y aislamiento de los iones se produce en el tiempo, mientras que para el analizador QqQ, este proceso se realiza en el espacio, presentando ambos analizadores ventajas y desventajas. En concreto, el QqQ está compuesto de tres cuadrupolos colocados consecutivamente, de los cuales el primero y el tercero seleccionan los iones de interés, y el segundo actúa como celda de colisión. Este segundo cuadrupolo es el que permite la realización de MS/MS, puesto que el ciclo ruptura-detección se realiza dos veces. Físicamente se aceleran los iones precursores desde el primer cuadrupolo hacia el segundo en presencia de un gas de colisión inerte (generalmente argón o helio), pudiendo obtenerse distintas fragmentaciones.

Garrido Frenich et al., 2008 realizaron una comparación entre los analizadores QqQ e IT acoplados a GC para la determinación de pesticidas en alimentos de consumo. En general, los analizadores IT ofrecen mayor información estructural que los QqQ además de una mayor robustez en las medidas. Mejor sensibilidad y selectividad presenta el QqQ respecto al IT para muestras más complejas, además de una mayor velocidad de *scan*,

permitiendo la inclusión de un mayor número de compuestos por método analítico.

Para llevar a cabo una correcta confirmación de la identidad de los compuestos detectados, en esta Tesis se seguirá el criterio de identificación por puntos propuesto por la Unión Europea (Commission Decision 2002/657/EC). En nuestro caso, trabajando en MS/MS por cada ión precursor se obtiene un punto de identificación (IP) y por cada ión producto 1,5 puntos. Trabajando en modo SIR, por cada ión adquirido obtendremos 1 punto de identificación. Para la confirmación de compuestos prohibidos es necesario conseguir 4 IPs mientras que para compuestos legales 3 IPs (Hernández et al., 2005).

3.2. Analizadores TOF. El analizador TOF es, en cuanto a fundamento, uno de los analizadores más simples de los que se utilizan hoy en día. Se basa en la medida del tiempo que tardan los iones generados y acelerados con igual energía en la fuente de iones, en alcanzar un electrodo colector situado a una distancia prefijada. Como los iones poseen la misma energía pero diferentes masas, éstos alcanzarán el colector a diferentes tiempos, dependiendo de su masa, carga y energía cinética. Los analizadores TOF destacan por su elevada resolución (~7000 FWHM) y exactitud de masa.

Estos analizadores son capaces de proporcionar información espectral completa de alta resolución permitiendo adquirir gran cantidad de información química en un único análisis. También permite aplicaciones de búsqueda de compuestos desconocidos mediante técnicas de *screening*. Además, el analizador TOF es capaz de analizar simultáneamente todos los analitos dentro de un rango de masas predeterminado, no así los analizadores de masas como el Q o IT, donde las masas son preseleccionadas, aisladas y analizadas secuencialmente. Gracias a estas posibilidades, la

combinación de GC junto a un analizador TOF ofrece una excelente sensibilidad en la adquisición de un espectro completo.

Como se ha comentado anteriormente, para la confirmación de la identidad de los compuestos detectados, se seguirá el criterio de identificación por puntos propuesto por la Unión Europea (Commission Decision 2002/657/EC).

4. Detector: Los iones generados llegan al detector que al recibir el impacto producido por las partículas cargadas emite electrones. Estos electrones son acelerados hacia un dínodo el cual emite varios electrones al recibir el impacto de cada electrón. Este proceso se repite varias veces hasta obtenerse una cascada de electrones que llega al colector lográndose una corriente fuertemente amplificada. La corriente obtenida puede amplificarse de nuevo por procedimientos electrónicos y se lleva a un sistema de procesamiento.

5. Procesamiento de datos: Los espectros de masas se almacenan y procesan en un ordenador. Es muy frecuente la construcción de bibliotecas de espectros de masas que permiten la identificación de compuestos químicos por comparación.

1.5. Acoplamiento cromatografía de gases a espectrometría de masas.

El acoplamiento GC-MS combinando la técnica de separación y la detección e identificación de los componentes de una mezcla o matriz compleja, es una técnica ampliamente utilizada en laboratorios analíticos de investigación. GC-MS es una de las técnicas más atractivas y potentes para el análisis rutinario de contaminantes orgánicos debido a que ofrece gran sensibilidad y selectividad. En un sistema GC-MS, las muestras son introducidas al GC pasando éstas a estado gas y mezclándose con un gas inerte que es empujado por una alta corriente de vacío hacia el analizador de MS (**Figura 1.3**).

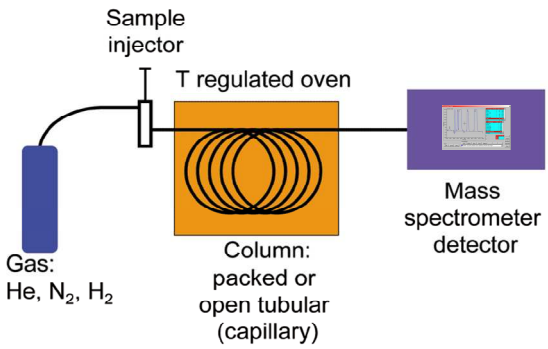

Figura 1.3. Sistema GC-MS.

Una vez producida la separación, los compuestos separados van llegando al MS gracias a la corriente de vacío. Los compuestos se ionizan y los iones son seleccionados en relación a su relación *m/z* (McMaster et al., 1998).

GC-MS es una técnica popular, potente, relativamente económica y frecuentemente utilizada para la realización de estudios analíticos ambientales. La GC es una técnica separativa que tiene la cualidad de conseguir la separación de mezclas muy complejas. Una vez separados los componentes individuales de una muestra problema por GC, el único dato que disponemos para la identificación de cada uno de ellos es el tiempo de retención de los correspondientes picos cromatográficos. Este dato no es suficiente para una identificación, sobre todo cuando analizamos muestras con un número elevado de componentes, que es el caso frecuente. De ahí la importancia del acoplamiento GC-MS.

Como se ha comentado anteriormente, los analizadores MS pueden identificar de manera casi inequívoca cualquier sustancia pura, pero normalmente no es capaz de identificar los componentes individuales de una mezcla sin haberlos separado previamente. Esto es debido a la extrema complejidad del espectro obtenido por superposición de los espectros particulares de cada componente. Por tanto, la asociación de las dos técnicas, da lugar a una técnica combinada GC-MS que permite la separación e identificación de compuestos químicos en mezclas complejas.

La utilización de la GC-MS requiere sistemas especiales de conexión. En principio, se trata de dos técnicas que trabajan en fase gaseosa y necesitan una muy pequeña cantidad de muestra para su análisis, por lo que son muy compatibles. El único obstáculo serio a la hora de realizar su acoplamiento es que el efluente que emerge de la columna cromatográfica sale a presión atmosférica y debe introducirse en el interior del MS a alto vacío. Actualmente, el acoplamiento directo resulta fácil cuando se utiliza la GC capilar, que es el caso más habitual (Kitson et al., 1996; Santos et al., 2003).

Una mezcla de compuestos inyectada en el GC se separa en la columna cromatográfica obteniendo la elución sucesiva de los componentes individuales aislados que pasan inmediatamente al MS. Cada uno de estos componentes se registra en forma de pico cromatográfico y se identifica mediante su respectivo espectro de masas. En este proceso también se registra la corriente iónica total generada en la fuente iónica, cuya representación gráfica constituye el cromatograma o "TIC" (cromatograma de iones totales). En efecto, la corriente iónica generada por todos los iones da lugar a un pico gaussiano de área proporcional a la concentración del compuesto detectado.

1.6. Aplicación de GC-MS a estudios de *screening* de contaminantes orgánicos en muestras ambientales y de salud pública.

Las diferentes enfoques analíticos que ofrece el acoplamiento GC-MS con analizadores TOF, hacen de esta técnica una herramienta realmente idónea para el *screening* medioambiental de contaminantes orgánicos (Hernández et al., 2007; Hernández et al., 2009). GC-TOF MS es una excelente técnica para la identificación de contaminantes orgánicos de interés en distintos tipos de muestras de interés ambiental y alimentario. Mediante GC-TOF MS es posible desarrollar metodología analítica avanzada para la rápida detección y confirmación inequívoca de diferentes contaminantes. Esta metodología permite el *screening* rápido y fiable de un amplio rango de contaminantes con poca manipulación de muestra y con bajo consumo de disolventes.

En nuestro caso, la utilización de GC-TOF MS para el *screening* de contaminantes orgánicos se llevó a cabo en muestras de sal, salmueras y aguas medioambientales. Mediante esta técnica ha sido posible identificar compuestos de creciente interés toxicológico. Entre ellos destacamos los alquilfosfatos y derivados (Reemtsma et al., 2008), compuestos farmacéuticos o de cuidado personal como el Galoxolide (Muñoz et al., 2008) así como compuestos filtrantes de la radiación UV como la Benzofenona (Jeon et al., 2006).

1.7. Aplicación de GC-MS a estudios de bioacumulación.

La bioacumulación es un proceso por el que un compuesto químico entra en un organismo acuático directamente desde el agua a través de las agallas, del tejido epitelial o directamente a través de la comida. Después de la entrada del compuesto químico en el organismo, éste se distribuye gracias al sistema circulatorio por todos los tejidos, tendiendo a depositarse preferentemente en órganos y tejidos grasos. La bioacumulación de un compuesto químico se cuantifica en función de sus factores de bioacumulación o bioconcentración o en el caso de no contar con estos valores se estima en función de su coeficiente de partición octanol-agua (K_{OW}). El valor de K_{OW} de una sustancia está relacionado con su potencial de bioacumulación. Un valor de K_{OW} bajo indica movilidad y transporte de ese compuesto, una fácil metabolización y biodegradación por lo que no tiende a bioacumularse. Por el contrario, un valor de K_{OW} alto indica posible bioacumulación y absorción. La movilidad y acumulación de un contaminante dependerán de sus características y de la naturaleza de los compartimientos ambientales.

La técnica de GC-MS ha sido y sigue siendo en la actualidad ampliamente utilizada para la determinación de POPs en muestras complejas a niveles de traza gracias a su excelente sensibilidad y selectividad. Para el estudio de las concentraciones de contaminantes orgánicos hidrófobos, el potencial de la técnica GC-MS presenta grandes aportaciones para su determinación proporcionando resultados satisfactorios (Serrano et al., 2008; Van Leeuwen et al., 2008). GC-MS es una técnica ampliamente reconocida para el análisis de compuestos bioacumulables dado su excelente potencial de separación,

identificación y cuantificación (Serrano et al., 2003 y 2008; Dórea et al., 2006; Hites et al., 2004).

Los PBDEs, PAHs y OCs, incluyendo los PCBs, son contaminantes orgánicos que muestran un carácter no polar, son altamente lipofílicos y presentan gran afinidad de bioacumulación en tejidos de organismos vivos (Chiu et al., 2000; Braune et al., 2005). El comportamiento bioacumulador se basa también en las funciones de los órganos excretores (riñón, pulmón, etc.) que en muchos casos, no son capaces de activar rutas detoxificadoras para reducir la presencia de estos contaminantes.

Las metodologías analíticas desarrolladas en esta Tesis se aplican al estudio de la posible bioacumulación de PAHs, OCs y PBDEs en los productos de la acuicultura marina, mediante su aplicación al análisis de piensos y filetes de pescado procedentes de un estudio experimental que incluye un ciclo completo de engorde de doradas (*Sparus aurata L*.) en el marco del Proyecto AQUAMAX (Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers. www.aquamaxip.eu). Las muestras consideradas incluyen materias primas de origen vegetal y animal, piensos con diferente composición y filetes de dorada cultivada. El objetivo básico de este proyecto ha sido establecer el nivel máximo de sustitución conjunta de proteínas y aceites de pescado en piensos de acuicultura elaborados con materias primas alternativas, sostenibles y con una baja carga de contaminantes, sin afectar al crecimiento, la conversión del alimento, la salud y bienestar animal. Todo ello sin perjuicio de obtener un pescado de alto valor nutritivo, garantizando la seguridad y calidad alimentaria, y con amplia aceptación por parte del consumidor.

1.8. Referencias.

Barker, J. 1998. Mass spectrometry: Analytical chemistry by open learning. New York, John Wiley & Sons.

Björklund, E.; Sporring, S.; Wiberg, K.; Haglund, P.; Holst, C.V. 2006. New strategies for extraction and clean-up of persistent organic pollutants from food and feed samples using selective pressurized liquid extraction. Trends Anal. Chem. 25, 318-325.

Braune, B.M., Outridge, P.M., Fisk, A.T., Muir, D.C.G., Helm, P.A., Hobbs, K., Hoekstra, P.F., (...), Stirling, I. 2005. Persistent organic pollutants and mercury in marine biota of the Canadian Arctic: An overview of spatial and temporal trends. Sci. Tot. Environ. 351-352, 4-56.

Cela, R.; Lorenzo, R.A.; Casais, M.C. 2002. Técnicas Analíticas de Separación en Química analítica. Ed. Síntesis.

Chhabil, D. 2007. Fundamentals of contemporary mass spectrometry. University of Memphis, Memphis, Tennessee, USA.

Chiu, A.; Chiu, N.; Beaubier, N.T.; Beaubier, J.; Nalesnik, R.; Singh, D.; Hill, W.R.; Lau, C.; Riebow, J. 2000. Effects and mechanisms of PCB ecotoxicity in food chains: Algae → fish → seal → polar bear. J Environ. Sci. Heal. C 18, 127-152.

Clement, R.E.; Yang, P.W.; Koester, C.J. 1999. Environmental analysis. Anal. Chem. 71, 257-292.

Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Available: eur-lex. europa.eu/pri/en/oj/dat/2002/l_221/l_22120020817en000-80036.pdf.

Daughton C. G.; Ternes T. A. 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change. Environ. Health Perspect. 107, 907-938.

De Koning, S.; Janssen, H.-G.; Brinkman, U.A.Th. 2009. Modern methods of sample preparation for GC analysis. Chromatographia 69, 33-78.

Document N° SANCO/3131/2007. Method validation and quality control procedures for pesticide residues analysis in food and feed.

Document N° SANCO/10684/2009. Method validation and quality control procedures for pesticide residues analysis in food and feed.

Dórea, J.G. 2006. Fish meal in animal feed and human exposure to persistent bioaccumulative and toxic substances. J.Food Prot. 69, 2777-2785.

Eljarrat, E.; Barceló, D. 2003. Priority lists for persistent organic pollutants and emerging contaminants based on their relative toxic potency in environmental samples. Trends Anal. Chem. 22, 655-665.

Fernández-González, V.; Concha-Graña, E.; Muniategui-Lorenzo, S.; López-Mahía, P.; Prada-Rodríguez, D. 2008a. Pressurized hot water extraction coupled to solid-phase microextraction-gas chromatography-mass spectrometry for the analysis of polycyclic aromatic hydrocarbons in sediments. J. Chromatogr. A 1196, 65-72.

Fernández-González, V.; Muniategui-Lorenzo, S.; López-Mahía, P.; Prada-Rodríguez, D. 2008b. Development of a programmed temperature vaporization-gas chromatography-tandem mass spectrometry method for polycyclic aromatic hydrocarbons analysis in biota samples at ultratrace levels. J Chromatogr. A 1207, 136-145.

Fidalgo-Used, N.; Blanco-González, E.; Sanz-Medel, A. 2007. Sample handling strategies for the determination of persistent trace organic contaminants from biota samples. Anal. Chim. Acta 590, 1-16.

Garrido Frenich, A.; Plaza-Bolaños, P.; Martínez Vidal, J.L. 2008. Comparison of tandem-in-space and tandem-in-time mass spectrometry in gas chromatography determination of pesticides: Application to simple and complex food samples. J. Chromatogr. A 1203, 229-238.

Griffitt, K.R.; Craun, J.C. 1974. Gel permeation chromatographic system: an evaluation. J. Assoc. Off. Ana. Chem. 57, 168-172.

Hernández, F.; Sancho, J.V.; Pozo, O.J. 2005. Critical review of the application of liquid chromatography/mass spectrometry to the determination of pesticide residues in biological samples. Anal. Bioanal. Chem. 382, 934-946.

Hernández, F.; Portolés, T.; Pitarch, E.; Lopez, F.J. 2007. Target and non target screening of organic micropollutants in water by solid-phase microextraction combined with gas chromatography/high-resolution time-of-flight mass spectrometry. Anal. Chem. 79, 9494-9504.

Hernández, F.; Portolés, T.; Pitarch, E.; López, F.J. 2009. Searching for anthropogenic contaminants in human breast adipose tissues using gas chromatography-time-of-flight mass spectrometry. J. Mass Spectrom. 44, 1-11.

Hites, R.A.; Foran, J.A.; Carpenter, D.O.; Hamilton, M.C.; Knuth, B.A.; Schwager, S.J. 2004. Global Assessment of Organic Contaminants in Farmed Salmon. Science 303, 226-229.

Jeon, H.-K.; Chung, Y.; Ryu, J.-C. 2006. Simultaneous determination of benzophenone-type UV filters in water and soil by gas chromatography-mass spectrometry. J. Chromatogr. A 1131, 192-202.

Kitson F.G. 1996. Gas chromatography and mass spectrometry. A practical guide. San Diego, Academic press.

McMaster, M.; McMaster, C. 1998. GC/MS. A practical user´s guide. United States of America, Wiley-VCH.

Meloan C.E. 1999. Chemical Separations. Principles, Techniques and Experimentes. New York, Ed. Wiley.

Muñoz, I.; Gómez, M. J.; Molina-Díaz, A.; Huijbregts, M. A. J.; Fernández-Alba, A. R.; García-Calvo, E. 2008. Ranking potential impacts of priority and emerging pollutants in urban wastewater through life cycle impact assessment. Chemosphere 74, 37-44.

Perugini, M.; Visciano, P.; Giammarino, A.; Manera, M.; Di Nardo, W.; Amorena, M. 2007. Polycyclic aromatic hydrocarbons in marine organisms from the Adriatic Sea, Italy. Chemosphere 66, 1904-1910.

Picó, Y.; Fernández, M.; Ruiz, M.J.; Font, G. 2007. Current trends in solid-phase-based extraction techniques for the determination of pesticides in food and environment. J. Biochem. Biophys. Methods 70, 117-131.

Pitarch, E.; Portolés, T.; Marín, J.M.; Ibáñez, M.; Albarrán, F.; Hernández, F. 2010. Analytical strategy based on the use of liquid chromatography and gas chromatography with triple-quadrupole and time-of-flight MS analyzers for investigating organic contaminants in wastewater. Anal. Bioanal. Chem. 397, 2763-2776.

Poster, D.L.; Schantz, M.M.; Sander, L.C.; Wise, S.A. 2006. Analysis of polycyclic aromatic hydrocarbons (PAHs) in environmental samples: A critical review of gas chromatographic (GC) methods. Anal. Bioanal. Chem. 386, 859-881.

Reemtsma, T., García-López, M., Rodríguez, I., Quintana, J.B., Rodil, R. 2008. Organophosphorus flame retardants and plasticizers in water and air I. Occurrence and fate. Trends Anal. Chem. 27, 727-737.

Rubio, S.; Pérez-Bendito, D. 2009. Recent advances in environmental analysis. Anal. Chem. 81, 4601-4622.

Serrano, R.; Blanes, M.A.: López, F.J. 2008. Biomagnification of organochlorine pollutants in farmed and wild gilthead sea bream (*Sparus aurata L.*) and stable isotope characterization of the trophic chains. Sci. Total Environ. 389, 340-349.

Serrano, R.; Barreda, M.; Pitarch, E.; Hernández, F. 2003. Determination of low concentrations of organochlorine pesticides and PCBs in fish feed and fish tissues from aquaculture activities by gas chromatography with tandem mass spectrometry. J. Sep. Sci. 26, 75-86.

Santos, F.J.; Galceran, M.T., 2003. Modern developments in gas chromatography-mass spectrometry-based environmental analysis. J. Chromatogr. A 1000, 125-151.

Schantz, M.M. 2006. Pressurized liquid extraction in environmental analysis. Anal. Bioanal. Chem. 386, 1043-1047.

Ternes, T.A.; Joss A; Siegrist, H. 2004.Scrutinizing pharmaceuticals and personal care products in wastewater treatment. Environ. Sci. Technol. 38, 392-399.

Tindle, R.C.; Stalling, D.L. 1972. Apparatus for automated gel permeation cleanup for pesticide residue analysis: Applications to fish lipids. Anal.Chem. 44, 1768-1773.

Van Leeuwen, S.P.J.; de Boer, J. 2008. Advances in the gas chromatographic determination of persistent organic pollutants in the aquatic environment. J. Chromatogr. A 1186, 161-182.

CAPÍTULO 2

OBJETIVOS

El objetivo principal de la presente Tesis Doctoral es el desarrollo de metodología analítica avanzada para la determinación de contaminantes orgánicos en muestras medioambientales complejas.

La primera parte de la Tesis, dedicada al desarrollo de metodologías basadas en GC-MS para la determinación de POPs en muestras marinas grasas, presenta los objetivos parciales que se detallan a continuación:

1. Desarrollo de metodología analítica basada en GC-(QqQ)MS/MS y GC-TOF MS para la determinación y confirmación de PAHs en matrices complejas.

2. Desarrollo de metodología analítica basada en el acoplamiento GC-MS para la determinación de PBDEs en matrices complejas.

En la segunda parte, se desarrollan metodologías basadas en GC-TOF MS para el análisis de la presencia de contaminantes orgánicos en muestras ambientales. Los objetivos parciales son los siguientes:

1. Desarrollo de metodología "*non-target*" par la identificación de contaminantes orgánicos en muestras de sal y agua de mar mediante GC-TOF MS.

2. Desarrollo y aplicación de metodología analítica avanzada para el estudio de OPEs en sales, salmueras y aguas medioambientales mediante GC-TOF MS.

Por último, la aplicación de metodología analítica previamente desarrollada se aplicó al análisis de muestras procedentes de un experimento de bioacumulación de contaminantes orgánicos persistentes, lo que permitió el estudio de la posible acumulación

de estos en dorada a lo largo de un ciclo de crecimiento completo. Los objetivos parciales son:

1. Estudio de la bioacumulación de PAHs en filetes de dorada.

2. Estudio de la bioacumulación de OCs en filetes de dorada.

CAPÍTULO 3

PLAN DE TRABAJO

Para alcanzar los objetivos propuestos en esta Tesis se ha seguido el siguiente plan de trabajo para el **análisis cuantitativo**:

- Selección de los contaminantes en función de su persistencia, toxicidad y carácter emergente en muestras procedentes de la acuicultura.
- Revisión bibliográfica de procedimientos analíticos para la determinación de contaminantes a niveles de traza en las matrices seleccionadas.
- Optimización de la separación cromatográfica mediante inyección en el equipo de patrones de referencia. Selección del gradiente de temperaturas óptimo para los compuestos seleccionados. Estudio del *dwell time* en función de la anchura de los picos, del número de transiciones y la sensibilidad requerida.
- Estudio de las condiciones óptimas de MS mediante inyección de patrones de referencia. Estudio de los espectros de ionización electrónica y selección de iones precursores para cada analito. Para los PAHs, aislamiento de los iones precursores seleccionados en el primer cuadrupolo y optimización de la energía de colisión para la obtención de iones producto característicos, en el segundo cuadrupolo. Selección de dos transiciones SRM por compuesto atendiendo a la sensibilidad y selectividad. Para el caso de los PBDEs, selección de los iones más sensibles para su adquisición en modo SIR (NCI-MS).
- Estudio y optimización de procedimientos de extracción y purificación para la determinación de los contaminantes orgánicos seleccionados a niveles de traza en las matrices seleccionadas.

- Estudio del efecto matriz en las muestras mediante el análisis de muestras fortificadas y patrones en solvente a la misma concentración.

- Validación de metodología analítica para la determinación de PAHs y PBDEs en muestras de peces, piensos y materias primas mediante el acoplamiento GC-MS con analizador QqQ. Estudio de los parámetros de linealidad, especificidad, exactitud, precisión y confirmación, mediante ensayos de recuperación en muestras fortificadas a varios niveles de concentración atendiendo las guías SANCO de la Unión Europea.

- Aplicación de la metodología validada para la determinación de PAHs y PBDEs en muestras procedentes de la acuicultura, entre las cuales destacan los filetes de dorada, piensos y materias primas, incluyendo aceites de pescado y aceites vegetales.

En términos de **análisis confirmativo** mediante GC-TOF MS, se ha seguido el siguiente plan de trabajo:

- Inyección de patrones y obtención del tiempo de retención de los fragmentos iónicos más representativos con su masa exacta, a partir de su espectro en ionización electrónica.

- Desarrollo de metodología de identificación mediante el software TargetLynx. Se incluirá en la metodología información relevante para la identificación de cada analito, como su tiempo de retención, composición elemental y masa exacta de los principales fragmentos de su espectro de ionización electrónica, así como la relación de intensidades entre los mismos.

- Inyección de las muestras en GC-TOF MS.

- Aplicación de la metodología analítica a muestras procedentes de la acuicultura. Comparación de los datos obtenidos con los obtenidos previamente mediante GC-(QqQ)MS/MS para la determinación de PAHs.

De manera general para el **análisis cualitativo** desarrollado en la presente Tesis Doctoral, se ha seguido el siguiente plan de trabajo:

- Revisión bibliográfica y recopilación de información de contaminantes de interés así como posibles contaminantes encontrados en muestras de agua de mar y sales marinas.

- Desarrollo de metodología *non-target* mediante GC-TOF MS basada en el software de deconvolución de datos ChromaLynx que proporciona, de manera automática, el espectro de iones de masa exacta, permitiendo su comparación con los espectros disponibles en librerías comerciales, para ionización electrónica, así como el estudio del error de masas de los fragmentos seleccionados.

- Aplicación de tratamientos de muestra adecuados para la realización de rápidos *screening* que incluyan una menor manipulación de la muestra, con el fin de conseguir elevada multirresidualidad y que sea compatible con la identificación a bajos niveles de concentración. Para ello se utilizará la técnica de SPE.

- Estudio de la presencia de contaminantes en muestras de sales marinas y agua de mar mediante metodología *non-target*.

- Desarrollo de metodología *target* mediante GC-TOF MS, para compuestos éster organofosforados (OPEs), mediante el uso del software de procesamiento de datos TargetLynx. La metodología contendrá información relevante para la identificación de cada analito, como su tiempo de retención, composición elemental y masa exacta de los principales fragmentos de su espectro de ionización electrónica, así como la relación de intensidades entre los mismos.

- Estudio de los parámetros críticos que pueden afectar al procesamiento de datos *target* como la selección de los iones, ventana de extracción de masa, interferencias con el *lock mass*, problemas de saturación del detector, entre otros.

- Aplicación de la metodología analítica de *screening* de OPEs desarrollada a muestras de sales.

- Aplicación de la metodología analítica de *screening* de OPEs a muestras de sal, salmueras y aguas medioambientales previamente analizadas mediante GC-TOF MS. Análisis retrospectivo.

Por último, el plan de trabajo incluye la aplicación de metodologías analíticas disponibles al estudio de la posible bioacumulación de OCPs, PAHs, PBDEs y PCBs en los productos de la acuicultura marina, mediante un estudio experimental que incluye un ciclo completo de engorde de doradas (*Sparus aurata L*.), y la interpretación de los resultados obtenidos.

El presente trabajo ha quedado reflejado en seis artículos científicos, ya publicados y pendientes de publicación, en revistas de carácter internacional.

CAPÍTULO 4

DETERMINACIÓN DE CONTAMINANTES ORGÁNICOS PERSISTENTES EN MUESTRAS COMPLEJAS CON ALTO CONTENIDO GRASO.

4.1. Introducción

El objetivo de este capítulo es el desarrollo de metodología analítica avanzada para la determinación de POPs a niveles traza en matrices complejas procedentes de la acuicultura marina. Las muestras consideradas incluyen piensos para el cultivo de peces, materias primas para su elaboración (aceites vegetales y de pescado) y peces cultivados. Debido a la complejidad de las muestras descritas y a la necesidad de alcanzar niveles de detección y cuantificación lo más bajos posible, se ha prestado especial atención al tratamiento de muestra, previo a la determinación instrumental mediante GC-MS.

En una primera parte se desarrolla metodología analítica para la determinación de PAHs. Para ello se utilizó la técnica de GC-MS con analizadores QqQ para la cuantificación y analizadores TOF para la confirmación adicional de los PAHs. La metodología desarrollada resultó ser suficientemente rápida, sensible y selectiva para la determinación de PAHs en muestras con alto contenido graso. Gracias a una eficiente extracción y purificación mediante saponificación y posterior SPE, fue posible la validación de la metodología en cuatro tipos de muestras complejas alcanzando LOQs a niveles bajos de concentración (μg/Kg). La combinación de analizadores QqQ y TOF resultó una excelente estrategia para la confirmación de la presencia de los PAHs en las muestras analizadas.

En la segunda parte del capítulo se desarrolla metodología para la determinación de PBDEs mediante GC-MS en modo NCI. Esta metodología permitió la cuantificación y confirmación de la identidad de 12 PBDEs en muestras con alto contenido graso. Como resultado del tratamiento de muestra mediante digestión con ácido sulfúrico y posterior SPE con Florisil, fue posible llevar a cabo la validación de la metodología en cuatro tipos de muestras complejas alcanzando LOQs a niveles bajos de concentración (μg/Kg). Cabe destacar que fue posible la determinación del BDE 209, uno de los retardantes de llama más complicados de determinar mediante GC-MS por requerir condiciones cromatográficas específicas. Trabajando con el analizador QqQ en modo SIR fue posible la adquisición de tres iones carácterísticos por compuesto lo que permitió una correcta identificación de los 12 PBDEs seleccionados.

4.2. Desarrollo de metodología analítica para la determinación de hidrocarburos policíclicos aromáticos mediante GC-(QqQ)MS/MS y GC-TOF MS.

4.2.1. Introducción.

Los PAHs son derivados poliméricos del benceno, causantes de reacciones adversas no deseadas en animales, en humanos y en el medio ambiente. Los PAHs se forman a partir de materia orgánica cuando ésta se somete a temperaturas elevadas durante un cierto tiempo. Fuentes naturales como las erupciones volcánicas o los incendios forestales también emiten PAHs al medio (Srogi et al., 2007). Otras fuentes de PAHs son las emisiones de gases procedentes de la combustión de petróleo y carbón, así como de calefacciones. A parte, el hábito de fumar, el consumo de alimentos ahumados o demasiado tostados por el efecto del fuego puede incrementar la exposición del hombre a los PAHs hasta superar niveles de riesgo. Los PAHs son compuestos ubicuos, por lo que la población está expuesta a ellos por diferentes vías. Como consecuencia, es necesaria una legislación adecuada que regule los niveles de estos contaminantes en los alimentos, con el fin de salvaguardar la salud pública. En este sentido, el reglamento de la Unión Europea Nº 1881/2006 de la comisión de 19 de diciembre de 2006, fija el contenido máximo de determinados contaminantes en los productos alimenticios. La toxicidad del contenido de PAHs en una muestra está referenciado al benzo(a)pireno. Así, se establecen contenidos máximos referidos a concentración en peso fresco de la muestra (µg/Kg), como por ejemplo, para filetes de pescado no ahumados, aceites y grasas, de 2 µg de benzo(a)pireno por Kg de muestra.

Los PAHs constituyen una familia de compuestos ampliamente distribuida en el medio ambiente, que se caracterizan por contener dos o más anillos de benceno unidos entre sí. En la **Figura 4.1.** se muestran 16 PAHs considerados de riesgo y altamente tóxicos por la Agencia de Protección Ambiental de los Estados Unidos (USEPA, www.epa.gov).

Los PAHs son compuestos lipófilos, con tendencia a acumularse en los tejidos grasos. La toxicidad de los PAHs depende en gran medida de su estructura. Entre los más peligrosos destacan los derivados del antraceno, así como las moléculas derivadas a las que se les añade algún anillo bencénico, como en el caso del benzo[a]pireno y el dibenzo[a,h]antraceno.

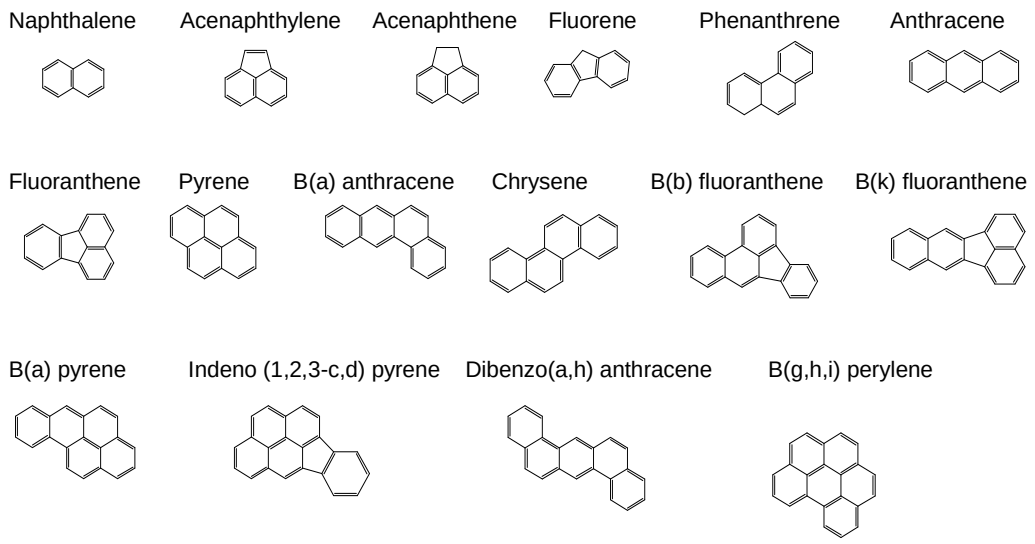

Figura 4.1. Estructuras de los 16 PAHs considerados cancerígenos por la USEPA.

La exposición humana a estos contaminantes se debe principalmente a la inhalación o ingestión, aunque también es posible la intoxicación vía cutánea. Tras la absorción se produce la distribución de estos hacia los órganos y tejidos, especialmente los de contenido graso. Una vez incorporados al organismo, experimentan una oxidación enzimática llevada a cabo en el hígado que los convierte en epóxidos y en dihidrodioles. Estas especies químicas constituyen la forma genotóxica activa de los PAHs, las cuales pueden formar aductos covalentes con proteínas y ácidos nucleicos celulares (Koss et al., 1999). Los aductos con ADN generan mutación genética, de graves consecuencias por la generación de tumores malignos para el individuo expuesto, sin olvidar la posibilidad de malformaciones en embriones.

Los alimentos constituyen una vía de exposición a los PAHs para la población. Los consumidores pueden estar expuestos a PAHs mediante la ingesta de alimentos como cereales contaminados, harina, pan y verduras o alimentos braseados. Algunos autores han encontrado PAHs a niveles de µg/kg en muestras de pescado y marisco comercializado en mercados (Srogi et al., 2007). También, algunos cereales que han sufrido un proceso de tostado, pueden presentar niveles elevados al igual que vegetales como el té que puede contener un total de PAHs a niveles de µg/Kg (Lin et al., 2005). Entre los PAHs detectados,

el de mayor interés es el benzo(a)pireno, ya que se ha determinado su toxicidad y, como se ha comentado anteriormente, es el compuesto utilizado como referencia para establecer límites legales en alimentos. En general, entre los alimentos no ahumados, los de mayor contenido graso son los que presentan niveles superiores de benzo(a)pireno (Bartle et al., 1991).

Como consecuencia de sus propiedades físico-químicas, los PAHs son compuestos persistentes en el medio marino acumulándose en la fracción lipídica de los tejidos grasos de los organismos marinos, dañando potencialmente los recursos marinos. Los mecanismos de acumulación de estos compuestos en los organismos acuáticos pueden ser muy diversos: por difusión pasiva del agua a través de las agallas o la piel (bioconcentración), a través de la ingestión de partículas suspendidas contaminadas o, en mayor proporción, por ingesta de alimento contaminado (procesos de bioacumulación y biomagnificación)(Van der Oost et al., 2003). La bioacumulación está influenciada por numerosos procesos biológicos como son la respiración, la capacidad de los organismos tanto de acumular como de eliminar los compuestos y del crecimiento de la especie.

En la actualidad la acuicultura marina ha sufrido una importante expansión debido a la sobreexplotación de los mares que ha supuesto la pesca extractiva y la creciente demanda de pescado en el mercado. De hecho, el esfuerzo pesquero ha alcanzado su techo y es urgente la búsqueda de fuentes alternativas de materias primas para los piensos de engorde de peces. Actualmente, la industria pesquera vive días difíciles puesto que no puede abastecer a la demanda global de pescado. Hasta ahora, la acuicultura ha logrado cubrir este déficit pero su crecimiento se hace cada vez más complicado debido a la necesidad de harinas y aceite de pescado, materias primas cada vez más limitadas y de las cuales depende. Dentro de la acuicultura, hay también una creciente preocupación por la presencia de compuestos tóxicos, tanto en materias primas como en los productos procedentes de la acuicultura. (Aquamax newsletter, 2008).

Como respuesta al interés que presenta el análisis de estos contaminantes en peces, piensos y materias primas utilizadas en la elaboración de estos piensos, se ha desarrollado una metodología analítica que permita la determinación de PAHs a niveles traza en

diferentes matrices procedentes de la acuicultura marina, bajo unos criterios estrictos de cuantificación y confirmación de la identidad de los mismos.

En el desarrollo de la metodología analítica se ha prestado especial atención a la etapa de extracción y purificación debido al elevado contenido lipídico de las muestras analizadas y la complejidad de las mismas.

En la etapa de la determinación instrumental se ha optimizado la cromatografía y la adquisición mediante dos analizadores de MS. En primer lugar se ha utilizado un analizador QqQ trabajando en modo MS/MS para la cuantificación a niveles de traza y en segundo lugar se ha utilizado un analizador TOF para la confirmación inequívoca de la identidad de los compuestos determinados con el analizador QqQ. La metodología desarrollada se ha aplicado para la determinación de PAHs en muestras reales procedentes de la acuicultura marina de manera fiable y precisa tanto para la cuantificación como para la identificación inequívoca de los mismos.

Las matrices incluidas son filetes de dorada cultivada, piensos con diferentes proporciones de aceites de origen animal y vegetal, aceite de pescado, aceite de lino, aceite de colza y aceite de palma. Las muestras proceden de un estudio experimental de crecimiento de doradas (*Sparus aurataL.*) en el marco del Proyecto AQUAMAX (www.aquamaxip.eu).

4.2.2. Artículo científico 1:

A reliable analytical approach based on gas chromatography coupled to triple quadrupole and time of flight analyzers for the determination and confirmation of polycyclic aromatic hydrocarbons in complex matrices from aquaculture activities.

J. Nácher-Mestre, R. Serrano, T. Portolés, F. Hernández, L. Benedito-Palos, J. Pérez-Sanchez

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RCM

A reliable analytical approach based on gas chromatography coupled to triple quadrupole and time-of-flight mass analyzers for the determination and confirmation of polycyclic aromatic hydrocarbons in complex matrices from aquaculture activities

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The potential of gas chromatography coupled to tandem mass spectrometry (GC/MS/MS) with a triple quadrupole analyzer (QqQ) has been investigated for the quantification and reliable identification of sixteen polycyclic aromatic hydrocarbons (PAHs) from the EPA priority list in animal and vegetable samples from aquaculture activities, whose fat content ranged from 5 to 100%. Matrices analyzed included fish fillet, fish feed, fish oil and linseed oil. Combining optimized saponification and solid-phase extraction led to high efficiency in the elimination of interfering compounds, mainly fat, from the extracts. The developed procedure minimized the presence of these interfering compounds in the extracts and provided satisfactory recoveries of PAHs. The excellent sensitivity and selectivity of GC/(QqQ)MS/MS in selected reaction monitoring (SRM) allowed to reach limits of detection at pg/g levels. Two SRM transitions were acquired for each analyte to ensure reliable identification of compounds detected in samples. Confirmation of positive findings was performed by GC coupled to high-resolution time-of-flight mass spectrometry (GC/TOFMS). The accurate mass information provided by GC/TOFMS in full acquisition mode together with its high mass resolution makes it a powerful analytical tool for the unequivocal confirmation of PAHs in the matrices tested. The method developed was applied to the analysis of real-

world samples of each matrix studied with the result of detecting and confirming the majority of analytes at the µg/kg level by both QqQ and TOF mass spectrometers.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants from both natural and anthropogenic origins, such as the partial combustion of organic compounds, or pollution from petrochemical activities.[1] PAHs are lipophilic contaminants and tend to accumulate in the biotic compartment of the environment.[1-6] Concern has arisen about their potential adverse effects on organisms, including human beings, and this has led to the inclusion of 16 PAHs in the list of priority contaminants by the United States Environmental Protection Agency.[7]

Marine aquaculture has undergone strong development in the last few decades as a consequence of increased fish consumption by the world population and decreasing wild stocks. Fish culture operates in parallel to traditional fisheries and nowadays both cultured and wild fish are important components of the Mediterranean diet.[8,9] Aquaculture products are subject to increasingly strict control and regulation. As an example, the European Commission Regulation (EC) 1881/2006[10] has fixed a maximum level of 2 µg/kg (wet weight) for benzo[a]pyrene in fish.

PAHs enter the marine environment through atmospheric depositions and surface runoff, and, due to their lipophilic character, they are accumulated by marine organisms.[2-4,6,11-13] As consequence of the artificial food chain in aquaculture activities, fish used as raw material for the manufacture of fish feed ingredients (fish oils and meals) are a potential source of PAHs in fish feed, which might be bioaccumulated by cultured fish.[14] Likewise, vegetable oils used in fish feed manufacture are another possible source of this family of contaminants.[5,15]

The determination of PAHs in aquaculture matrices is difficult due to their complexity and the presence of interfering substances, mainly fats that are co-extracted with the analytes when using techniques such as Soxhlet, microwave-assisted extraction,

pressurized liquid extraction, etc.[6,16-22] making an efficient cleanup before analysis essential. Traditionally, the cleanup step is performed by solid-phase extraction (SPE) or preparative column chromatography using Florisil, silica, alumina or other available absorbents.[6,18,21,23] Other techniques are now applied, such as accelerated solvent extraction followed by gel-permeation chromatography,[19] solid-phase microextraction,[22] or microwave-assisted extraction.[21]

High-performance liquid chromatography (HPLC) with fluorescence detection has been widely used for determination of PAHs.[16] However, in the analysis of complex matrices, gas chromatography (GC) rather than LC is often preferred for separation, identification and quantification because GC generally affords greater selectivity, resolution and sensitivity for determination of PAHs. GC/single quadrupole mass spectrometry has been widely used for the determination of organic compounds in environmental samples.[13,15,19] In recent years, tandem mass spectrometry (MS/MS) has been increasingly used because of its higher sensitivity and selectivity that minimize or even remove many interferences. GC/MS/MS, using ion trap or triple quadrupole (QqQ) analyzers, has been successfully applied to the analysis of PAHs in a variety of matrices.[21,24] The use of two stages of mass analysis in MS/MS systems based on QqQ analyzers offers the possibility of applying selected reaction monitoring (SRM), one of the most selective and sensitive approaches for quantification and confirmation, especially at trace levels. Thus, the number of GC/MS/MS (QqQ) applications in the environmental and food analysis fields has increased in the last few years.

In recent years, the use of high-resolution time-of-flight mass spectrometry (TOFMS) has become prevalent in environmental analysis. This technique can provide conclusive information for the reliable confirmation of target analytes and also for the structural elucidation of non-target compounds due to its unrivalled full spectrum sensitivity together with its greater mass resolution and mass accuracy.[25,26]

The aim of this study is to develop a reliable and sensitive methodology based on the use of advanced GC/MS techniques for the quantitative determination and safe identification of the 16 EPA PAH priority contaminants in complex matrices from aquaculture activities. We have studied the extraction and cleanup steps, in order to obtain

extracts with minimal fat content. The developed methodology has been applied to real-world samples of fish feed, raw materials and fish specimens from feeding trials performed with gilthead sea bream (Sparus aurata L.) carried out as a part of the European Union project Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers (AQUAMAX; Contract number: 016249-2). The acquisition of two selective SRM transitions for each target analyte by GC/(QqQ)MS/MS has allowed the quantification and identification of PAHs at the low µg/kg level. The positive GC/(QqQ)MS/MS findings have been confirmed by GC/TOFMS, taking advantage of the high mass resolution and mass accuracy provided by this technique.

EXPERIMENTAL

Materials and reagents

A PAH analytical standard mixture (PAH-Mix 9) containing naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene and benzo[g,h,i]perylene was purchased from Dr Ehrenstorfer (Promochem, Wesel, Germany) with a purity 97-99.8% as a 10 µg/mL solution in cyclohexane. Stock solutions (1 µg/mL) were prepared by dissolving the reference standard in n-hexane and stored in a freezer at -20°C. Working solutions were prepared by diluting the stock solution in n-hexane for sample fortification and calibration curves.

In addition, benzo[a]anthracene-D_{12} from Dr Ehrenstorfer was used as a surrogate internal standard in the validation study and real-world samples analysis. Working solutions of the labeled standards were prepared by dilution of the commercial solutions with n-hexane and stored at 4°C.

Methanol, dichloromethane, n-hexane and ethyl acetate (ultratrace quality) were purchased from Scharlab (Barcelona, Spain). Anhydrous sodium sulfate of pesticide residue quality (Scharlab) was dried for 18 h at 300°C before use. Potassium and sodium hydroxide were purchased from Scharlab. The 0.2 µm filters were from Serviquimia (Castellón, Spain).

Silica cartridges (Strata 0.5 g and 1 g; Phenomenex, Torrance, CA, USA) and Supelclean LC-Florisil SPE tubes (0.5 g and 1 g; from Sigma-Aldrich, Madrid, Spain) were used in SPE experiments.

Sample material

Gilthead sea bream (*Sparus aurata L.*) specimens of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were cultured at the Instituto de Acuicultura de Torre la Sal, Castellón, Spain (IATS, CSIC) and collected when the fish reached commercial size (500 g). The left-side fillets (denuded from skin and bone) were excised and stored at -20°C until analysis. Samples of the fish feed supplied to sea bream during feeding trials from AQUAMAX project experiments were stored at -20°C until analysis. Fish oil and linseed oil used in fish feed manufacture, usually as raw materials of fish feed, were also stored at -20°C until analysis. The ingredients and the chemical composition of the fish feed are shown in Table 1.

GC instrumentation

Two GC systems (Agilent 6890N; Agilent, Palo Alto, CA, USA) equipped with an autosampler (Agilent 7683) were coupled to (1) a triple quadrupole mass spectrometer (Quattro Micro GC; Micromass, Boston, MA, USA) and (2) a time-of-flight mass spectrometer (GCT; Waters Corp., Manchester, UK), both operating in electron ionization (EI) mode. In both cases, the GC separation was performed using a fused-silica HP-5MS capillary column (length 30 m, i.d. 0.25 mm, film thickness 0.25 µm; J&W Scientific, Folson, CA, USA). The injector temperature was set to 250°C. Splitless injections of 1 µL of the sample were carried out. Helium (99.999%; Carburos Metálicos, Valencia, Spain) was used as carrier gas at a flow rate of 1 mL/min. The interface and source temperatures were set to 250°C in both systems and a solvent delay of 3 min was selected.

Table 1. Ingredients and chemical composition of fish feed analyzed.

Ingredient (%)	FO	33VO	66VO
Fish meal (CP 70%) [1]	15	15	15
CPSP 90 [2]	5	5	5
Corn gluten	40	40	40
Soybean meal	14.3	14.3	14.3
Extruded wheat	4	4	4
Fish oil [3]	15.15	10.15	5.15
Rapeseed oil	0	0.85	1.7
Linseed oil	0	2.9	5.8
Palm oil	0	1.25	2.5
Soya lecithin	1	1	1
Binder	1	1	1
Mineral premix [4]	1	1	1
Vitamin premix [5]	1	1	1
CaHPO4.2H2O (18%P)	2	2	2
L-Lys	0.55	0.55	0.55
Composition			
Dry matter (DM, %)	93.13	92.9	92.77
Protein (% DM)	53.2	52.81	52.62
Fat (% DM)	21.09	21	20.99
Ash (% DM)	6.52	6.69	6.57

[1]Fish meal (Scandinavian LT)
[2]Fish soluble protein concentrate (Sopropêche, France)
[3]Fish oil (Sopropêche, France)
[4]Supplied the following (mg · kg diet^{-1}, except as noted): calcium carbonate (40% Ca) 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0.3.
[5]Supplied the following (mg · kg diet^{-1}): retinyl acetate 2.58, DL-cholecalciferol 0.037, DL-α tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5, riboflavin 15, pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium pantothenate 2.5, vitamin B_{12} 0.025, ascorbic acid 250, inositol 500, biotin 1.25 and choline chloride 500.
FO: Reference fish feed; 33VO: fish feed with 33% fish oil replacement; 66VO: fish feed with 66% fish oil replacement

The oven temperature program in the GC/QqQ system was as follows: 90°C (1 min); 10°C/min to 250°C; 5°C/min to 300°C (3min). The oven temperature in the GC/TOF analysis

was programmed as follows: 90°C (1min); 5°C/min to 300°C (2 min). In both cases helium was used as the carrier gas at a flow rate of 1 mL/min. The QqQ system was operated in MS/MS mode using 99.995% argon (Carburos Metálicos) as the collision gas at a pressure of 0.28 Pa in the collision cell, and a dwell time per channel of between 0.1 and 0.3 s. The TOF mass spectrometer was operated at 1 spectrum/s, acquisition rate over the mass range m/z 50-300, using a multichannel plate voltage of 2650 V. The mass resolution of the TOFMS instrument was approximately 7000 (FWHM). Heptacosa standard, used for the daily mass calibration and as lock mass, was injected via a syringe in the reference reservoir at 30°C, monitoring the ion at m/z 218.9856. The application managers TargetLynx and QuanLynx (Waters) were used to process the qualitative and quantitative data obtained from calibration standards and from sample analysis.

Analytical procedure

Before analysis, samples were thawed at room temperature and, in the case of fish fillet and fish feed, were carefully ground using a Super JS mill (Moulinex, Ecully Cedex, France). Approximately 2 g of sample were homogenized with 6 g of anhydrous sodium sulfate and the blend was spiked with 100 µL of surrogate solution (25 ng/mL). The 10 mL of methanolic 1 M KOH solution were added to the mixture and saponified for 3 h at 80°C. The analytes were then extracted twice with 8 mL of n-hexane and the solution was filtered through a 0.2 µm filter and concentrated under a gentle stream of nitrogen at 40°C to a volume of 1 mL (in the case of oils, extracts were concentrated to 5 mL). The 1mL extract was passed through a Florisil SPE cartridge, previously conditioned with 6 mL of n-hexane, and eluted with 8 mL dichloromethane/hexane (DCM/Hx) (20:80). The eluate was evaporated under a gentle stream of nitrogen at 40°C and the final residue was redissolved in 0.25 mL of n-hexane. The final extracts obtained after cleanup were analyzed by GC/QqQ-MS under the experimental conditions shown in Table 2. Quantification of the samples was carried out by means of calibration curves with standard in solvent using the internal standard method. Positive real samples were reanalyzed by GC/TOFMS for additional confirmation of the compounds detected by QqQ-MS.

Table 2. Experimental conditions of the optimized GC-EI(SRM) method.

t_R (min)	Window (min)	Compounds	Precursor Ion (m/z)	Product Ion (m/z)	Q/q	Dwell time (sec)	Collision Energy (eV)	Q/q[c] Ratio
5.84	3-7	Naphthalene	128	102	Q	0.1	30	1.20 (5)
			128	77	q		30	
8.8	7-9.1	Acenaphthylene	152	126	Q	0.2	20	1.09 (6)
			152	150	q		30	
9.2	8.9-10.5	Acenaphthene	154	152	Q	0.2	35	3.58 (5)
			153	126	q		30	
10.44	9.7-11.5	Fluorene	165	115	Q	0.15	30	1.03 (10)
			166	164	q		35	
12.67	11.5-14	Phenanthrene[b]	178	152	Q	0.1	20	3.12 (4)
12.8		Anthracene[b]	178	176	q	0.1	35	2.62 (9)
15.51	14-15.9	Fluoranthene	202	200	Q	0.1	35	3.41 (5)
			202	150	q		45	
16.02	15.8-17	Pyrene	202	200	Q	0.1	30	28.17 (3)
			202	152	q		20	
18.9		B(a)Anthracene-D12[a]	240	236		0.1	30	
19.1	17-21	B(a)Anthracene[b]	228	226	Q	0.1	20	4.00 (11)
19.2		Chrysene[b]	228	224	q	0.1	55	3.48 (12)
22.33	21-23	B(b)Fluoranthene[b]	252	250	Q	0.2	35	4.38 (7)
22.42		B(k)Fluoranthene[b]	250	248	q	0.2	35	4.02 (10)
23.31	23-25	B(a)Pyrene	252	250	Q	0.2	35	4.24 (4)
			250	248	q		30	
26.75	25-27.6	Indeno(1,2,3-cd)Pyrene	276	274	Q	0.1	40	2.89 (10)
			276	272	q		60	
26.9		Dibenzo(a,h)Anthracene	278	276	Q	0.1	30	3.27 (9)
			278	274	q		30	
27.5	27.2-28.5	B(g,h,i)Perylene	276	274	Q	0.3	30	3.54 (7)
			274	272	q		30	

[a]Internal Standard used as surrogate. [b]The same transitions for both compounds. [c]Average value calculated from standard solutions at eight concentration levels each injected three times and RSD, in brackets. Q: Quantification transition, q: confirmation transition.

Validation

Statistical validation of the GC/(QqQ)MS/MS method was performed by evaluating the following parameters.

–Linearity. The calibration curves were obtained by injecting reference standard solutions in triplicate. The concentration range tested was 0.5-200 ng/mL (eight

points). Linearity was assumed when the regression coefficient was greater than 0.99 with residuals randomly distributed and less than 30%.

–Accuracy. The accuracy was evaluated by means of recovery experiments, analyzing blank samples of each matrix spiked at three concentration levels in fish fillet and fish feed (0.125, 1.25 and 2.5 μg/kg, in sextuplicate) and two levels (1.25 and 2.5 μg/kg) in fish oil and linseed oil (in triplicate). Previously, blank samples were analyzed to determine the concentration of the analytes present in the matrices (fish fillet and fish feed in sextuplicate, and oils in triplicate) (see Tables 3 and 4).

–Precision. The precision, expressed as the repeatability of the method, was determined in terms of relative standard deviation in percentage (RSD, %) from recovery experiments at each fortification level.

–Limit of quantification (LOQ). The LOQ was established as the lowest concentration that was validated following the overall analytical procedure with satisfactory recovery (between 70-110%) and precision (RSD <20%).

–Limit of detection (LOD). The LOD was statistically estimated, from the quantification transition, as the analyte concentration giving a peak signal of three times the background noise from the chromatograms at the lowest fortification level tested. When the analytes were present in the blank, LODs were calculated from the chromatogram of the analyzed blank sample. The LOD was calculated using the software option for estimating the signal-to-noise (S/N) ratio and referring this value to an S/N value of 3.[27]

–Confirmation criteria. The Q/q ratio, defined as the ratio between the intensity of the quantification (Q) and the confirmation (q) transitions, was used to confirm the identity of the compounds detected in samples. A safe confirmation was assumed by taking into account of the European Commission Decision (2002/657/CE).[28] Briefly, to confirm a finding as an actual positive, a maximum ratio tolerance ±20% was accepted when the relative intensity of the confirmative transition was >50% of the quantitative one (Q/q ratio 1-2). For higher Q/q ratios, the tolerances increased. Thus, deviations ±25% (relative intensity 20-50%, Q/q ratio 2-5), ±30% (relative intensity 10-20%, Q/q

Table 3. Validation parameters obtained for the analysis of PAHs in fish fillets and fish feeds (n=6, at each fortification level). Analysis performed by GC-(QqQ)MS/MS.

			Recoveries (%) (n=6)							
			Fortification levels (µg/kg)							
	"Blank" (µg/kg) (n=6)		0.125		1.25		2.5		LOD (µg/kg, fresh weight)	
Compound	Fish Fillet	Fish Feed	Fish Fillet	Fish Feed	Fish Fillet	Fish Feed	Fish Fillet	Fish Feed	Fish Fillet	Fish Feed
Naphthalene	2.4 (6)	2.9 (8)	-*	-*	105(36)	76(36)	107(37)	83(37)	0.06	0.09
Acenaphthylene	0.2(16)	0.2(2)	112(10)	80(20)	85(16)	85(17)	80(12)	61(17)	0.05	0.09
Acenaphthene	0.2 (17)	0.3 (15)	106(20)	102(27)	73(13)	108(5)	64(9)	87(13)	0.02	0.1
Fluorene	0.8 (6)	1.7 (6)	108(22)	82(16)	91(12)	104(13)	89(10)	108(12)	0.1	0.1
Phenanthrene	2.6 (20)	3.9 (3)	-*	-*	71(9)	92(4)	71(9)	102(8)	0.1	0.1
Anthracene	0.6 (14)	0.8 (1)	90(6)	100(22)	88(7)	101(7)	91(4)	96(12)	0.03	0.1
Fluoranthene	1.7 (1)	4(4)	-*	-*	76(14)	124(5)	86(7)	105(10)	0.1	0.1
Pyrene	4.4 (1)	7(1)	-*	-*	84(18)	105(2)	89(4)	99(6)	0.1	0.1
Benzo(*a*)anthracene	0.06 (19)	0.7 (4)	88(17)	83(15)	103(2)	99(2)	104(4)	108(4)	0.02	0.1
Chrysene	0.1 (19)	1.5 (3)	83(7)	98(25)	101(4)	101(2)	100(2)	108(9)	0.04	0.1
Benzo(*b*)fluoranthene	-	0.8 (5)	93(9)	93(19)	100(4)	100(5)	100(3)	108(8)	0.06	0.1
Benzo(*k*)fluoranthene	-	0.3 (17)	93(13)	103(6)	99(4)	102(2)	101(3)	102(3)	0.09	0.1
Benzo(*a*)pyrene	-	0.45 (6)	98(8)	95(9)	101(3)	100(6)	101(3)	103(5)	0.09	0.09
Indeno (*1,2,3-cd*)pyrene	-	0.3 (6)	97(13)	97(13)	96(6)	98(6)	97(7)	98(9)	0.09	0.07
Dibenzo(*a,h*)anthracene	-	0.2 (16)	98(12)	92(14)	94(11)	81(3)	93(8)	74(9)	0.09	0.06
Benzo(*g,h,i*)perylene	-	0.4 (8)	105(10)	109(9)	99(4)	101(4)	100(3)	104(10)	0.07	0.1

*- not validated due to the high analyte content in the "blank" sample.

Table 4. Validation parameters obtained for the analysis of PAHs in fish oil and linseed oil (n=3, at each fortification level). Analysis performed by GC-(QqQ)MS/MS.

	Recoveries (%) (n=3)							
			Fortification levels (µg/kg)					
	"Blank" (µg/kg) (n=3)		1.25		2.5		LOD (µg/kg, lipid weight)	
Compound	Fish Oil	Linseed Oil	Fish Oil	Linseed Oil	Fish Oil	Linseed Oil	Fish Oil	Linseed Oil
Naphthalene	-	-	78(28)	75(24)	80(21)	71(22)	0.4	0.1
Acenaphthylene	-	-	116(1)	74(21)	89(29)	82(24)	1	0.9
Acenaphthene	0.3 (8)	-	97(8)	88(15)	105(28)	90(13)	0.2	1.25
Fluorene	4.3 (5)	2.6(4)	112(3)	87(13)	100(25)	115(4)	0.8	1.25
Phenanthrene	38.2 (2)	15.2(1)	-*	-*	113(9)	93(3)	1.25	1.25
Anthracene	-	-	94(11)	71(4)	104(8)	93(7)	1.25	1.25
Fluoranthene	13.3(7)	16.7(4)	-*	-*	98(3)	105(1)	1.25	1.25
Pyrene	9(1)	11.3(1)	-*	-*	92(1)	111(2)	1.25	1.25
Benzo(a)anthracene	-	-	113(7)	104(15)	112(4)	100(1)	0.6	1.25
Chrysene	-	-	119(3)	108(9)	104(5)	111(4)	0.6	1.25
Benzo(b)fluoranthene	0.3(22)	0.5(4)	111(6)	112(6)	111(4)	120(4)	0.3	0.4
Benzo(k)fluoranthene	0.3(2)	0.4(5)	108(6)	115(8)	107(4)	103(9)	0.3	0.4
Benzo(a)pyrene	-	0.3(8)	80(5)	87(11)	110(4)	95(4)	0.7	0.3
Indeno (1,2,3-cd)pyrene	-	-	84(10)	107(14)	97(3)	103(7)	0.5	0.4
Dibenzo(a,h)anthracene	-	-	89(7)	99(19)	98(7)	117(2)	0.15	0.2

*- not validated due to the high analyte content in the "blank" sample.

ratio 5-10) and 50% (relative intensity 10%, Q/q ratio >10) were accepted. This criterion was originally defined on measures to monitor certain substances and residues thereof in live animals and animal products, and it is being increasingly used in other fields such as environmental and biological samples analysis.[29,30] Obviously, agreement in the retention time for sample and reference standard was also required to confirm a positive finding. The Q/q ratio for each compound was empirically determined as the average value calculated from eight standard solutions injected in triplicate.

RESULTS AND DISCUSSION

Cleanup optimization.

Cleanup is an important step in environmental and food analysis due to the complexity of the matrices and the selectivity required. Traditionally, analytical methods for PAHs in fatty samples include an alkaline saponification. This treatment provides a satisfactory lipid removal of the extracts.[5,6,31] In our work, as a consequence of the complexity of the matrices analyzed (especially fish and vegetable oils), modification of the commonly applied saponification procedures was necessary in order to obtain complete reaction of the lipids.[6,32] Different alkaline solutions were investigated, including NaOH/MeOH 1M, NaOH/EtOH 1M, NaOH/MeOH saturated, NaOH/EtOH saturated and KOH/MeOH 1M, KOH /EtOH 1M, KOH/MeOH saturated, and KOH/EtOH saturated. The effect of adding hexane or water was also tested but poor results were observed, so their addition was discarded. In all cases, heating for more than 2 h at a temperature higher than 60°C was required to remove lipid interferences and to hydrolyze the lipids and esters produced in the process. As can be seen in Fig. 1, fluorene, fluoranthene and pyrene were masked when the saponification time was less than 3 h. Under those conditions, esters and free fatty acids were not hydrolyzed, thus producing matrix effects. On the contrary, when the sample was submitted to saponification for 3 h or more at 80°C, the matrix effect decreased, and the selectivity and sensitivity improved. Thus, poor saponification led to unsatisfactory data. These results are in agreement with previous data reported for low recoveries of organic compounds in marine matrices, suggesting poor saponification as the

cause.[6,31] We obtained the most satisfactory results when using KOH/MeOH 1 M for 3 h at 80°C (Fig. 1). Under these conditions, the saponification process removed most of the fats after extraction with n-hexane and filtration (see analytical procedure).

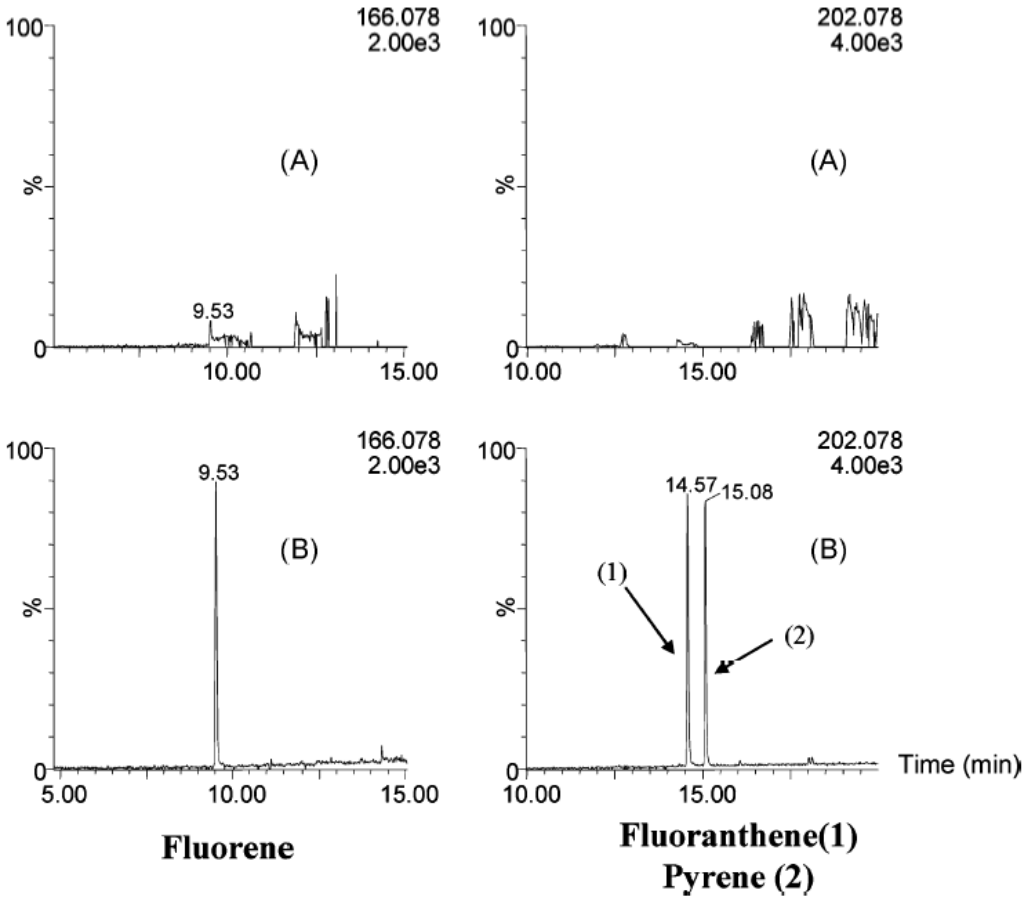

Figure 1. GC/TOFMS extracted ion chromatograms (mass window 0.02 m/z units) for fluorene (m/z 166.0782), and fluoranthene and pyrene (m/z 202.0782) in fortified fish fillet extracts (50 µg/kg) submitted to saponification at 80°C for (A) 1 h and (B) 3 h.

An additional step using SPE cleanup was still necessary to remove several interfering compounds. Silica and Florisil cartridges with 0.5 g and 1 g stationary phase, and different elution procedures, were compared with regard to their efficiency in lipid removal, the recovery of analytes, elution volume and elution time.

Florisil cartridges (1 g) were finally selected, and the elution was performed with DCM/Hx (20:80), which led to cleaner extracts and better recoveries than other mixtures assayed, such as n-hexane, DCM or ethyl acetate. Using DCM/Hx (20:80), interfering compounds seemed to be more retained on the cartridge, while PAHs eluted in cleaner eluates.

Other SPE purification procedures reported, using silica, Florisil[18] or C18 cartridges,[33] did not allow the determination of light PAHs such as naphthalene and acenaphthylene, this being a notable difference with the methodology developed in the present work.

GC/MS/MS optimization

High-resolution GC coupled to MS using either a single quadrupole or an ion trap for detection has been widely used in environmental applications.[16,19,34] However, there is little information about the use of tandem mass spectrometry with QqQ for the determination of PAHs in complex aquaculture samples. Optimization of the GC/MS/MS method was performed in this study by injecting hexane standard solutions into the GC/(QqQ)MS/MS system operating in EI mode. Full-scan EI mass spectra for all the PAH congeners showed basically the molecular ion, with poor fragmentation. Thus, the molecular ion was selected as the precursor ion for fragmentation purposes in the collision cell. Different values of collision energy (between 20 and 60 eV) were tested to perform the subsequent fragmentation of the selected precursor ion. Two MS/MS transitions were selected for each compound, normally the most sensitive ones, in order to have a reliable confirmation of the identity of the analyte. In most cases, the losses from the molecular ion of two or four hydrogen atoms were chosen as the quantitative and/or confirmative transitions for the determination of PAHs with improved selectivity and sensitivity. The dwell time parameter was also optimized between 0.1 and 0.3 s in order to obtain at least ten points across the peaks and thus maintain satisfactory sensitivity for each compound. Table 2 shows the precursor and product ions corresponding to the quantitative and confirmation transitions monitored. Linearity of the relative response of analytes was established by analyzing hexane standard solutions in the ranges 0.5-200 ng/mL. Regression coefficients above

0.995 were obtained for all the compounds with residuals lower than 30% and without any clear trend in their distribution.

GC/TOF MS optimization

In our study, GC/TOFMS was used for additional confirmation of the PAHs detected in samples by QqQ-MS. For these purpose a TargetLynx processing method was employed, using reference standard solutions in solvent. The MS spectrum for each compound was obtained and four ions were selected, for which elemental compositions were proposed (see Table 5). Narrow mass windows of 0.02 m/z units were chosen as a compromise between sensitivity, peak shape and accurate mass measurements. Q/q intensity ratios

Table 5. Experimental ions selected for the confirmation of PAHs by GC-TOF MS.

Compound	Ion $_1$	m/z $_1$	Ion $_2$	m/z $_2$	Ion $_3$	m/z $_3$	Ion $_4$	m/z $_4$
Naphthalene	$C_{10}H_8$	128.0626	$C_{10}H_7$	127.0548	$C_{10}H_6$	126.0452	C_8H_6	102.047
Acenaphthylene	$C_{12}H_8$	152.0626	$C_{12}H_7$	151.0548	$C_{12}H_6$	150.047	$C_{10}H_6$	126.047
Acenaphthene	$C_{12}H_9$	153.0707	$C_{12}H_{10}$	154.0782	$C_{12}H_8$	152.0626	$C_{10}H_6$	126.047
Fluorene	$C_{13}H_9$	165.0704	$C_{13}H_{10}$	166.0782	$C_{13}H_8$	164.0621	$C_{11}H_7$	139.0544
Phenanthrene	$C_{14}H_{10}$	178.0782	$C_{14}H_8$	176.0626	$C_{12}H_8$	152.0626	$C_{12}H_6$	150.047
Anthracene	$C_{14}H_{10}$	178.0774	$C_{14}H_8$	176.0626	$C_{12}H_8$	152.0626	$C_{12}H_6$	150.047
Fluoranthene	$C_{16}H_{10}$	202.0782	$C_{16}H_8$	200.0626	$C_{14}H_6$	174.047	$C_{12}H_6$	150.047
Pyrene	$C_{16}H_{10}$	202.0782	$C_{16}H_9$	201.0621	$C_{16}H_8$	200.0621	$C_{14}H_6$	174.047
Benzo(a)anthracene	$C_{18}H_{12}$	228.0939	$C_{18}H_{10}$	226.0783	$C_{16}H_8$	202.0626	C_9H_6	114.047
Chrysene	$C_{18}H_{12}$	228.0939	$C_{18}H_{10}$	226.0783	$C_{16}H_8$	202.0626	C_9H_6	114.047
Benzo(b)fluoranthene	$C_{20}H_{12}$	252.0939	$C_{20}H_{10}$	250.0783	$C_{10}H_6$	126.047	C_9H_5	113.0391
Benzo(k)fluoranthene	$C_{20}H_{12}$	252.0939	$C_{20}H_{10}$	250.0783	$C_{10}H_6$	126.047	C_9H_5	113.0391
Benzo(a)pyrene	$C_{20}H_{12}$	252.0939	$C_{20}H_{10}$	250.0783	$C_{10}H_6$	126.047	C_9H_5	113.0391
Indeno (1,2,3-cd)pyrene	$C_{22}H_{12}$	276.0939	$C_{22}H_{10}$	274.0783	$C_{11}H_6$	138.047	$C_{10}H_5$	125.0391
Dibenzo(a,h)anthracene	$C_{22}H_{12}$	276.0939	$C_{22}H_{10}$	274.0783	$C_{11}H_7$	139.0548	$C_{10}H_5$	125.0391
Benzo(g,h,i)perylene	$C_{22}H_{12}$	276.0939	$C_{22}H_{10}$	274.0783	$C_{11}H_6$	138.047	$C_{10}H_5$	125.0391

were used as confirmation parameter. Theoretical Q/q ratios were calculated from solvent standard solutions as the ratio between the most sensitive ion (Q, quantitative) and each of the other measured ions (q, confirmative). The selection of four ions provided up to three Q/q intensity ratios that could be used for the reliable confirmation of compounds in

samples. The tolerances accepted were in accordance with the European Commission Decision (2002/657/CE).[28] Agreement in the retention times of the analyte in sample and of the reference standard was also required to confirm a positive result (relative error ±0.5%).

Analytical parameters

Blank samples used for validation purposes contained appreciable concentrations of several PAHs. Therefore, it was necessary to accurately calculate this concentration in order to correct the quantitative results in recovery experiments.[27] One labelled standard (benzo[a]anthracene-D12) was added at the initial stage of the procedure as a quality control (surrogate) in order to correct for possible losses during the overall procedure and instrumental deviations for all the compounds except for naphthalene and acenaphthylene which were processed without a surrogate.

Fortified samples of the four matrices studied in this work were analyzed applying the developed methodology with satisfactory results (see Tables 3 and 4).

Fish fillet and fish feed

Pools of fish fillet and fish feed, blending 325 g of left-hand fillets and 50 g of fish feed, respectively, were used for the statistical validation experiments. First, six replicates of each matrix were analyzed by applying the analytical procedure proposed to determine the content of selected analytes in these samples. These showed the presence of trace levels of most of them, as could be expected because of the usual presence of PAHs in marine samples,[2,3,5] especially in fish feed due to the concentration of contaminants during the manufacturing process with fish derivatives.[35] Fluorene, phenanthrene, fluoranthene and pyrene presented the highest concentrations in these blank samples. As can be seen in Table 3, samples were fortified at 0.125, 1.25 and 2.5 μg/kg (n = 6) and submitted to the developed procedure. In general, recoveries were satisfactory, with average values between 80 and 110% and RSDs of less than 30%, except for naphthalene which presented poor precision. LODs were found to be at sub-μg/kg level in both sample matrices (all 0.1

µg/kg). Such low LODs were achieved thanks to the efficient cleanup applied, together with the selectivity and sensitivity provided by QqQ-MS/MS in SRM mode.

The average Q/q intensity ratios calculated from reference standards in solvent (see Table 2) were compared with those experimentally obtained from spiked sample extracts to test the robustness of these values and for the presence of potential matrix interferences that might affect Q/q ratios and, consequently, the confirmation process. The average deviations obtained were in all cases in accordance with the criteria indicated in the validation section.

As an example, Fig. 2 shows representative chromatograms of fish fillet (naphthalene, dibenzo[a,h]anthracene) and fish feed (acenaphthene and benzo[g,h,i]perylene) fortified at 0.125 µg/kg (except for naphthalene, 1.25 µg/kg).

Q/q ratios for all analytes were between 1 and 5, except for pyrene, which showed by far the highest Q/q ratio with a value of 28.17 (Table 2). This allows the determination of pyrene at the LOQ level, but makes its confirmation difficult at low concentrations below the LOQ. No other transitions with the selected precursor ion (m/z 202) or with other precursor ions were found to lead to better sensitivity.

Fish and Linseed oil

Frequently, oils added in fish feed, such as fish oil and linseed oil, are a source of contamination.[36] These ingredients are the main part of the fish feed compositions, necessary to reach a healthy composition of the diets (Table 1). For these reasons, data obtained in the analysis of the blank sample used for validation reveal appreciable concentrations of PAHs. As reported by several authors, fats and oils represent one of the major sources of contamination in the diet because of their lipophilic nature.[37] Table 4 shows the analytical parameters for the oil matrices studied, with validation data expressed as µg/kg lipid weight. Fluorene, phenanthrene, fluoranthene and pyrene were, similarly to fish fillet and fish feed, the most abundant PAHs found in the oils. Blanks were fortified at 1.25 and 2.5 µg/kg and submitted to the developed procedure with satisfactory recoveries in all cases, and with a RSD lower than 30%. The limits of detection were at the low µg/kg

levels in fish oil and linseed oil. The LODs, LOQs and Q/q ratios were calculated in the same way as for the fish fillet and fish feed, obtaining in all cases satisfactory results in accordance with the criteria established.

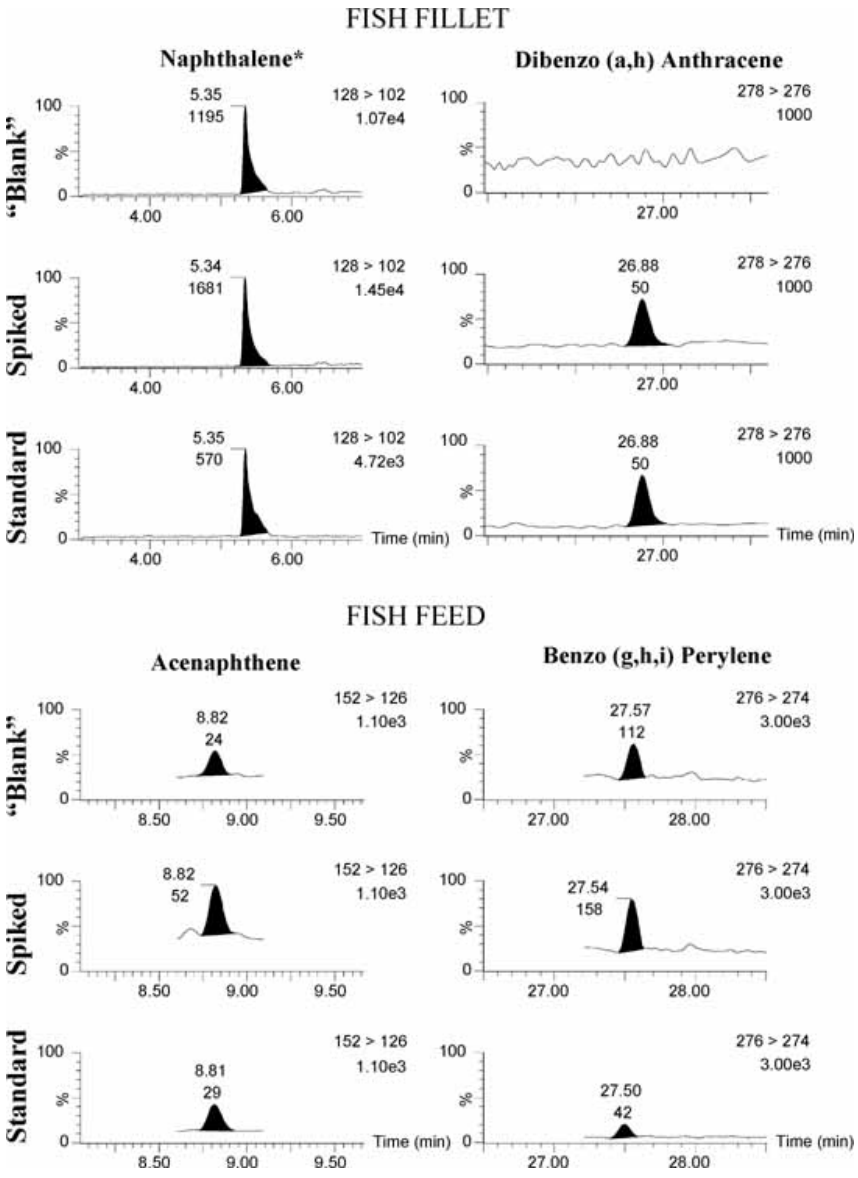

Figure 2. Blank sample, spiked sample (0.125 µg/kg) and standard (1 ng/mL, equivalent to 0.125 µg/kg in sample) GC/MS/MS chromatograms from fish fillet and fish feed. SRM transitions monitored: naphthalene (m/z 128 > 102), dibenzo[a,h]anthracene (m/z 278 > 276), acenaphthene (m/z 152 > 126) and benzo[g,h,i]perylene (m/z 276 > 274). *Spiking level for naphthalene in fish fillet was 1.25 µg/kg, and the reference standard was 10 ng/mL, equivalent to 1.25 µg/kg in sample.

Application to real samples

The optimized sample procedure followed by GC/(QqQ)MS/MS and GC/TOFMS was applied (in triplicate) to the analysis of raw materials, fish feeds and fillet samples from AQUAMAX long-term feeding trials with gilthead sea bream (Sparus aurata L.) as part of a European project. Fish were exposed through the entire productive cycle to experimental diets with graded levels of fish oil replacement, studying the health and welfare of the farmed fish, and maximizing the health-promoting properties, safety, quality and acceptability of the final product to the consumer.[35] PAHs were determined in 19 fish fillet samples, 8 fish feeds, 1 fish oil, 1 linseed oil, 1 rapeseed oil and 1 palm oil from fish exposed through the productive cycle (14 months) to experimental diets with different percentages of fish oil replacement with vegetable oils.

For the GC/QqQ-MS analysis, Table 6 shows the PAH concentrations detected in aquaculture samples. All PAHs studied were found in fish feed in the concentration range of 0.2-12.7 μg/kg. The only exception was naphthalene in six fish feed samples, where it was found at concentrations around 200 μg/kg fresh weight. All fish fillet samples analyzed were positive for phenanthrene, fluoranthene and pyrene (range of 0.2-11.4 μg/kg). Benzo[a]pyrene was detected only in one sample of fish fillet, but at a concentration (3.9μg/kg) above the maximum established by the European Commission Regulation (EC) No 1881/2006. Rapeseed and palm oils presented lower total loads of PAHs (12 μg/kg) than fish and linseed oils, which had total loads of 65.7 and 47 μg/kg, respectively, principally due to the major presence of fluorene, phenanthrene, fluoranthene and pyrene (see values in Table 4).

Illustrative chromatograms for real samples analyzed are shown in Fig. 3, where the quantification and confirmation transitions monitored for several PAHs can be seen in the different matrices analyzed. As shown in the chromatograms, both quantification and confirmation were feasible at sub-μg/kg levels with satisfactory peak shape. Benzo[a]pyrene was not detected in fish fillet, whereas it was quantified and confirmed in the diets.

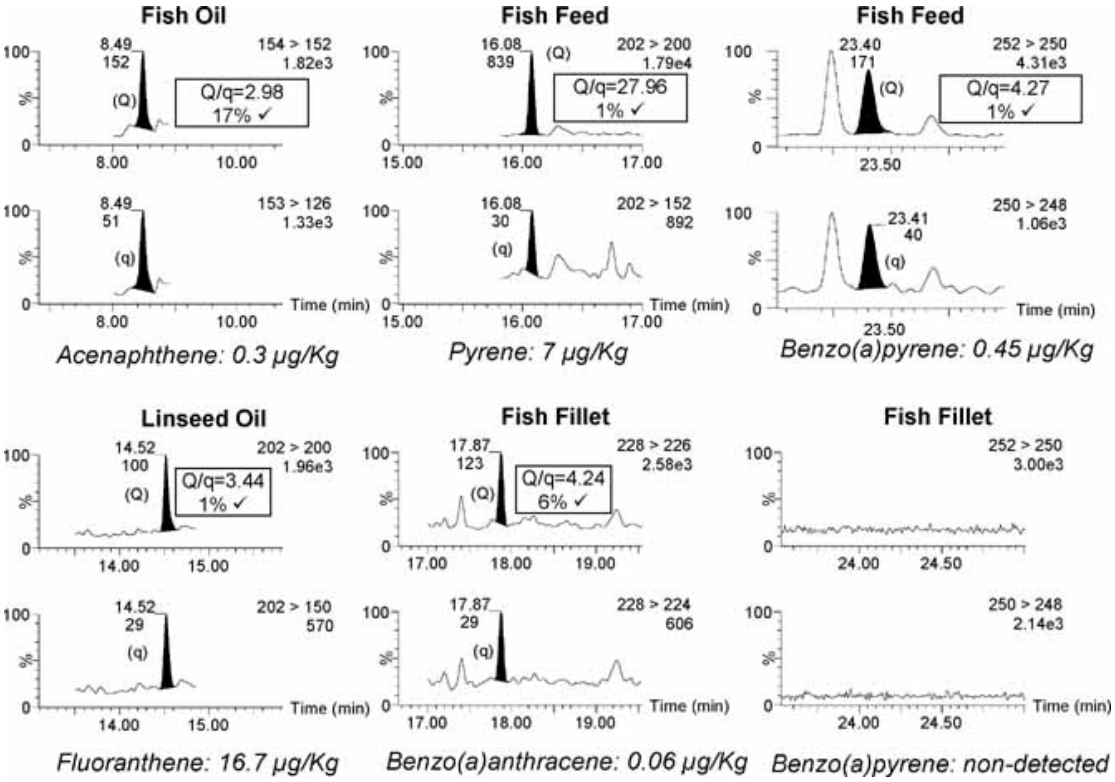

Figure 3. GC/MS/MS chromatograms for selected PAHs in real aquaculture samples. Q quantification transition, q confirmation transition. : Q/q ratio within tolerance limits. SRM transitions monitored: acenaphthene (m/z 154 > 152, 153 > 126), pyrene (m/z 202 > 200, 202 > 152), benzo[a]pyrene (m/z 252 > 250, 250 > 248), fluoranthene (m/z 202 > 200, 202 > 150), benzo[a]anthracene, (m/z 228 > 226, 228 > 224).

GC/TOFMS was used for the additional confirmation of PAHs previously detected by QqQ-MS. The GC/TOF detection and identification of target PAHs in the samples were carried out by obtaining up to four narrow-window eXtracted Ion Chromatograms (nw-XICs) (0.02 m/z units) at selected m/z values for every compound (Table 5). The TargetLynx software automatically processed data and reported qualitative data. Analyte confirmation was carried out by comparison of the Q/q intensity ratios obtained in samples with those obtained from reference standards in solvent. In all cases, the presence of chromatographic peaks at the expected retention time and the attainment of all Q/q ratios when comparing with the reference standard allowed the confirmation of these findings in samples. In

addition, EI-TOFMS accurate mass spectra were obtained and the mass errors for representative ions were calculated, giving high confidence in the confirmation process.

As an example, Fig. 4 shows GC/TOF chromatograms (nw-XICs) for pyrene and naphthalene detected in fish fillet and fish feed. Reliable confirmation was feasible as all the Q/q ratios were in agreement with the European Commission Decision (2002/657/CE).[28] In addition, EI accurate mass spectra provided mass errors for the four ions monitored of below 2.9 m m/z units.

Most of the positives found by triple quadrupole MS could be confirmed by TOFMS. When the findings could not be confirmed by TOFMS, the reason was the absence of the chromatographic peak in the nw-XIC. All these cases occurred when the analyte concentration was very low (generally 1 μg/kg), surely as a consequence of the lower sensitivity of the TOF mass spectrometer than of the triple quadrupole instrument working in SRM mode.

However, in the full-analysis field, the capability of the TOF mass spectrometer to allow interrogation of full-spectrum data after acquisition is interesting, as it allows us to screen for unexpected compounds and metabolites at the time of injection without re-analyzing the sample.[38,39] The main advantage is the huge number of compounds that might be investigated, with the obvious restrictions deriving from the requirements of GC and MS analysis. High-occurrence metabolites, or new compounds that have been properly identified by TOFMS, might then be included in analytical methods using QqQ-MS if the commercial reference standards are available. This attractive approach is under study at present in our laboratories for the samples analyzed in this work.

Table 6. PAH concentrations in aquaculture samples analyzed by the application of the developed method.

Compound	Fish Fillet[(a)]		Fish Feed[(b)]		Rapeseed Oil[(c)]		Palm Oil[(d)]	
	Positives	conc. range (μg/kg)	Positives	conc. range (μg/kg)	Positives	conc. range (μg/kg)	Positives	conc. range (μg/kg)
Naphthalene	2	2.4-3.9	7	2.9-242	1	1.9	1	0.8
Acenaphthylene	1	0.2	7	0.2-1.6	1	0.2	1	0.2
Acenaphthene	1	0.2	7	0.2-1.7	1	0.6	1	0.5
Fluorene	18	0.2-0.8	7	1.7-6.4	1	0.9	1	0.4
Phenanthrene	19	0.5-3.7	8	0.4-12.7	1	1.2	1	1.3
Anthracene	2	0.6-1	8	0.4-2.1	1	1.6	-	-
Fluoranthene	19	0.2-3.9	7	2-4	-	-	-	-
Pyrene	19	0.2-11.4	8	0.8-7	-	-	-	-
Benzo(a)anthracene	6	0.06-4.9	8	0.4-0.9	1	0.9	1	1.2
Chrysene	12	0.1-6.9	8	0.6-1.5	1	0.8	-	-
Benzo(b)fluoranthene	1	5.9	8	0.6-2.7	1	0.4	1	1.3
Benzo(k)fluoranthene	1	5.9	8	0.3-2.3	1	0.4	1	1.3
Benzo(a)pyrene	1	3.9	2	0.45-0.5	1	0.7	1	1.4
Indeno (1,2,3-cd)pyrene	-	-	1	0.3	1	0.7	1	1.1
Dibenzo(a,h)anthracene	-	-	1	0.2	1	0.9	1	1.3
Benzo(g,h,i)perylene	1	3.4	1	0.4	1	0.8	1	1.2

Concentrations detected from fish oil and linseed oil are presented in Table 4. Total number of samples analyzed: (a) 19, (b) 8, (c) 1 and (d) 1.

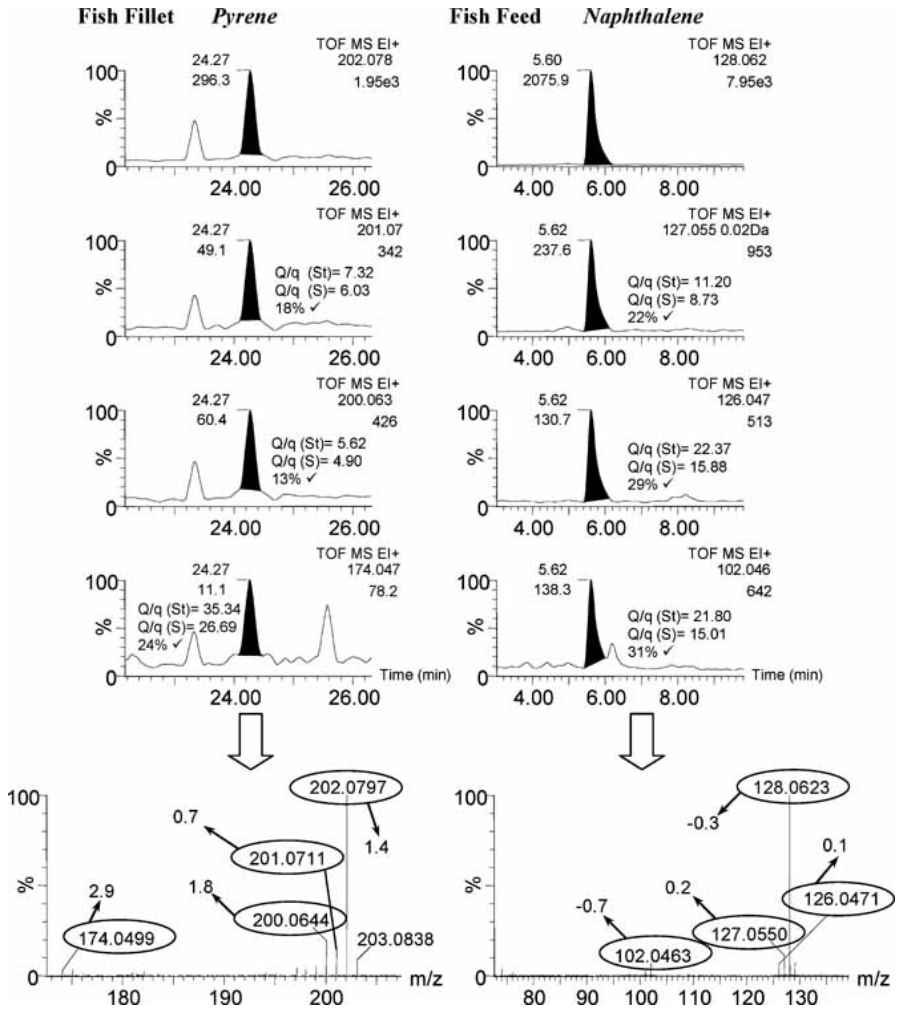

Figure 4. GC/TOFMS extracted ion chromatograms at four m/z values (mass window 0.02 m/z units) for pyrene detected in fish fillet and naphthalene detected in fish feed. Accurate mass spectra showing the most relevant ions together with the experimental mass errors, in m m/z units (bottom). : Q/q ratio within tolerance limits. St: reference standard; S: sample. Ions selected are shown in Table 5.

CONCLUSIONS

A rapid, sensitive and selective analytical methodology for the determination of PAHs in high lipid content aquaculture samples has been developed, reaching low quantification levels by means of an efficient cleanup step combining saponification and SPE procedures

prior to the injection of the extracts into a gas chromatograph coupled to QqQ and TOF mass spectrometers. Especial attention was paid to naphthalene, the earliest eluting chromatographic peak, which was a major problem in part due to the high levels found in the blank fish fillet and fish feed samples used for validation experiments. The combined use of triple quadrupole and time-of-flight analyzers gives an extraordinary reliability to the confirmation process of the compounds detected in samples.

ACKNOWLEDGEMENTS

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REFERENCES

1.Menzie CA, Potocki BB, Santodonato J. Environ. Sci. Technol. 1992; 26: 1278.

2.Baumard P, Budzinski H, Garrigues P, Sorbe JC, Burgeot T, Bellocq J. Mar. Pollut. Bulletin 1998; 36: 951.

3.Filipkowska A, Lubecki L, Kowalewska G. Anal. Chim. Acta 2005; 547: 243.

4.Hellou J, Leonard J, Collier TK, Ariese F. Mar. Pollut. Bull. 2006; 52: 433.

5.Moret S, Conte LS. J. Chromatogr. A 2000; 882: 245.

6.Perugini M, Visciano P, Giammarino A, Manera M, Di Nardo W, Amorena M. Chemosphere 2007; 66: 1904.

7.USEPA. Toxicological profile for polycyclic aromatic hydrocarbons (PAHs). Available: http://www.usepa.gov/S.

8.Martí-Cid R, Bocio A, Llobet JM, Domingo JL. Food Chem. Toxicol. 2007; 45: 1968.

9.Martí-Cid R, Llobet JM, Castell V, Domingo JL. Food Chem Toxicol. 2008; 46: 3163.

10.Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Available: eurlex.europa.eu/LexUriServ/site/en/oj/2006/l_364/l_36420061220en00050024.pdf.

11.Baumard P, Budzinski H, Garrigues P, Narbonne JF, Burgeot T, Michel X, Bellocq J. Mar. Environ. Res. 1999; 47: 415.

12.Hellou J, Haya K, Steller S, Burridge L. Aquat. Conserv. 2005; 15: 349.

13.Malik A, Ojha P, Singh KP. B. Environ. Contam. Tox. 2008; 80: 134.

14.Hellou J, Haya K, Steller S, Burridge L. Aquat. Conserv. 2005; 15: 349.

15.Patel K, Fussell RJ, Hetmanski M, Goodall DM, Keely BJ. J. Chromatogr. A 2005; 1068: 289.

16.Poster DL, Schantz MM, Sander LC, Wise SA. Anal. Bioanal Chem. 2006; 386: 859.

17.Pena T, Pensado L, Casais C, Mejuto C, Phan-Tan-Luu R, Cela R. J. Chromatogr. A 2006; 1121:163.

18.Navarro P, Cortazar E, Bartolomé L, Deusto M, Raposo JC, Zuloaga O, Arana G, Etxebarria N. J. Chromatogr. A 2006; 1128: 10.

19.Liguori L, Heggstad K, Hove HT, Julshamn K. Anal. Chim. Acta 2006; 573-574: 181.

20.Fernández-González V, Concha-Graña E, Muniategui-Lorenzo S, López-Mahía P, Prada-Rodríguez D. J. Chromatogr. A 2007; 1176: 48.

21.Fernández-González V, Muniategui-Lorenzo S, López-Mahía P, Prada-Rodríguez D. J. Chromatogr. A 2008; 1207: 136.

22.Campíns-Falcó P, Verdú-Andrés J, Sevillano-Cabeza A, Molins-Legua C, Herráez-Hernández R. J. Chromatogr. A 2008; 1211: 13.

23.Loutfy N, Fuerhacker M, Tundo P, Raccanelli S, Ahmed MT. Chemosphere 2007; 66: 1962.

24.Pitarch E, Medina C, Portolés T, López FJ, Hernández F. Anal. Chim. Acta 2007; 583: 246.

25.Portolés T, Pitarch E, López FJ, Sancho JV, Hernández F. J. Mass Spectrom. 2007; 42: 1175.

26.Căljka T, Hajlová J. J. Chromatogr. A 2004; 1058: 251.

27.Hernández F, Portolés T, Pitarch E, López FJ, Beltrán J, Vázquez C. Anal. Chem. 2005; 77: 7662.

28.Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Available: eur- lex.europa.eu/pri/en/oj/dat/2002/l_221/l_221200 20817en00080036.pdf.

29.Hernández F, Sancho JV, Pozo OJ. Anal. Bioanal. Chem. 2005; 382: 934.

30.Ferrer I , Thurman EM (eds). Liquid Chromatography Mass Spectrometry/Mass Spectrometry, MS/MS and Time-of-Flight MS: Analysis of Emerging Contaminants, American Chemical Society Symposium Series 850, Oxford University Press: New York, 2003.

31.Numata M, Yarita T, Aoyagi Y, Yamazaki M, Takatsu A. Chemosphere 2005; 58: 865.

32.Baumard P, Budzinski H, Garrigues P. Polycycl. Aromat. Comp. 1996; 9: 241.

33.Barranco A, Alonso-Salces RM, Bakkali A, Berrueta LA, Gallo B, Vicente F, Sarobe M. J. Chromatogr. A 2003; 988: 33.

34.Santos FJ, Galceran MT. TrAC - Trends Anal. Chem. 2002; 21: 672.

35.Nácher-Mestre J, Serrano R, Benedito-Palos L, Navarro JC, Lopez FJ, Pérez-Sanchez J. Chemosphere 2009; accepted.

36.Dennis MJ, Massey RC, Cripps G, Venn I, Howarth N, Lee G. Food Addit. Contam. 1991; 8: 517.

37.Van der Lee MK, van der Weg G, Traag WA, Mol HGJ. J. Chromatogr. A 2008; 1186: 325.

38.Hernández F, Portolés T, Pitarch E, López FJ. Anal. Chem. 2007; 79: 9494.

39.Hernández F, Portolés T, Pitarch E, López FJ. J. Mass Spectrom. 2009; 44: 1.

4.2.3. Discusión de los resultados.

La USEPA propuso una lista de 16 PAHs como contaminantes prioritarios debido a su potente toxicidad sobre la población y su presencia en lugares de riesgo. En nuestro caso, la elección de los PAHs a analizar se llevó a cabo considerando la lista propuesta por la EPA por incluir aquellos PAHs utilizados como marcadores de bioacumulación en muestras de pescado (EPA 1993)(EPA 2000). Asimismo, la lista de la EPA considera PAHs con 2, 3, 4, 5 y 6 anillos de benceno con características carcinogénicas y mutagénicas. Del mismo modo, se incluye al naftaleno, que es considerado como un contaminante altamente ubicuo y encontrado en concentraciones elevadas en sedimentos y tejidos de peces (Liang et al., 2007). La presencia de fenantreno, antraceno, fluoranteno y pireno en esta lista también fue un factor determinante ya que suelen presentar valores del orden de µg/Kg en muestras de origen marino (Cheung et al., 2007; Berntssen et al., 2010).

La autoridad europea "European Food Safety Authority" (EFSA) publicó en 2002 una lista con 15 PAHs prioritarios para su control y determinación en muestras alimentarias. Sólo algunos de estos PAHs estaban incluidos previamente en la lista propuesta por la EPA y se incluyen otros por su toxicidad (EFSA 2002). En 2005, las organizaciones "Food and Agriculture Organization" (FAO) y la "World Health Organization" (WHO) añadieron a la lista de la EFSA un nuevo PAH considerado como tóxico: "benzo[c]fluorine" (FAO/WHO, 2005) con el que la lista quedaba ampliada a 16 PAHs pero fue nombrada como "15+1" (15+1 PAH list) para diferenciarla de la lista de la EPA (16 PAH list). Aún así, en todos los casos sigue tomándose como referencia la presencia de benzo(a)pireno, incluido en ambas listas como marcador de la toxicidad por PAHs.

Los 16 PAHs incluidos en la lista de la EPA fueron seleccionados en nuestra investigación como indicadores de este grupo de contaminantes en organismos marinos. Estudios recientes, como el realizado por Marti-Cid (2007), sobre la ingesta de PAHs debido al consumo de pescado y mariscos contaminados por niños (Martí-Cid et al., 2007), se han basado también en el análisis de los 16 PAHs propuestos por la EPA.

- Optimización del método de extracción/purificación mediante saponificación

Para la extracción de los PAHs en muestras complejas, siguen utilizándose hoy en día técnicas de extracción tradicionales como el reflujo y Soxhlet debido a su buen funcionamiento. Actualmente también se utilizan técnicas automatizables. Como se ha comentado en la introducción de esta Tesis, técnicas de extracción como la PLE, ASE, MAE y la SPME (Schantz et al., 2006; Fidalgo-Used et al., 2007) muestran también buenos resultados (van Leeuwen et al., 2008; Björklund et al., 2006). Yusà et al. (2005) propuso una lista general de procedimientos analíticos para la determinación de PAHs en muestras de pescado y marisco. En esta lista se incluyen diferentes procesos de extracción/purificación mediante saponificación (Yusà et al., 2005). Tras la extracción, es frecuente la necesidad de una etapa adicional de purificación del extracto, con el fin de obtener los mejores parámetros analíticos en la determinación instrumental. En nuestro caso, el alto contenido en grasa hizo necesario la optimización de un exhaustivo tratamiento de la muestra previo al análisis por GC-MS/MS y GC-TOF MS.

En primer lugar se aplicó una etapa de saponificación para eliminar la gran cantidad de grasa presente en las muestras. La saponificación resultó ser eficaz por eliminar un elevado porcentaje de grasa y no degradar los PAHs. Como los PAHs son ácido-lábiles no se pudo realizar el tratamiento con ácido por observarse degradación de los mismos. El tratamiento con ácido es muy eficaz para eliminar grasa y es utilizado frecuentemente en metodologías analíticas para la determinación de PBDEs y PCBs (Covaci et al., 2003; Serrano et al., 2003). Las condiciones de saponificación se optimizaron con el objeto de eliminar la mayor cantidad posible de ácidos grasos derivados de los lípidos de las matrices. Para ello, se ensayaron diferentes temperaturas, disolventes y tiempos de reacción. Se realizaron diferentes experimentos que incluían la mezcla, agitación y posterior extracción líquido-líquido: saponificación inmediata, saponificación durante una hora, dos y tres horas en condiciones de temperatura ambiente, 60°C y 80°C. Para todas ellas se utilizaron los alcalinizantes KOH y NaOH en diferentes disolventes: agua, EtOH, MeOH, preparando disoluciones a diferentes concentraciones y volúmenes: 1M, 2M y saturada. Además se añadió NaCl, agua y hexano individualmente para mejorar la separación de las fases.

Los mejores resultados se obtuvieron realizando la saponificación durante tres horas a una temperatura de 80°C como se puede observar en la **Figura 1** (**Artículo científico 1**).

- Optimización de la purificación mediante SPE

En nuestro caso la saponificación no fue suficiente para obtener extractos lo suficientemente limpios como para conseguir resultados óptimos en la determinación mediante GC-MS/MS, por lo que se aplicó una etapa adicional de purificación basada en SPE.

Otros autores también han propuesto metodologías para la determinación de PAHs acoplando diferentes técnicas de purificación dado que, en muestras complejas, una única etapa de purificación suele resultar insuficiente para determinar cuantitativamente los analitos a niveles de concentración suficientemente bajos (Fromberg et al., 2007).

A pesar de los avances en la separación y la detección de los sistemas cromatográficos, la etapa de tratamiento de muestra resulta trascendental para obtener unos datos fiables. Hoy en día todavía existe una necesidad de búsqueda de metodologías de tratamiento de muestras complejas, que sean eficaces, económicas y rápidas para la determinación de compuestos orgánicos arraigados a matrices con alto contenido graso (Gilbert-López et al., 2009).

Entre las técnicas ampliamente utilizadas hoy en día, la SPE es utilizada frecuentemente por presentar grandes posibilidades en la purificación y concentración de las muestras con el fin de obtener una gran sensibilidad en la medida analítica. Varios autores han utilizado la SPE en metodologías analíticas para la determinación de contaminantes orgánicos, incluyendo PAHs, en muestras medioambientales a niveles de ng/L (Matamoros et al., 2010). Otra técnica ampliamente utilizada es la GPC ya que es una potente herramienta de separación y concentración de los analitos en muestras complejas. Este tipo de técnica se utiliza frecuentemente para separar compuestos de tamaño molecular elevado de la matriz, como por ejemplo los lípidos, ofreciendo resultados satisfactorios (Fromberg et al., 2007; Jira et al., 2008).

En nuestro caso, la aplicación de SPE a la purificación de los extractos de las diferentes matrices consideradas mostró una elevada eficacia en la eliminación de los lípidos y permitió la concentración de los PAHs, alcanzando LOQs a niveles de μg/Kg.

Para ello, fue indispensable optimizar el proceso de SPE, ensayando diferentes fases estacionarias, disolventes y procedimientos de elución. Hay que considerar que, dependiendo del disolvente empleado, el patrón de elución de los PAHs variaba considerablemente. Así se probaron diferentes disolventes, entre ellos el hexano, diclorometano, acetato de etilo y mezclas entre ambos. Al final se decidió utilizar una mezcla de diclorometano/hexano (20:80) como mejor disolvente para la elución de los PAHs debido a la elución correcta de los PAHs manteniendo la fracción lipídica retenida en la fase estacionaria de Florisil.

- Optimización de GC-MS/MS

Con el objetivo de conseguir LOQs lo más bajos posible, se empleó la GC acoplada a un analizador de masas QqQ. Este analizador permite trabajar en MS/MS obteniendo un alto grado de fiabilidad en la identificación de los analitos. En modo de adquisición "SRM", los analizadores QqQ resultan excelentes para la cuantificación y confirmación de la identidad de los compuestos a niveles traza, especialmente en matrices complejas. Para la determinación de PAHs se seleccionaron dos transiciones por compuesto atendiendo a su mayor sensibilidad. A raíz de la pobre fragmentación de los PAHs, el pico molecular fue escogido como precursor para la transición de cuantificación en la mayoría de los casos (**Tabla 2, Figura 3. Artículo Científico 1**).

- Optimización de GC-TOF MS

GC-TOF MS se utilizó para realizar una confirmación de la identidad adicional de los PAHs detectados con el GC-MS/MS. Se seleccionaron cuatro iones para la identificación de cada uno de los compuestos analizados. La relación *m/z* más intensa fue seleccionada para la cuantificación (Q) mientras que las tres restantes fueron adquiridas para la confirmación

(q) y estudio de las relaciones Q/q (**Tabla 5, Figura 4. Artículo de Investigación 1**). El estudio de los valores Q/q se realizó atendiendo al reglamento emitido por la Unión Europea sobre metodología analítica e interpretación de resultados (Commission Decision 2002/657/EC).

- Validación de la metodología analítica

La validación de la metodología analítica se desarrolló para cuatro matrices (filete de dorada cultivada, pienso, aceite de pescado y aceite de lino). Se llevó a cabo mediante experimentos de recuperación en muestras blanco seleccionadas fortificadas a tres niveles de concentración en filetes de pescado y pienso (0.125, 1.25 and 2.5µg/Kg, n=6) y dos niveles en aceite de pescado y aceite de lino (1.25 and 2.5µg/Kg, n=3). En general se obtuvieron recuperaciones satisfactorias (80-110%), así como valores óptimos de precisión (RSD<30%). Naftaleno, fenantreno, fluoranteno y pireno presentaron concentraciones apreciables en el blanco por lo que fueron validados a 1.25 µg/kg.

No se encontraron materiales de referencia certificados de las matrices consideradas a los niveles de concentración más bajos estudiados en el presente trabajo (0.125µg/kg en filete y pienso; 1.25 µg/kg en aceites) por lo que la validación estadística mediante matrices "blanco" fortificadas a diferentes concentraciones fue seleccionada como la mejor opción considerando todos los parámetros propuestos por la guía SANCO vigente (Document N° SANCO/2007/3131).

4.2.4. Referencias.

Aquamax Issue N° 01. 2008. Aquamax Project: Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers. www.aquamaxip.eu.

Bartle, K.D. 1991. En "Food contaminants: Sources and Surveillance" (Creaser C, Purchase R. eds). The Royal Society of chemistry, Cambridge. 41-60.

Berntssen, M.H.G.; Julshamn, K.; Lundebye, A. 2010. Chemical contaminants in aquafeeds and Atlantic salmon (*Salmo salar*) following the use of traditional- versus alternative feed ingredients. Chemosphere 78, 637-646.

Cheung, K.C.; Leung, H.M.; Kong, K.Y.; Wong, M.H. 2007. Residual levels of DDTs and PAHs in freshwater and marine fish from Hong Kong markets and their health risk assessment. Chemosphere 66, 460-468.

Chou, P.; Matsui, S. 2006. Contamination of polycyclic aromatic hydrocarbons and their implication for environmental hazard. Water Sci. Technol. 6, 165-174.

Commission Regulation 1881/2006/EC of 19December 2006 setting maximum levels for certain contaminants in foodstuffs. Available: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:EN:PDF

Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Available: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF

Covaci, A.; Voorspoels, S.; de Boer, J. 2003. Determination of brominated flame retardants, with emphasis on polybrominated diphenyl ethers (PBDEs) in environmental and human samples. Environ. Int. 29, 735-756.

Document N° SANCO/2007/3131. 2007. Method validation and quality control procedures for pesticide residues analysis in food and feed.

EFSA. 2002. Opinion of the Scientific Committee on Food on the Risks to Human Health of Polycyclic Aromatic Hydrocarbons in Food, 2002, SCF/CS/CNTM/PAF/29/Final.

EPA. 1993. Provisional guidance for quantitative risk assessment of polycyclic aromatic hydrocarbons. EPA 600/R-93/089. US Environmental Protection Agency, Washington DC.

EPA. 2000. Guidance for assessing chemical contaminant data for use in fish advisories. EPA. 823-B-00-008. US Environmental Protection Agency, Washington DC.

FAO/WHO. 2005. Summary and Conclusions of the Joint FAO/WHO Expert Committee on Food Additives, Sixty-Fourth meeting, Rome, 2005, JECFA/64/SC.

Fromberg, A.; Højgård, A.; Duedahl-Olesen, L. 2007. Analysis of polycyclic aromatic hydrocarbons in vegetable oils combining gel permeation chromatography with solid-phase extraction clean-up. Food Addit. Contam. 24, 758-767.

Gilbert-López, B.; García-Reyes, J.F.; Molina-Díaz, A. 2009. Sample treatment and determination of pesticide residues in fatty vegetable matrices: A review. Talanta. 79, 109-128.

Jira, W.; Ziegenhals, K.; Speer, K. 2008. Gas chromatography-mass spectrometry (GC-MS) method for the determination of 16 European priority polycyclic aromatic hydrocarbons in smoked meat products and edible oils. Food Addit. Contam. A 25, 704-713.

Koss, G. & I. Tesseraux. (1999) En "Toxicology" (Marquardt H. et al., eds). Academic Press, San Diego. 603-644

Liang, Y.; Tse, M.F.; Young, L.; Wong, M.H. 2007. Distribution patterns of polycyclic aromatic hydrocarbons (PAHs) in the sediments and fish at Mai Po Marshes Nature Reserve, Hong Kong. Water Res. 41, 1303-1311.

Lin, D.; Tu, Y.; Zhu, L. 2005. Concentrations and health risk of polycyclic aromatic hydrocarbons in tea. Food Chem. Toxicol. 43, 41-48.

Martí-Cid, R.; Bocio, A.; Llobet, J.M.; Domingo, J.L. 2007. Intake of chemical contaminants through fish and seafood consumption by children of Catalonia, Spain: Health risks. Food Chem. Toxicol. 45, 1968-1974.

Matamoros, V.; Jover, E.; Bayona, J.M. 2010. Part-per-Trillion determination of pharmaceuticals, pesticides, and related organic contaminants in river water by solid-phase extraction followed by comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry. Anal. Chem. 82, 699-706.

Serrano, R.; Barreda, M.; Pitarch, E.; Hernández, F. 2003. Determination of low concentrations of organochlorine pesticides and PCBs in fish feed and fish tissues from aquaculture activities by gas chromatography with tandem mass spectrometry. J. Sep. Sci. 26, 75-86.

Srogi, K. 2007. Monitoring of environmental exposure to polycyclic aromatic hydrocarbons: A review. Environ. Chem. Lett. 5, 169-195.

Talsness, C.E. 2008. Overview of toxicological aspects of polybrominated diphenyl ethers: A flame-retardant additive in several consumer products. Environ. Res. 108, 158-167.

United States Environmental Protection Agency, Polynuclear Hydrocarbons, vol. 10, US-EPA, Washington, DC, 1984.

Van der Oost, R.; Beyer, J.; Vermeulen, N.P.E. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environ. Toxicol. Pharmacol. 13, 57-149.

Vonderheide, A.P.; Mueller, K.E.; Meija, J.; Welsh, G.L. 2008. Polybrominated diphenyl ethers: Causes for concern and knowledge gaps regarding environmental distribution, fate and toxicity. Sci. Total Environ. 400, 425-436.

Yusà, V.; Pardo, O.; Martí, P.; Pastor, A. 2005. Application of accelarated solvent extraction followed by gel performance chromatography and high-performance liquid chromatography for the determination of polycyclic aromatic hydrocarbons in mussel tissue. Food Addit. Contam. 22, 482-489.

4.3. Metodología analítica para la determinación de difenil éter polibromados en muestras complejas.

4.3.1. Introducción.

Existe una creciente preocupación como consecuencia de la presencia en el medio ambiente de PBDEs debido a su alto poder de bioacumulación y a su toxicidad (Vonderheide et al., 2008). La razón de la presencia de estos compuestos en diferentes ambientes es debida a su uso como retardantes de llama en materiales que forman parte de una gran variedad de artículos de consumo. Ejemplos de estos artículos serían las espumas de poliuretano flexibles utilizadas en el acolchado de muebles, las carcasas de aparatos eléctricos y electrónicos, principalmente en televisores y ordenadores. Los PBDEs son liberados en el medio ambiente a través del polvo, por ordenadores personales, televisores, alfombras, tapizados y a través de las aguas residuales (procedentes de vertederos). En el medioambiente, los PBDEs entran a la cadena alimentaria y se pueden encontrar en la grasa del ganado y los peces. Las rutas de exposición más comunes en seres humanos son el consumo de comida contaminada (carnes, pescado, productos lácteos) o respirar aire contaminado en el interior de casas o edificios (Frederiksen et al., 2009). Además, los niños recién nacidos pueden estar expuestos a los PBDEs a través de la leche materna contaminada (Talsness et al., 2008).

El término PBDEs engloba a todos los compuestos que presentan la fórmula molecular recogida en la **Figura 4.2**. Existen 209 congéneres posibles según el número y la posición de los átomos de bromo en la molécula de difenil éter. Los congéneres con el mismo número de átomos de bromo en diferentes posiciones reciben el nombre de homólogos, formando todos ellos la familia de homólogos correspondiente. Así, el término PentaBDE hace referencia a todos los homólogos de difenil éter bromado con 5 átomos de bromo.

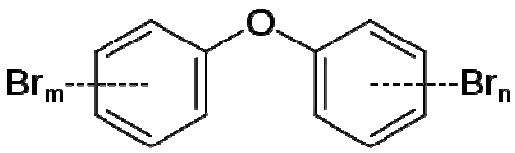

Figura 4.2. Estructura molecular de los PBDEs.

Las propiedades físico-químicas más relevantes de estos compuestos son similares a las de los PCBs, presentando baja presión de vapor y solubilidad, así como un alto valor de la constante de reparto octanol/agua (expresada como log K_{ow}), que determinan su comportamiento ambiental. A medida que aumenta el grado de bromación, los valores de presión de vapor y solubilidad disminuyen, aumentando el valor de log K_{ow}. Este parámetro nos advierte de su capacidad para acumularse en los organismos vivos y su potencial de absorción sobre la materia orgánica de los suelos. Los contaminantes con valores de K_{ow} elevados (superiores a 4) tienen un potencial de bioacumulación muy alto, y por tanto, se trata de compuestos que si se utilizan en cantidades elevadas pueden llegar a transferirse a lo largo de las cadenas tróficas.

Como consecuencia de la necesidad de controlar la presencia de PBDEs en diferentes matrices ambientales y, concretamente en los productos procedentes de la acuicultura marina, el presente trabajo pretende desarrollar una metodología analítica para el análisis de muestras reales de manera fiable y precisa tanto para la identificación como la cuantificación de los PBDEs seleccionados. En el desarrollo de la metodología analítica se prestó especial atención a la etapa de purificación de los interferentes presentes en las muestras, debido a la problemática que presenta el análisis de extractos con un elevado contenido lipídico.

Las matrices incluidas en el estudio son filetes de dorada cultivada, piensos con diferente composición, aceite de pescado y aceites vegetales. Las muestras proceden de un estudio experimental en el marco del proyecto AQUAMAX (Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers. www.aquamaxip.eu).

4.3.2. Artículo científico 2:

Gas chromatography-mass spectrometric determination of polybrominated diphenyl ethers in complex fatty matrices from aquaculture activities.

J. Nácher-Mestre, R. Serrano, F. Hernández, L. Benedito-Palos, J. Pérez-Sánchez.

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Gas chromatography–mass spectrometric determination of polybrominated diphenyl ethers in complex fatty matrices from aquaculture activities

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Abstract

Gas chromatography coupled to mass spectrometry in negative chemical ionization mode (GC-(NCI)MS) has been applied to the quantification and reliable identification of polybrominated diphenyl ethers (PBDEs) in animal and vegetable samples from aquaculture activities. Matrices analyzed included fish fillet, fish feed, fish oil and linseed oil, their fat content ranged from 5% to 100%. Solid-phase extraction (SPE) (using Florisil and silica cartridges) and normal-phase high performance liquid chromatography were tested for an efficient clean-up in order to obtain sample extracts free of interfering compounds. Combining sulphuric acid digestion and SPE with Florisil led to the highest efficiency in the elimination of interferences from the extracts. The sample procedure developed, together with the application of GC-(NCI)MS for measurement, led to the satisfactory determination of PBDEs at µg kg^{-1} levels in complex aquaculture matrices with high lipid content. The use of a short and thin film-thickness fused-silica capillary column allowed to determine the problematic BDE 209 with satisfactory results. Three m/z ions were acquired for each analyte, which ensured a reliable identification of compounds detected in samples.

Keywords: Polybrominated diphenyl ethers; Fatty samples; Gas chromatography; Mass spectrometry; Clean-up; Aquaculture matrices

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are anthropogenic chemicals that are added to a wide variety of consumer/commercial products in order to improve their fire resistance. For this reason, PBDEs are commonly named as brominated flame retardants (BFRs) due to their capability in reducing fire in a widespread list of products. BFR's frequent employment is to reduce successfully deaths related to the fire, injuries and property damage [1]. This fact, together with their hydrophobic properties, contributes to the presence and bioaccumulation of BFRs in environmental samples [2]. Analytical studies on BFRs have been focused on environmental samples [3], as air, sewage sludge, sediments, soils and water [4], and on biota for fish derivatives [5], and they are normally found in biological tissues at $\mu g kg^{-1}$ levels [6,7].

Marine aquaculture has experienced strong development in the last decades as a consequence of increased fish consumption by the world population and decreasing wild stocks. Fish used as raw material for manufacture of fish feed ingredients (fish oils and meals) are a potential source of PBDEs in fish feed, which can be bioaccumulated by aquatic species [5,8]. PBDEs' determination in samples and resources from aquaculture is important in order to establish the contamination origin, for a dietary exposure assessment and for protection of public health [5,9,10].

The determination of PBDEs in marine matrices is difficult due to the low analyte levels normally present and the presence of interfering compounds (e.g. fats), which are co-extracted with the analytes. This makes an efficiency clean-up necessary before instrumental measurement. Extraction of PBDEs in these samples can be carried out using Soxhlet, sonication, matrix solid-phase dispersion, solid-phase microextraction or pressurized liquid extraction [11-13]. The purification of fatty marine extracts is crucial to get satisfactory analytical data and to reach the sensitivity required. An efficient clean-up can be performed using gel permeation chromatography, solid-phase extraction (SPE) or normal-phase high performance liquid chromatography (NPLC), as well as by acid treatment or the use of acidified silica for lipid removal [13,14].

As regards analytical measurement, gas chromatography coupled to mass spectrometry (GC-MS) is normally the preferred technique for PBDEs, although LC-MS

under different ionization modes has been also tested [1,15]. GC with electron capture detector has been applied, but it has important limitations in selectivity because the detection and identification is based only on retention time and matrix interferences can appear [16]. Some authors have reported PBDEs measurements in environmental analysis by GC-MS under selected ion monitoring mode (SIM) [17-19]. In the light of data reported, one of the most sensitive approaches seems to be high resolution GC with negative chemical ionization (NCI) coupled to a single quadrupole MS in SIM mode, while electron ionization (EI) may improve the selectivity thanks to the most abundant fragmentation. NCI-MS may be less specific than EI-MS due to the Br^- ions formation, which can be confused from interfering compounds in complex samples [16]. However, GC-(NCI)MS increases sensitivity and accuracy in the determination of BDE 209, the most difficult BDE from an analytical point of view, due to its particular behavior on degradation and sensitivity [20].

Analytical methodologies reported for the determination of PBDEs in marine fatty samples are normally focused on tri, tetra, penta and hexa PBDEs congeners. Thus, BDE 47 (tetra-BDE), 99 and 100 (penta-BDEs), have been usually detected in this kind of samples [21-23]. High-brominated compounds with seven or more bromine atoms have proven to be more difficult to be determined correctly. Thus, high PBDEs, such as BDE 209, are not often included in PBDE studies [24,25], possibly due to their tendency to degrade in injectors and during gas chromatographic separation process [23]. Nevertheless, most of the commercial mixtures of PBDEs reference standards also contain high-brominated congeners, as they are relevant as a consequence of their higher lipophilic character and consequently their greater tendency to be bioaccumulated in fatty animal tissues.

As regards the determination of PBDEs in fish feed and their ingredients, Fajar et al. [26] proposed amethodology for the determination of seven PBDEs in fish feed and molluscs, at $\mu g kg^{-1}$ levels. However, fish and vegetal oils used in the fish feed manufacture and farmed fish are normally scarcely investigated in the bibliography.

In this work, we have developed a sensitive method for the reliable quantification and identification of 12 PBDEs (including tri, tetra, penta, hexa, hepta PBDEs congeners and the deca congener BDE 209), in aquaculture samples with fat contents ranging from 5% to 100%. The method is based on the use of GC-MS working in NCI mode and selected ion

recording (SIR). Working in SIR means that only the first quadrupole in the triple quadrupole analyzer is acquiring. SIR is the nomenclature used by the manufacturer (Waters) and it is equivalent to SIM, employed by other manufacturers. In order to obtain extracts with very low content of fats and other interference compounds, the efficiency of SPE and of NPLC as clean-up techniques has been studied after applying a first clean-up step by sulphuric acid digestion. The developed methodology has been applied to real-world samples of fish feed, vegetable and fish oils, and fish fillets from feeding trials with gilthead sea bream (Sparus aurata L.) as a part of the European Union project "Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for consumers" (AQUAMAX), Contract number: 016249-2. The acquisition of three selective m/z ions for each analyte has allowed quantification and reliable identification of PBDEs, at low $\mu g kg^{-1}$ level, in the different fatty samples analyzed.

2. Experimental

2.1. Materials and reagents

Standards of PBDEs (50 mg L^{-1} in isooctane) were obtained from Chiron As (Trondheim, Norway) with purity higher than 99.5%: tri-BDE: BDE 28 (2,4,4'-tribromodiphenyl ether), tetra-BDEs: BDE 71 (2,3',4',6-tetrabromodiphenyl ether), BDE 47 (2,2',4,4'-tetrabromodiphenyl ether), BDE 66 (2,3',4,4'-tetrabromodiphenyl ether), penta-BDEs: BDE 100 (2,2',4,4',6-pentabromodiphenyl ether), BDE 99 (2,2',4,4',5-pentabromodiphenyl ether), BDE 85 (2,2',3,4,4'-pentabromodiphenyl ether), hexa-BDEs: BDE 154 (2,2',4,4',5,6'-hexabromodiphenyl ether), BDE 153 (2,2',4,4',5,5'-hexabromodiphenyl ether), BDE 138 (2,2',3,4,4',5'-hexabromodiphenyl ether), hepta-BDE: BDE 183 (2,2',3,4,4',5',6-heptabromodiphenyl ether) and the deca-BDE: BDE 209 (decabromodiphenyl ether). A standard mixture, which contained the 12 PBDEs (5 mg L^{-1}) was prepared by dilution of individual reference standard solutions with n-hexane, and it was stored in a freezer at −20°C.

Working solutions of 12 PBDEs were prepared by diluting the standard mixture solution with n-hexane, and they were used for sample fortification and for preparation of calibration curves.

4,4-DDE D_8 from Promochem (Wesel, Germany) was used as surrogate internal standard (IS). Working solutions (100 μg L^{-1} and 250 μg L^{-1}) were prepared by dilution of the stock solution (100 mg L^{-1}) with n-hexane and were stored at 4°C.

Ethyl acetate and n-hexane (ultratrace quality) were purchased from Scharlab (Barcelona, Spain). Sulphuric acid 95-98% was purchased from Scharlab. Anhydrous sodium sulphate of pesticide residue quality (Scharlab) was dried for 18 h at 300°C before use. Strata silica cartridges (1 g) from Phenomenex (Torrance, CA, USA) and Supelclean LC-Florisil SPE tube (1 g) from Sigma-Aldrich (Madrid, Spain) were used in SPE experiments.

2.2. Sample material

Gilthead sea bream (Sparus aurata L.) specimens of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were cultured at the Instituto de Acuicultura de Torre la Sal, Spain (IATS, CSIC) and collected when fish accomplished commercial size (≈500 g). The left-side fillets (denuded from skin and bone) were excised and stored at −20°C until analysis. Fish feed supplied to sea bream during feeding trials was stored at −20°C until analysis. Fish oil and linseed oil used in fish feed manufacture, were also stored at −20°C until analysis.

2.3. NPLC clean-up procedure

NPLC clean-up system previously applied by Serrano et al. [14] was tested to remove the interferences in the sample extracts. The system consisted of an LC Pump Master 305 from Gilson (Middleton, WI, USA), two VICI Valco six-way high pressure valves from Europe instruments (Schenkon, Switzerland), a Rheodyne sampler injector valve from Rheodyne (Colati, CA, USA) with 1mL loop, a Novapack silica column (150 mm×3.9 mm i.d., 4μm particle size) from Waters (Mildford, MA, USA), and a Gilson FC 203B fraction collector. The mobile phase was hexane at a flow rate of 1 mL min^{-1}. The volume injected (sample extracts and/or standard solutions) was 1 mL. The sample extract injected into the NPLC system was previously treated with sulphuric acid to remove most of the fat content.

Firstly, 1-mL LC fractions after injecting PBDE standards were collected and analyzed by GC-(NCI)MS in order to know their elution patterns in the LC clean-up. Results showed that all PBDEs eluted in the fractions between 2 min and 8 min. Therefore, the whole

fraction eluting from 2 min to 8 min was collected when injecting sample extracts and it was adjusted to 0.25 mL by evaporation under N_2 stream at 40°C. The overall procedure was controlled from the LC pump, programming the times of valve activation automatically.

2.4. SPE clean-up procedure

SPE was also tested as clean-up procedure for fatty samples using Florisil and silica cartridges. 1-mL of PBDEs mix standard solution was loaded into the cartridge, previously conditioned with 6mL of n-hexane. Elution was carried with n-hexane and individual fractions of 1mL were collected and analyzed by GC-(NCI)MS. Results showed that all PBDEs eluted in the first 8mL using Florisil cartridges. In the case of silica SPE cartridges, 10mL n-hexane was necessary for elution of all 12 PBDEs.

Once optimized the SPE-Florisil and SPE-silica clean-up procedures, 1-mL of the cleaned-up hexanic extract sample (after acid treatment) was introduced in the SPE cartridge (Florisil and silica, respectively) and fractions were collected. The whole eluate, containing all analytes, was evaporated under a gentle nitrogen stream at 40°C, and the final residue was dissolved in 0.25mL n-hexane in both cases and injected in the GC system.

2.5. GC instrumentation

A GC system (Agilent 6890N) from Agilent Technologies (Palo Alto, CA, USA) equipped with an autosampler (Agilent 7683) was coupled to a triple quadrupole (QqQ) mass spectrometer, Quattro Micro GC from Micromass (Boston, MA, USA), operating in NCI mode. The GC separation was performed using a fused-silica DB-1HT capillary column with a length of 15 m, an internal diameter of 0.25 mm and a film thickness of 0.1 μm from J&W Scientific (Folsom, CA, USA). The injector temperature was set to 260°C. Splitless injections of 1 μL were carried out. Helium (99.999%) from Praxair (Madrid, Spain) was used as carrier gas at a flow rate of 1 mL min^{-1}. The source temperature was set to 200°C and a solvent delay of 3 min was selected. QqQ system operated in SIR mode. Methane (99.9995%) from Praxair (Madrid, Spain) was used as reagent gas with an optimal flow of 60%. Experimental conditions selected for GC-(NCI)MS measurement are shown in **Table 1**. The oven temperature program was as follows: 120 °C (1 min); 10 °C min^{-1} to 220 °C; 20 °C min^{-1} to

300 °C (3 min), 40 °C min^{-1} to 340 °C (3 min). Helium was used as carrier gas at 1 mL min^{-1}. A dwell time per channel between 0.05 s and 0.1 s was chosen. The application manager TargetLynx was used to process the quantitative data obtained from calibration standards and samples.

2.6. Recommended procedure

Before analysis, samples were thawed at room temperature and carefully ground using a mill Super JS from Moulinex (Bagnolet Cedex, France). Approximately 16 g of fish fillet or fish feed was homogenized with the amount of anhydrous sodium sulphate necessary to remove water (around 20 g Na_2SO_4), and the blend was spiked with 800 µL of surrogate IS solution (250 µg L^{-1} 4,4-DDE-D_8). Extraction was performed by refluxing in n-hexane for 4 h at 80 °C. After filtration (0.45 µm), the extract was pre-concentrated using a Kuderna-Danish apparatus until ca. 8 mL (2 g sample per mL n-hexane). A sulphuric acid digestion [14] was firstly applied to 1 mL of the hexanic extract, in order to remove most of the fats.

In the case of oils, 0.5 g of fish oil or linseed oil was mixed with 1.5 mL of n-hexane and was directly digested. Digestion was carried by adding 2 mL of conc. H_2SO_4 and shaking 1 min in a Vortex. The hexanic layer was collected, and the remaining sulphuric phase was washed with 1 mL hexane twice. Finally, the around 3 mL cleaned-up hexanic phase was adjusted to 1 mL at 40 °C under a gentle N_2 stream.

Table 1. GC-(NCI)MS measurement conditions selected for PBDEs determination.

PBDE number	t_R (min)	Quantification Ion (Q) (m/z)	Confirmation Ion (q) (m/z)	q	Q/q_1	Q/q_2	Dwell time (s)
p,p'-DDE-D_8[a]	7.4	289 $[C_{14}D_8{}^{35}Cl_3]^-$					0.1
28	7.9	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	1.01(5)	>20	0.05
71	9.6	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	1.01(1)	>20	0.05
47	9.8	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	1.01(1)	>20	0.05
66	10.1	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	1.05(4)	>20	0.05
100	11.2	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	1.02(1)	>20	0.05
99	11.6	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	0.99(7)	>20	0.05
85	12.1	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	0.98(10)	>20	0.05
154	12.5	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	0.99(8)	>20	0.05
153	12.9	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	0.96(13)	>20	0.05
138	13.3	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	0.96(9)	>20	0.05
183	13.8	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 161 $[H^{79}Br^{81}Br]^-$	q_1 q_2	1(7)	>20	0.05
209	16.5	79 $[^{79}Br]^-$	81 $[^{81}Br]^-$ 487$[C_6O^{79}Br_3{}^{81}Br_2]^-$	q_1 q_2	0.98(6)	12.85(17)	0.05

[a] *Internal Standard used as surrogate.*
[b] *Average value calculated from standard solutions at seven concentration levels (n=2 each). RSD (%) shown in brackets.*

The 1-mL extract resulting from sulphuric acid digestion (neither pre-concentration nor dilution of the sample extracts takes place) was passed through a 1 g Florisil SPE cartridge, previously conditioned with 6 mL of n-hexane, and it was eluted with 8 mL n-hexane. The eluate was evaporated under a gentle nitrogen stream at 40 °C and the final residue was reconstituted in 0.25 mL of n-hexane. Along the whole analytical procedure 8-fold pre-concentration (fish fillet and fish feed) or 2-fold pre-concentration (oil samples) takes place.

The final cleaned-up extracts obtained were injected into the Quattro Micro GC system working in NCI(MS) mode under the optimized conditions shown in **Table 1**. Quantification of samples was carried out using calibration with standards in solvent, using 4,4-DDE D_8 as surrogate IS for all congeners.

2.7. Validation

Validation of the method developed was performed by evaluating the following parameters:

- Linearity. The calibration curves were obtained by injecting reference standard solutions in n-hexane in duplicate. Linearity of relative response (analyte versus IS) was tested by injecting standard solutions in duplicate. All PBDEs were studied in the concentration range 0.5-50 μg L^{-1}, while BDE 209 was tested between 2.5 μg L^{-1} and 250 μg L^{-1} due to its lower sensitivity. Linearity was assumed when the regression coefficient was greater than 0.99 with residuals randomly distributed and being lower than 30%.

- Accuracy. It was evaluated by means of recovery experiments, analyzing "blank" reference samples (n = 3) of each matrix investigated, which were spiked at two concentration levels: 0.25 μg kg^{-1} and 2.5 μg kg^{-1} (1.25 μg kg^{-1} and 12.5 μg kg^{-1} for BDE 209) in fish fillet and fish feed, and 1 μg kg^{-1} and 10 μg kg^{-1} (5 μg kg^{-1} and 50 μg kg^{-1} for BDE 209) in fish and linseed oils. Previously, several samples were analyzed in triplicate to determine the concentration of the analytes in the different sample matrices. Data showed that BDE 28 and BDE 47 were most frequently detected in fish fillet and fish feed, while in oils PBDEs were always below the limits of quantification. From these analyses, we selected the

"blank" samples with the lowest levels of PBDEs, in such a way that PBDEs concentrations in the four blanks were lower than the limits of quantification.

- Precision. The precision, expressed as the repeatability of the method, was determined in terms of relative standard deviation (RSD, %) from recovery experiments at each fortification level.

- Limit of quantification (LOQ) objective. It was established, from the quantification ion, as the lowest concentration that was validated in spiked samples following the overall procedure with satisfactory recovery (between 80% and 120%) and precision (RSD < 20%) (0.25 μg kg^{-1} fish fillet and fish feed, 1 μg kg^{-1} fish oil and linseed oil. For BDE 209 these values were 1.25 and 5 μg kg^{-1}, respectively).

- Limit of detection (LOD). LOD of the method was statistically estimated, from the quantification ion, as the analyte giving a peak signal of three times the background noise from the sample chromatograms at the lowest fortification level tested. Instrumental LODs were estimated from the lowest standard in solvent injected (e.g. 0.5 μg L^{-1}, except for BDE 209 which was 2.5 μg L^{-1}).

- Confirmation criteria: The Q/q ratio, defined as the intensity ratio between the quantification ion (Q) and the confirmation ion (q), was used to confirm the identity of the compounds detected in samples. Confirmation criteria were based on the European Commission Decision (2002/657/CE) [27]. Briefly, to confirm a finding as an actual positive, a maximum ratio tolerance ±20% was accepted when the relative intensity of the confirmative transition was >50% as regards the quantitative one (Q/q ratio 1-2). For higher Q/q ratios, the tolerances increased. Thus, maximum deviations of ±25% (relative intensity 20-50%, Q/q ratio 2-5), ±30% (relative intensity 10-20%, Q/q ratio 5-10) and ±50% (relative intensity ≤ 10%, Q/q ratio > 10) were accepted. This criterion was originally defined on measures to monitor certain substances and residues thereof in live animals and animal products, and it is being increasingly used in other fields like environmental and biological samples analysis [28-31]. Obviously, the agreement in the retention time in sample and reference standard was also required to confirm a positive finding. The theoretical average Q/q ratios for each compound were obtained as the mean value calculated from seven standard solutions injected twice.

3. Results and discussion

3.1. Comparison between NPLC and SPE clean-up procedures

Firstly, the linearity of the method was evaluated with standard solutions using responses relative to IS. The response was linear along the whole concentration range tested, i.e. 0.5-50 μg L^{-1}. For BDE 209, linearity was satisfactory between 2.5 μg L^{-1} and 250 μg L^{-1}.

As a consequence of the high lipid content and complexity of the matrices studied, first a clean-up of the extracts by means of acid digestion using conc. sulphuric acid was necessary, which allowed us to remove around 90% of lipids. In spite of this, an amount of lipids and may be other interfering compounds still remained in extracts making a second clean-up step necessary to improve sensitivity and selectivity. The application of NPLC or SPE was found to be an efficient way to reduce interferences, and allowed us to achieve the low LODs pursued in this research.

NPLC and SPE (Florisil and silica) were tested in order to know the improvements of the method in terms of LODs, LOQs, recoveries and matrix effects in the four sample matrices fortified at 0.25 μg kg^{-1} (fish fillet and fish feed) or at 1 μg kg^{-1} (fish oil and linseed oil). BDE 209 fortification level was 1.25 μg kg^{-1} and 5 μg kg^{-1}, respectively.

All sample extracts were treated with sulphuric acid before applying the SPE or NPLC clean-up. Recoveries obtained corresponded to the whole analytical procedure, including extraction, sulphuric acid treatment and SPE/NPLC clean-up, and GC-(NCI)MS measurement. The sample volume injected in the SPE/NPLC system was in all experiments 1 mL. The sample amount that can be loaded in SPE is normally higher than in NPLC. For this reason, we performed several experiments to optimize the sample volume injected in the NPLC system in order to avoid overloading. Finally, 1 mL was injected (notice that sample extract was previously treated by sulphuric acid).

Although the three clean-up techniques were optimized post acid treatment, it was found that SPE was more efficient as NPLC due to the SPE cartridges allowed the purification of several sample extracts at the same time while, in the case of NPLC, only one sample extract is introduced in the system. Moreover, more volume of solvents is in use in

NPLC in 26 min. of automatic purification program [14] in comparison with SPE-Florisil or SPE-silica.

Recoveries of PBDEs using SPE clean-up with Florisil (**Table 2**) were typically between 80% and 120%, and mostly better than those obtained after SPE-silica or NPLC clean-up. In some particular cases, recoveries slightly higher than 120% were achieved suggesting a poor correction when using DDE-D8 as surrogate, specially after silica SPE and NPLC clean-up. Working with Florisil, only one exception was observed; BDE 154 in fish oil at 1 μg kg^{-1} spiked level (recovery 133%). RSDs were lower than 15% with the exception of the lowest level (0.25 μg kg^{-1}) in fish fillet, where values around 25% were obtained for several compounds. In general, RSDs were also better compared with SPE-silica and NPLC.

Similar LODs were obtained with the three clean-up approaches, except for BDE 209 where the lowest LODs were reached in all matrices using Florisil SPE cartridges. **Fig. 1** shows a comparison between BDE 209 chromatograms obtained when applying different clean-up procedures to fish fillet, fish feed, linseed and fish oil samples at the lowest fortified level (1.25 μg kg^{-1} for fish fillet and fish feed, 5 μg kg^{-1} for oils). As expected, oil matrices presented more interferences. LODs after applying sulphuric acid treatment and Florisil SPE clean-up are shown in **Table 2**. These values ranged for the eleven selected PBDEs (in μg kg^{-1}) from 0.03 to 0.10 (fish fillet), 0.03 to 0.19 (fish feed), 0.06 to 0.25 (fish oil) and 0.05 to 0.20 (linseed oil). LODs for BDE 209 varied from 0.70 μg kg^{-1} to 0.90 μg kg^{-1}. Instrumental LODs were estimated from the lowest standard injected and were in the range of 0.03-0.16 μg L^{-1}, except for BDE 209 that was 0.52 μg L^{-1}.

The analytical methodology developed presents high selectivity and sensitivity as consequence of the efficient clean-up procedure applied, and the instrumental determination by GC-(NCI)MS, reaching low LODs for all PBDEs selected in the four matrices studied.

Table 2. Recoveries (%) of the overall analytical procedure for GC-(NCI)MS analysis of PBDEs in fish fillet, fish feed, fish oil and linseed oil (n=3, at each fortification level) when using SPE-Florisil clean-up. Limits of detection.

	Recoveries (%)								LOD (μg kg $^{-1}$)			
	0.25μg kg $^{-1}$ [a]		2.5μg kg $^{-1}$ [b]		1μg kg $^{-1}$ [c]		10μg kg $^{-1}$ [d]					
BDE	Fish Fillet	Fish Feed	Fish Fillet	Fish Feed	Fish Oil	Linseed Oil	Fish Oil	Linseed Oil	Fish Fillet	Fish Feed	Fish Oil	Linseed Oil
28	107(28)	92(6)	104(2)	99(11)	87(3)	79(11)	86(24)	108(9)	0.04	0.04	0.15	0.09
71	99(25)	95(16)	102(4)	103(15)	87(8)	86(13)	89(16)	87(23)	0.04	0.10	0.07	0.05
47	98(7)	80(4)	102(10)	98(9)	92(14)	89(19)	96(7)	108(3)	0.10	0.12	0.24	0.16
66	107(19)	103(3)	98(3)	103(2)	115(9)	105(7)	93(12)	112(5)	0.10	0.19	0.25	0.20
100	115(25)	104(6)	101(9)	116(8)	107(14)	102(18)	104(10)	119(3)	0.03	0.12	0.10	0.05
99	98(4)	119(5)	103(9)	114(10)	99(7)	119(14)	104(8)	117(5)	0.07	0.07	0.08	0.12
85	106(10)	115(4)	95(5)	119(8)	103(10)	98(16)	101(5)	116(12)	0.06	0.07	0.13	0.12
154	110(5)	117(9)	101(11)	126(6)	133(11)	101(10)	98(6)	111(5)	0.05	0.03	0.08	0.08
153	95(26)	108(13)	100(15)	107(8)	108(10)	108(3)	107(7)	118(15)	0.06	0.05	0.11	0.09
138	100(25)	102(6)	102(16)	132(6)	113(9)	111(3)	102(11)	119(1)	0.08	0.05	0.15	0.16
183	100(21)	110(5)	75(12)	100(8)	103(10)	109(2)	81(9)	99(7)	0.04	0.05	0.06	0.07
209	110(16)	84(15)	80(13)	116(8)	104(12)	106(9)	84(8)	109(8)	0.70	0.90	0.90	0.85

[a] 1.25 μg kg $^{-1}$ for BDE 209, [b] 12.5 μg kg $^{-1}$ for BDE 209.
[c] 5 μg kg $^{-1}$ for BDE 209, [d] 50 μg kg $^{-1}$ for BDE 209.
RSD (%) shown in brackets.

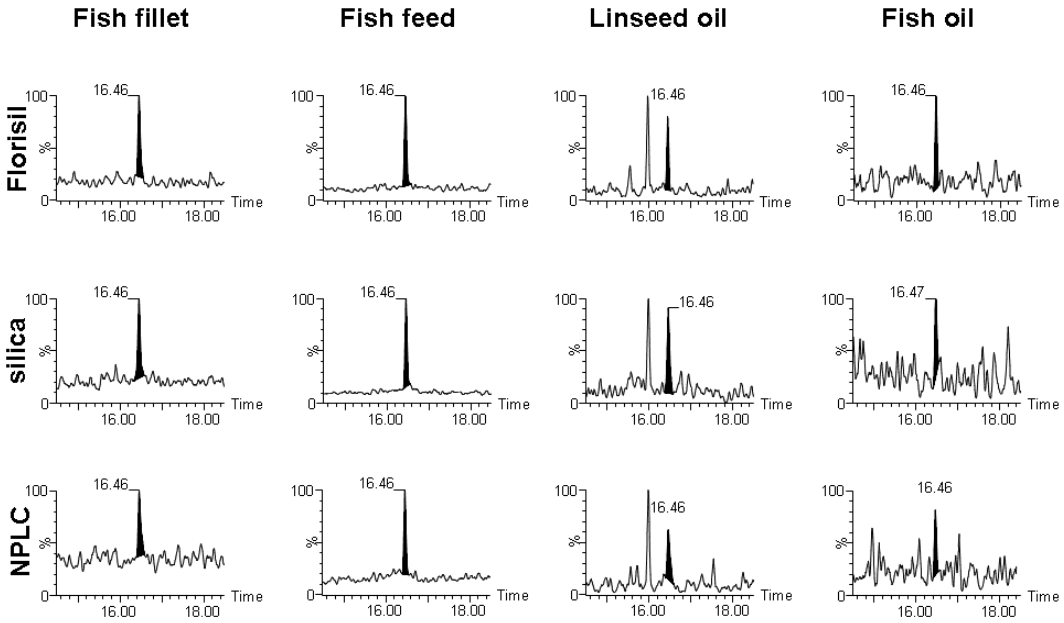

Fig. 1. GC-(NCI)MS chromatograms of BDE 209 for fish fillet, fish feed, linseed and fish oils spiked at the lowest fortification level (1.25 μg kg^{-1} for fish fillet and fish feed, 5 μg kg^{-1} for oils). Comparison of different clean-up procedures: SPE-Florisil (top), SPE-silica (medium), and NPLC (bottom) applied after sulphuric acid treatment.

Fig. 2 and **Fig. 3** show illustrative GC-(NCI)MS chromatograms at the lowest level validated for the sample matrices studied after applying the overall recommended analytical procedure. **Fig. 2** shows chromatograms for tri-BDE (28) and tetra-BDEs (47, 66, 71) and **Fig. 3** shows chromatograms for penta-BDEs (85, 99, 100), hexa-BDEs (138, 153, 154), hepta-BDE (183) and deca-BDE (209). As it can be seen, satisfactory sensitivity was obtained at the LOQ level when using the quantification ion (Q) and the first confirmation ion (q_1), while the lower abundance of the second confirmation ion (q_2) led to a notable decrease in sensitivity. In spite of this, q_2 was also acquired to help in the confirmation of the identity of positive findings.

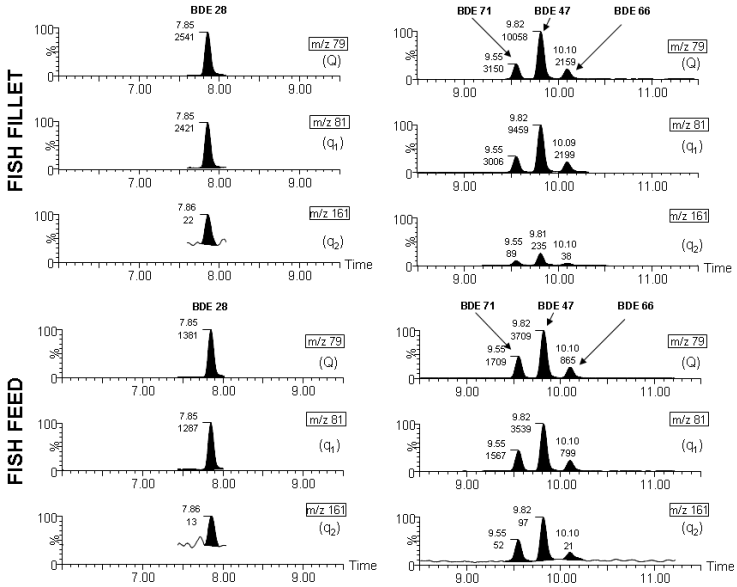

Fig. 2. GC-(NCI)MS illustrative chromatograms at 0.25 μg kg^{-1} level for fish fillet (top) and fish feed (bottom) following the overall recommended procedure. "Q" quantification ion, "q_1 and q_2" confirmation ions. Q/q were in agreement with the criteria established. Numbers on top of the peak are retention time (top) and peak area (bottom).

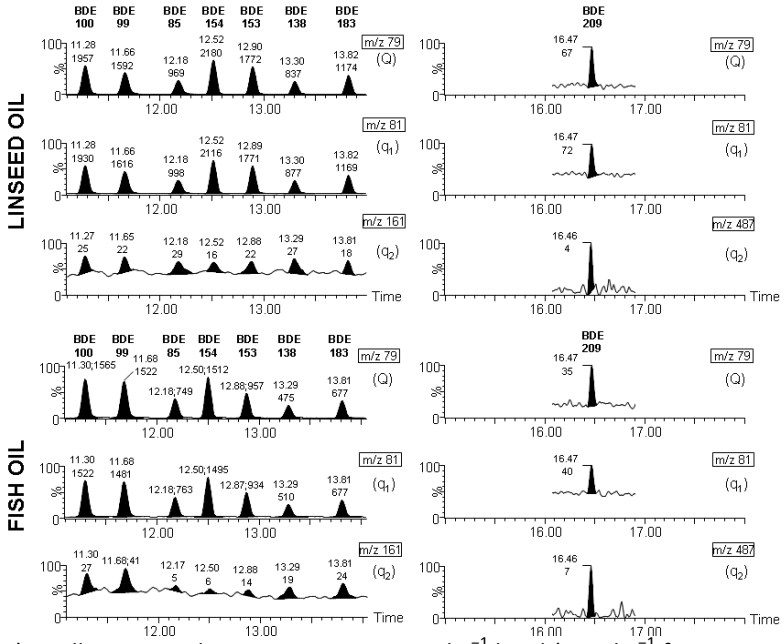

Fig. 3. GC-(NCI)MS illustrative chromatograms at 1 μg kg^{-1} level (5 μg kg^{-1} for BDE 209) for linseed oil (top) and fish oil (bottom) following the overall recommended procedure. "Q" quantification ion, "q_1 and q_2" confirmation ion. Q/q were in agreement with the criteria established. Numbers on top of the peak are retention time (top) and peak area (bottom).

3.2. GC-(NCI)MS conditions

GC coupled to mass spectrometry is, in most cases, the preferred technique for the analysis of organic pollutants in environmental samples [3,32]. In addition, the QqQ mass analyzer working in tandem MS/MS mode provides high selectivity and sensitivity in the analysis of complex environmental samples, which is an evident advantage in environmental analysis. The use of two stages of mass analysis in MS/MS systems based on QqQ analyzers offers the possibility of applying selected reaction monitoring, one of the most selective and sensitive approaches. In spite of this, there are still some compounds that cannot be determined with enough sensitivity with this technique. Thus, the development of MS/MS method in NCI mode for PBDEs was not feasible because of the poor MS spectra, which only showed the clusters from the fragments $[Br]^-$ and $[HBr_2]^-$ [6]. The molecular cluster was not observed or only constituted a minor peak. Therefore, the unique transition reasonable for the majority of compounds would have been the fragmentation of $[HBr_2]^-$ to give a bromine atom, with low sensitivity and poor selectivity. Consequently, a SIR method was optimized by monitoring the three most intense peaks of the mass spectra, which corresponded to m/z 79 ($[^{79}Br]^-$), m/z 81 ($[^{81}Br]^-$), m/z 161 ($[H^{79}Br^{81}Br]^-$) and m/z 487 ($[C_6O^{79}Br_3{}^{81}Br_2]^-$) for BDE 209.

Chromatographic conditions for PBDEs in GC-MS were optimized by injecting standard solutions in solvent. We studied the temperature program in order to achieve satisfactory resolution and peak shape for the IS and 12 PBDEs, including the BDE 209. The deca-BDE 209 should receive special attention because of its sensitivity for the higher susceptibility for degradation in the GC system [33]. Due to its unique chemical and physical properties, including high molecular weight, accurate determination of BDE 209 still remains a challenge, especially at low analyte levels. The different oven programs tested in our work indicated that reducing the column residence time of BDE 209, by using a short 15 m column, in combination with fast ramp rates, improved the peak sensitivity. Increasing oven temperature up to 340 °C and setting the injection temperature to 260 °C, to ensure complete vaporization, resulted in a consistent and more sensitive response, notably

reducing the BDE 209 retention time. Therefore, a short and thin capillary column was used to prevent degradation of PBDEs and to improve accuracy and precision of the analysis. The overall procedure was tested for the analytical characteristics shown in the validation section, obtaining satisfactory results. Due to its special characteristics, BDE 209 is not commonly included in multi-analyte PBDEs methods [34, 35].

The coelution of PBDE congeners under certain chromatographic conditions has been reported. Thus, BDE 49 and BDE 71 are close-eluting on 100% Dimethylpolysiloxane GC capillary column and might coelute in our DB-1HT column. Covaci et al. [13] have performed a detailed study of PBDEs chromatographic behavior using different GC capillary columns, but they reported no coelution of the two mentioned congeners. We did not test this fact as BDE 49 was not studied in our work.

NCI ionization in SIR acquisition mode was applied to monitoring bromine ions: $[^{79}Br]^-$, $[^{81}Br]^-$ and secondary fragments such $[H^{79}Br^{81}Br]^-$ and $[C_6O^{79}Br_3{}^{81}Br_2]^-$. GC-(NCI)MS was proven to notably increase the selectivity, sensitivity and accuracy for PBDEs determination, specially for BDE 209 [13] and [16]. The $[Br]^-$ mass fragment was the most intense ion, while the molecular ion was normally missing. $[H^{79}Br^{81}Br]^-$ and $[C_6O^{79}Br_3{}^{81}Br_2]^-$ fragments were less abundant than $[Br]^-$. Thus, $[Br]^-$ (m/z = 79) was selected for quantification due to the higher sensitivity reached, and $[Br]^-$ (m/z = 81) was selected as confirmation ion. $[H^{79}Br^{81}Br]^-$ and $[C_6O^{79}Br_3{}^{81}Br_2]^-$ were selected for a more reliable confirmation of PBDEs in samples, but they presented some difficulties due to the lower abundance compared with $[Br]^-$ ions. Due to the notable differences in abundances, we obtained Q/q ratios around one for the relation with 79/81 (Q/q_1), but much higher Q/q ratios for 79/161 and 79/487 (Q/q_2). **Fig. 2**, **Fig. 3** and **Fig. 4** illustrate in fact the limitations for confirmation when using Q/q_2 ratio in samples at low concentration levels. In spite of this, we decided to acquire m/z = 161 and 487 although in the most of cases the Q/q_2 ratios were higher than 20. Using the second confirmation ion was useful when the analyte was present at higher concentrations. Agreement in Q/q ratios between samples and reference standards is required to confirm positive findings [27]. The agreement in the retention time of a compound in the sample and in reference standard is also required to confirm a positive result.

3.3. Application to samples from bioaccumulation experiments

The overall optimized procedure was applied to the analysis (in triplicate) of 20 fish fillet samples, 11 fish feed, 1 fish oil, 1 linseed oil, 1 rapeseed oil and 1 palm oil used in aquaculture experiments. The results obtained are shown in **Table 3**. Most PBDE congeners were below the limit of detection and only a few samples presented positive findings in this study. The most frequently detected congeners were PBDE 28 and PBDE 47. **Fig. 4** reveals positive findings of tetra, penta- and hexa-PBDEs in fish fillets. Concentration levels found in fish fillets ranged from 0.25 μg kg^{-1} to 4.7 μg kg^{-1}. Some authors have also reported low PBDE levels in wild aquatic species (mud carp and crucian carp), with individual values from 1.3 μg kg^{-1} to 400 μg kg^{-1} [5]. Shaw et al. [18] reported mean concentrations of ∑PBDEs in farmed salmon samples from Maine (0.95 μg kg^{-1} ww, 7.3 μg kg^{-1} lw) and eastern Canada (0.85 μg kg^{-1} ww, 6.3 μg kg^{-1} lw) lower than data reported in our paper for fish fillet.

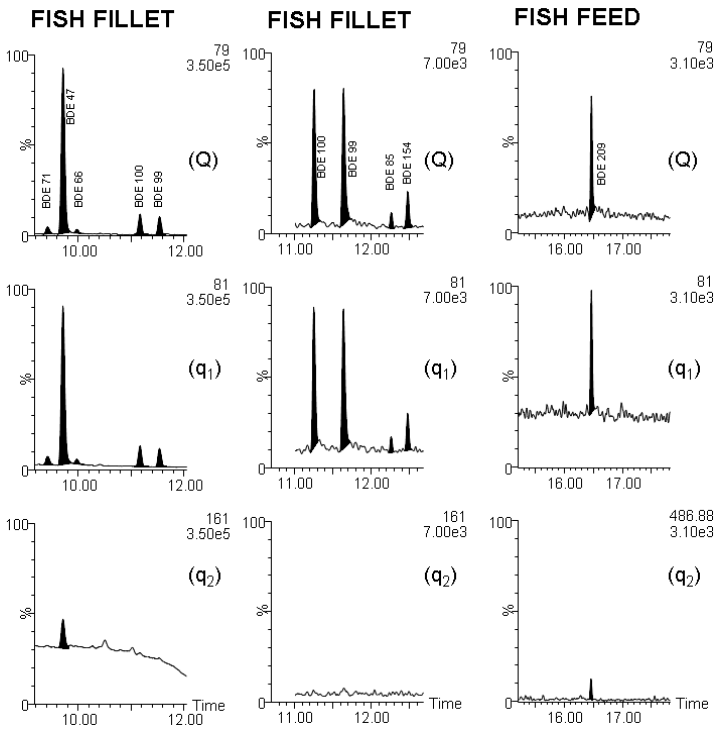

Fig. 4. Positive findings of PBDEs in samples of fish fillet, fish feed. Q/q were in agreement with the criteria established.

PBDEs in fish feed ranged from 0.5 µg kg^{-1} to 2.8 µg kg^{-1}, except for one sample that contained BDE 209 at a 15.5 µg kg^{-1} (**Fig. 4**). This high value corresponds to the only commercial fish feed analyzed, as the remaining samples were home-made for the AQUAMAX project after a careful selection of the ingredients. The high BDE 209 level was confirmed by additional analysis, concluding that there was a remarkable contamination in this sample. Some authors reported low contamination by PBDEs in aquatic species compared with the samples in our work [18] and [36]. Dietary intake studies of lower PBDEs have shown that fish and animal products are important vectors of human exposure, but data on higher brominated BDEs are scarce [37].

Table 3. PBDEs detected in aquaculture samples (n=3).

	Fish Fillet		**Fish Feed**	
BDE Congener	Number of positives	conc. range (µg kg^{-1})	Number of positives	conc. range (µg kg^{-1})
28	7	0.25-0.5	4	0.25-0.3
71	3	0.25-0.5	4	0.25-0.8
47	14	0.25-4.7	4	0.25-2.8
66	1	0.6	3	0.25-0.3
100	2	0.4-0.7	4	0.25-0.7
99	2	0.6-0.9	4	0.25-0.9
85	2	0.4-0.7	4	0.25-0.6
154	2	0.4-0.6	4	0.25-0.6
153	2	0.4-0.6	4	0.4-0.9
138	1	0.7	2	0.25-0.3
183	1	0.6	2	0.25-0.3
209	-		1	15.5

Total number of samples analyzed: 20 fish fillet, 11 fish feed. Although some PBDEs were detected in the oil samples, their levels were below the limit of quantification.

We did not find PBDEs in the oil samples analyzed at levels higher than the LOQ objective (1 µg kg^{-1}; 5 µg kg^{-1} BDE 209). This fact agrees with data reported by Domingo et al. [9], where total load of PBDEs from marine species, oils and fats and other components from the normal diet was low; e.g. the highest PBDE levels found in oils and fats was 0.59 µg kg^{-1}.

4. Conclusions

An efficient and sensitive analytical methodology for the determination of PBDEs in fat aquaculture matrices has been developed using an efficient clean-up step combining acid digestion and SPE-Florisil procedure previous to the injection of the extracts in GC-MS system operating in NCI ionization and SIR mode acquisition. The procedure applied allowed to reach LODs as low as 0.03-0.06 μg kg^{-1} in samples. The most difficult task in this work was to determine the BDE 209, as it required special chromatographic conditions to reach good peak shape and satisfactory sensitivity. The acquisition of three m/z ions in SIR mode offers a reliable confirmation of the identity of the PBDEs detected in samples. BDE 28 and 47 were the most commonly found in fish fillet and fish feed. Individual concentrations were always below 5 μg kg^{-1}, except for BDE 209 in one fish feed sample that reached up to 15.5 μg kg^{-1}.

Acknowledgements

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References

[1] S.D. Richardson, Anal. Chem. 80 (2008), pp. 4373-4402.

[2] A. Binelli, S.K. Sarkar, M. Chatterjee, C. Riva, M. Parolini, B.d. Bhattacharya, A.K. Bhattacharya and K.K. Satpathy, Mar. Pollut. Bull. 54 (2007), pp. 1220-1229.

[3] F. Hernández, T. Portolés, E. Pitarch and F.J. López, Anal. Chem. 79 (2007), pp. 9494-9504.

[4] S.D. Shaw and K. Kannan, Rev. Environ. Health 24 (2009), pp. 157-229.

[5] J. Wu, X. Luo, Y. Zhang, Y. Luo and S. Chen, Environ. Int. 34 (2008), pp. 1109-1113.

[6] C.M. Medina, E. Pitarch, F.J. López, C. Vázquez and F. Hernández, Anal. Bioanal. Chem. 390 (2008), pp. 1343-1354.

[7] C.M. Medina, E. Pitarch, T. Portolés, F.J. López and F. Hernández, J. Sep. Sci. 32 (2009), pp. 2090-2102.

[8] B.C. Kelly, M.G. Ikonomou, J.D. Blair and F.A.P.C. Gobas, Sci. Total Environ. 401 (2008), pp. 60-78.

[9] J.L. Domingo, R. Martí-Cid, V. Castell and J.M. Llobet, Toxicology 248 (2008), pp. 25-32.

[10] G.T. Yogui and J.L. Sericano, Environ. Int. 35 (2008), pp. 655-666.

[11] S. Voorspoels, A. Covaci and P. Schepens, Environ. Sci. Technol. 37 (2003), pp. 4348-4357.

[12] M.N. Jacobs, A. Covaci, A. Gheorghe and P. Schepens, J. Agric. Food Chem. 52 (2004), pp. 1780- 1788.

[13] A. Covaci, S. Voorspoels, L. Ramos, H. Neels and R. Blust, J. Chromatogr. A 1153 (2007), pp. 145-171.

[14] R. Serrano, M. Barreda, E. Pitarch and F. Hernández, J. Sep. Sci 26 (2003), pp. 75-86.

[15] R. Cariou, J.-P. Antignac and L. Debrauwer, J. Chromatogr. Sci 44 (2006), pp. 489-497.

[16] H.M. Stapleton, Anal. Bioanal. Chem 386 (2006), pp. 807-817.

[17] S. Bayen, H.K. Lee and J.P. Obbard, J. Chromatogr. A 1035 (2004), pp. 291-294.

[18] S.D. Shaw, M.L. Berger, D. Brenner, D.O. Carpenter, L. Tao, C. Hong and K. Kannan, Chemosphere 71 (2008), pp. 1422-1431.

[19] A.R. Fontana, M.F. Silva, L.D. Martínez, R.G. Wuilloud and J.C. Altamirano, J. Chromatogr. A 1216 (2009), pp. 4339-4346.

[20] J. Björklund, P. Tollbäch and C. Östman, J. Mass Spectrom. 38 (2003), pp. 394-400.

[21] A. Covaci, J. De Boer, J.J. Ryan, S. Voorspoels and P. Schepens, Anal. Chem. 74 (2002), pp. 790-798.

[22] A.M. Carro, R.A. Lorenzo, F. Fernández, R. Phan-Tan-Luu and R. Cela, Anal. Bioanal. Chem. 388 (2007), pp. 1021-1029.

[23] L. Roosens, A.C. Dirtu, G. Goemans, C. Belpaire, A. Gheorghe, H. Neels, R. Blust and A. Covaci, Environ. Int. 34 (2008), pp. 976-983.

[24] J. Hajšlová, J. Pulkrabová, J. Poustka, T. Cájka and T. Randák, Chemosphere 69 (2007), pp. 1195-1203.

[25] M. Frederiksen, K. Vorkamp, M. Thomsen and L.E. Knudsen, Int. J. Hyg. Environ. Health 212 (2009), pp. 109-134.

[26] N.M. Fajar, A.M. Carro, R.A. Lorenzo, F. Fernandez and R. Cela, Food Addit. Contam. A 25 (2008), pp. 1015-1023.

[27] Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (eur-lex.europa.eu/pri/en/oj/dat/2002/l_221/l_22120020817en00080036.pdf).

[28] F. Hernández, J.V. Sancho and O.J. Pozo, Anal. Bioanal. Chem. 382 (2005), pp. 934-946.

[29] I. Ferrer, E.T. Furlong and E.M. Thurman In: I. Ferrer and E.M. Thurman, Editors, Liquid Chromatography Mass Spectrometry/Mass Spectrometry, MS/MS and Time-of-Flight MS: Analysis of Emerging Contaminants, American Chemical Society Symposium Series 850, Oxford University Press, New York (2003), pp. 376-393.

[30] O.J. Pozo, J.V. Sancho, M. Ibáñez, F. Hernández and W.M.A. Niessen, Trends Anal. Chem. 25 (2006), pp. 1030-1042.

[31] M.W.F. Nielen, M.C. van Engelen, R. Zuiderent and R. Ramaker, Anal. Chim. Acta 586 (2007), pp. 122-129.

[32] J. Nácher-Mestre, R. Serrano, T. Portolés, F. Hernández, L. Benedito-Palos and J. Pérez-Sánchez, Rapid Commun. Mass Spectrom. 23 (2009), pp. 2075-2086.

[33] A. Covaci, S. Voorspoels and J. De Boer, Environ. Int. 29 (2003), pp. 735-756.

[34] Y.P. Liu, J.G. Li, Y.F. Zhao, Y.N. Wu and L.Y. Zhu, Food Addit. Contam. A 26 (2009), pp. 1180-1184.

[35] S. Losada, F.J. Santos and M.T. Galcerán, Talanta 80 (2009), pp. 839-845.

[36] D. Hayward, J. Wong and A.J. Krynitsky, Environ. Res. 103 (2007), pp. 46-54.

[37] A. Kierkegaard, L. Asplund, C.A. De Wit, M.S. McLachlan, G.O. Thomas, A.J. Sweetman and K.C. Jones, Environ. Sci. Technol. 41 (2007), pp. 417-423.

SUPPORTING DATA

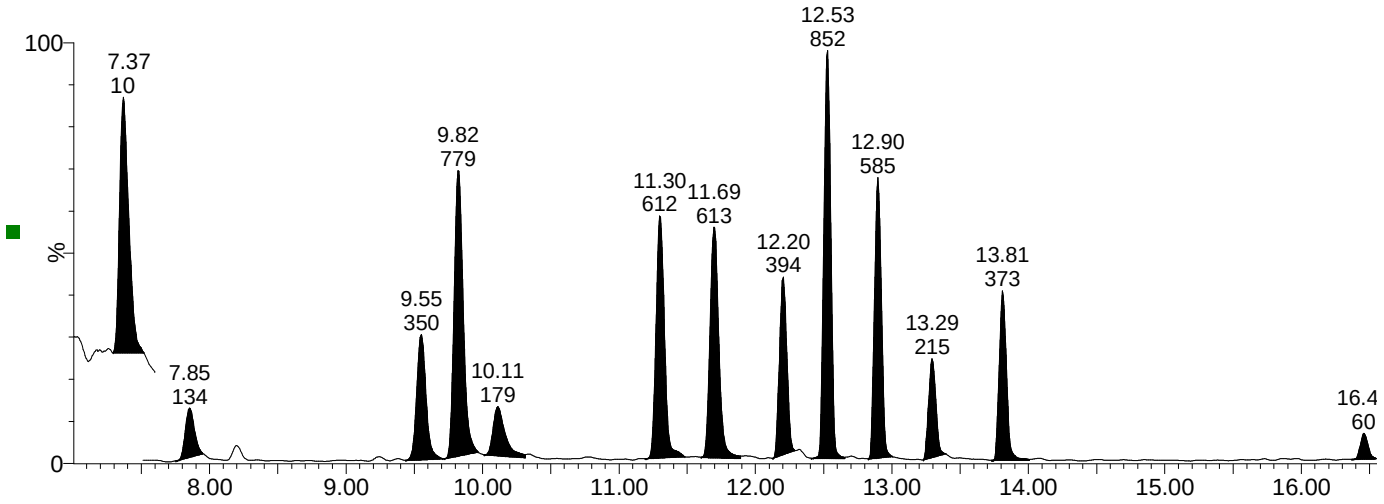

Figure 1. SIR chromatogram for the IS (DDE-D_8, m/z 289) and 12 PBDEs (m/z 79) spiked at the lowest level in fish feed (0.25μg/kg, and 1.25μg/kg for BDE 209).

SUPPORTING DATA

Table1. Recoveries of the overall analytical procedure for spiked samples when using different SPE or NPLC clean-up.

	Florisil				Silica				NPLC			
	0.25 µg/kg		1 µg/kg		0.25 µg/kg		1 µg/kg		0.25 µg/kg		1 µg/kg	
BDE	Filete	Pienso	Aceite pescado	Aceite lino	Filete	Pienso	Aceite pescado	Aceite lino	Filete	Pienso	Aceite pescado	Aceite lino
28	107(28)	92(6)	87(3)	79(11)	118(23)	115(16)	63(9)	73(12)	95(15)	82(4)	63(20)	102(15)
71	99(25)	95(16)	87(8)	86(13)	97(24)	145(9)	68(21)	78(15)	100(23)	105(11)	60(14)	102(12)
47	98(7)	80(4)	92(14)	89(19)	60(17)	129(18)	60(11)	78(23)	56(29)	64(28)	55(14)	116(13)
66	107(19)	103(3)	115(9)	105(7)	112(22)	122(10)	89(4)	98(7)	107(26)	73(8)	94(9)	135(16)
100	115(25)	104(6)	107(14)	102(18)	87(16)	137(11)	73(16)	93(20)	95(21)	107(2)	68(10)	126(14)
99	98(4)	119(5)	99(7)	119(14)	96(22)	130(25)	108(16)	118(21)	115(17)	105(14)	93(10)	172(17)
85	106(10)	115(4)	103(10)	98(16)	91(27)	175(13)	94(12)	90(14)	95(24)	110(5)	80(11)	104(9)
154	110(5)	117(9)	133(11)	101(10)	75(24)	184(9)	104(17)	108(20)	102(14)	110(5)	85(21)	144(15)
153	95(26)	108(13)	108(10)	108(3)	84(8)	170(8)	84(5)	110(22)	96(11)	117(3)	75(12)	155(15)
138	100(25)	102(6)	113(9)	111(3)	97(11)	145(6)	99(14)	113(24)	110(6)	93(3)	80(13)	140(14)
183	100(21)	110(5)	103(10)	109(2)	95(7)	165(11)	94(15)	113(20)	95(26)	120(4)	80(26)	149(25)
209	110(16)	84(15)	104(12)	106(9)	134(24)	159(25)	94(14)	118(17)	125(7)	73(28)	63(21)	107(16)

SUPPORTING DATA

Table 2. LODs obtained in the four sample matrices analyzed using different clean-up procedures.

	Florisil				Silica				NPLC			
Compound	Fish Fillet	Fish Feed	Fish Oil	Linseed Oil	Fish Fillet	Fish Feed	Fish Oil	Linseed Oil	Fish Fillet	Fish Feed	Fish Oil	Linseed Oil
28	0.04	0.04	0.15	0.09	0.05	0.07	0.13	0.16	0.08	0.04	0.16	0.12
71	0.04	0.10	0.07	0.05	0.04	0.20	0.08	0.05	0.07	0.09	0.06	0.05
47	0.10	0.12	0.24	0.16	0.06	0.16	0.19	0.21	0.15	0.09	0.16	0.27
66	0.10	0.19	0.25	0.20	0.10	0.21	0.19	0.24	0.30	0.06	0.28	0.28
100	0.03	0.12	0.10	0.05	0.05	0.16	0.08	0.05	0.06	0.10	0.06	0.05
99	0.07	0.07	0.08	0.12	0.05	0.08	0.14	0.11	0.10	0.09	0.08	0.13
85	0.06	0.07	0.13	0.12	0.06	0.12	0.13	0.12	0.20	0.07	0.12	0.11
154	0.05	0.03	0.08	0.08	0.04	0.07	0.05	0.05	0.04	0.10	0.04	0.07
153	0.06	0.05	0.11	0.09	0.07	0.07	0.12	0.08	0.09	0.07	0.07	0.12
138	0.08	0.05	0.15	0.16	0.08	0.08	0.16	0.13	0.10	0.04	0.15	0.19
183	0.04	0.05	0.06	0.07	0.06	0.06	0.05	0.05	0.05	0.06	0.06	0.09
209	0.70	0.90	0.90	0.85	3.30	1.23	2.90	2.30	2.41	2.23	2.15	2.50

4.3.3. Discusión de resultados.

Debido al gran uso de los PBDEs durante años y aún utilizados en gran cantidad de materiales en servicio hoy en día, su presencia en diferentes matrices ambientales ha sido puesta de manifiesto por varios autores (Kierkegaard et al., 2009). A pesar de que existen hasta 209 congéneres de PBDEs, no todos son frecuentemente encontrados en muestras ambientales. Generalmente los congéneres mayoritariamente identificados y cuantificados son el BDE-47 (*2,2',4,4'-tetrabromodiphenyl ether*), el BDE-99 (*2,2',4,4',5-pentabromodiphenyl ether*), BDE-100 (*2,2',4,4',6-pentabromodiphenyl ether*) y en muchos casos BDE-153 (*2,2',4,4',5,5'-hexabromodiphenyl ether*), BDE-154 (*2,2',4,4',5,6'-hexabromodiphenyl ether*) y el BDE (209) (*decabromodiphenyl ether*) (Lacorte et al., 2010; Zhang et al., 2010; Boon et al., 2002). Aunque son los PBDEs de mayor bromación los que presentan mayor tendencia a la bioacumulación y biomagnificación (BDEs con k_{ow}>4), los PBDEs de menor bromación (tetra-BDEs y penta-BDEs) suelen presentar niveles altos en muestras ambientales y por eso es también importante su análisis. La lista de PBDEs propuesta en nuestra metología ha sido elaborada en virtud de la presencia de estos PBDEs en las matrices seleccionadas procedentes de la acuicultura marina. Así pues, PBDEs con diferente grado de bromación han sido seleccionados desde el tri-BDE-28 hasta el deca-BDE-209. La metodología desarrollada permite la determinación de un mayor número de congéneres en comparación con algunas metodologías recientes en las que el estudio de PBDEs se centra únicamente en un grupo reducido de éstos (Fontana et al., 2009; Liu et al., 2009; Montes et al., 2009; Chen et al., 2010).

- Estudio de la etapa de purificación.

Como se ha indicado con anterioridad, gran parte de los interferentes presentes en las muestras de estudio, principalmente lípidos, dificultan el análisis de los compuestos orgánicos y concretamente de los PBDEs. La elección de un procedimiento de purificación adecuado que mejore la selectividad y sensibilidad del método analítico para la determinación de estos contaminantes en las muestras de estudio será un factor decisivo.

Es necesario un sistema de purificación eficaz que elimine las sustancias interferentes en la determinación cromatográfica, evitando daños en la columna y la contaminación de los detectores de masas. En este sentido, el tratamiento con ácido resulta una técnica que permite eliminar prácticamente la gran mayoría de los lípidos presentes en la matriz (Covaci et al., 2003). Serrano et al. (2003) ya propuso una metodología similar a la del presente estudio para eliminar la fracción lipídica de muestras procedentes de la acuicultura. Más del 90% de las grasas eran eliminadas mediante un tratamiento con H_2SO_4 (Serrano et al., 2003). A pesar de este primer tratamiento, la gran cantidad de lípidos presentes en las muestras inicialmente hace necesaria una etapa adicional de purificación. En el presente estudio, se han ensayado tres procedimientos de purificación diferentes, comparándose la eficacia de cada uno en la eliminación de interferentes de la matriz.

En concreto se ensayaron un sistema automatizado de purificación mediante NPLC, además de la SPE mediante cartuchos con fase estacionaria de Florisil (SPE-Florisil) y de silica (SPE-silica). Los tres procedimientos se aplicaron sobre extractos de las muestras de estudio después de su extracción y posterior tratamiento con ácido. Se realizaron tres réplicas de cada muestra a un nivel de fortificación de 0.25 µg/kg (filete de dorada y pienso) y de 1 µg/kg (aceite de pescado y aceite de lino). Estos extractos purificados se analizaron mediante GC-(NCI)MS y se compararon los resultados atendiendo a los LOD, LOQ, recuperación absoluta y efecto matriz.

Los resultados mostraron que la etapa de purificación mediante SPE-Florisil resultó más eficaz que las otras técnicas por conseguir una mayor eficacia de purificación. Además el proceso consume menos tiempo y menor gasto de disolventes. Igualmente, los LODs globales obtenidos para las diferentes etapas fueron óptimos en todos los casos excepto para el BDE 209 en donde los mejores LODs fueron obtenidos con SPE-Florisil (**ver Tabla 2, Supporting Data**, **Artículo científico 2**). Las recuperaciones absolutas obtenidas para las diferentes muestras fortificadas al nivel bajo, mediante los tres procedimientos de purificación, pueden verse en la **Tabla 1** (**Supporting Data**, **Artículo científico 2**). Como se puede observar, estos valores son mejores mediante el procedimiento de SPE-Florisil en comparación con los obtenidos utilizando SPE-silica y NPLC que, para el caso de algunos PBDEs, presentaban valores muy elevados.

La aplicación de una segunda etapa de purificación posterior al tratamiento con H_2SO_4 resultó muy eficaz. Se redujeron de una manera notable los interferentes, lo que permitió alcanzar LODs a los niveles deseados.

- Optimización GC-MS.

Respecto a la determinación instrumental, se seleccionó el modo de ionización NCI en lugar de EI, puesto que la sensibilidad en modo CI era mayor. Este hecho fue contrastado con bibliografía disponible (Eljarrat et al., 2002; Covaci et al., 2003). El desarrollo de una metodología con fragmentación en MS/MS en modo NCI fue rechazada puesto que el espectro de masas resultante era muy poco significativo por presentar únicamente los fragmentos $[Br]^-$ y $[HBr_2]^-$. La única transición posible para la mayoría de PBDEs resultó ser la fragmentación de $[HBr_2]^-$ para observar $[Br]^-$. Adquiriendo en modo SIR los iones $[Br]^-$ y $[HBr_2]^-$ se obtuvo mayor sensibilidad que para la fragmentación en MS/MS por lo que se decidió trabajar en modo SIR adquiriendo tres iones por compuesto (**ver Tabla 1, Artículo científico 2**). La adquisición de tres iones en modo SIR ofreció una buena confirmación de la identidad de los PBDEs en las muestras. Cabe destacar que los iones m/z_1=79, m/z_2=81 procedentes del fragmento $[Br]^-$ eran los más sensibles en comparación a m/z_3=161 del fragmento $[HBr_2]^-$.

- Determinación del PBDE 209.

El PBDE 209 es el difenil éter polibromado de mayor bromación lo que hace que tenga unas características físico-químicas específicas como son su pobre volatilidad y la posibilidad de degradación en los inyectores y en las columnas del sistema cromatográfico. Como consecuencia, este compuesto presenta más dificultades que ningún otro PBDE en su análisis mediante GC-MS (Stapleton et al., 2006; Covaci et al., 2007). Por este motivo no se ha incluido frecuentemente al PBDE 209 en metodologías publicadas (Borghesi et al., 2009; Liu X. et al., 2009; Bianco et al., 2010) o se obtienen recuperaciones no satisfactorias en algunos casos (Labadie et al., 2010a). El PBDE 209 es también el compuesto que presenta

una menor sensibilidad por lo que, comparándolo con los demás PBDEs de la lista, puede llegar a ser incluso 5 veces menos sensible (**Figura 4.3**). Esto hizo necesario el estudio de la validación de este compuesto a un nivel 5 veces mas concentrado que para el resto de los PBDEs. Por su degradabilidad en el sistema cromatográfico es conveniente disminuir, en la medida de lo posible, el tiempo de residencia en el sistema. Aplicando una rampa de temperaturas rápida y alcanzando los 340°C se consiguió, mediante una columna DB-1HT de 15 m, que el tiempo de elución y el tiempo de residencia fuera reducido. Otros autores también han sugerido la necesidad de utilizar columnas cortas para la determinación de los PBDEs con mayor índice de bromación, obteniendo resultados satisfactorios (Lacorte et al., 2010; Labadie et al., 2010b).

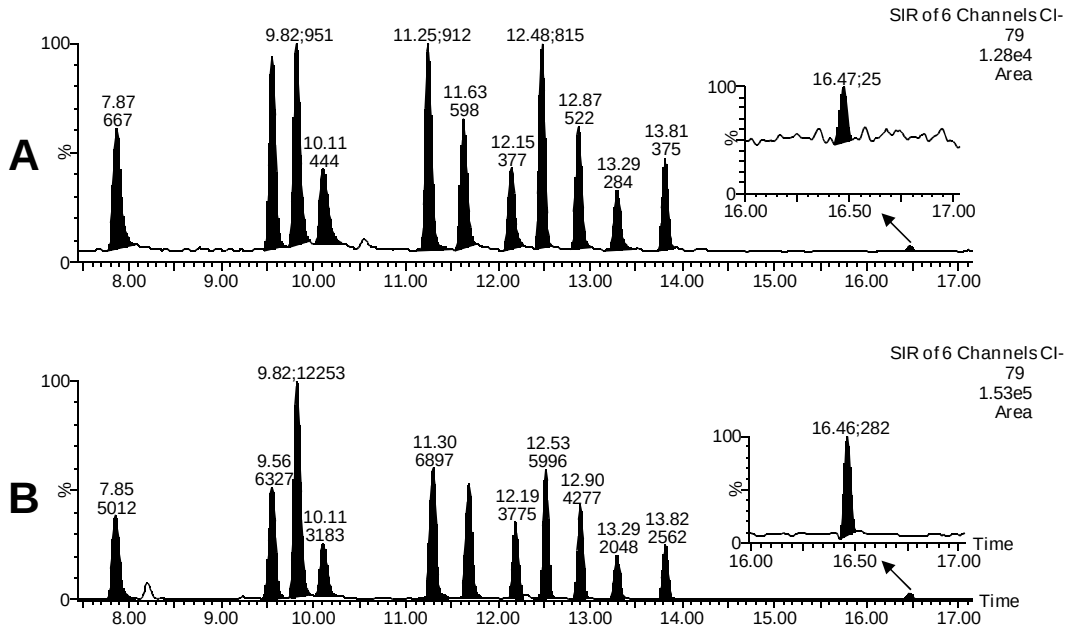

Figura 4.3. Cromatogramas GC-(NCI)MS. Mezcla patrón de referencia a 0.5 µg/L (2.5 µg/L en BDE 209) en hexano (A). Blanco de pienso fortificado a 0.25 µg/kg (1.25 µg/kg en BDE 209)(B).

- Validación del método analítico.

La metodología desarrollada fue validada en cuatros matrices: filete de dorada, pienso, aceite de pescado y aceite de lino. Como el contenido lipídico en aceites era aproximadamente cuatro veces mayor que en filetes y pienso y durante el procedimiento los extractos de muestras de aceite iban a ser diluidos para minimizar el efecto matriz, la valización en aceites se llevó a cabo a una concentración cuatro veces mayor: 1 μg kg^{-1} y 10 μg kg^{-1} (5 μg kg^{-1} y 50 μg kg^{-1} para BDE 209). El proceso de validación se llevó a cabo atendiendo a los parámetros propuestos por la guía SANCO vigente (Document N° SANCO/2007/3131).

- Aplicación de la metodología desarrollada a muestras reales.

Una vez desarrollado y validado el método analítico mediante GC-(NCI)MS, éste se aplicó al análisis de muestras reales. Se analizaron 20 muestras de filete de dorada que habían sido alimentadas mediante piensos con diferente composición procedentes del proyecto "AQUAMAX". También se analizaron 11 piensos con diferente carga lipídica y composición de aceites vegetales y de origen animal. Por último, la metodología también se aplicó para el análisis de cuatro aceites: aceite de pescado, aceite de lino, aceite de colza y aceite de palma.

En general los valores de PBDEs encontrados en nuestro estudio fueron muy bajos (0.25-4.7 μg/kg en filete, 0.5-15.5 μg/kg en piensos). En algunos casos, las muestras no fueron cuantificadas por presentar concentraciones por debajo del LOQ propuesto (0.25 μg/kg en filete de pescado y pienso, 1 μg/kg en aceites; para el BDE 209 estos valores fueron 1.25 y 5 μg/kg, respectivamente).

Los ingredientes de origen marino, tradicionalmente usados en la alimentación de pescado comercial, son la fuente de contaminantes en el pescado cultivado. El uso de materias primas sostenibles y seleccionadas con niveles bajos de PBDEs da lugar a piensos y peces cultivados con niveles bajos de estos compuestos. Los valores propuestos en este estudio (**ver Tabla 3, Artículo científico 2)** son comparables con los valores encontrados

por otros autores que revelan poca contaminación en peces cultivados utilizando materias primas con baja carga de contaminantes (Berntssen et al., 2010).

4.3.4. Referencias.

Berntssen, M.H.G.; Julshamn, K.; Lundebye, A.-K. 2010. Chemical contaminants in aquafeeds and Atlantic salmon (Salmo salar) following the use of traditional- versus alternative feed ingredients. Chemosphere 78, 637-646.

Bianco, G., Novario, G., Anzilotta, G., Palma, A., Mangone, A., Cataldi, T.R.I. 2010. Polybrominated diphenyl ethers (PBDEs) in Mediterranean mussels (Mytilus galloprovincialis) from selected Apulia coastal sites evaluated by GC-HRMS. J. Mass Spectrom. 45, 1046-1055.

Boon, J.P.; Lewis, W.E.; Tjoen-A-Choy, M.R.; Allchin, C.R.; Law, R.J.; De Boer, J.; Ten Hallers-Tjabbes, C.C.; Zegers, B.N. 2002. Levels of polybrominated diphenyl ether (PBDE) flame retardants in animals representing different trophic levels of the North Sea food web. Environ. Sci. Technol. 36, 4025-4032.

Borghesi, N., Corsolini, S., Leonards, P., Brandsma, S., de Boer, J., Focardi, S. 2009. Polybrominated diphenyl ether contamination levels in fish from the Antarctic and the Mediterranean Sea. Chemosphere 77, 693-698.

Chen, M.Y.Y.; Tang, A.S.P.; Ho, Y.Y.; Xiao, Y. 2010. Dietary exposure of secondary school students in Hong Kong to polybrominated diphenyl ethers from foods of animal origin. Food Addit. Contam. 27, 521-529.

Covaci, A.; Voorspoels, S.; de Boer, J. 2003. Determination of brominated flame retardants, with emphasis on polybrominated diphenyl ethers (PBDEs) in environmental and human samples. Environ. Int. 29, 735-756.

Covaci, A.; Voorspoels, S.; Ramos, L.; Neels, H.; Blust, R. 2007. Recent developments in the analysis of brominated flame retardants and brominated natural compounds. J. Chromatogr. A 1153, 145-171.

Document N° SANCO/2007/3131. 2007. Method validation and quality control procedures for pesticide residues analysis in food and feed.

Eljarrat, E.; Lacorte, S.; Barcelo, D. 2002. Optimization of congener-specific analysis of 40 polybrominated diphenyl ethers by gas chromatography/mass spectrometry. J. Mass Spectr. 37, 76-84.

Fontana, A.R.; Silva, M.F.; Martínez, L.D.; Wuilloud, R.G.; Altamirano, J.C. 2009. Determination of polybrominated diphenyl ethers in water and soil samples by cloud point extraction-ultrasound-assisted back-extraction-gas chromatography-mass spectrometry. J. Chromatogr. A 1216, 4339-4346.

Frederiksen, M.; Vorkamp, K.; Thomsen, M.; Knudsen, L.E. 2009. Human internal and external exposure to PBDEs - A review of levels and sources. Int. J. Hyg. Envir. Heal. 212, 109-134.

Kierkegaard, A.; Sellström, U.; McLachlan, M.S. 2009. Environmental analysis of higher brominated diphenyl ethers and decabromodiphenyl ethane. J. Chromatogr. A 1216, 364-375.

Labadie, P.; Tlili, K.; Alliot, F.; Bourges, C.; Desportes, A.; Chevreuil, M. 2010a. Development of analytical procedures for trace-level determination of polybrominated diphenyl ethers and tetrabromobisphenol A in river water and sediment. Anal. Bioanal. Chem. 396, 865-875.

Labadie, P.; Alliot, F.; Bourges, C.; Desportes, A.; Chevreuil, M. 2010b. Determination of polybrominated diphenyl ethers in fish tissues by matrix solid-phase dispersion and gas chromatography coupled to triple quadrupole mass spectrometry: Case study on European eel (Anguilla anguilla) from Mediterranean coastal lagoons. Anal. Chim. Acta 675, 97-105.

Lacorte, S.; Ikonomou, M.G.; Fischer, M. 2010. A comprehensive gas chromatography coupled to high resolution mass spectrometry based method for the determination of polybrominated diphenyl ethers and their hydroxylated and methoxylated metabolites in environmental samples. J. Chromatogr. A 1217, 337-347.

Liu, X., Hu, J., Huang, C., Wang, H., Wang, X. 2009. Determination of polybrominated diphenyl ethers in aquatic animal tissue using cleanup by freezing-dispersive liquid-liquid microextraction combined with GC-MS. J. Sep. Sci. 32, 4213-4219.

Liu, Y.P.; Li, J.G.; Zhao, Y.F.; Wu, Y.N.; Zhu, L.Y. 2009. Rapid determination of polybrominated diphenyl ethers (PBDEs) in fish using selective pressurized liquid extraction (SPLE) combined with automated online gel permeation chromatography-gas chromatography mass spectrometry (GPC-GC/MS). Food Addit. Contam. A 26, 1180-1184.

Montes, R.; Rodríguez, I.; Cela, R. 2010. Solid-phase microextraction with simultaneous oxidative sample treatment for the sensitive determination of tetra- to hexa-brominated diphenyl ethers in sediments. J. Chromatogr. A 1217, 14-21.

Serrano, R.; Barreda, M.; Pitarch, E.; Hernández, F. 2003. Determination of low concentrations of organochlorine pesticides and PCBs in fish feed and fish tissues from aquaculture activities by gas chromatography with tandem mass spectrometry. J. Sep. Sci. 26, 75-86.

Stapleton, H.M. 2006. Instrumental methods and challenges in quantifying polybrominated diphenyl ethers in environmental extracts: A review. Anal. Bioanal. Chem. 386, 807-817.

Talsness, C.E. 2008. Overview of toxicological aspects of polybrominated diphenyl ethers: A flame-retardant additive in several consumer products. Environ. Res. 108, 158-167.

Vonderheide, A.P.; Mueller, K.E.; Meija, J; Welsh, G.L. 2008. Polybrominated diphenyl ethers: Causes for concern and knowledge gaps regarding environmental distribution, fate and toxicity. Sci. Total Environ. 400, 425-436.

Zhang, B.-Z.; Ni, H.-G.; Guan, Y.-F.; Zeng, E.Y. 2010. Occurrence, bioaccumulation and potential sources of polybrominated diphenyl ethers in typical freshwater cultured fish ponds of South China. Environ. Pollut. 158, 1876-1882.

CAPÍTULO 5

DESARROLLO DE METODOLOGÍAS *SCREENING* BASADAS EN CROMATOGRAFÍA DE GASES ACOPLADA A ESPECTROMETRÍA DE MASAS CON ANALIZADOR DE TIEMPO DE VUELO PARA LA IDENTIFICACIÓN DE CONTAMINANTES ORGÁNICOS.

5.1. Introducción

El mar, los ríos y, en general, todas las aguas superficiales han sido usadas tradicionalmente como medio de evacuación de los residuos humanos. Los ciclos biológicos del agua aseguran la reabsorción de estos restos orgánicos. Actualmente, la cantidad de materia orgánica generada por el hombre sobrepasa, en ocasiones, el potencial depurador de los mecanismos naturales. Además, ya no son solamente estos residuos orgánicos los que son arrojados a los ríos y a los mares sino que también se desechan productos químicos nocivos que destruyen la vida animal y vegetal acuática. Estos compuestos orgánicos pueden llegar a depositarse en cualquier organismo o superficie asociado al agua, por lo que es probable la presencia de diferentes familias de compuestos orgánicos, distribuidos en el medio ambiente.

El presente capítulo muestra el gran potencial del acoplamiento GC-TOF MS para investigar la presencia de contaminantes orgánicos en muestras ambientales. En los últimos años, el uso de instrumentos de elevada resolución de masa como el TOF-MS ha ido creciendo paulatinamente, siendo utilizado y reconocido como instrumento de medida en el análisis de muestras ambientales, biológicas y alimentos. Esta técnica ofrece la posibilidad de estudiar la presencia de compuestos *target* (compuestos diana) y también de obtener información estructural sobre compuestos *non-target* (compuestos desconocidos) presentes en la muestra analizada. Para ello, los analizadores TOF MS presentan una gran sensibilidad y velocidad de adquisición en modo de adquisición de espectros completos *(full spectra acquisition)* junto a medidas de masa con elevada exactitud (errores de masa del orden de mDa).

Desde el punto de vista analítico, se pretende afrontar la investigación de contaminantes orgánicos, trabajando con GC-TOF MS, mediante tres metodologías de *screening*:

- *Target screening*: los analitos son seleccionados previamente a la adquisición por MS. Únicamente se identifican los compuestos seleccionados a priori.

- *Post-target screening*: se adquiere el espectro completo de todos los compuestos eluidos del sistema cromatográfico mediante MS. Los fragmentos iónicos seleccionados se extraen a partir del cromatograma completo de la muestra.

- *Non-target screening*: El objetivo es investigar la presencia de compuestos de los que se desconoce su identidad y presencia en las muestras problema.

En la primera parte de este capítulo, se aplica metodología "*non-target*" a partir de la cual se pretende realizar una búsqueda de contaminantes orgánicos, no seleccionados previamente, y que pueden estar presentes en las muestras de análisis (apartado 5.2).

En la segunda parte del capítulo se va a desarrollar y evaluar una metodología *target* para el estudio de OPEs en sales y posteriormente la metodología desarrollada se aplica al análisis de éstos en muestras de sal y muestras acuosas mediante GC-TOF MS (apartado 5.3).

5.2. *Screening* de contaminantes orgánicos en muestras de sal y agua de mar

5.2.1. Introducción.

En este capítulo, se ha empleado el acoplamiento GC-TOF MS para la caracterización de la contaminación presente en sales marinas procedentes de salinas de la costa mediterránea. Tal y como se explicó en la introducción de esta Tesis, el detector TOF MS proporciona una alta sensibilidad y elevada resolución de masa proporcionando espectros de iones completos medidos con buena exactitud de masa (<3mDa). Las cualidades de estos equipos sofisticados han permitido aplicaciones relevantes dentro de estudios medioambientales tanto con GC o LC obteniendo resultados satisfactorios (Ferrer et al., 2003; Čajka et al., 2004; Portolés et al., 2007). Recientemente los analizadores de masas TOF han sido utilizados para el *screening* de plaguicidas en muestras ambientales (Feo et al., 2010; Grimalt et al., 2010). Estos trabajos resaltan la capacidad de los analizadores TOF para trabajar con diferentes enfoques analíticos (*target*, *non-target*). Uno de los inconvenientes de este tipo de analizadores es su elevado precio y la necesidad de ser manipulado por personal realmente especializado. También su menor rango lineal para la cuantificación, así como su menor sensibilidad en comparación con los analizadores QqQ en modo SRM, podrían ser inconvenientes a añadir. Estas deficiencias parecen mejorarse con los analizadores TOF de nueva generación.

Hoy día, han sido publicadas metodologías analíticas suficientemente selectivas y precisas para la identificación de compuestos diana (análisis *target*) mediante la utilización de un analizador TOF (Dallüge et al., 2002; Hernández et al., 2009). Estas metodologías permiten una identificación inequívoca de la presencia de estos compuestos en las muestras estudiadas. Es por ello que se considera una herramienta muy adecuada para el análisis de muestras procedentes del campo medioambiental, biológico y alimentario, que a menudo contienen cientos, o aún miles, de compuestos orgánicos.

Actualmente, la lista de compuestos analizados y controlados mediante metodologías *target* equivale a una fracción muy pequeña de la amplia lista de contaminantes químicos existentes (Muir et al., 2006; Howard et al., 2010). Las prestaciones de un analizador TOF permiten ampliar nuestro conocimiento sobre la posible

presencia de estos contaminantes en muestras ambientales, obteniendo resultados satisfactorios. El procesamiento y tratamiento de datos con el analizador TOF es complejo ya que se genera mucha información que debe ser interpretada correctamente.

Para el análisis de compuestos desconocidos, el analizador TOF presenta una serie de herramientas de procesamiento de datos que permiten buscar, comparar e identificar rápidamente los compuestos en matrices complejas. Este procesamiento de datos se basa en una serie de algoritmos que permiten una exploración de datos exhaustiva para obtener un nivel máximo de fiabilidad en la identificación. TOF MS permite la detección de un gran número de compuestos y garantiza una elevada fiabilidad para la identificación de éstos gracias a la adquisición de información espectral completa.

En el artículo que se incluye a continuación, se presenta una aplicación *non-target* para el estudio de la posible presencia de contaminantes orgánicos en muestras salinas y de agua de mar mediante la técnica GC-TOF MS.

5.2.2. Artículo de investigación 3.

Non-target screening of organic contaminants in marine salts by gas chromatography coupled to high-resolution time-of-flight mass spectrometry

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Non-target screening of organic contaminants in marine salts by gas chromatography coupled to high-resolution time-of-flight mass spectrometry

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Abstract

Gas chromatography coupled to high-resolution time-of-flight mass spectrometry (GC-TOF MS) has been applied to characterize the organic pollution pattern of marine salt samples collected in saltworks from the Spanish Mediterranean coast. After dissolving the samples in water, the solution was subjected to solid-phase extraction reaching an overall 250-preconcentration factor. The screening proposed allowed detecting sample components, which identity was established by accurate mass measurements and by comparison of the full-acquisition spectrum with theoretical MS libraries. Several organic pollutants were identified in the marine salt samples. Some plasticizers, potentially toxic to humans, and fragrances, included among the group of pharmaceuticals and personal care products were detected. The contamination pattern present in the samples suggests a notable input of pollutants from industries, farms and/or urbanized areas running off into the coastal environment where sea water is pumped. The presence of these pollutants in the marine salt samples suggests that they remain after the crystallization process. GC-TOF MS was proven to be a powerful technique for wide-scope screening of organic contaminants in order to monitoring the quality of marine salts thanks to the sensitive full-spectrum acquisition at accurate mass, which facilitates the reliable identification of many GC-MS ionizable compounds in the samples.

Keywords: marine salt, saltworks, saltpans, gas chromatography, mass spectrometry, non-target screening.

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1. Introduction

Marine salt is obtained by evaporation of sea water due to the combined effect of wind blow and sunlight heat in the solar saltworks. Saltpans are located near the sea, becoming peculiar environments inhabited by wildlife species associated to high salinity conditions. Concern has arisen as consequence of the vulnerability of these environments to anthropogenic pollution. Run-off from farms and industries may contain high concentrations of pesticides and industrial sub-products and reach these vulnerable coastal locations, with a deleterious impact on the briny aquatic systems (Ferrando et al., 1991). This fact can also affect to the quality of the marine salt produced. Several authors have reported the presence of contaminants in coastal sea and saline waters, such as pesticides (Hernández et al., 1996), halocarbons, aliphatic and aromatic hydrocarbons and ketones (Silva et al., 2009). Badoil and Benanou et al. (2009) have detected phenols, phosphates and other volatile and semi-volatile compounds in waste landfill leachates, which reach coastal waters. Contaminants produced by anthropogenic activities are transported by rivers and water flows from wastewater treatment plants and are frequently deposited on coastal locations like salt marshes or river estuaries and deltas. Several authors have detected a variety of contaminants in these vulnerable areas (Wang et al., 2001; Navarro et al., 2009). The marine salts obtained from saltpans can contain the contaminants present in sea water, provided that they remain after the concentration and crystallisation processes. As a consequence, monitoring the presence of organic contaminants in marine salts seems necessary to have a realistic knowledge of their quality.

Hyphenation of gas chromatography (GC) with mass spectrometry (MS) is the most widely used and accepted technique for determination of volatile and semivolatile compounds of low-medium polarity in aquatic ecosystems, particularly in surface coastal water and marine environments. Different MS analyzers have been applied for this purpose, from single quadrupole to ion-trap or triple quadrupole, although the two later allow working under tandem MS mode (Hernández et al., 1996; Silva et al., 2009; Bravo-Linares et al., 2007; Pitarch et al., 2007). Recently, Silva et al. (2009) reported a methodology based

on head space solid phase microextraction and GC-quadrupole mass spectrometry for the analysis of volatile compounds in marine salt, able to detect 40 volatile compounds belonging to different chemical groups.

The wide majority of methods reported until now in the environmental field are focused on a limited list of target contaminants. Even in the case that target pollutants investigated belong to priority lists, target methods do not allow the wide-scope screening required to investigate a large number of compounds that might be present in the samples. In most target methods, other non-selected contaminants would not be detected due to the specific-analyte information acquired. Although conventional MS analyzers can also work under scan mode, their capability to detect organic contaminants at low levels in complex-matrix samples is rather limited due to their low sensitivity and selectivity and their nominal mass measurements.

The recent irruption of modern high-resolution time-of-flight (TOF) analyzers opens new perspectives to develop wide-scope screening methodologies. GC-TOF MS offers interesting features for this purpose, as it combines high full-spectrum sensitivity and elevated mass resolution making feasible the accurate mass measurements of the molecular and/or fragments ions of any GC-amenable compound present in the sample. This technique allows searching organic contaminants in a post-target (i.e. searching for selected compounds after MS acquisition) and also in a non-target way (i.e. searching for unknowns, without any kind of compounds selection) (Hernández et al., 2010). GC-TOF MS has been successfully applied for screening, identification and elucidation of organic pollutants in environmental water and biological samples (Hernández et al., 2007, 2009), and also for confirmation of pollutants in highly complex matrix like wastewater (Ellis et al., 2007).

In this work, we have applied GC-TOF MS for the rapid and wide-scope screening of organic pollutants in sea water and in marine salts obtained from solar saltworks and from a pristine sea shore salt marsh sited along the Spanish Western Mediterranean coast. The identity of the sample components detected in a non-target way was established by means of exact mass measurements and by comparison with theoretical spectral libraries. In

addition, the organophosphate esters (OPEs) identified were confirmed by injecting reference standards.

2. Material and methods

2.1. Sampling points.

Marine salt samples from four solar saltworks sited in the Spanish Mediterranean shore (see **Figure 1**) were collected directly from the crystallized salt stock in saltpans (samples 3 and 5) or purchased from the producers (samples 1 and 4). A seawater sample was also collected from the sea shore in front of a pristine salt marsh located in Torre la Sal, neighbouring a natural protected area (Natural Park of Ribera de Cabanes, Spain), sited close to the city of Castellon (Sampling point 2). Sampling point 1 is a solar saltwork sited in the Alfaques bay, south of the Ebro River delta. This river receives domestic and industrial wastewater from numerous minor settlements along its way. Discharges into the Ebro River vary at different locations, showing an increase downstream, probably due to inputs from the tributaries or natural recharge of the stream, and finally it flows into the Mediterranean sea after crossing through the Ebro Delta (Bouza-Deaño et al., 2008). Sampling point 3 is a solar saltwork located in the vicinity of an important fishing and middle trade harbour, surrounded by a highly urbanized area. Sampling points 4 and 5 are solar saltworks sited in high valuable natural areas but neighbouring important summer touristic areas. All samples were stored at -20°C until analysis.

2.2. Reagents.

HPLC-grade water was obtained from a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). Acetone, Ethyl Acetate, Dichlorometane (DCM) and n-Hexane (ultra trace quality) used in solid-phase extraction (SPE) experiments were purchased from Scharlab (Barcelona, Spain). Bond Elut cartridges C18 (500 mg) (Varian, Harbor City, CA, USA) were used for SPE. Triphenyl phosphate (TPhP) and 2-Ethylhexyl diphenyl phosphate (EHDPP) reference standards were purchased from TCI Europe (Zwijndrecht, Belgium). Tri-

n-buthyl phosphate (TBP) and Tris(1-chloro-2-propyl) phosphate (TCPP) reference standards were purchased from Sigma-Aldrich (Madrid, Spain).

2.3. GC-TOF MS instrumentation.

GC system (Agilent 6890N; Agilent Palo Alto, USA) equipped with an autosampler (Agilent 7683) was coupled to a time-of-flight mass spectrometer (GCT, Waters Corporation, Manchester, U.K.), operating in electron ionization (EI). GC separation was performed using a fused silica HP-5MS capillary column with a length of 30 m, an internal diameter of 0.25 mm and a film thickness of 0.25 µm (J&W Scientific, Folson, CA, USA). The injector temperature was set to 280°C. Splitless injections of 1 µL samples were carried out. Helium (99.999%; Carburos Metálicos, Valencia, Spain) was used as carrier gas at a flow rate of 1 mL/min. The interface and source temperature were set to 250°C and a solvent delay of 4 min was selected.

The oven program in GC-TOF MS analysis was programmed as follows: 90 °C (1min); 5 °C/ min to 300 °C (2 min). The TOF MS operated at 1 spectrum/s, acquisition rate over the mass range m/z 50-650, using a multichannel plate voltage of 2850 V. TOF-MS resolution was approximately 7000 (FWHM). Heptacosa standard, used for the daily mass calibration and as lock mass, was injected via syringe in the reference reservoir at 30°C for this purpose; the m/z ion monitored was 218.9856. The application manager ChromaLynx and TargetLynx was used to process the qualitative data obtained from standards and from sample analysis. Library search was performed using the commercial NIST library.

2.4. Recommended analytical procedure.

The sample procedure was based on the previously applied for the determination of around 50 compounds, including organochlorine and organophosphorus insecticides, herbicides, polychlorinated biphenyls, polyciclic aromatic hydrocarbons, brominated diphenyl ethers, octyl/nonyl phenols and pentachlorobenzene with some modifications (Pitarch et al., 2007). Briefly, 62.5 g of salt were diluted with water to a final volume of 250 mL and

filtered. The filtered solution was passed through the C_{18} SPE cartridge, previously conditioned by passing 6 mL methanol, 6 mL ethyl acetate:DCM (50:50), 6 mL methanol and 6 mL water avoiding dryness. After loading the sample (250 mL), the cartridges were washed with 3 mL water and dried by passing air under vacuum for at least 15 min. The elution was performed by passing 5 mL ethyl acetate:DCM (50:50). The extract collected was evaporated under a gentle nitrogen stream at 40°C and redisolved in 0.25 mL of n-hexane. The overall procedure also involved a method blank to test that no contamination was introduced in the extracts along the analysis.

2.5. GC-TOF MS methodology for non-target screening.

GC-TOF MS non-target screening was carried out by using the ChromaLynx Application Manager. This software was used to detect the presence of multiple components and to show its deconvoluted MS spectra to be submitted to library search routine (in our case NIST library). Components are reduced to a list of possible candidates by using the list factor from the mass library search (library match >700). Then, accurate mass confirmation is automatically performed. The formula from the library list is submitted to an Elemental Composition calculator and accurate mass measurements of (up to) 5 abundant ions are evaluated for confirmation/rejection of the finding (for more details see Hernández et al., 2007, 2010).

3. Results and Discussion

The analytical methodology described was applied to the analysis of one sea water and four marine salt samples collected from different solar saltworks located along the Spanish Mediterranean coast. The sensitive and reliable qualitative analysis was feasible thanks to the 250-fold pre-concentration in the sample, with low sample handling as corresponds to the SPE procedures, which was combined with the advantages offered by GC-TOF MS. The procedure applied allowed the confirmation of the compounds' identity of at estimated concentrations around sub-ng/Kg, which was tested for some reference standards of the

compounds detected. As shown in this paper and confirmed in our previous works (Hernández et al., 2007, 2009), the non-target methodology applied for screening organic contaminants is able to detect and identify a large number of GC-amenable compounds belonging to different chemical families. However, a genuine non-target analysis is a laborious and time-consuming task, as a consequence of the huge amount of chromatographic peaks from the sample components and to the lack of list of compounds to be searched. Therefore, the use of advanced processing software is required to facilitate this task. This software should be able to detect relevant/abundant sample components and to confirm their identity making use of the accurate full-spectrum data provided by TOF MS. Although a part of the process can be performed (almost) in an automated way, the expertise of the analyst on MS spectra interpretation and the knowledge of the MS fragmentation rules are needed for a successful analysis (Hernández et al., 2010).

The samples analyzed contained volatile and semi volatile compounds, including industrial sub-products, pesticides, flame retardants, plasticizers and personal care products (**Table 1**). This kind of contaminants have been also found in other studies related to water pollution, and they are into the environment as a consequence of anthropogenic activities (Badoil et al., 2009; Navarro et al., 2009; Hernández et al., 2007; Martínez-Carballo et al., 2007; Reemtsma et al., 2008; Matamoros et al., 2009).

Table 1 shows the contamination pattern observed in the marine salt and seawater samples studied in this work. The seawater sample collected from the sea shore at Torre la Sal (S2), considered as a protected natural area relatively free of contaminant sources, was almost free of the contaminants found in marine salt, and only two alkyl phenols and one organic acid were identified. On the contrary, the marine salt samples were more contaminated. The type of compounds detected seemed to vary according to the geographical location of the saltwork.

As an illustrative example, **Table 2** shows the confirmation of identity of the compounds detected in “Santa Pola” salt sample (S3). The elemental composition could be proposed for at least 4 m/z fragment ions based on accurate mass measurements. In addition, the experimental accurate mass for the main ions was compared with the

theoretical ones. In general, mass errors were below 3 mDa, except for a few low-abundant ions. An example of the non-target detection of TCPP in "San Pedro del Pinatar" salt sample is given in **Figure 2**. Five ions were selected from the EI spectrum for the accurate mass confirmation of the identity of TCPP, with mass errors always below 2.6 mDa. In addition, the chemical structure suggested for these ions was in agreement with that of TCPP.

It is worth to notice that several of the compounds detected belong to the OPEs family. These chemicals are produced in large quantities for their use as flame retardants, plasticizers and also as pesticides. Their widespread use and presence in host materials led to a continuous discharge and distribution through wastewaters (Reemtsma et al., 2008), and coastal areas are the fate of wastewaters from industrial and urban activities containing these and other pollutants. As a consequence of the toxicity and environmental persistence of OPEs, their presence in marine salt intended for human consumption should be under control to assure its right quality.

Considering the interest of OPEs, reference standards of TCPP, TBP, TPhP and EHDPP were acquired in a subsequent step to perform additional experiments for confirmation. We could not find the reference standard of bis(1-chloro-2-propyl) (3-chloro-1-propyl)phosphate, which was also detected in the non-target screening. Using reference standards it was feasible to test the retention time and to obtain their TOF MS spectrum to unequivocally confirm the presence of these compounds in the samples. The experiments with reference standards allowed us to confirm all positives previously reported by TOF MS, demonstrating the excellent potential of this technique for identificative purposes, even without reference standards.

As an illustrative example, **Figure 3** shows the eXtracted Ion Chromatograms (XICs) for the positive of EHDPP detected in "La Trinitat Saltwork" salt sample (S1) which could be additionally confirmed using the reference standard. The presence of the chromatographic peaks in the XICs, at the expected retention time, and the attainment of all Q/q ratios when comparing with the reference standard allowed the confirmation of the finding in the sample. The corresponding EI accurate mass spectra generated by TOF MS are also shown. Mass errors for four representative ions were below 3.2 mDa, which gave more confidence

to the confirmation process. Chemical structures for the most abundant fragment ions were suggested based on the elemental compositions proposed accordingly to the accurate mass measurements given by the instrument.

Apart from OPEs, the most abundant compounds detected were alkyl phenols. Fragrances and plasticizers were also identified in some salt samples. The presence of alkylphenols in aquatic environments has been previously reported by several authors (Hernández et al., 2010; Sheldon et al., 1978; During et al., 2002). They are degradation products from alkylphenol polyethoxylates, mainly applied to pesticide formulations and as plastic additives, among other uses (Badoil et al., 2009). The persistence and accumulation properties of alkylphenols have led to their wide distribution in different environmental compartments (Vazquez-Duhalt et al., 2006; Correa-Reyes et al., 2007). The sources of these pollutants are commonly the wastewaters from industrial and municipal treatment plants (USEPA, 2005) and their accumulation has been observed in sediments receiving contaminated water flows (Petrovic et al., 2002). Data obtained in the present work suggest that these compounds have been accumulated in salts after the crystallization process. The presence of these pollutants might pose a threat to the quality of the salt produced in saltworks sited in environments like deltaic and estuarine locations receiving water flows from industrial and/or urbanized areas. In fact, most detections of alkylphenols corresponded to sampling points 1 and 3 (which accomplish these characteristics; see description in Experimental section). Recently, Navarro et al. (Navarro et al., 2009), making use of GC-MS with single quadrupole, have detected several of these compounds in the Ebro River sediments, in which delta the sampling point 1 is sited, as indicated above.

The presence of Di-(2ethylhexyl)adipate in marine salts is also of concern. This compound is used as plasticizer for food packaging, and it is considered as carcinogen and endocrine disruptor (Rahman et al., 2009; Ghisari et al., 2009). Another plasticizer detected, and also considered as endocrine disruptor, was benzyl butyl phthalate. This compound has been previously reported to be present in marine sediments (Xu et al., 2009). Butylated hydroxytoluene (BHT) was detected also in samples 3 and 4. This compound is an antioxidant widely used as food aditive and in biological samples for storage before

analysis, as well as in cosmetics, pharmaceuticals, jet fuels, among other uses (Badoil et al., 2009), and it has been found in aquatic environments (Hernández et al., 2007; Fries et al., 2002; Gómez et al., 2009).

2-Oxohexamethylenimine (caprolactam) -the monomer of nylon-6- has been identified in marine salt samples probably due to the use of ammonium sulphate (a sub product obtained during the manufacture of the polymer) in growing crops as fertilizer. Methyl dihydrojasmonate and galaxolide were other compounds detected. They are used as fragrances, and are included in the group of water contaminants called Pharmaceuticals and Personal Care products (PPCPs), which are suspected to be an environmental problem still not well known (Daughton et al., 1999). Similarly to other organic contaminants, these compounds could be removed entirely or partly by means of adequate technologies of wastewater treatment (Matamoros et al., 2009). Dihydroactinidiolide, detected in sample 4, is a volatile terpene occurring naturally in a variety of plants and insects, but it has also been prepared synthetically for its use as a fragrance (Yao et al., 1998).

Other relevant compounds detected in marine salt were benzophenone and 3-methyl-benzophenone, used as photoinitiator in UV-curing applications and as UV filter (Badoil et al., 2009); cyclic octaatomic sulfur, indicator of microbiological activity (Badoil et al., 2009); and nonanoic acid, used in the preparation of plasticizers and lacquers, and also as herbicide.

All identifications reported in this work were supported by accurate mass measurements of several EI ions (up to five in most of the cases), by the low mass errors observed in relation to their theoretical exact masses, and by the compatibility of the chemical structures proposed for these ions with the chemical structure of the compound identified.

The contamination pattern observed in the marine salt samples includes up to 25 organic compounds, with around 12 of them being present in every sample. Sources of these contaminants surely are run offs from industries, farms and urbanized areas. Our findings suggest an important input of these pollutants into the environments around the

saltpans, which is in agreements with data reported in similar areas (Daughton et al 1999; Ternes et al., 2004; Muñoz et al., 2008). The presence of the compounds identified in the marine salt samples indicates that they are concentrated and that they persist along the crystallization process.

4. Conclusion

Without using any previous list of compounds to be investigated, the non-target methodology applied in this work has allowed the detection and reliable identification of several relevant contaminants of anthropogenic origin, belonging to quite different chemical groups. The strong potential of GC-TOF MS for qualitative purposes comes from the full spectrum acquisition at accurate mass, with satisfactory sensitivity, provided by this instrument. Making an appropriate use of all relevant information given by this technique it has been feasible to identify many contaminants in a reliable way, even without reference standards being available, as illustrated in this work. Surely, several of the compounds detected in marine salt would not had been detected using a target approach, as although relevant they might not have been included in a target screening, which is typically focused on a limited list of priority pollutants.

In the light of the results reported, we can conclude that priority pollutants, typically subjected to strict control, constitute only part of the large chemical pollution puzzle. There is a diverse group of unregulated pollutants, including industrial sub-products, PPCPs, and an increasing concern on the risks that they pose on humans and on the environment.

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References

Badoil, L., Benanou, D., 2009. Characterization of volatile and semivolatile compounds in waste landfill leachates using stir bar sorptive extraction-GC/MS. Analytical and Bioanalytical Chemistry 393, 1043-1054.

Bravo-Linares, C. M., Mudge, S. M., Loyola-Sepulveda, R. H., 2007. Ocurrence of volatile organic compounds (VOCs) in Liverpool Bay, Irish Sea. Marine Pollution Bulletin 54, 1742-1753.

Bouza-Deaño R., Ternero-Rodríguez, M., Fernández-Espinosa A. J., 2008.Trend study and assessment of surface water quality in the Ebro River (Spain). Journal of Hydrology 361, 227-239.

Correa-Reyes, G., Viana, M. T., Marquez-Rocha, F. J., Licea, A. F., Ponce, E., Vazquez-Duhalt, R., 2007. Nonylphenol algal bioaccumulation and its effect through the trophic chain. Chemosphere 68, 662-670.

Daughton C. G., Ternes T. A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change. Environmental Health Perspectives 107, 907-938.

During, R. A., Krahe, S., Gath, S., 2002. Sorption Behaviour of nonylphenol in terrestrial soils. Environmental Science & Technology 36, 4052-4057.

Ellis, J., Shah, M., Kubachka, K. M., Caruso, J. A., 2007. Determination of organophosphorus fire retardants and plasticizers in wastewater samples using MAE-SPME with GC-IPCMS and GC- TOF MS detection. Journal of Environmental Monitoring 9, 1329-1336.

Ferrando, M. D., Andreu-Moliner, E., 1991. Acute lethal toxicity of some pesticides to Brachionus calyciflorus and Brachionus plicatilis. Bulletin of Environmental contamination and Toxicology 47, 479-484.

Fries, E., Püttmann, W., 2002.Analysis of the antioxidant butylated hydroxytoluene (BHT) in water by means of solid phase extraction combined with GC/MS. Water Research 36, 2319-2327.

Ghisari, M., Jorgensen, E. C. B., 2009.Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicology Letters 189, 67-77.

Gómez, M. J., Gómez-Ramos, M. M., Agüera, A., Mezcua, M., Herrera, S., Fernández-Alba, A.R., 2009. A new gas chromatography/mass spectrometry method for the simultaneous analysis of target and non-target organic contaminants in waters. Journal of Chromatography A 1216, 4071-4082.

Hernández, F., Serrano, R., Miralles, M. C., Font, N., 1996.Gas and liquid chromatography and enzyme linked immunosorbent assay in pesticide monitoring of surface water from the Western Mediterranean (Comunidad Valenciana, Spain). Chromatographia 42, 151-158.

Hernández, F., Portolés, T., Pitarch, E., Lopez, F. J., 2007.Target and non target screening of organoc micropollutants in water by solid-phase microextraction combined with gas chromatography/high-resolution time-of-flight mass spectrometry. Analytical Chemistry 79, 9494-9504

Hernández, F., Portolés, T., Pitarch, E., López, F. J., 2009. Searching for anthropogenic contaminants in human breast adipose tissues using gas chromatography-time-of-flight mass spectrometry. Journal of Mass Spectrometry 44, 1-11.

Hernández, F., Portolés, T., Pitarch, E., Lopez, F. J., 2010. Gas Chromatography/High-resolution mass spectrometry: An advanced analytical tool to investigate the presence of organic compounds at trace levels in environmental, food safety and toxicology fields. Submitted to TRAC-Trends in Analytical Chemistry. September.

Martínez-Carballo, E., González-Barreiro, C., Sitka, A., Scharf, S., Gans, O., 2007. Determination of selected organophosphate esters in the aquatic environment of Austria. Science of the Total Environment 388, 290-299.

Matamoros, V., Arias, C., Brix, H., Bayona, J. M., 2009. Preliminary screening of small-scale domestic wastewater treatment systems for removal of pharmaceuticals and personal care products. Water Research 43, 55-62.

Muñoz, I., Gómez, M. J., Molina-Díaz, A., Huijbregts, M. A. J., Fernández-Alba, A. R., García-Calvo, E., 2008. Ranking potential impacts of priority and emerging pollutants in urban wastewater through life cycle impact assessment. Chemosphere 74, 37-44.

Navarro, A., Endo, S., Gocht, T., Barth, J. A. C., Lacorte, S., Barceló, D., Grathwohl, P., 2009. Sorption of alkylphenols on Ebro River sediments: Comparing isotherms with field observations in river water and sediments. Environmental Pollution 157, 698-703.

Petrovic, M., Lacorte, S., Viana, P., Barceló, D., 2002. Pressured liquid extraction followed by liquid chromatography-mass spectrometry for the determination of alkylphenolic compounds in river sediment. Journal of Chromatography A 959, 15-23.

Pitarch, E., Medina, C., Portolés, T., López, F.J., Hernández, F., 2007. Determination of priority micro-polutants in water by gas chromatography coupled to triple quadrupole mass spectrometry. Analytica Chimica Acta 583, 246-258.

Rahman, M. B. A., Chaibakhsh, N., Basri, M., Salleh, A. B., Rahman, R. N. Z. R. A., 2009. Application of artificial network for yield prediction of lipase-catalyzed synthesis of dioctyl adipate. Applied Biochemistry and Biotechnology 158, 722-735.

Reemtsma, T., Quintana, J. B., Rodil, R., Garcia-Lopez, M., Rodriguez, I., 2008. Organophosphorus flame retardants and plasticizers in water and air. I. Occurrence and fate. TRAC-Trends in Analytical Chemistry 27, 727-737.

Sheldon, L. S., Hites, R. A., 1978. Organic compounds in the Delaware River. Environmental Science & Technology 12, 1188-1194.

Silva, I., Rocha, S. M., Coimbra, M. A., 2009. Head solid phase microextraction and gas chromatography-quadrupole mass spectrometry methodology for analysis of volatile compounds of marine salt as potential origin biomarkers. Analytica Chimica Acta 635, 167-174.

Ternes, T. A., Joss A., Siegrist, H., 2004.Scrutinizing pharmaceuticals and personal care products in wastewater treatment. Environmental Science & Technology 38, 392-399.

USEPA. 2005. Aquatic Life Ambient Water Quality Criteria – Nonylphenol. U.S. Environmental Protection Agency, Office of Water: Washington, DC.

Vazquez-Duhalt, R., Marquez-Rocha, F. J., Ponce, E., Licea, A. F., Viana, M. T., 2006. Nonylphenol, an integrated vision of a pollutant. Applied Ecology and Environmental Research 4, 1-25.

Wang, Z., Fingas, M., Owens, E. H., Sigouin, L., Brown, C. E., 2001. Long-term fate and persistence of the spilled Metula oil in a marine salt marsh environment. Degradation of petroleum biomarkers. Journal of Chromatography A. 926, 275-290.

Xu, X-R., Li, X. Y., 2009. Sorption behaviour of benzyl butyl phthalate on marine sediments: Equilibrium assessments, effects of organic carbon content, temperature and salinity. Marine Chemistry 115, 66-71.

Yao, S., Johannsen, M., Hazell, R. G., Jorgensen, K. A., 1998. Total synthesis of (R)-Dihydroactiniolide and (R)-Actiniolide using asymmetric catalytic hetero-diels-alder methodology. Journal of Organic Chemistry 63, 118-121.

Table 1. Compounds identified in marine salt and seawater sample.

Compound	CAS Number	S1	S2(w)	S3	S4	S5	Observations[a]
1-[4-(1-methylethenyl)phenyl]-Ethanone	5359-04-6	X			X	X	Industrial sub-product
2,4-di-tert-butylphenol	96-76-4		X	X			Toxic and dangerous for the environment, highly flammable, harmful and irritant.
2-[(Z)-3-hydroxy-3-methyl-1-butenyl]phenol	17235-14-2		X				Industrial sub-product
2-phenoxyethanol	122-99-6				X		Anesthesic
2-oxohexamethylenimine (Caprolactam)	105-60-2	X		X	X	X	Toxic by ingestion, inhalation, or absorption through the skin.
3,5-di-tert-butylphenol	1138-52-9				X		Antioxidants and light-protection agents
3,6-di-tert-butyl-4-ethylphenol	4130-42-1	X		X		X	Non toxic
3-methyl-benzophenone	134-84-9				X	X	UV filter. UV-curing applications
4,5,7-trichloro-2-methyl- benzofuran	18628-11-0				X	X	Pesticide
4-tert-amylphenol	80-46-6			X			Intermediate for organic mercury germicides pesticides and chemicals used in rubber and petroleum industries
4 -tert-octylphenol	140-66-9	X		X			Acutely very toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment
Benzophenone	119-61-9	X			X		UV filter. UV-curing applications
Benzyl butyl phthalate	85-68-7					X	Plasticizer. Toxic effects such as cellular necrosis
Bis (1-chloro-2-propyl) (3-chloro-1-propyl) phosphate	137909-40-1	X		X			Pesticide, toxic and irritating
BHT	128-37-0			X	X		Synthethic antioxidant
Cyclic octaatomic sulfur	10544-50-0				X		Microbiological activity indicator[a]
Di (2-ethylhexyl) adipate	103-23-1					X	human carcinogen
Dihydroactinidiolide	17092-92-1				X		Volatile terpene (large structure hydrocarbon)
EHDPP	1241-94-7	X		X			Pesticide, toxic to aquatic organisms
Galaxolide	1222-05-5	X			X		Musk fragrance
Methyl dihydrojasmonate	24851-98-7	X	X	X			Musk fragrance
Nonanoic acid	112-05-0			X			Irritant
TBP	126-73-8	X		X	X	X	Used as a herbicide and fungicide
TCPP	13674-84-5	X		X	X	X	Pesticide, flame retardant
TPhP	115-86-6	X		X			Pesticide, plasticizer and flame retardant

S1: Delta del Ebro (La Trinitat Saltworks), Tarragona; S2(w): Sea water from Torre la Sal Sea shore, Castellón; S3: Santa Pola Saltworks, Alicante; S4: Torrevieja Saltworks, Alicante; S5: San Pedro del Pinatar Saltworks, Murcia; [a] International Chemical Safety Cards: www.inchem.org/documents

Table 2. Confirmation of organic compounds in S3 salt sample.

Compound	N° CAS	Molecular peak		Ion 1		Ion 2		Ion 3		Ion 4		Ion 5	
		Molecular formula	Molecular mass	Elemental composition	Experimental m/z (error in mDa)	Elemental composition	Experimental m/z (error in mDa)	Elemental composition	Experimental m/z (error in mDa)	Elemental composition	Experimental m/z (error in mDa)	Elemental composition	Experimental m/z (error in mDa)
2,4-di-tert-butylphenol	96-76-4	$C_{14}H_{22}O$	206.1671	$C_{14}H_{22}O$	206.1660 (-1.1)	$C_{13}H_{20}O$	192.1483 (-3.1)	$C_{13}H_{19}O$	191.1443 (0.7)	$C_{11}H_{15}O$	163.1120(-0.3)	$C_9H_{11}O$	135.0802 (-0.8)
3,6-di-tert-butyl-4-ethylphenol	4130-42-1	$C_{16}H_{26}O$	234.1984	$C_{16}H_{26}O$	234.2013 (2.9)	$C_{15}H_{24}O$	220.1836 (0.9)	$C_{15}H_{23}O$	219.1760 (1.1)	C_6H_6	78.0442 (-2.8)		
4-tert-octylphenol	140-66-9	$C_{14}H_{22}O$	206.1671	$C_9H_{12}O$	136.0857 (-3.1)	$C_9H_{11}O$	135.0798 (-1.2)	C_8H_7O	119.0475 (-2.2)	C_7H_7O	107.0496(-0.1)		
4-tert-amylphenol	80-46-6	$C_{11}H_{16}O$	164.1201	$C_{10}H_{13}O$	149.0969 (0.3)	$C_9H_{11}O$	135.0810 (0)	C_8H_9O	121.0666 (1.3)	C_7H_7O	107.0486(-1.1)	C_6H_7O	95.0477 (-2.0)
TCPP	13674-84-5	$C_9H_{18}Cl_3O_4P$	326.0008	$C_5H_{11}O_4PCl$	201.0100 (1.6)	$C_3H_7O_3PCl$	156.9818 (-0.3)	$C_2H_6O_4P$	125.0001 (-0.3)	H_4O_4P	98.9826 (-2.1)	C_2H_5OP	76.0058 (-3.0)
TPhP	115-86-6	$C_{18}H_{15}O_4P$	326.0708	$C_{18}H_{15}O_4P$	326.0737 (2.9)	$C_{18}H_{14}O_4P$	325.0656 (2.6)	C_6H_6O	94.0458 (3.9)	C_6H_5	77.0375 (-1.6)		
TBP	126-73-8	$C_{12}H_{27}O_4P$	266.1647	$C_4H_{12}O_4P$	155.0480 (0.7)	$C_2H_6O_4P$	125.0019 (1.5)	H_4O_4P	98.9830 (-1.7)	C_4H_8	56.0567 (-5.9)	$C_8H_{20}O_4P$	211.1109 (1.0)
Bis (1-chloro-2-propyl) (3-chloro-1-propyl) phosphate	137909-40-1	$C_9H_{18}Cl_3O_4P$	326.0008	$C_3H_7ClO_3P$	156.9818 (-0.3)	$C_2H_6O_4P$	125.0003 (-0.1)	$C_2H_3Cl_2O_3P$	116.9509 (0.1)	H_4O_4P	98.9828 (-1.9)	C_2H_5OP	76.0048 (3.0)
EHDPP	1241-94-7	$C_{20}H_{27}O_4P$	362.1647	$C_{12}H_{12}O_4P$	251.0457(-1.6)	$C_{12}H_{10}O$	170.0750 (1.8)	C_8H_{16}	112.1253 (0.1)	C_6H_6O	94.0412 (-0.7)	C_6H_5	77.0366 (-2.5)
BHT	128-37-0	$C_{15}H_{24}O$	220.1827	$C_{15}H_{24}O$	220.1861 (3.4)	$C_{14}H_{21}O$	205.1611 (1.9)	$C_{11}H_{13}O$	161.0978 (1.2)	$C_{11}H_{13}$	145.0983(-3.4)		
2-oxohexamethylenimine (Caprolactam)	105-60-2	$C_6H_{11}NO$	113.0841	$C_6H_{11}NO$	113.0822 (-1.9)	C_4H_7NO	85.0555 (2.7)	C_5H_8O	84.0547 (-2.8)	C_2H_2NO	56.0169 (3.3)	C_2HNO	55.0116 (5.8)
Methyl dihydrojasmonate	24851-98-7	$C_{13}H_{22}O_3$	226.1569	$C_{13}H_{22}O_3$	226.1601 (3.2)	$C_8H_{12}O_3$	156.0773(-1.3)	$C_{10}H_{17}O$	153.1274(-0.5)	C_5H_7O	83.0504 (0.7)		
Nonanoic acid	112-05-0	$C_9H_{18}O_2$	158.1307	$C_7H_{13}O_2$	129.0920 (0.4)	$C_6H_{11}O_2$	115.0756(-0.3)	$C_3H_5O_2$	73.0258 (-3.2)	$C_2H_4O_2$	60.0182 (-2.9)		

Figure captions

Figure 1. Area of study and sampling points

Figure 2. Detection of TCPP in the salt sample S5 by GC-TOF MS non-target screening. (A) Extracted ion Chromatograms for five fragment ions. (B) Library mass spectrum of TCPP at nominal mass. (C) Experimental EI accurate mass spectrum of the positive finding of TCPP. Chemical structures proposed for the five most abundant EI fragment ions and mass errors.

Figure 3. GC-TOF MS extracted ion chromatograms (top) at different m/z (mass window 0.02 Da) and accurate mass spectrum (bottom) for EHDPP for the reference standard (left) and for one positive salt sample (right). Q, qualitative ion; q, confirmative ion; St, reference standard; S, sample; Q/q ratio within tolerance limits. Chemical structures proposed for the most abundant fragment ions.

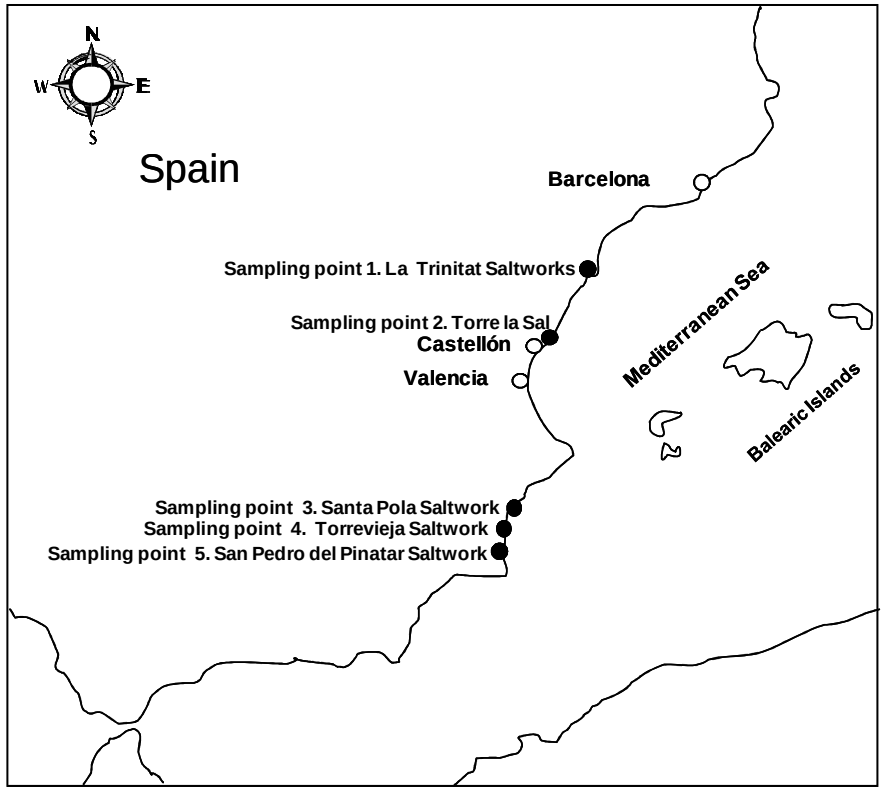
N
W
E
S
Spain
Barcelona
Sampling point 1. La Trinitat Saltworks
Sampling point 2. Torre la Sal
Castellón
Valencia
Mediterranean Sea
Balearic Islands
Sampling point 3. Santa Pola Saltwork
Sampling point 4. Torrevieja Saltwork
Sampling point 5. San Pedro del Pinatar Saltwork

Figure 1.

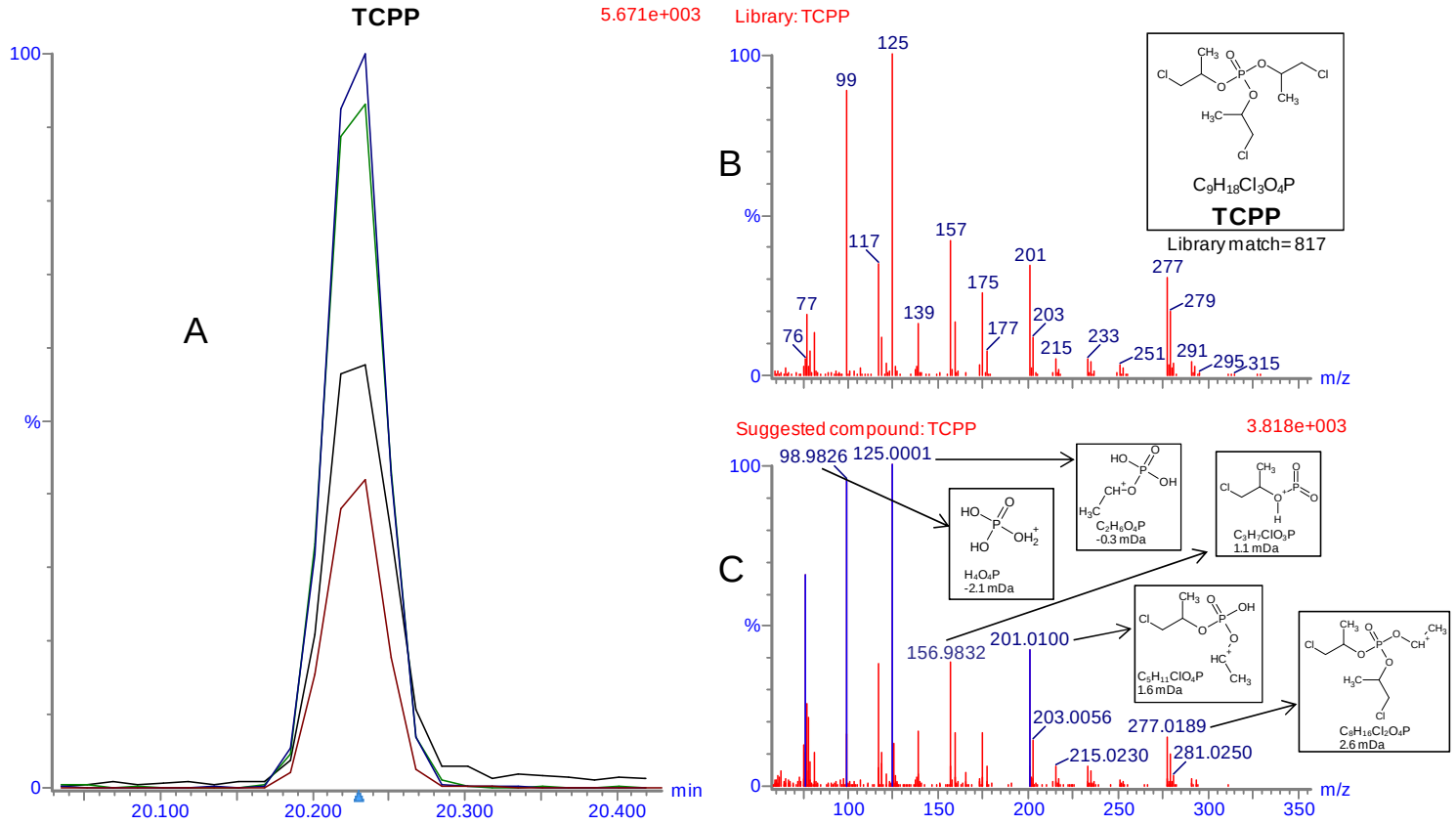
TCPP
5.671e+003
A
100
%
0
20.100
20.200
20.300
20.400
min
Library: TCPP
B
99
125
117
157
201
175
77
139
177
203
215
233
251
277
279
291
295
315
76
C9H18Cl3O4P
TCPP
Library match= 817
Suggested compound: TCPP
3.818e+003
C
98.9826
125.0001
H4O4P
-2.1 mDa
C2H6O4P
-0.3 mDa
C3H7ClO3P
1.1 mDa
156.9832
201.0100
C5H11ClO4P
1.6 mDa
203.0056
277.0189
C8H16Cl2O4P
2.6 mDa
215.0230
281.0250
100
150
200
250
300
350
m/z

Figure 2

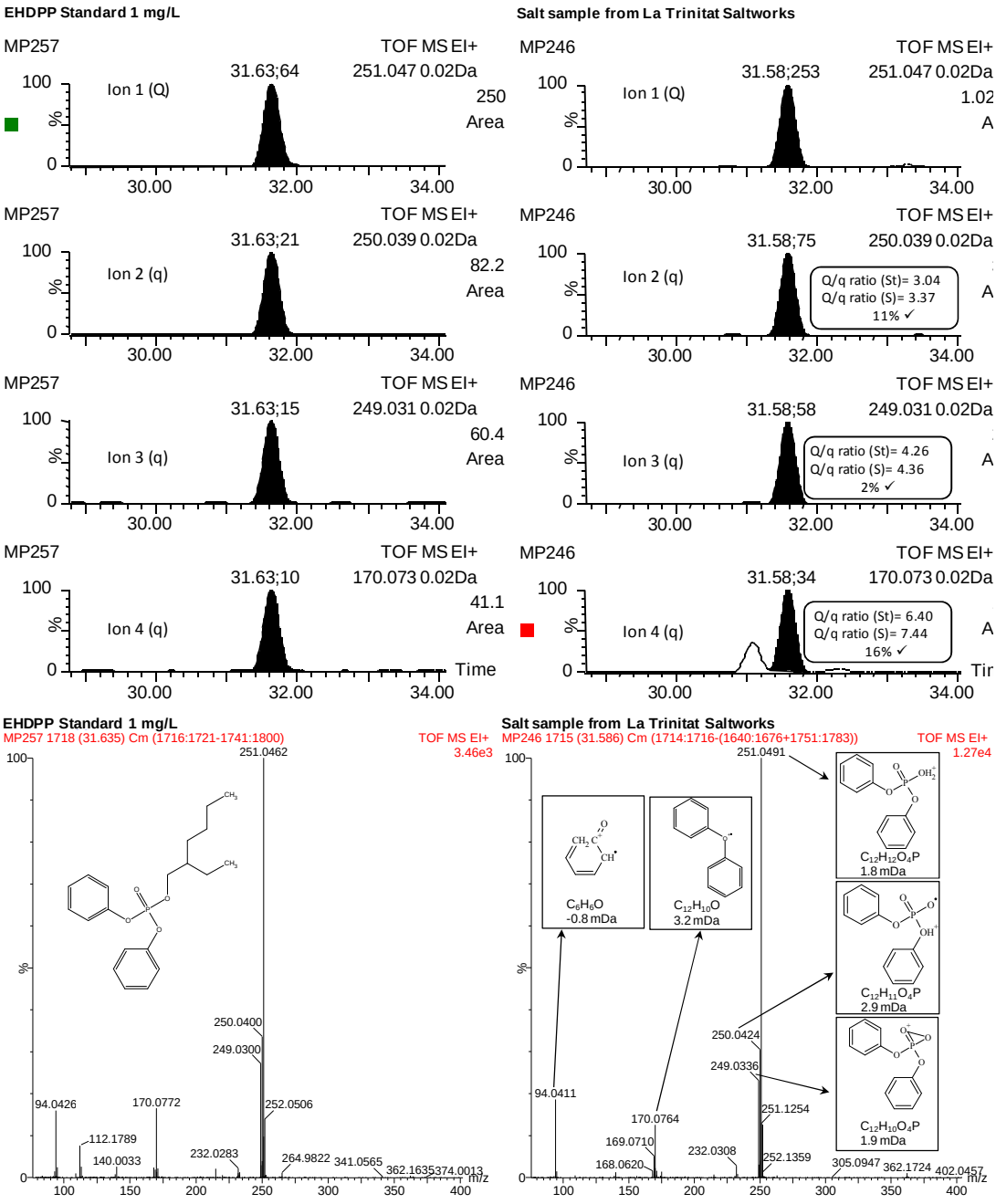
EHDPP Standard 1 mg/L
MP257
TOF MS EI+
Ion 1 (Q)
31.63;64
251.047 0.02Da
Ion 2 (q)
31.63;21
250.039 0.02Da
Ion 3 (q)
31.63;15
249.031 0.02Da
Ion 4 (q)
31.63;10
170.073 0.02Da
Area
Time
Salt sample from La Trinitat Saltworks
MP246
31.58;253
31.58;75
31.58;58
31.58;34
Q/q ratio (St)= 3.04
Q/q ratio (S)= 3.37
11% ✓
Q/q ratio (St)= 4.26
Q/q ratio (S)= 4.36
2% ✓
Q/q ratio (St)= 6.40
Q/q ratio (S)= 7.44
16% ✓
MP257 1718 (31.635) Cm (1716:1721-1741:1800)
3.46e3
251.0462
250.0400
249.0300
94.0426
170.0772
252.0506
MP246 1715 (31.586) Cm (1714:1716-(1640:1676+1751:1783))
1.27e4
251.0491
C6H6O
-0.8 mDa
C12H10O
3.2 mDa
C12H12O4P
1.8 mDa
C12H11O4P
2.9 mDa
C12H10O4P
1.9 mDa
250.0424
249.0336
94.0411
170.0764
169.0710
168.0620
232.0308
251.1254
252.1359
305.0947
362.1724
402.0457
m/z

Figure 3

5.2.3. Discusión de los resultados.

La metodología analítica desarrollada fue llevada a cabo para el análisis de contaminantes en muestras con elevado contenido salino, concretamente en sales marinas y agua de mar. El tratamiento de las muestras se basó en una purificación y posterior concentración mediante SPE. Partiendo de una muestra con elevado contenido salino (aproximadamente 25% sal) se obtenía un extracto final concentrado 250 veces (**ver Figura 5.1**). Posteriormente, el extracto se introducía en el sistema GC-TOF MS para el *screening* de contaminantes orgánicos.

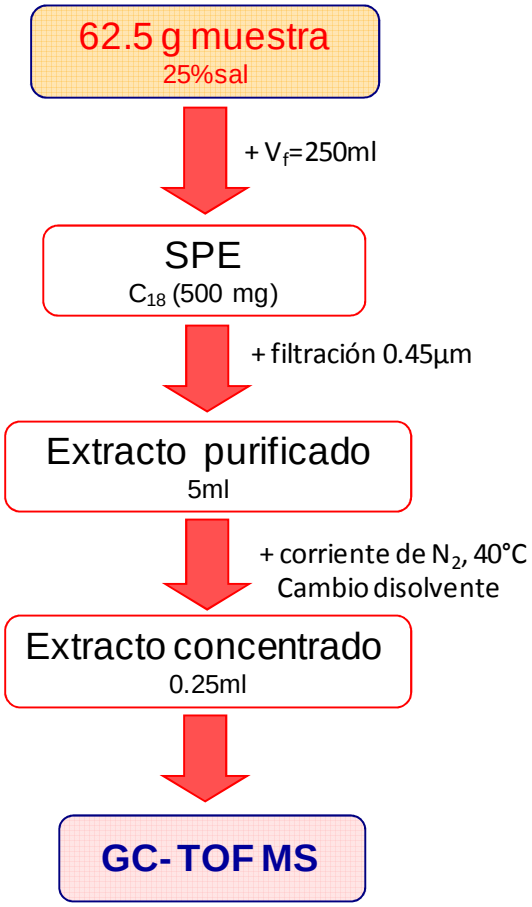

Figura 5.1. Proceso experimental para el *screening* de contaminantes orgánicos.

Utilizando la metodología anterior, ha sido posible identificar un gran número de contaminantes. Cabe destacar la identificación de compuestos presentes en derivados plásticos procedentes de la industria como son los compuestos fenólicos y también compuestos OPs. El *di(2-ethylhexyl) adipate* ha sido encontrado en una muestra salina, componente generalmente asociado a productos plásticos. De hecho, este compuesto ha sido encontrado en diferentes alimentos, incluyendo alimento infantil, debido a la migración de los componentes plásticos que envuelven a los alimentos (Petersent y Breindahl et al., 2000). El *di(2-ethylhexyl) adipate*, al igual que el *benzyl butyl phthalate*, encontrados en la misma muestra, son compuestos considerados como disruptores endocrinos y su estudio es de gran importancia por ser compuestos de riesgo para la salud humana (Ghisari et al., 2009). Recientemente, la presencia de compuestos como el *galaxolide* y los OPs también ha sido reportada por otros autores señalando que se tratan de compuestos ampliamente extendidos en el medio ambiente y por ello se debe controlar su presencia (Schwarzbauer y Ricking et al., 2010). Por otro lado, cabe destacar que no fueron detectados en las muestras compuestos POPs del tipo PAH, PBDE, OC o PCB mediante el *screening non-target*. Entre los compuestos detectados, algunos presentan diferentes riesgos medioambientales y en humanos debido a su toxicidad (**ver Tabla 1. Artículo científico 5**).

- *Non-target screening* mediante GC-TOF MS.

La metodología de *screening* desarrollada en este estudio permitió la identificación de contaminantes presentes en las muestras y la posibilidad de obtener su composición elemental. La elevada exactitud de masa de los analizadores TOF junto a su excelente sensibilidad y selectividad en el análisis de compuestos orgánicos a niveles de traza, resulta una herramienta esencial para llevar a cabo una identificación satisfactoria. Como ejemplo, la **Figura 5.2.** muestra información relevante para un posible positivo de TPhP en una muestra salina localizada en el Delta del Ebro. En la Figura se presenta una superposición de los iones principales, seleccionados automáticamente por el software para la identificación del compuesto propuesto (**A**). El software identifica un pico cromatográfico y propone su espectro de deconvolución que es comparado con un espectro en masa nominal de librería

(**B, C**). Los parámetros "Forward Fit" y "Reverse Fit" representan la concordancia del espectro experimental con el de librería. Cuanto mayor sean estos valores, más semejante es el espectro experimental del compuesto propuesto con el teórico de librería.

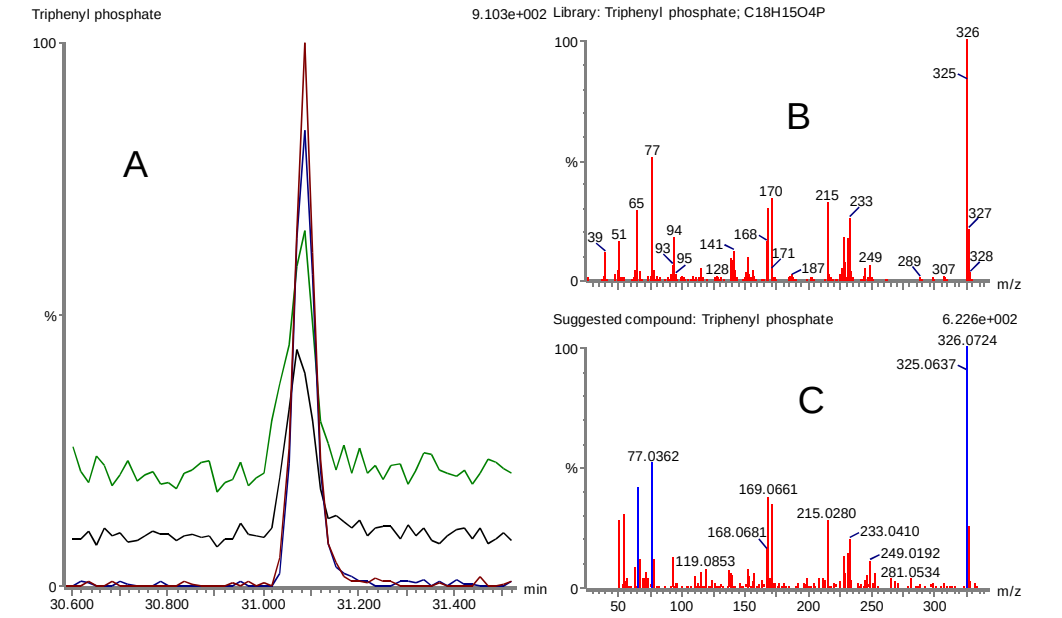

Figura 5.2. Información obtenida a partir del software ChromaLynx para la identificación de compuestos *non-target*. (A) Identificación de TPhP en una muestra salina. (B) Espectro nominal teórico del TPhP a partir de librería. (C) Espectro de deconvolución experimental para el TPhP. (D) *Match* de librería y errores de masas (mDa) de los principales fragmentos del espectro.

Para el caso del TPhP, estos valores son 788 y 856 respectivamente, demostrando una gran similitud entre el espectro de deconvolución que ofrece el software comparado con el espectro de librería. Posteriormente, se presenta información acerca de los errores de masas de fragmentos del espectro (**D,** error obtenido entre $m/z_{experimental}$-$m/z_{teórica}$). El

software compara la masa experimental obtenida para un ión seleccionado con su masa teórica (obtenida a partir de la herramienta *Molecular Weight Calculator*). Cuanto más bajo sea el error de masa, la identificación es más fiable. Para el caso del TPhP mostrado anteriormente, la identificación se pudo llevar a cabo satisfactoriamente obteniendo errores de masa <3mDa para cinco iones principales del espectro.

El software asigna una composición elemental a cada ión *m/z* encontrado. Este proceso se realiza teniendo en cuenta todas aquellas combinaciones de átomos posibles, sin considerar si esta composición elemental tiene sentido como fragmento de la estructura del compuesto identificado. Este proceso constituye una limitación a tener en cuenta en el proceso de confirmación. Por ello, es importante revisar cada una de las composiciones elementales asignadas y estudiar si estas pueden corresponder a fragmentos del compuesto identificado.

Exclusivamente para el caso de los OPs encontrados, se llevó a cabo una confirmación adicional con patrones de referencia. De esta manera fue posible comparar los resultados obtenidos con los mimos obtenidos con patrones. Las desviaciones entre el tiempo de retención y relación de Q/q fue atendida siguiendo las instrucciones de la reglamentación vigente (Commission Decision 2002/657/EC) para evitar reportar falsos positivos.

Previamente a nuestro estudio ya se habían publicado trabajos utilizando analizadores TOF para el *screening non-target* de compuestos orgánicos de interés. García-Reyes et al., (2007) discuten las distintas posibilidades de trabajo en modo *screening* con LC-TOF MS para el análisis de residuos de plaguicidas en muestras alimentarias. El trabajo manifiesta la excelente sensibilidad en modo de adquisición de espectro completa y la posibilidad de realizar medidas de masa exacta trabajando con analizadores TOF para la identificación de plaguicidas a niveles de concentración bajos. La utilidad de los analizadores TOF ha crecido paulatinamente y se puede encontrar en bibliografía trabajos excelentes tanto para aplicaciones con LC-QTOF MS o GC-TOF MS gracias a sus excelentes

prestaciones para la búsqueda de compuestos desconocidos mediante metodología *non-target* (Hernández et al., 2007; Ibañez et al., 2008; Zhou et al., 2009; Guthery et al., 2010).

5.2.4. Referencias.

Čajka, T.; Hajšlová, J. 2004. Gas chromatography-high-resolution time-of-flight mass spectrometry in pesticide residue analysis: Advantages and limitations. J. Chromatogr. A 1058, 251-261.

Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Available: http://eur-ex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN: PDF.

Dallüge, J.; Van Rijn, M.; Beens, J.; Vreuls, R.J.J.; Brinkman, U.A.Th. 2002. Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection applied to the determination of pesticides in food extracts. J. Chromatogr. A 965, 207-217.

Feo, M.L.; Eljarrat, E.; Barceló, D.; Barceló, D. 2010. Determination of pyrethroid insecticides in environmental samples. Trens Anal. Chem. 29, 692-705.

Ferrer, I.; Thurman, E.M. 2003. Liquid chromatography/time-of-flight/mass spectrometry (LC/TOF/MS) for the analysis of emerging contaminants. Trends Anal. Chem. 22, 750-756.

García-Reyes, J.F.; Hernando, M.D.; Molina-Díaz, A.; Fernández-Alba, A.R. 2007. Comprehensive screening of target, non-target and unknown pesticides in food by LC-TOF-MS. Trends Anal. Chem. 26, 828-841.

Grimalt, S.; V.Sancho, J.; Pozo, O.J.; Hernández, F. 2010. Quantification, confirmation and screening capability of UHPLC coupled to triple quadrupole and hybrid quadrupole time-of-flight mass spectrometry in pesticide residue analysis. J. Mass Spectrom. 45, 421-436.

Ghisari, M.; Bonefeld-Jorgensen, E.C. 2009. Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol. Lett. 189, 67-77.

Guthery, B.; Bassindale, T.; Bassindale, A.; Pillinger, C.T.; Morgan, G.H. 2010. Qualitative drug analysis of hair extracts by comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry. J. Chromatogr. A 1217, 4402-4410.

Hernández, F.; Portolés, T.; Pitarch, E.; López, F.J. 2007. Target and nontarget screening of organic micropollutants in water by solid-phase microextraction combined with gas chromatography/high-resolution time-of-flight mass spectrometry. Anal. Chem. 79, 9494-9504.

Hernández, F.; Portolés, T.; Pitarch, E.; López, F.J. 2009. Searching for anthropogenic contaminants in human breast adipose tissues using gas chromatography-time-of-flight mass spectrometry. J. Mass Spectrom. 44, 1-11.

Howard, P.H.; Muir, D.C.G. 2010. Identifying new persistent and bioaccumulative organics among chemicals in commerce. Environ. Sci. Technol. 44, 2277-2285.

Ibáñez, M.; Sancho, J.V.; Hernández, F.; McMillan, D.; Rao, R. 2008. Rapid non-target screening of organic pollutants in water by ultraperformance liquid chromatography coupled to time-of-light mass spectrometry. Trends Anal. Chem. 27, 481-489.

Muir, D.C.G.; Howard, P.H. 2006. Are there other persistent organic pollutants? A challenge for environmental chemists. Environ. Sci. Technol. 40, 7157-7166.

Petersent, J.H.; Breindahl, T. 2000. Plasticizers in total diet samples, baby food and infant formulae. Food Addit. Contam. 17, 133-141.

Portolés, T.; Pitarch, E.; López, F.J.; Sancho, J.V.; Hernández, F. 2007. Methodical approach for the use of GC-TOF MS for screening and confirmation of organic pollutants in environmental water. J. Mass Spectrom. 42, 1175-1185.

Schwarzbauer, J.; Ricking, M. 2010. Non-target screening analysis of river water as compound-related base for monitoring measures. Environ. Sci. Pollut. R. 17, 934-947.

Zhou, J.-L.; Qi, L.-W.; Li, P. 2009. Herbal medicine analysis by liquid chromatography/time-of-flight mass spectrometry. J. Chromatogr. A 1216, 7582-7594.

5.3. Desarrollo de metodología analítica para la identificación de OPEs en muestras medioambientales.

5.3.1. Introducción.

Como se ha indicado anteriormente, en la actualidad una gran cantidad de los compuestos químicos utilizados industrialmente o asociados a materiales de diferente naturaleza como plásticos, pinturas, productos de limpieza, etc, provocan efectos perjudiciales sobre la salud y el medioambiente. Legislación europea reciente pretende proporcionar un marco legal para tratar estos productos químicos con el fin de asegurar un alto nivel de seguridad y protección para el medio ambiente (http://echa.europa.eu/home_es.asp). Dentro de la amplia lista de compuestos susceptibles de provocar algún efecto adverso se encuentran los POPs, que incluyen a los PCBs, dioxinas (PCDDs), dibenzofuranos (PCDFs), OCs, PAHs, PBDEs, entre otros. También aparecen nuevos compuestos de reciente interés como ftalatos, fosfatos retardantes de llama, perfumes, pesticidas, biocidas, y alquilos perfluorados (Garcia-Jares et al., 2009; Richardson et al., 2010). Sobre todos estos compuestos existe un creciente interés por su presencia en el medio ambiente y los efectos adversos que pueden producir.

Dentro de nuestra investigación, a partir de la realización de un *screening* de compuestos desconocidos en muestras de sal, se identificaron cuatro compuestos OPEs: TCPP, TBP, TPhP y EHDPP. Estos compuestos pueden ser un claro ejemplo de contaminantes emergentes dado que han sido frecuentemente identificados en muestras ambientales en los últimos años (Reemtsma et al., 2008). La mayor parte de los OPEs han sido producidos y siguen produciéndose en proporciones elevadas y son ampliamente utilizados como retardantes de llama (Reemtsma et al., 2008), por lo que son objeto de una creciente preocupación y hace necesario su control en alimentos y diferentes recursos naturales.

En nuestro caso, la identificación y detección de estos compuestos en muestras analizadas previamente nos llevó a desarrollar una metodología avanzada basada en GC-TOF MS para la identificación de 12 compuestos OPEs en muestras acuosas, sales y salmueras.

En el artículo que se presenta a continuación se muestran diferentes enfoques analíticos utilizando el GC-TOF MS para el *screening* de contaminantes orgánicos. En primer lugar se llevó a cabo la selección de los iones para la identificación de cada compuesto. Se seleccionaron como mínimo cuatro iones por compuesto para una correcta identificación. En segundo lugar, se realizó un análisis *target* de muestras seleccionadas para el análisis de OPEs. Por último, se realizó un análisis *post-target* de OPEs en muestras analizadas en el equipo con anterioridad gracias a la adquisición de espectro completo del TOF MS.

5.3.2. Artículo científico 4:

Investigation of organophosphate esters in fresh water, salt and brine samples by GC-TOF MS.

J. Nácher-Mestre, R. Serrano, T. Portolés, F. Hernández.

Submitted to Analytical and Bioanalytical Chemistry

Investigation of OPEs in fresh water, salt and brine samples by GC-TOF MS.

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Abstract

Advanced analytical methodology based on gas chromatography coupled to a high resolution time-of-flight mass analyzer (GC-TOF MS) is proposed for investigation of organophosphate esters (OPEs) in environmental water, marine salts and brine samples. The target screening was carried out for 12 OPEs by evaluating the presence of up to four m/z ions for every compound. The identification criterion was the presence of, at least, two m/z ions at expected retention time, measured at their accurate mass, and the agreement of the Q/qi ratio when compared to reference standards. This procedure allows the detection and reliable identification of the compounds in the samples at very low concentration levels (ng/g). The developed methodology was applied to commercial marine salt, brines and different environmental water samples obtaining positive findings for several OPEs such as tri(chloro-propyl)phosphate, tributhyl phosphate, triphenyl phosphate among other organophosphate compounds. Thanks to the full-spectrum acquisition performed in GC-TOF MS, it is possible to make a retrospective data evaluation without the need of performing additional analysis. This allowed in this work to re-evaluate data acquired in a previous GC-TOF MS non-target analysis of some marine salt and water samples with the result of discovering several of the OPEs investigated in those samples.

Keywords: marine salt, brine, water, gas chromatography, time-of-flight mass spectrometer, qualitative screening, organic contaminants.

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Introduction

Nowadays, there is a rising concern about toxicity and environmental effects of compounds that are not included in commission regulations, legislations or directives which inform about the control levels of these called "new contaminants" in environmental matrices [1]. Organophosphorus compounds (OPs) are a family of organic compounds which are widespread found in the environment because they have been used in different applications such as plastizers, flame retardants, pesticides, antifouling or textiles. Several authors have reported high levels of organophosphate esters (OPEs), a sub-family of OPs, in environmental samples like environmental and waste water, air or sediments [2-6]. OPEs have been recently under review due to their adverse effects as neurotoxics and carcinogens and tend to be present in the environment at high concentrations. These properties have increased the concern about the potential negative impact of these substances in the environment and for this reason they were classified as "re-emerging contaminants" [2]. Chlorinated OPEs such as tris(2-chloroethyl)phosphate (TCEP), tri(chloro-propyl)phosphate (TCPP) and tri(dichloropropyl)phosphate (TDCP) have been considered as carcinogenic. Moreover, non-chlorinated OPEs such trimethyl phosphate (TMP), tributhyl phosphate (TBP), triphenyl phosphate (TPhP) and tributoxyethyl phosphate (TBEP) have also toxic effects. A widespread report of toxicity and ecotoxicology for other OPEs was reported by Reemtsma et al. (2008) [2].

OPEs have been monitored in different environmental samples and there is a tendency to develop new sensitive methodologies to determine low concentrations of OPEs in the environmental water, air, sediments, urban dust samples, and also in human biological samples, like urine [6-11]. Chromatographic techniques, both gas chromatography (GC) and liquid chromatography (LC), coupled to mass spectrometry (MS) are normally the techniques of choice. Quintana et al. have recently reported wide information on the determination and outcomes of GC and LC coupled to MS and other minor sophisticated techniques [7].

GC coupled to high resolution (HR) time-of-flight mass analyzer (TOF MS) is an advanced technique very powerful from a qualitative point of view, able to provide a highly reliable identification of GC-amenable contaminants in different sample matrices [12]. TOF MS has high sensitivity in full-spectrum acquisition mode and high mass resolution, which provides high mass accuracy. These characteristics lead to high selectivity by using narrow-window eXtracted Ion Chromatograms (nw-XICs) [13]. This analyzer allows obtaining an extraordinary amount of chemical information in a single injection making this technique particularly attractive for the investigation of non-target compounds and also for searching analytes in a target way [14]. Despite its excellent potential in relevant applied fields, like environmental pollution, food safety, toxicology or doping control analysis, HRTOF MS has been scarcely used until now [12, 15], and it is expected it will definitely grow to be the essential, sophisticated analytical tool for the screening of organic pollutants in environmental samples, among other applied fields.

In a previous work, we applied a non-target screening by GC-TOF MS in sea water and marine salts from saltworks located at the Spanish Mediterranean coast [3]. The objective was to investigate the presence in these samples of any type of organic GC-amenable compounds that might be relevant from an environmental point of view. Without any kind of previous analyte selection, several non-targeted compounds such as di-(2ethylhexyl)adipate, benzyl butyl phthalate, butylated hydroxytoluene and galaxolide, were detected. In addition, four OPEs, the most commonly reported in the literature, were identified in several samples. This fact, considering the relevance of these contaminants, encouraged us to widen the number of OPEs investigated in a further research. The aim of this work is to develop a GC-TOF MS target screening method for the identification of 12 OPEs in salt from saltworks and marine salt used in aquaculture, food and feeding purposes. The method was applied to the analysis of several samples, including commercial marine salts, brine samples obtained from different saltworks and also to different environmental waters (ground and surface water).

Experimental

Materials and reagents

Individual organophosphate reference standards were obtained from TCI Europe (Zwijndrecht, Belgium) and Sigma Aldrich (Madrid, Spain). TMP, TPhP, 2-Ethylhexyl Diphenyl Phosphate (EHDPP), Tris(2-ethylhexyl)Phosphate (TEHP), TBEP, Triethyl Phosphate (TEP), TDCP, TCEP and Tricresyl Phosphate (TCrP) were purchased from TCI Europe. TBP, Tripropyl Phosphate (TPrP) and TCPP were purchased from Sigma-Aldrich.

Individual stock solution (1 and 10 µg/ml) were prepared by dissolving reference standards in acetone and stored in a freezer at –20 ºC. Working solutions were prepared by diluting stock solutions in acetone for sample fortification and in n-hexane for injection in the chromatographic system.

HPLC-grade water was obtained from a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). Ultratrace quality solvents such as Acetone, Ethyl Acetate, Dichlorometane (DCM) and n-Hexane purchased from Scharlab (Barcelona, Spain), were used in solid phase extraction (SPE) experiments. Bond Elut cartridges C18 (500 mg) purchased from Varian (Harbor City, CA, USA), were also used for SPE.

Sample material

Samples involved in this work were the following: Nine marine salt samples (five commercial from supermarkets and four obtained directly from manufacturers); nine brine samples obtained from saltworks sited in the Ebro river estuary (Mediterranean coast); six ground water and five surface water samples (both from Spanish Mediterranean area). All samples were stored at -20ºC until analysis.

GC instrumentation

GC system (Agilent 6890N; Agilent Palo Alto, USA) equipped with an autosampler (Agilent 7683) were coupled to a time-of-flight mass spectrometer (GCT, Waters Corporation, Manchester, U.K.), operating in electron ionization mode (EI). GC separation was performed

using a fused silica HP-5MS capillary column with a length of 30 m, an internal diameter of 0.25 mm and a film thickness of 0.25 µm (J&W Scientific, Folson, CA, USA). The injector temperature was set to 280°C. Splitless injections of 1 µL of the sample were carried out. Helium (99.999%; Carburos Metálicos, Valencia, Spain) was used as carrier gas at a flow rate of 1 mL/min. The interface and source temperature were set to 250°C and a solvent delay of 4 min was selected.

The oven program in the GC system was programmed, working for target analysis of OPEs, as follows: 60°C (1min); 8°C/ min to 300°C (2 min). The TOF MS was operating at 1 spectrum/s, acquisition rate over the mass range m/z 50-650, using a multichannel plate voltage of 2850 V. TOF MS resolution was approximately 7000 (FWHM). Heptacosa standard, used for the daily mass calibration and as a lock mass, was injected via syringe in the reference reservoir at 30 °C for this purpose; the m/z ion monitored was 218.9856. The application manager TargetLynx was used to process the qualitative data obtained from standards and from sample analysis.

Analytical procedure

The analytical procedure applied was based on the method previously developed by Pitarch et al. (2007) [16] for the determination of priority organic pollutants in water by GC-MS/MS, with some modifications in the pre-concentration step. Approximately 62.5g of salt were dissolved in 250 mL of water. In the analysis of brines and environmental waters, 250 mL of sample were taken. In all cases, the filtered solution was passed through the C_{18} SPE cartridge, previously conditioned by passing 6 mL methanol, 6 mL ethyl acetate:DCM (50:50), 6 mL methanol and 6 mL water avoiding dryness. After loading the sample (250mL), cartridges were washed with 3 mL water. Then, the cartridge was dried by passing air, using vacuum for at least 15 min, and the elution was performed by passing 5 mL ethyl acetate:DCM (50:50). Finally, the extract collected was evaporated under a gentle nitrogen stream at 40°C and redisolved in 0.25 mL n-hexane and analyzed by GC-TOF MS.

GC-TOF MS target screening

Full spectrum acquisition data generated by TOF MS were treated using an automated processing method. Briefly, the detection and identification of target OPEs was carried out by obtaining between 2 and 4 nw-XICs (mass window 0.02 Da) at selected *m/z* exact masses for every compound (**Table 1**). The presence of at least two ions at expected retention time measured at their accurate masses was required together to the attainment of the Q/q ratio within specified tolerances. This methodology has been previously applied by our group and further details in development of automatic processing method can be found in the literature [12-14].

Target analytical methodology evaluation

Prior sample analysis, this methodology was tested in marine salts that were spiked with target OPEs at different levels. For this purpose, five marine salt samples were analyzed using the developed procedure to identify the presence of OPEs. None of the samples was a true blank, as all the samples analyzed contained one or more OPE. Therefore, we selected one of these samples (the less contaminated) for spiking.

The evaluation of the overall procedure, including sample treatment and GC-TOF MS measurement was carried out by analysis of 5 replicates of the marine salt sample spiked at three different concentration levels: 0.4, 2 and 4 ng/g of each of the 12 OPEs selected. As the salt was dissolved in 250 mL of HPLC-grade water, these levels would correspond to 0.1, 0.5 and 1 µg/L in brines and water samples. The evaluation of the overall procedure also involved the analysis of two replicates of the "blank" sample used for spiking and two method blanks to assure that no contamination was introduced in the procedure by the routine analysis.

To consider a finding of OPE as positive, the criteria established was the presence of at least two *m/z* ions at expected retention time, measured at their accurate mass, and the agreement of their Q/q ratios within specific tolerances. The confirmation of the identity

was assumed by taking into account of the European Commission Decision (2002/657/CE) [17].

The limit of identification (LOI) was established as the lowest concentration level tested at which all the five replicates were positive, accordingly with the identification criterion indicated above.

Results and discussion

Qualitative target investigation of OPEs

The analytical strategy developed in this paper was focused on the detection and reliable identification of target OPEs in environmental samples more than on quantification, as the latest can be successfully made by using the most commonly applied techniques of GC-NPD, GC-MS and LC-MS/MS [7].

After a non-target GC-TOF MS analysis previously applied to marine salts [3], we discovered the presence in the samples of up to 4 OPEs among other relevant contaminants. Considering the interest of investigating OPEs in this type of samples, we extended the research to a higher number of OPEs by developing a GC-TOF MS target screening focused on 12 relevant OPEs. The acquisition of reference standards allowed as to test the qualitative method in spiked samples and to perform the unequivocal confirmation of the identity of these compounds in the samples analyzed.

The use of GC-TOF MS allows performing a retrospective analysis of the full spectrum acquisition data acquired. Based on this, we have explored in this paper the potential of GC-TOF MS to perform a retrospective analysis of previous data obtained in a non-target way [3]. Data re-evaluation has been made in a post-target way, i.e. searching for selected analytes after MS data acquisition without the need of any additional sample injection into the GC-TOF MS [12, 18]. This possibility is not accessible using single- or triple-quadrupole analyzers in SIM or SRM mode, respectively, or using ion trap analyzers in the MS/MS mode where the selection of analytes must be done before the acquisition, as only analite-specific information is acquired.

The target screening processing method was developed by injecting reference standards in solvent. In general, the structures suggested for the main fragments from EI-TOF MS spectra of the compounds, were in accordance to those available in the literature [19, 20] (see **Table 1**). Many of the main *m/z* ions are produced by the rearrangement of more than one hydrogen atom, giving an even-electron ion as the stable product. In the EI mode, alkyl phosphate esters tend to lose alkyl groups by McLafferty rearrangements [21]. Basically, this means that alkyl groups are sufficiently long to donate two hydrogens to the phosphate interior structure during the fragmentation process. As an example, tri-alkylated phosphate esters will afford *m/z* 98.9847 as the base ion [$H_4O_4P^+$].

Evaluation of the target methodology

As indicated in section 2.6., the GC-TOF MS screening methodology was tested in marine salt spiked at 0.4, 2 and 4 ng/g for each OPE selected. The LOI was established as the lowest concentration level tested for which the identification criterion was achieved in the five replicates tested (**Table 2**). Consequently, a compound was considered as satisfactory identified at a certain concentration level, only when the five replicates spiked at that level were positive by the accomplishment of the identification criteria established [22].

As **Table 2** shows, several OPEs were identified in a reliable way at the lowest fortification level tested. Thus, the compounds TEP, TPrP, TCEP, TDCP and TPhP achieved the established identification criteria at 0.4 ng/g. The LOI for TBEP was found to be 2 ng/g in salt samples, and the remaining OPES, TMP, EHDPP, TEHP and TCrP could only be correctly identified at the highest concentration level assayed (4ng/g). It is worth to mention that TBP and TCPP could be correctly identified at the lowest level, but no LOI could be proposed since they were present in the “blank” sample selected for this evaluation. **Table 2** also shows the equivalence of LOI in water samples.

Application to marine salt, brine and water samples. Retrospective analysis

The methodology developed in this paper was applied to the screening of OPEs in commercial marine salt and brine samples. **Table 3** shows the OPEs identified using the

methodology proposed. With regards to commercial marine salts, six OPEs were identified (TEP, TPrP, TBP, TCEP, TCPP and EHDPP), three of them (TEP, TPrP and TCEP) none previously detected using a non-target screening for the four marine salts from saltworks [3]. Moreover, three positive findings for TBP and one for TCEP were also found in brine samples. TCPP was present in all samples investigated. As an illustrative example, **Figure 1** shows the positive finding of TBP in one commercial marine salt using the target screening developed. In this case, 5 characteristic ions at expected retention time in the nw-XIC were observed. The attainment of all Q/q_i ratios (in this case 4) criteria within specified tolerances led to the unequivocal confirmation of the identity of the compounds selected. The accurate mass spectrum of the sample peak is shown together with mass errors for the four ions (the lowest fragment was not present in the spectrum). These mass errors were below 3mDa. In addition, chemical structures for the most abundant EI fragment ions were suggested based on the elemental compositions proposed for those ions accordingly to the accurate mass measurements given by the instrument in the target methodology applied (see Table 1). All structures proposed for the fragments were compatible with the chemical structure of TBP, making the identification still more reliable.

TOF MS allows an excellent advantage in order to reprocess data files without a new GC-TOF MS running due to its full acquisition mode. In this sense, the developed methodology was applied in order to investigate the presence of other OPEs in the four salt samples previously analyzed, where four OPEs had been previously detected (TBP, TCPP, TPhP and EHDPP) [3]. These four OPEs detected in the first study were also identified with the new strategy but no more OPEs from the target list were found in the four salt samples satisfying our confirmation criteria. Besides the salts, 6 ground waters and 5 surface waters were also retrospectively analyzed. TCPP was detected in all these waters and TCEP was identified in only one surface water.

As an illustrative example**, Figure 2** shows nw-XICs for the OPEs found in the waters. This figure shows the positive findings of TCPP, TCEP and TCPP in ground water and surface water respectively. Five characteristic ions at expected retention time in the nw-XIC were also observed. The attainment of all (in these case 4) Q/q_i ratios criteria within specified

tolerances led to the unequivocal confirmation of these compounds. The qualitative screening to these samples shows that the OPEs identified were at least over the LOI proposed (**Table 2)**. The presence of these compounds in these samples was in accordance with several authors who found TCPP and other OPEs in environmental samples such wastewater, surface water and sub-surface water [5].

Conclusions

A rapid sensitive screening with little sample manipulation is described in this work for detection and identification of OPEs in different type of samples, based on the use of SPE pre-concentration step and GC-TOF MS analysis. The screening method has been evaluated in qualitative terms at different concentration levels for a total of 12 OPEs allowing the study of these compounds in samples frequently used for aquaculture and alimentation purposes like marine salts. For the majority of the studied analytes, LOIs were very low (from 0.4 ng/g to 4 ng/g in salts and from 0.1 ng/mL to 1 ng/mL in waters) except for TBP and TCPP which no LOI could be proposed due to these compounds were present in the blank.

The screening methodology has been applied to investigate the presence of 12 OPEs in different samples including marine salts from saltworks and commercial salts, brine, ground water and surface water. Some of these samples were simply subjected to a retrospective data evaluation without the need of performance an additional analysis. This was possible because those samples were previously analyzed by GC-TOF MS and full-spectrum accurate mass data remained available for re-evaluation, making the searching of OPEs feasible in a post-target way. The developed methodology allowed the reliable identification of several OPEs such as TBP, TCPP and EHDPP in marine salts, together with TPhP in salts from saltworks, and other OPEs, such as TEP, TPrP and TCEP in commercial salt samples used for human nutrition and alimentation purposes. All samples monitored, salts, brines and waters, were positive for TCPP. In addition to TCPP, TBP was identified in three brines and TCEP was identified in one brine sample and in one surface water sample.

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References.

[1] Richardson SD (2008) Environmental mass spectrometry: Emerging contaminants and current issues. Anal Chem 80:4373-4402

[2] Reemtsma T, García-López M, Rodríguez I, Quintana JB, Rodil R (2008) Organophosphorus flame retardants and plasticizers in water and air I. Occurrence and fate. TrAC – Trend Anal. Chem 27: 727-737

[3] Serrano R, Nácher-Mestre J, Portolés J, Amat F, Hernández F (2010) Non-target screening of organic contaminants in marine salts by gas chromatography coupled to high-resolution time-of-flight mass spectrometry. Submitted to Talanta.

[4] Martínez-Carballo E, González-Barreiro C, Sitka A, Scharf S, Gans O (2007) Determination of selected organophosphate esters in the aquatic environment of Austria. Sci Total Environ 388: 290-299

[5] Marklund A, Andersson B, Haglund P (2005) Organophosphorus flame retardants and plasticizers in Swedish sewage treatment plants. Environ Sci Technol 39:7423-7429

[6] García-López M, Rodríguez I, Cela R, Kroening KK, Caruso JA (2009) Determination of organophosphate flame retardants and plasticizers in sediment samples using microwave-assisted extraction and gas chromatography with inductively coupled plasma mass spectrometry. Talanta 79:824-829

[7] Quintana JB, Rodil R, Reemtsma T, García-López M, Rodríguez I (2008) Organophosphorus flame retardants and plasticizers in water and air II. Analytical methodology. TrAC – Trend Anal Chem 27:904-915

[8] Quintana JB, Rodil R, López-Mahía P, Muniategui-Lorenzo S, Prada-Rodríguez D (2007) Optimisation of a selective method for the determination of organophosphorous triesters in outdoor particulate samples by pressurised liquid extraction and large-volume injection gas chromatography-positive chemical ionisation-tandem mass spectrometry. Anal Bioanal Chem 388:1283-1293

[9] García-López M, Rodríguez I, Cela R (2009) Pressurized liquid extraction of organophosphate triesters from sediment samples using aqueous solutions. J Chromatogr A, 1216:6986-6993

[10] Schindler BK, Förster K, Angerer J (2009) Determination of human urinary organophosphate flame retardant metabolites by solid-phase extraction and gas chromatography-tandem mass spectrometry. J Chromatogr B 877:375-381

[11] García-López M, Rodríguez I, Cela R (2010) Mixed-mode solid-phase extraction followed by liquid chromatography-tandem mass spectrometry for the determination of tri- and di-substituted organophosphorus species in water samples. J Cromatogr A 1217:1476-1484

[12] Hernández F, Portolés T, Pitarch E, Lopez FJ (2011) Gas chromatography coupled to high-resolution time-of-flight mass spectrometry to analyze trace-level organic compounds in environment, food safety and toxicology. TRAC-Trend Anal Chem. DOI 10.1016/j.trac.2010.11.007

[13] Portolés T, Pitarch E, López FJ, Sancho JV, Hernández F (2007) Methodical approach for the use of GC-TOF MS for screening and confirmation of organic pollutants in environmental water. J Mass Spectrom 42:1175-1185

[14] Hernández F, Portolés T, Pitarch E, López FJ (2007) Target and nontarget screening of organic micropollutants in water by solid-phase microextraction combined with gas chromatography/high-resolution time-of-flight mass spectrometry. Anal Chem 79:9494-9504

[15] Peters RJB, Oosterink JE, Stolker AAM, Georgakopoulos C, Nielen MWF (2010) Generic sample preparation combined with high-resolution liquid chromatography-time-of-flight mass spectrometry for unification of urine screening in doping-control laboratorios. Anal Bioanal Chem 396:2583-2598

[16] Pitarch E, Medina C, Portolés T, López FJ, Hernández F (2007) Determination of priority organic micro-pollutants in water by gas chromatography coupled to triple quadrupole mass spectrometry. Anal Chim Acta 583:246-258

[17] Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Available: eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF

[18] Hernández F, Pozo OJ, Sancho JV, López FJ, Marín JM, Ibáñez M (2005) Strategies for quantification and confirmation of multi-class polar pesticides and transformation products in water by LC-MS2 using triple quadrupole and hybrid quadrupole time-of-flight analyzers. TrAC – Trend Anal Chem 24:596-612

[19] Rodil R, Quintana JB, Reemtsma T (2005) Liquid chromatography-tandem mass spectrometry determination of nonionic organophosphorus flame retardants and plasticizers in wastewater samples. Anal Chem 77:3083-3089

[20] Ellis J, Shah M, Kubachka KM, Caruso JA (2007) Determination of organophosphorus fire retardants and plasticizers in wastewater samples using MAE-SPME with GC-ICPMS and GC-TOFMS detection. J Environ Monitor 9:1329-1336

[21] McLafferty FW (1959) Mass spectrometric analysis: Molecular rearrangements Anal Chem 31:82-87

[22] Portolés T, Pitarch E, López FJ, Hernández F (2011) Development and validation of a rapid and wide-scope qualitative screening method for detection and identification of organic pollutants in natural water and wastewater by gas chromatography time-of-flight mass spectrometry. J Chromatogr A 1218:303-315

Figure Captions.

Figure 1. Extracted-ion chromatograms (mass window 0.02 Da) showing a positive finding of TBP in commercial marine salt. Experimental EI accurate mass spectrum. Chemical structures proposed for the most abundant EI fragment ions. St: reference standard; S: sample; Q: qualitative ion; q: confirmation ion; ✓: Q/q ratio within tolerance limits.

Figure 2. Extracted ion chromatogram at different *m/z* (mass window 0.02 Da) for TCPP in ground water and, TCEP and TCPP in surface waters. St: reference standard; S: sample; Q: qualitative ion; q: confirmation ion; ✓: Q/q ratio within tolerance limits.

Table1. *m/z* ions selected for the identification of OPEs.

Compound		CAS	tR	Formula	MW	Ion_1	m/z_1	Ion_2	m/z_2	Ion_3	m/z_3	Ion_4	m/z_4	Ion_5	m/z_5
Trimethyl phosphate	TMP	512-56-1	4.17	$C_3H_9O_4P$	140.0238	$C_3H_9O_4P$	140.0238	$C_2H_7O_3P$	110.0133	$C_2H_6O_3P$	109.0055	CH_5O_2P	80.0027	CH_4O_2P	78.9949
Triethyl phosphate	TEP	78-40-0	7.38	$C_6H_{15}O_4P$	182.0708	$C_4H_{12}O_4P$	155.0473	$C_3H_8O_4P$	139.0160	$C_2H_8O_4P$	127.0160	$C_2H_6O_3P$	109.0055	H_4O_4P	98.9847
Tripropyl phosphate	TPrP	513-08-6	11.83	$C_9H_{21}O_4P$	224.1177	H_4O_4P	98.9847	$C_6H_{16}O_4P$	183.0786	$C_4H_{10}O_4P$	153.0317	$C_3H_{10}O_4P$	141.0317	$C_3H_8O_3P$	123.0211
Tributyl phosphate	TBP	126-73-8	15.85	$C_{12}H_{27}O_4P$	266.1647	H_4O_4P	98.9847	$C_8H_{20}O_4P$	211.1099	$C_4H_{12}O_4P$	155.0473	$C_2H_6O_4P$	125.0004	C_4H_8	56.0626
Tris-(2-chloroethyl)phosphate	TCEP	115-96-8	17.43	$C_6H_{12}Cl_3O_4P$	283.9539	$C_4H_8Cl_2O_3P$	204.9588	$C_6H_{12}Cl_2O_4P$	248.9850	H_4O_4P	98.9847	$C_2H_5ClO_3P$	142.9665	C_2H_4Cl	63.0002
Tris(1-chloro-2-propyl) phosphate	TCPP	13674-84-5	17.95	$C_9H_{18}Cl_3O_4P$	326.0008	H_4O_4P	98.9847	$C_5H_{11}ClO_4P$	201.0084	$C_3H_7ClO_3P$	156.9821	$C_3H_9ClO_4P$	174.9927	$C_8H_{16}Cl_2O_4P$	277.0163
Tris(1,3-dichloro-2-propyl)phosphate	TDCP	13674-87-8	24.19	$C_9H_{15}Cl_6O_4P$	427.8839	$C_3H_6Cl_2O_3P$	190.9432	$C_3H_8Cl_2O_4P$	208.9537	$C_3H_5ClO_3P$	154.9665	$C_3H_4Cl_2$	109.9690	C_3H_4Cl	75.0002
Triphenyl phosphate	TPhP	115-86-6	24.85	$C_{18}H_{15}O_4P$	326.0708	$C_{18}H_{15}O_4P$	326.0707	$C_{12}H_8O_2P$	215.0262	C_6H_6O	94.0419	C_6H_5	77.0391	-	-
Tri(butoxyethyl)phosphate	TBEP	78-51-3	24.94	$C_{18}H_{39}O_7P$	398.2433	C_5H_9O	85.0653	C_6H_6O	94.0419	$C_2H_6O_4P$	125.0004	$C_6H_{12}O$	100.0888	-	-
2-Ethylhexyl diphenyl phosphate	EHDPP	1241-94-7	25.14	$C_{20}H_{27}O_4P$	362.1647	$C_{12}H_{12}O_4P$	251.0473	$C_{12}H_{11}O_4P$	250.0395	$C_{12}H_{10}O_4P$	249.0317	$C_{12}H_{10}O$	170.0732	-	-
Tris(ethylhexyl)phosphate	TEHP	78-42-2	25.52	$C_{24}H_{51}O_4P$	434.3525	H_4O_4P	98.9847	C_8H_{16}	112.1252	C_8H_{17}	113.1330	C_6H_{11}	83.0861	C_5H_{10}	70.0783
Tricresyl phosphate	TCrP	1330-78-5	27.29	$C_{21}H_{21}O_4P$	368.1177	$C_{21}H_{21}O_4P$	368.1177	$C_{21}H_{20}O_4P$	367.1099	C_7H_8O	108.0575	C_7H_7O	107.0497	-	-

Table 2. Evaluation of the procedure when applied to spiked samples (n=5). Limits of identification.

Compound		Nº CAS	Formula	LOI Marine Salt (ng/g)	LOI Water [a] (µg/L)
Trimethyl phosphate	TMP	512-56-1	$C_3H_9O_4P$	4	1
Triethyl phosphate	TEP	78-40-0	$C_6H_{15}O_4P$	0.4	0.1
Tripropyl phosphate	TPrP	513-08-6	$C_9H_{21}O_4P$	0.4	0.1
Tributyl phosphate	TBP	126-73-8	$C_{12}H_{27}PO_4$	(b)	(c)
Tris-(2-chloroethyl)phosphate	TCEP	115-96-8	$C_6H_{12}Cl_3O_4P$	0.4	0.1
Tris(1-chloro-2-propyl) phosphate	TCPP	13674-84-5	$C_9H_{18}Cl_3O_4P$	(b)	(c)
Tris(1,3-dichloro-2-propyl)phosphate	TDCP	13674-87-8	$C_9H_{15}Cl_6O_4P$	0.4	0.1
Triphenyl phosphate	TPhP	115-86-6	$C_{18}H_{15}O_4P$	0.4	0.1
Tri(butoxyethyl)phosphate	TBEP	78-51-3	$C_{18}H_{39}O_7P$	2	0.5
2-Ethylhexyl diphenyl phosphate (Octizicer)	EHDPP	1241-94-7	$C_{20}H_{27}O_4P$	4	1
Tris(2-ethylhexyl)phosphate	TEHP	78-42-2	$C_{24}H_{51}O_4P$	4	1
Tricresyl phosphate	TCrP	1330-78-5	$C_{21}H_{21}O_4P$	4	1

a: Values that would correspond to water samples considering the treatment applied to salt samples.

b: Compounds identified at 0.4 ng/g level, although they were present in the "blank" sample used in the experiments.

c: Compounds identified at a level that would correspond to 0.1 µg/L, although they were also present in the "blank" sample used in the experiments.

Table 3. Identification of OPEs in different type of samples using the proposed methodology.

Number of positives / number of samples analyzed						
Compound		**New samples analyzed**		**Retrospective analysis of samples previously injected into GC-TOF MS**		
		5 commercial marine salts	**9 brine water**	**4 marine salts from saltworks**	**6 ground waters**	**5 surface waters**
Trimethyl phosphate	TMP	0/5	0/9	0/4	0/6	0/5
Trimethyl phosphate	TMP	0/5	0/9	0/4	0/6	0/5
Triethyl phosphate	TEP	4/5	0/9	0/4	0/6	0/5
Tripropyl phosphate	TPrP	1/5	0/9	0/4	0/6	0/5
Tributyl phosphate	TBP	5/5	3/9	4/4	0/6	0/5
Tris-(2-chloroethyl)phosphate	TCEP	1/5	1/9	0/4	0/6	1/5
Tris(1-chloro-2-propyl) phosphate	TCPP	5/5	9/9	4/4	6/6	5/5
Tris(1,3-dichloro-2-propyl)phosphate	TDCP	0/5	0/9	0/4	0/6	0/5
Triphenyl phosphate	TPhP	0/5	0/9	2/4	0/6	0/5
Tri(butoxyethyl)phosphate	TBEP	0/5	0/9	0/4	0/6	0/5
2-Ethylhexyl diphenyl phosphate (Octizicer)	EHDPP	2/5	0/9	2/4	0/6	0/5
Tris(2-ethylhexyl)phosphate	TEHP	0/5	0/9	0/4	0/6	0/5
Tricresyl phosphate	TCrP	0/5	0/9	0/4	0/6	0/5

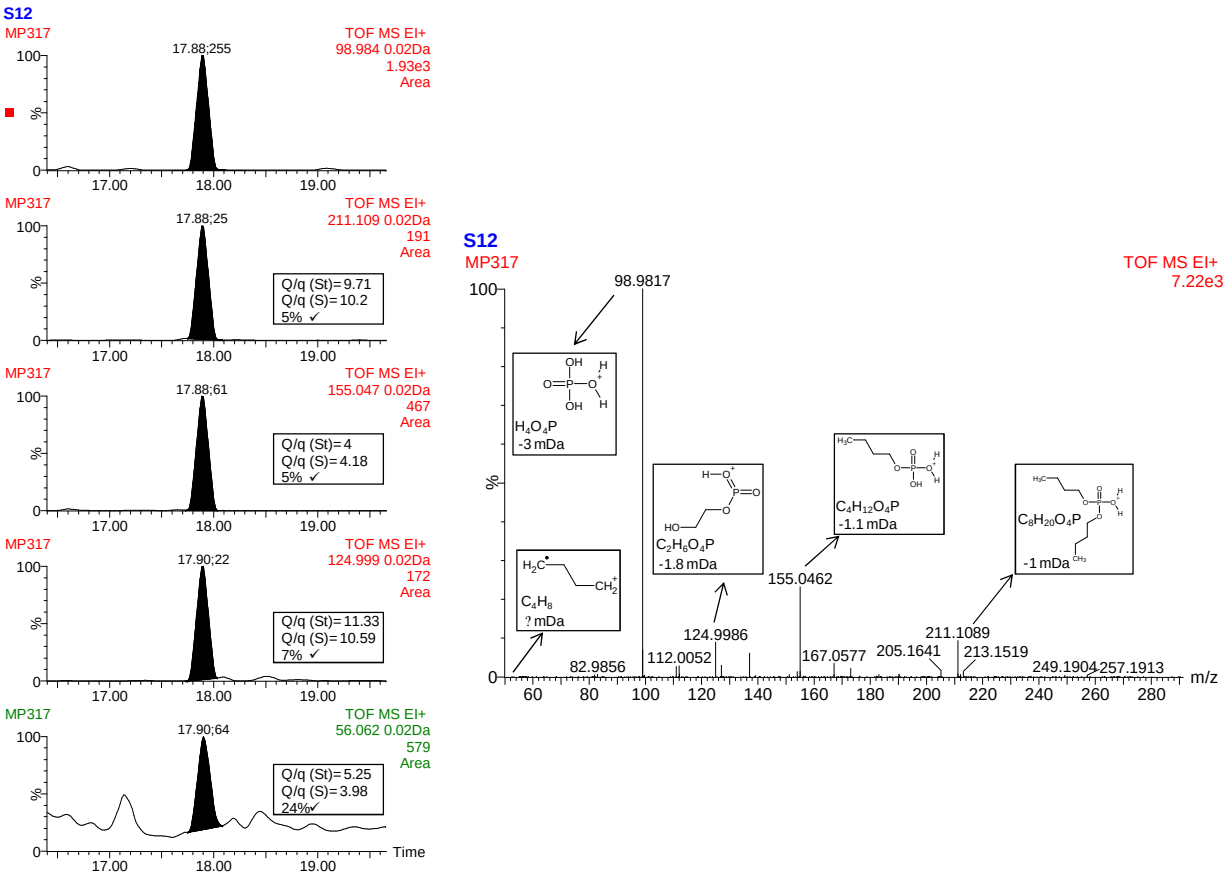
S12
MP317
TOF MS EI+
98.984 0.02Da
1.93e3
Area
17.88;255
Q/q (St)= 9.71
Q/q (S)= 10.2
5% ✓
211.109 0.02Da
191
17.88;25
155.047 0.02Da
467
17.88;61
Q/q (St)= 4
Q/q (S)= 4.18
124.999 0.02Da
172
17.90;22
Q/q (St)= 11.33
Q/q (S)= 10.59
7% ✓
56.062 0.02Da
579
17.90;64
Q/q (St)= 5.25
Q/q (S)= 3.98
24%✓
Time
7.22e3
98.9817
H4O4P
-3 mDa
C2H6O4P
-1.8 mDa
C4H8
? mDa
C4H12O4P
-1.1 mDa
C8H20O4P
-1 mDa
155.0462
124.9986
211.1089
82.9856
112.0052
167.0577
205.1641
213.1519
249.1904
257.1913
m/z

Figure 1.

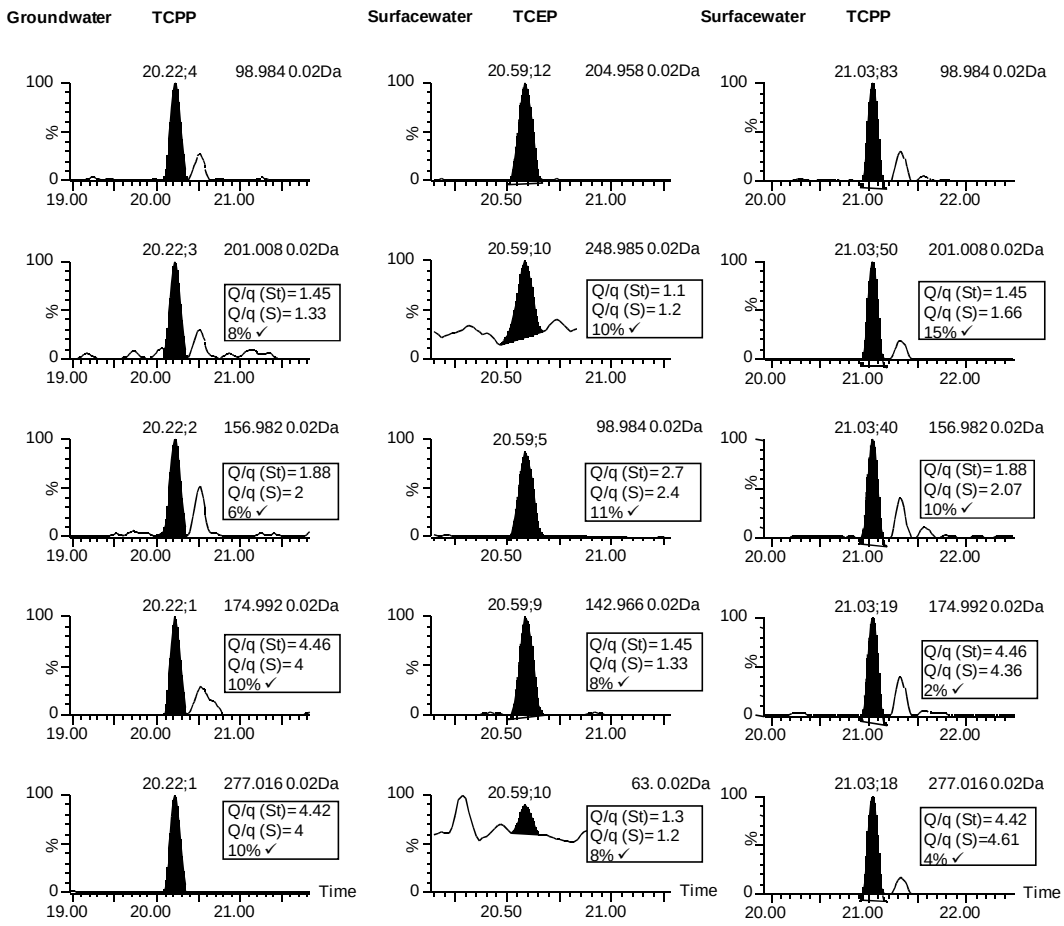
Groundwater TCPP
Surfacewater TCEP
Surfacewater TCPP
20.22;4 98.984 0.02Da
20.59;12 204.958 0.02Da
21.03;83 98.984 0.02Da
20.22;3 201.008 0.02Da
Q/q (St)= 1.45
Q/q (S)= 1.33
8% ✓
20.59;10 248.985 0.02Da
Q/q (St)= 1.1
Q/q (S)= 1.2
10% ✓
21.03;50 201.008 0.02Da
Q/q (St)= 1.45
Q/q (S)= 1.66
15% ✓
20.22;2 156.982 0.02Da
Q/q (St)= 1.88
Q/q (S)= 2
6% ✓
20.59;5 98.984 0.02Da
Q/q (St)= 2.7
Q/q (S)= 2.4
11% ✓
21.03;40 156.982 0.02Da
Q/q (St)= 1.88
Q/q (S)= 2.07
10% ✓
20.22;1 174.992 0.02Da
Q/q (St)= 4.46
Q/q (S)= 4
10% ✓
20.59;9 142.966 0.02Da
Q/q (St)= 1.45
Q/q (S)= 1.33
8% ✓
21.03;19 174.992 0.02Da
Q/q (St)= 4.46
Q/q (S)= 4.36
2% ✓
20.22;1 277.016 0.02Da
Q/q (St)= 4.42
Q/q (S)= 4
10% ✓
20.59;10 63. 0.02Da
Q/q (St)= 1.3
Q/q (S)= 1.2
8% ✓
21.03;18 277.016 0.02Da
Q/q (St)= 4.42
Q/q (S)=4.61
4% ✓
100
%
0
Time
19.00
20.00
21.00
20.50
22.00

Figure 2.

5.3.3. Discusión de resultados.

- Metodología *screening* de OPEs.

Para llevar a cabo una correcta identificación de la presencia de OPEs en las muestras mediante GC-TOF MS, se seleccionaron al menos cuatro iones característicos por compuesto. Se consideró la masa exacta de dos iones por compuesto como criterio mínimo para alcanzar una buena identificación atendiendo a los criterios de identificación propuestos por la legislación europea (Commission Decision 2002/657/EC). El tiempo de retención además de las relaciones Q/q fueron comparadas entre muestras y patrones certificados para cada compuesto con el fin de asegurar una correcta identificación (Nácher-Mestre et al., 2009).

La masa teórica experimental de los OPEs seleccionados fue obtenida a partir de la adquisición del espectro completo mediante GC-TOF MS al inyectar en el equipo patrones certificados de cada compuesto. De esta forma ha sido posible obtener picos cromatográficos para una masa exacta determinada y poder confirmar la presencia inequívoca de OPEs en las muestras analizadas. La exactitud de masa resulta ser un parámetro crítico para la identificación de compuestos puesto que permite la confirmación de la identidad de los OPEs con errores de masa normalmente bajos (Ferrer et al., 2006). Así pues, a cada relación *m/z* le corresponde una composición elemental lo que limita extraordinariamente la posibilidad de confusión con otro compuesto. Adquiriendo más de un ión identificativo por compuesto mediante GC-TOF MS se obtiene una correcta identificación y exactitud de masa con hasta 3 ó 4 puntos de identificación (IPs), requeridos para compuestos autorizados o prohibidos, respectivamente (Commission Decision 2002/657/EC).

La utilización de GC-TOF MS ha resultado ser una herramienta muy útil para la identificación de OPEs en matrices medioambientales y de consumo humano. Los analizadores TOF han sido también utilizados para obtener una confirmación adicional de la presencia de contaminantes gracias a su elevada resolución y exactitud de masa (Nácher-Mestre et al., 2009; Pitarch et al., 2010). Recientemente, Ellis et al. (2007) ha desarrollado

una metodología para la determinación de OPEs mediante GC-MS con fuente de plasma de acoplamiento inductivo (GC-ICP MS) y con la utilización de GC-TOF MS mostrando así un gran poder de identificación de los analitos.

- Evaluación de la metodología.

El método para la identificación de OPEs fue evaluado con el fin de realizar una estimación real de la identificación de OPEs a concentraciones por encima de los LOI establecidos. Los resultados fueron satisfactorios pudiendo proponer LOIs a niveles de ng/g para sales marinas y a niveles de µg/L para las aguas, para todos los OPEs seleccionados. Únicamente para los compuestos TBP y TCPP no se pudieron establecer LOIs puesto que estos analitos estaban presentes en la matriz "blanco". El criterio para reportar un positivo fue la identificación de al menos dos fragmentos iónicos a su masa exacta sin que presentaran desviaciones apreciables en la relación Q/q (Commission Decision 2002/657/EC). Así se obtuvo un alto grado de confirmación para la presencia de OPEs en muestras acuosas y muestras con elevado contenido salino a niveles de concentración extraordinariamente bajos.

- *Screening post-target* de OPEs en muestras reales. Análisis retrospectivo.

Gracias a que el analizador TOF presenta una elevada capacidad resolutiva y también gracias a su modo de adquisición en *full scan* con alta sensibilidad, es posible realizar una búsqueda de un analito específico con posterioridad a la adquisición de datos por MS, siempre conociendo sus fragmentos iónicos teóricos. Así, contaminantes orgánicos diferentes a los seleccionados en muestras previamente adquiridas en analizadores TOF pueden ser identificados a posteriori (Rubio et al., 2009). En nuestro caso, la información espectral completa adquirida por los analizadores TOF nos permitió identificar analitos seleccionados en modo *post-target*. Así pues, fue posible llevar a cabo el análisis de OPEs en muestras previamente analizadas mediante GC-TOF MS, muestras en las que no se había llevado a cabo el estudio de la presencia de OPEs previamente. Igualmente, una de las ventajas fue el hecho de no ser necesario realizar un nuevo tratamiento a las muestras puesto que el analizador TOF adquiere todo el espectro de masas y es posible identificar la

presencia de cualquier compuesto ionizable en un sistema de GC a posteriori. El procesado de la información mediante el software TargetLynx para los compuestos seleccionados, permitió su identificación en diferentes muestras.

Previamente a nuestro trabajo, Portolés et al. (2007) realizó una aplicación con TOF MS que permitió la investigación de la presencia de varios analitos en muestras acuosas mediante análisis *post-target* mostrando la gran ventaja de no reanalizar las muestras de nuevo. Esta posibilidad de análisis no es posible con otros analizadores del tipo Q o QqQ trabajando en modos SIM o SRM o trabajando con analizadores IT en modo MS/MS puesto que la selección de los iones se realiza antes de la adquisición en MS. Recientemente, Grimalt et al. (2010) realizó una comparativa de tres analizadores de MS, QqQ, TOF y QTOF en la que el analizador TOF mostró mayores ventajas que los demás para el *screening* por su mejor sensibilidad trabajando en modo de adquisición de espectro completo y medidas de masa exacta.

5.3.4. Referencias.

Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Available: eur-lex. europa.eu/pri/en/oj/dat/2002/l_221/l_22120020817en000-80036.pdf.

Ellis, J.; Shah, M.; Kubachka, K.M.; Caruso, J.A. 2007. Determination of organophosphorus fire retardants and plasticizers in wastewater samples using MAE-SPME with GC-ICPMS and GC-TOFMS detection. J. Environ. Monitor. 9, 1329-1336.

Ferrer, I.; Fernandez-Alba, A.; Zweigenbaum, J.A.; Thurman, E.M. 2006. Exact-mass library for pesticides using a molecular-feature database. Rapid Commun. Mass Spectrom. 20, 3659-3668.

Garcia-Jares, C.; Regueiro, J.; Barro, R.; Dagnac, T.; Llompart, M. 2009. Analysis of industrial contaminants in indoor air. Part 2. Emergent contaminants and pesticides. J. Chromatogr. A 1216, 567-597.

Grimalt, S.; V.Sancho, J.; Pozo, O.J.; Hernández, F.E. 2010. Quantification, confirmation and screening capability of UHPLC coupled to triple quadrupole and hybrid quadrupole time-of-flightmass spectrometry in pesticide residue analysis. J. Mass Spectrom. 45, 421-436.

McLafferty, F.W. 1959. Mass spectrometric analysis: Molecular rearrangements. Anal. Chem. 31, 82-87.

Nácher-Mestre, J.; Serrano, R.; Portolés, T.; Hernández, F.; Benedito-Palos, L.; Pérez-Sánchez, J., 2009. A reliable analytical approach based on gas chromatography coupled to triple quadrupole and time-of-flight mass analyzers for the determination and confirmation of polycyclic aromatic hydrocarbons in complex matrices from aquaculture activities. Rapid Commun. Mass Spectrom. 23, 2075-2086.

Pitarch, E.; Portolés, T.; Marín, J.M.; Ibáñez, M.; Albarrán, F.; Hernández, F. 2010. Analytical strategy based on the use of liquid chromatography and gas chromatography with triple-quadrupole and time-of-flight MS analyzers for investigating organic contaminants in wastewater. Anal. Bioanal. Chem. Article in Press.

Portolés, T.; Pitarch, E.; López, F.J.; Sancho, J.V.; Hernández, F. 2007. Methodical approach for the use of GC-TOF MS for screening and confirmation of organic pollutants in environmental water. J. Mass Spectrom. 42, 1175-1185.

Reemtsma, T., García-López, M., Rodríguez, I., Quintana, J.B., Rodil, R. 2008. Organophosphorus flame retardants and plasticizers in water and air I. Occurrence and fate. Trends Anal. Chem. 27, 727-737.

Rubio, S.; Pérez-Bendito, D. 2009. Recent advances in environmental analysis. Anal. Chem. 81, 4601-4622.

CAPÍTULO 6

ESTUDIO DE LA BIOACUMULACIÓN DE CONTAMINANTES ORGÁNICOS PERSISTENTES EN PRODUCTOS DE LA ACUICULTURA MARINA.

6.1. Introducción

En este capítulo de la Tesis Doctoral se presentan dos estudios sobre la bioacumulación de contaminantes orgánicos en productos de la acuicultura marina.

En los últimos tiempos, la creciente demanda de aceites de pescado por parte de la acuicultura, unido a un decrecimiento de las capturas en las pesquerías, ha puesto en peligro la sostenibilidad de esta industria. Esto ha hecho necesaria la búsqueda de fuentes alternativas de lípidos para los piensos utilizados en la cría de peces.

Actualmente se estudia la posibilidad de sustituir el aceite de pescado presente en las formulaciones de piensos, al menos en parte, por aceites de origen vegetal. Esto hace necesario conocer las consecuencias que esta sustitución puede tener en la calidad del producto final, como por ejemplo en el perfil de ácidos grasos de los peces cultivados con estos piensos o la carga de contaminantes en el producto final.

En las instalaciones del Instituto de Acuicultura de Torre la Sal (IATS, CSIC), en el marco del proyecto europeo "Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers" (Codigo n° 016249-2), se llevó a cabo un experimento que consistió en el cultivo de doradas (*Sparus aurata L.*) a lo largo de un ciclo de crecimiento completo, utilizando dietas con composiciones que diferían en la proporción de aceites de pescado, sustituido por aceites de origen vegetal. A partir de este experimento, se estudiaron paralelamente parámetros de crecimiento, perfiles de ácidos grasos y otros indicadores bioquímicos, junto con el estudio de la presencia de POPs en los diferentes productos utilizados o producidos durante todo el proceso de cultivo.

Concretamente se estudió la presencia de PAHs, OCPs, PBDEs y PCBs en los piensos que fueron suministrados a los peces durante el experimento, en los ingredientes utilizados en la formación de estos, y en los filetes de los peces a lo largo de todo el proceso de engorde.

El estudio de la bioacumulación de PAHs y OCs dio lugar a los artículos científicos que se presentan y comentan a continuación. La presencia de PBDEs en las muestras analizadas presentó valores de concentración por debajo del LOQ en la mayoría de los casos, no observándose bioacumulación de estos compuestos a lo largo del proceso de engorde (**Artículo científico 6**).

6.2. Estudio de la concentración y bioacumulación de hidrocarburos policíclicos aromáticos en doradas procedentes de la acuicultura.

6.2.1. Introducción.

Como se ha comentado con anterioridad, los PAHs son compuestos orgánicos ampliamente distribuidos en el medio acuático como resultado de numerosas actividades humanas y de procesos naturales (Menzie et al., 1992). Estos compuestos orgánicos también han adquirido una notable importancia como contaminantes debido a su persistencia en el medio ya que muchos de ellos son potentes tóxicos, mutágenos y teratógenos para los organismos acuáticos e incluso para el hombre (IARC, 1987). En consecuencia, los PAHs han sido considerados contaminantes prioritarios por la Agencia Americana para la Protección del Medio Ambiente (EPA 1987, 1993) y por la USEPA (Directiva 2000/60/EC).

Los PAHs llegan al medio ambiente marino mediante dos vías: a partir de deposición atmosférica y procedentes de las aguas que contienen material particulado capaz de adsorber PAHs (Latimer and Zheng, 2003). Normalmente, en los procesos de combustión, los PAHs más ligeros con 2-3 anillos aromáticos están en forma gaseosa, mientras que los que contienen más anillos bencénicos son o bien semivolátiles o están totalmente adsorbidos a las partículas de hollín. Posteriormente se transfieren desde la atmósfera al suelo o al agua superficial tanto por deposición como por sedimentación de las partículas. Dependiendo de la temperatura, se puede producir reemisión desde el suelo. La movilidad de estos compuestos en el suelo está determinada por el transporte acuático.

Debido a su persistencia, volatilidad y su fuerte absorción, los PAHs tienden a acumularse en aquellas matrices afines a sus características lipófilas. Su naturaleza lipofílica hace que sean fácilmente ingeridos y acumulados por organismos acuáticos mediante la alimentación.

Las materias primas de origen marino son utilizadas frecuentemente en la acuicultura para aportar ácidos poliinsaturados. Estas matrices son susceptibles de presentar PAHs que pueden ser acumulados por los peces y transferidos a niveles superiores dentro de la cadena trófica. Por lo tanto, en la acuicultura marina, la alimentación de peces con materias primas de origen marino puede suponer la acumulación de PAHs y otros contaminantes lipófilos en las especies cultivadas.

La producción de peces, moluscos y otros invertebrados en la acuicultura ha aumentado rápidamente durante las últimas décadas como consecuencia del elevado consumo de estos y la pesca excesiva que sufren las especies salvajes. En los últimos años la demanda de productos procedentes de la acuicultura ha tenido un crecimiento muy pronunciado. Las materias primas, sobre todo harinas de pescado y aceites de pescado, tuvieron alzas significativas en los precios, debido a la mayor demanda mundial. Pero no hay que olvidar que éstos se producen a partir de productos pesqueros, que son limitados y que en el futuro se van a ver enfrentados a una mayor escasez.

Es necesario asegurar un futuro sostenible de la acuicultura y actuar para el desarrollo y la salud del entorno marítimo. Para ello, es imprescindible aumentar el conocimiento científico de los aspectos eco-toxicológicos de esta actividad. Para evaluar la toxicidad de los PAHs se utilizan criterios establecidos sobre factores de equivalencia tóxica (TEFs) (EPA 2000, Nisbet and Lagoy, 1992) y la referencia de toxicidad expresada como equivalentes del Benzo(a)Pireno (BaPEs). Estos equivalentes tóxicos suponen una estimación adecuada para conocer la toxicidad de la suma de los PAHs encontrados en las muestras del estudio.

En el presente estudio se determinó la concentración de PAHs en materias primas, piensos y filetes de dorada. Todas las muestras provienen de un estudio experimental que incluye un ciclo completo de engorde de doradas (*Sparus aurata L*.) en el marco del Proyecto AQUAMAX (Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers. www.aquamaxip.eu). La información generada a partir de estos estudios es de gran importancia pues permitirá identificar cuáles son las concentraciones de estos compuestos y si se produce biomagnificación (BMF) en el individuo durante su crecimiento, hasta llegar al tamaño comercial. También permitirá conocer cuáles son las concentraciones finales que puede presentar un filete de dorada una vez alcanzado su tamaño comercial cuando es destinado para el consumo humano.

La determinación de PAHs en las muestras se llevó a cabo mediante metodología analítica desarrollada como parte de esta Tesis (**ver Capítulo 4. Artículo científico 1**). Gracias a esta metodología fue posible eliminar gran parte de los interferentes procedentes

de las muestras y determinar PAHs a niveles de µg/kg mediante GC-(QqQ)MS/MS y GC-TOF MS.

6.2.2. Artículo científico 5:

Bioaccumulation of Polycyclic Aromatic Hydrocarbons in Gilthead Sea Bream (Sparus aurata L.) Exposed to Long Term Feeding Trials with Different Experimental Diets.

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Bioaccumulation of Polycyclic Aromatic Hydrocarbons in Gilthead Sea Bream (*Sparus aurata* L.) Exposed to Long Term Feeding Trials with Different Experimental Diets

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Abstract Polycyclic aromatic hydrocarbons (16 EPA list) were determined in oils, fish feed, and fillets from gilthead sea bream fed through a full production cycle (14 months) with feed containing different proportions of fish oil replaced by vegetable oils, followed by a finishing phase with fish oil. At the beginning of the study, fish presented 46.6 µg/kg fresh weight of the sum of PAHs in fillet and a benzo[a]pyrene equivalent value of 9.1 µg/kg fresh weight. These levels decreased after 330 days of rearing to values around 2 µg/kg. Although the concentration increased again during the finishing phase, they remained low. These low concentrations of PAHs could be the result of a dilution process associated with fish growth and with the detoxification pathways, both favored by the low levels of PAHs present in the feeds and the lack of any other potential source of contamination during the whole rearing period.

Polycyclic aromatic hydrocarbons (PAHs) are compounds formed by two or more fused aromatic rings originating from natural and anthropogenic sources, such as incomplete combustions, industrial incinerations, transport, or uncontrolled spills. Sixteen of them have been included in the list of priority pollutants by the US Environmental Protection Agency (EPA) as a consequence of their potential adverse effects on organisms, including human health (EPA 1987). Over recent years, a concern has arisen about the impact of these contaminants in coastal environments and on human

consumers, especially as a consequence of accidental spills (Cortazar et al. 2008). They are ubiquitous in the marine environment, and due to their hydrophobic nature, they tend to be absorbed rapidly on suspended materials and sediment, becoming bioavailable to fish and other marine organisms through the food chain (Latimer and Zheng 2003; Liang et al. 2007; Perugini et al. 2007).

Polycyclic aromatic hydrocarbons are carcinogenic in mammalian species and in some fish species (Hendricks et al. 1985; Schultz and Schultz 1982). Many chemical carcinogens are procarcinogens requiring biotransformation frequently by oxidative metabolism through the cytochrome P-450 monooxygenase system (Stegeman and Lech 1991). Metabolization and depuration ability of PAHs by fish has been investigated and stated by several authors (Ferreira et al. 2006; Kennedy et al. 2004; Martin-Skilton et al. 2008).

Marine aquaculture has seen strong development in the last few decades in order to meet the increased fish consumption by the world population and the decreasing wild stocks. In populations having high seafood consumption, both farmed and wild seafood products can also contribute toward contaminant load of the food basket (Martí-Cid et al. 2007, 2008). Fish used as raw material for the manufacture of fish oil (FO) and fish meal as feed ingredients are a possible source of PAHs in fish feed that could possibly be bioaccumulated by farmed fish (Hellou et al. 2005). Likewise, vegetable oils used in fish feed manufacture are another possible source of PAHs (Moret and Conte 2000). Aquaculture products are subject to increasing strict control and regulation. The European Commission Regulation (EC) 1881/2006 of December 2006 has fixed maximum levels of 2 µg/kg fresh weight for benzo[a]pyrene (BaPy) in fish. Additionally, some countries have adopted a legal limit of 1 µg/kg for BaPy content in smoked fish foodstuff (Bories 1988). However, there appear to be no legal limits for vegetable oils (Moret and Conte 2000).

In order to estimate the toxicity of PAHs, it is common to apply toxic equivalent factors (TEFs) for each individual congener in comparison with the carcinogenic activity of BaPy. For comparative purposes, the PAH concentrations are summed and also expressed as benzo[a]pyrene equivalents (BaPEs), their relative concentrations being weighted in relation to the carcinogenic potential of individual PAH compounds using equivalency factors (Law et al. 2002). The BaPEs values calculated represent the toxicity of the charge load of PAHs in each sample (EPA 2000; Nisbet and LaGoy 1992).

A wide variety of environmental pollutants have been detected in farmed fish (Bordajandi et al. 2006; Easton et al. 2002; Hites et al. 2004; Liang et al. 2007; Maule et al. 2007; Perugini et al. 2007; Santerre et al. 2000; Serrano et al. 2008a, 2008b). Fish meal and FO commonly used as feed ingredients, especially from the northern hemisphere, are known to contain persistent organic

pollutants (POPs) at different levels and a reduction of these POPs is an issue of concern (Oterhals and Nygård 2008; Oterhals et al. 2007).

The use of plant-based ingredients as alternatives to fish meal and FO can potentially reduce the charge load of lipophilic contaminants in aquafeeds, and thereby in farmed fish, improving at the same time the sustainability of marine fishery resources (Bell et al. 2005; Berntssen et al. 2005; Bethune et al. 2006). Although the presence and accumulation of organochlorine compounds in farmed fish have been studied rather extensively (Bordajandi et al. 2006; Easton et al. 2002; Hites et al. 2004; Jacobs et al. 1998; Santerre et al. 2000; Serrano et al. 2008a, 2008b), very little information is available on the behavior of PAHs in the aquaculture food chain, apart from guidances on risk assessment for animal and humans (Commission Regulation 2006; EPA 1993, 2000).

This work is part of a broader study aiming at analyzing the effects of FO substitution by vegetable oils in low-fish-meal diets. Whereas other parts of the study have dealt with fatty acid metabolism (Benedito-Palos et al. 2009) and the bioaccumulation of organochlorine compounds (Nácher-Mestre et al. 2009a), the main objective of the present study was to follow the potential accumulation of PAHs in fast-growing juveniles of the gilthead sea bream (Sparus aurata L.), a major farmed fish in the Mediterranean aquaculture, exposed through the entire productive cycle to feeds with graded levels of FO replaced by a mixture of vegetable oils and then switched for 3 months to a finishing feed based on FO. The levels of PAHs and their equivalence as BaPEs were determined in oils, feeds, and fish fillets in order to gather information on the effects of dietary oil sources on end-product quality and safety.

Materials and Methods

Experimental Diets

Three (isoproteic, isolipidic, and isoenergetic) feeds were formulated with a low inclusion level (20%) of fish meal and fish soluble protein concentrates. Fish oil from the southern hemisphere was the only lipid source in the control diet (FO), which was also used during the finishing period. The two remaining diets contained a blend of vegetable oils (2.5 rapeseed oil: 8.8 linseed oil: 3 palm oil), replacing 33% (33VO) and 66% (66VO) of the FO. All diets were manufactured using a twin-screw extruder (Clextral, BC 45) at the Institut National de la Recherche Agronomique (INRA) experimental research station of Donzacq (Landes, France), dried under hot air, sealed, and kept in air-tight bags until use. Ingredients and approximate composition of the feeds are reported in Table 1.

Bioaccumulation Experiment

Gilthead sea bream obtained from Ferme Marine de Douhet, Ile d'Oléron, France were acclimatized at the Instituto de Acuicultura de Torre de la Sal (IATS) for 20 days before the start of the study. Fish of ~18 g initial mean body weights were allocated into 9 fiberglass tanks (3000 L) in groups of 150 fish per tank. Water flow was 20 L/min and the oxygen content of outlet water remained above 85% saturation. The growth study was undertaken over 14 months (July 11, 2006 to September 2, 2007). The photoperiod and water temperature (10–26°C) varied over the course of the trial following natural changes at IATS latitude (40°5′N; 0°10′ E).

During the first 11 months of the trial, the three diets were randomly allocated to triplicate groups of fish and feed was offered by hand to apparent visual satiety. During the finishing feeding phase (12 weeks, June 6, 2007 to September 2, 2007), two tanks of the 33VO and two of the 66VO groups were fed with the FO diet. These groups were then renamed 33VO/FO and 66VO/FO, respectively. Fish fed the FO diet and one tank each of fish fed 33VO and 66VO diets were maintained on the initial diets until the end of the study. This led to five treatments in the bioaccumulation study: FO, 33VO, 66VO, 33VO/FO, and 66VO/FO (Fig. 1).

At the beginning and at regular intervals throughout the finishing feeding phase (0, 330, 360, 390, and 420 days), randomly selected fish (eight per treatment) were killed by a blow on the head prior to tissue sampling, and the left-side fillet (with skin and bone removed) was excised and stored at −20°C until analysis. Body weight and fillet yield were not affected by the dietary treatments over the course of the feeding trial (Benedito-Palos et al. 2009). Seawater for fish culture was checked using the methodology for PAHs described by Pitarch et al. (2007) and no PAH was detected (limit of detection: 5 and 100 ng/L), thus ensuring that no known PAH exposure other than that from feeds was expected during the study.

Analytical Method

Polycyclic aromatic hydrocarbons included in the list of priority contaminants by the US EPA (EPA 1987) were analyzed in oils, fish feed, and fish fillets following the method described by Nácher-Mestre et al. (2009b). Samples were collected and stored at −20°C until analysis. Eight fish from each

Table 1. Ingredients and chemical composition of experimental diets.

	FO	*33VO*	*66VO*
Ingredient (%)			
Fish meal (CP 70%) [a]	15	15	15
CPSP 90 [b]	5	5	5
Corn gluten	40	40	40
Soybean meal	14.3	14.3	14.3
Extruded wheat	4	4	4
Fish oil [c]	15.15	10.15	5.15
Rapeseed oil	0	0.85	1.7
Linseed oil	0	2.9	5.8
Palm oil	0	1.25	2.5
Soya lecithin	1	1	1
Binder	1	1	1
Mineral premix [d]	1	1	1
Vitamin premix [e]	1	1	1
$CaHPO_4.2H_2O$ (18%P)	2	2	2
L-Lysine	0.55	0.55	0.55
Composition			
Dry matter (DM %)	93.13	92.9	92.77
Protein (% DM)	53.2	52.81	52.62
Fat (% DM)	21.09	21	20.99
Ash (% DM)	6.52	6.69	6.57

[a]Fish meal (Scandinavian LT)
[b]Fish soluble protein concentrate (Sopropêche, France)
[c]Fish oil (Sopropêche, France)
[d]Supplied the following (mg · kg /diet, except as noted): calcium carbonate (40% Ca) 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0.3.
[e]Supplied the following (mg · kg /diet): retinyl acetate 2.58; DL-cholecalciferol, 0.037; DL-α tocopheryl acetate, 30; menadione sodium bisulphite, 2.5; thiamin, 7.5; riboflavin, 15; pyridoxine, 7.5; nicotinic acid, 87.5; folic acid, 2.5; calcium pantothenate, 2.5; vitamin B_{12}, 0.025; ascorbic acid, 250; inositol, 500 ; biotin, 1.25; choline, chloride 500

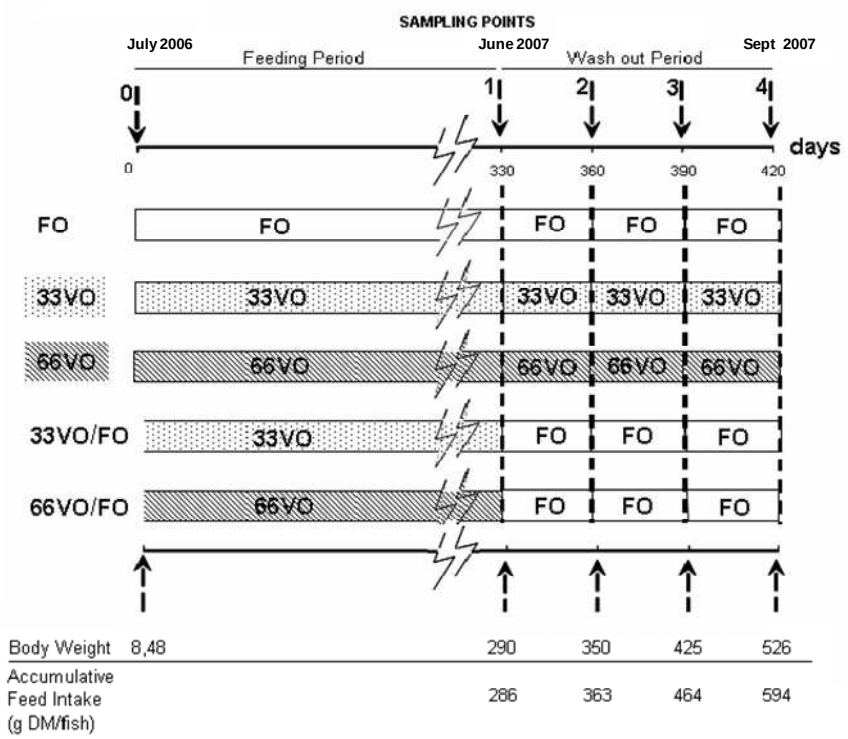

Fig. 1 Schematic representation of the long-term feeding trial over 14 months

treatment were randomly selected to obtain three composite samples of three, three, and two fillets. Fish diets and oils were homogenized and analyzed in triplicate.

Before analysis, samples were thawed at room temperature and were carefully ground (fillets and feeds) using a kitchen grinder (Super JS, Moulinex, France). Approximately 2 g of sample were homogenized with 6 g of anhydrous sodium sulfate and the blend was spiked with 100 µL of surrogate solution (25 ng/mL) Ten milliliters of methanolic solution of 1 M KOH was added to the mixture and saponified for 3 h at 80°C. Then analytes were extracted twice with 8 mL of n-hexane and the solution filtered through 0.2 µm and concentrated under gentle nitrogen stream at 40°C to 1 mL (in the case of oils, extracts were concentrated to 5 mL). One milliliter of the extract was passed through the Florisil SPE cartridge previously conditioned with 6 mL of n-hexane and eluted with 8 mL dichloromethane:n-hexane (20:80) solution. The eluate was evaporated and redissolved in 0.25 mL of n-hexane. The final extracts obtained after the cleanup procedure were analyzed by means of a triple quadrupole analyzer [details in Nácher-Mestre et al. (2009b)]. Quantification of samples was carried out by means of external calibration curves using the internal standard method. Statistical validation of the method developed was performed by evaluating analytical parameters. The method was applied to the four matrixes studied at different fortification levels offering satisfactory

recoveries (between 70% and 120%) and precisions (<30%) in all cases [more details in Nácher-Mestre et al. (2009b)].

The whole analytical process was carried out in Good Laboratory Practices-certified laboratories of the Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain.

Determination of Fat

The total fat content in the sample extracts was determined gravimetrically, after evaporation at 95°C until constant weight.

Data Analysis

The PAH concentrations are expressed as micrograms per kilogram of fresh weight and as micrograms per kilogram of lipid base in fillets. Bioaccumulation factors (BAFs) were calculated as the ratio between lipid-based concentrations of PAHs both in the fish fillets and in the fish feed at each sampling point. Because two different fish feed were used in the 33VO/FO and 66VO/FO groups, calculations of BAFs were made correcting for respective feed intakes. Thus, the arithmetic mean of PAH concentrations in the diet was calculated on the basis of the gram of dry matter ingested per fish, and the concentration of PAHs in each fish feed. BaPEs were calculated for fish fillets using the TEFs proposed by the US EPA (EPA 2000). The concentrations of individual PAHs (µg/kg fresh weight) are expressed as equivalents of BaPy, and these values are summed to obtain a BaPEs value. Data for concentrations of PAHs were compared by means of ANOVA I and a posteriori Scheffe's test ($p < 0.05$). All data were log-transformed before statistical analysis to achieve normality. Homoscedasticity of variances was tested by means of Bartlett's test ($p < 0.05$). All of the statistical analyses were conducted using STATGRAPHICS plus for Windows version 4.1 (Statistical Graphics Corporation).

Results and Discussion

PAHs in Oils and Feeds

The analytical method showed excellent sensitivity and selectivity as a consequence of the use of gas chromatography-coupled tandem mass spectrometry (GC–MS/MS) together with the efficiency of

the saponification followed by solid-phase extraction clean up. It has allowed analyte quantification at concentrations as low as picograms per gram of fresh weight and micrograms per kilogram of lipid weight in the case of oils, with acceptable precisions (below 30%) (Nácher-Mestre et al. 2009b).

Concentrations of PAHs present in the oils used in the feeds are shown in Table 2. Up to 14 of the 16 PAHs considered were detected only at trace levels. Both FO and vegetable oils had similar levels and patterns, with individual PAHs concentrations up to 38.2 µg/kg fresh weight and total loads (Σ16PAH) of 56.7 µg/kg fresh weight in FO and between 12 and 47 µg/kg fresh weights in vegetable oils. The presence of PAHs in the marine environment has been reported by several authors (Liang et al. 2007; Oros and Ross 2005; Perugini et al. 2007). Vegetable derivatives can accumulate PAHs from different sources such as atmospheric deposition of contaminated dust and particulate matter in the plants and the processing for oil production (Moret and Conte 2000).

The PAH concentrations in the feeds did not correlate with the amounts present in the FO or vegetable oils (VOs) used. We find here that replacement of FO by VOs in the feed did not allow one to minimize the PAHs charge load in complete fish feed. It then appears that the PAHs found in the feed originated from ingredients other than the oils used. The contamination pattern was similar in the different feeds with a predominance of 2-ring PAH naphthalene (up to 215 µg/kg fresh weight) and 3- and 4-ring PAHs, whereas 5- and 6-ring PAHs were less abundant. Naphthalene is the PAH with the lowest K ow and bioconcentration factor (BCF) values. Liang et al. (2007) have reported a similar PAH pattern contamination in fish, with trace concentrations of individual PAHs but naphthalene at concentrations higher than 100 µg/kg fresh weight. In that particular case, this was related to the high accumulation factor biota-sediment calculated for this compound. In our case, the sources and behavior of these compounds are very complex. The different ingredients used in the feeds are processed products from fishery, plant, and industrial sources and the proportions of the different individual PAHs, which could change during the manufacturing process. Even the drying of raw materials by means of hot air could be a potential source of contamination (Moret and Conte 2000). This process is always followed by a considerable increase in PAH content. Moret et al. (2000) reported that oils could have dangerous levels of PAHs for human health due to the drying process. Specifically, BaPy presented an increased concentration in oil samples after drying, ranging from 8.6 to 44.3 ppb (Moret et al. 2000).

Table 2 Concentration of polycyclic aromatic hydrocarbons (PAHs, µg/kg) in the oils used and in the complete feeds

Ingredients	**Fish oil**		**Linseed oil**		**Rapeseed oil**		**Palm oil**		**FO**		**33VO**		**66VO**	
Compound	*Mean*	*CV*	*Mean*	*CV*	*Mean*	*CV*	*Mean*	*CV*	*Mean*	*C.V.*	*Mean*	*C.V.*	*Mean*	*C.V.*
Naphthalene	-		-		1.9	22	0.8	24	215	11	242	10	161	9
Acenaphthylene	-		-		-		-		0.6	16	0.6	4	0.8	18
Acenaphthene	0.3	8	-		0.6	13	0.5	15	1.7	7	1.4	6	1.2	8
Fluorene	4.3	5	2.6	4	0.9	4	0.4	8	3.8	4	4	10	3.5	8
Phenanthrene	38.2	2	15.2	1	1.2	3	1.3	3	6.1	3	5.6	1	5	5
Anthracene	-		-		1.6	7	-		1.1	21	1	12	1.3	3
Fluoranthene	13.3	7	16.7	4	-		-		2.1	3	2	7	2	4
Pyrene	9	1	11.3	1	-		-		2.1	15	1.9	11	1.8	10
Benzo(a)Anthracene	-		-		0.9	1	1.2	1	-		-		-	
Chrysene	-		-		0.8	4	-		0.6	6	0.8	22	0.6	15
Benzo(b)Fluoranthene	0.3	22	0.5	4	0.4	4	1.3	3	0.7	13	0.6	20	0.7	16
Benzo(k)Fluoranthene	0.3	2	0.4	5	0.4	9	1.3	7	0.6	25	0.6	9	0.5	5
Benzo(a)Pyrene	-		0.3	8	0.7	4	1.4	3	-		-		-	
Indeno(1,2,3-cd)Pyrene	-		-		0.7	7	1.1	6	-		-		-	
Dibenzo(a.h)Anthracene	-		-		0.9	2	1.3	1	-		-		-	
Benzo(g.h.i)Perylene	-		-		0.8	8	1.2	6	-		-		-	
ΣPAH16	56.7	31	47	31	12	2	12	2	234.4	44.2	260.5	39.1	178.4	34.5

CV: coefficient of variation. Compounds were quantified with CV<30%. -: non detected. FO: Fish oil diet. 33VO: 33 % vegetable oil diet. 66VO: 66% vegetable oil diet.

PAHs in Fish Fed the Different Feeds

Table 3 presents the individual concentrations and Σ16PAHs in fillets from fish fed the different experimental diets during the study. Initial fish had 46.6 μg/kg fresh weight of Σ16PAHs (1084 μg/kg lipid). After 330 days of feeding with the diets, values descended to 1.1–1.9 μg/kg fresh weight (14.6–24.4 μg/kg lipid) in the different groups. These low concentrations of PAHs remained at trace levels until the end of the study. Naphthalene was not detected after 11 months of rearing, probably as consequence of its physicochemical characteristics, which could explain the low bioaccumulation ability found here. Similarly, anthracene, benzo[a]anthracene and all 5- and 6-ring PAHs initially present in fish at time 0 were not detected after 330 days of exposure.

Data on BaPEs in the fillets from fish fed the different feeds are also reported in Table 3. BaPE values were below 0.02 μg/kg fresh weight, except for initial fish, which had a value of 9.1 μg/kg fresh weight, which is higher than the maximum level of 2 μg/kg allowed in fish by the Commission Regulation (EC) 1881/2006 (Commission Regulation 2006).

The decrease of PAH concentrations (expressed per unit fresh weight or unit lipid) during the first 11 months suggests the action of a dilution process linked to body mass increase (from 18 to 300 g) in the absence of PAH sources other than the low load from diets. This process does not preclude a contaminant biodepuration of PAHs present in initial fish through detoxification metabolic routes. The load of organochlorine pollutants during the same growth study was also monitored (Nácher-Mestre et al. 2009a) and did not reveal any accumulation during the first 11 months of feeding with the different feeds.

As reported in Fig. 2, in spite of the low levels of PAHs found in fish fillets during the study, the ∑16PAHs concentration appreciably increased after 360 days of exposure in all groups, coinciding with the increase in feed intake and the growth spurt of summer (from 290 to 530 g in 90 days). These differences were significant at 390 days (Scheffe's test, $p < 0.05$), especially in groups refed with diet FO and were followed by a plateau by the end of the finishing phase.

The load of POPs, including organochlorine pesticides, polychlorinated byphenyls, PAHs, and polybrominated diphenyl ethers, has also been characterized in the feeds as part of the project. The total load of contaminants (POPs) in diet FO (Nácher-Mestre et al. 2009a) was higher than that of the other feeds. This could lead to a decrease in the efficacy of the PAH metabolism in fish from groups refed FO the last 3 months. On the other hand, the stabilization of the concentrations during the last month of the trial suggests the adaptation of the fish to the uptake levels of pollutants presented the last 3 months of the experiment and the recovery of the ability to biodepurate the PAHs up taken via

Table 3 Concentrations of PAHs (µg/kg fresh weight; n = 3) in fillets from gilthead sea bream fed the different diets

Diet			**FO**								**33VOFO**		**66VO**				**66VOFO**	
Time of exposure	**T0**		**T1**		**T4**		**T1**		**T4**		**T4**		**T1**		**T4**		**T4**	
	Mean	*CV*	*Mean*	*CV*	*Mean*	*CV*	*Mean*	*CV*	*Mean*	*CV*	*Mean*	*CV*	*Mean*	*CV*	*Mean*	*CV*	*Mean*	*CV*
Naphthalene	3.9	1	-		-		-		-		-		-		-		-	
Acenaphthylene	-		-		-		-		-		-		-		-		-	
Acenaphthene	-		-		-		-		-		-		-		-		-	
Fluorene	-		0.36	2	0.51	8	0.32	17	0.63	29	0.52	28	0.22	14	0.58	6	0.41	5
Phenanthrene	2.0	1	0.86	26	1.83	7	0.79	29	2.11	27	2.40	10	0.49	7	2.21	18	2.13	17
Anthracene	1.0	1	-		-		-		-		-		-		-		-	
Fluoranthene	3.9	1	0.28	12	0.99	13	0.22	16	1	7	1.8	22	0.18	3	0.64	29	1.61	11
Pyrene	4.9	1	0.4	27	2.8	19	0.31	19	2.07	1	4.71	27	0.25	9	1.35	29	4.21	12
Benzo(a)Anthracene	4.9	1	-		-		-		0.09	13	-		-		0.14	2	0.15	13
Chrysene	6.9	1	-		0.14	13	-		0.17	14	0.16	8	-		0.16	4	0.18	20
Benzo(b)Fluoranthene	5.9	1	-		-		-		-		-		-		-		-	
Benzo(k)Fluoranthene	5.9	1	-		-		-		-		-		-		-		-	
Benzo(a)Pyrene	3.9	1	-		-		-		-		-		-		-		-	
Indeno(1,2,3-cd)Pyrene	-		-		-		-		-		-		-		-		-	
Dibenzo(a.h)Anthracene	-		-		-		-		-		-		-		-		-	
Benzo(g.h.i)Perylene	3.4	1	-		-		-		-		-		-		-		-	
ΣPAH16 (µg/kg resh	46.6	3	1.9	20	6.3	7	1.6	19	6.06	7	9.6	26	1.1	12	5.1	14	8.7	6
ΣPAH16 (µg/kg lipid	1084	36	24.4	9	68.2	10	21.6	9	60.6	9	85.8	7	14.6	3	50.8	9	79.7	9
BaPEs (µg/kg fresh	9.1	2	0.002	10	0.007	15	0.002	13	0.002	19	0.013	16	0.001	6	0.006	15	0.010	21

T0 : July/06. T1 : June/07. T4 : Sept/07. Mean: arithmetic mean. CV: coefficient of variation (%). For the rest of abbreviations, see Figure 1.

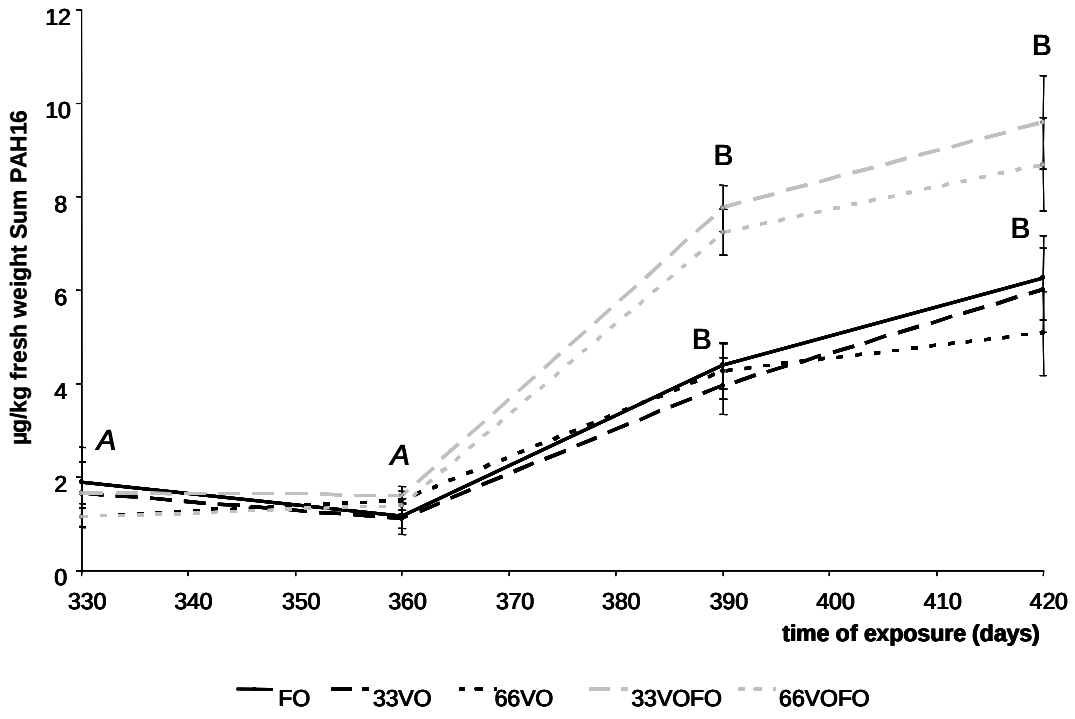

Fig. 2 Concentration of $\sum_{16}$PAH in fillets of fish fed different experimental diets with varying levels of fish oil substitution, during the feeding trial. Letters denote significant differences among sampling times for all groups; Scheffe's test; $p < 0.05$

diet. The extent to which this biodepuration process coexists with the above-mentioned dilution mechanism and the relative efficiency of both, especially foreseen in shorter periods of higher toxicant intake, like the finishing phase, remains to be ascertained.

Although it should be kept in mind that the concentrations present in fish are at the trace level, the increase of the PAH content at T4 could be explained in light of the increase of feed intake (Fig. 1) during the finishing phase and the reaching of a PAH intake level higher than the detoxification route capability.

In comparison with concentrations reported in the literature regarding seafood samples (Fontcuberta et al. 2006; Llobet et al. 2006; Saeed et al. 1995), the ∑PAHs values determined in gilthead sea bream as found here after 330 days of culture were low (~2 µg/kg). Although the concentration increased again during the finishing phase, as we have indicated earlier, it remained low, below 10 µg/kg in all groups, and similar to those found in fish from unpolluted areas. DouAbdul et al. (1997) indicated concentrations from <0.5 to 148 µg/kg fresh weight in fish fillets from

unpolluted areas. Bordajandi et al. (2004) reported levels between 8.2 and 71.4 µg/kg fresh weight in marine food samples from Spain, whereas Loufty et al. (2007) found levels in market fish samples from Egypt ranging from 0.78 to 19.70, similar to those observed in gilthead sea bream in this study and very low in comparison to polluted areas.

As reported in Table 4, individual PAHs found in fish fillets show variable BAF values. Phenanthrene, fluoranthene, and pyrene showed BAFs over 1 in almost all groups of fish. Phenanthrene, fluoranthene, and pyrene are bioaccumulated probably as a consequence of their high K ow (Table 4) because possible sources of these compounds other than diet, like combustions or petroleum, can be discarded. Nevertheless, chrysene had BAF values below 1 in spite of its higher K ow, which could be interpreted in terms of a more efficient degradation or depuration of this compound. On the basis of data obtained here, the different BAF values found for the different individual PAHs can be attributed to the variety of structures and physicochemical properties of the different components of the family.

Table 4 Bioaccumulation factors (BAF) for the polycyclic aromatic hydrocarbons (PAHs) detected after 420 days of the growth trial with the different diets (from lipid based concentrations in fish fillets and diets, n=3)

Diet	**FO**		**33VO**		**33VOFO**		**66VO**		**66VOFO**		**Kow**
Compound	*BAF*	*CV*	*BAF*	*CV*	*BAF*	*CV*	*BAF*	*CV*	*BAF*	*CV*	
Fluorene	0.29	8	0.32	29	1.10	6	0.26	28	0.98	5	4.18
Phenanthrene	0.65	7	0.75	27	2.45	18	1.21	10	1.63	17	4.57
Fluoranthene	1.02	13	1.00	7	2.28	29	1.80	22	2.42	11	5.22
Pyrene	2.90	19	2.18	1	4.05	29	6.01	27	3.60	12	5.18
Chrysene	0.51	13	0.43	14	0.52	8	0.49	4	0.91	20	5.86

Sources

The study of three PAH isomer ratios—anthracene/anthracene + phenanthrene (An/178), benz[a]anthracene/benz[a]anthracene + chrysene (BaA/228), and fluoranthene/fluoranthene +

pyrene [Fl/(Fl + Py)—was applied to identify the possible major sources of these contaminants in the different matrixes considered in this work (Table 5). These PAH ratios can be used as indicators of the different formation processes of the PAH contamination and of the possible sources of contamination in sediments (Yunker et al. 2002). FO and VOs have An/178 ratios below 0.1, which suggest unburned petroleum as one of the sources of the contamination present in these products. These An/178 ratios determined in the oils are similar to those found by Yunker et al. (2002) in sediments from remote or light-urban areas. The FO used in this research was from South American origin, which could explain the presence of PAHs from unburned petroleum. Fl/(Fl + Py) and BaA/228 ratios indicate nonpetroleum combustion sources of the PAHs found in the samples, supporting the idea of PAH contamination from light-urbanized areas.

Table 5 PAH isomer ratios (mean ± SD, n = 6) in oils, diets, and fish fed the different diets

PAH isomer ratio **Product**	**An/178**[a]	**BaA/228**[b]	**FL/FL+Py**[c]	**Phe/An**[d]	**Flu/Pyr**[e]
Vegetable oils	0.01±0.01	0.56±0.06	0.60±0.05	76 ± 10	1.5 ± 0.2
FO diet	0.15±0.02	0	0.50±0.06	5.5 ± 1.1	1.0 ± 0.2
33VO diet	0.15±0.01	0	0.51±0.05	5.6 ± 0.7	1.0 ± 0.1
66VO diet	0.21±0.02	0	0.53±0.06	3.8 ± 0.2	1.1 ± 0.1
Fish fillets T0[f]	0.33±0.02	0.42±0.05	0.44±0.06	2.0 ± 0.02	0.8 ± 0.008
Fish fillets T1	0	0	0.41±0.07	0	0.7 ± 0.2
Fish fillets T2	0	0	0.43±0.06	0	0.8 ± 0.01
Fish fillets T3	0	0	0.35±0.05	0	0.2 ± 0.001
Fish fillets T4	0	0	0.26±0.07	0	0.3 ± 0.06

[a] *An/178: anthracene/anthracene+phenanthrene,*
[b] *BaA/228: benzo[a]anthracene/benzo[a]anthracene+chrysene*
[c] *FL/FL + Py: fluoranthene/fluoranthene+pyrene,*
[d] *Phe/An: phenanthrene/anthracene,*
[e] *Flu/Pyr: fluoranthene/pyrene*
[f] *data for fish fillets are the mean of all experimental groups*

The feeds manufactured with the above-studied ingredients showed higher An/178 values, probably as a consequence of the manufacture process itself that could provoke degradations and

contaminations of the different individual PAHs. However, it is interesting to note that benzo[a]anthracene was not detected in the feed, possibly due to the degradation process during manufacture, as this PAH is one of the less stable (Yunker et al. 2002). The Fl/(Fl + Py) ratios found in the feeds are similar to those of the oils used: below 0.5.

Initial fish showed values for An/178, BaA/228, and Fl/(Fl + Py) of 0.333, 0.415, 0443, respectively. These values indicate both petroleum combustion and other combustions as sources of the PAHs in sediments (Yunker et al. 2002) and bivalves (Oros and Ross 2005), accumulated prior to the beginning of the present study. The An/178 and BaA/228 ratios decreased to 0 after 11 months of culture, whereas Fl/(Fl + Py) values decreased continuously during the whole growth period, suggesting a decrease of PAH exposure during the experimental period.

Conclusions

The levels of PAHs found in fish fillets grown with different diets over a full production cycle were very low and similar to those reported in foods from unpolluted marine areas. These low levels correspond to low levels of PAHs found in the diets and the lack of other potential sources of contamination in the rearing facilities over the experimental period. These conditions together with dilution and biodepuration mechanisms result in a net decrease of total PAH loads present in experimental fish at the start of the experiment and, thus, in the production of market size fish of good quality for human consumption.

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References

Bell JG, McGhee F, Dick JR, Tocher DR (2005) Dioxin and dioxin-like polychlorinated biphenyls (PCBs) in Scottish farmed salmon (Salmo salar): effects of replacement of dietary marine fish oil with vegetable oils. Aquaculture 243:305–314

Benedito-Palos L, Navarro JC, Bermejo-Nogales A, Saera-Vila A, Kaushik S, Pérez-Sánchez J (2009) The time course of fish oil wash-out follows a simple dilution model in gilthead sea bream (Sparus aurata L.) fed graded levels of vegetable oils. Aquaculture 288:98–105

Berntssen MHG, Lundebye AK, Torstennsen BE (2005) Reducing the levels of dioxins and dioxin-like PCBs in farmed Atlantic salmon by substitution of fish oil with vegetable oil in the feed. Aquacult Nutr 11:219–231

Bethune C, Seierstad SL, Seljeflot I, Johansen O, Arnesen H, Meltzer HM, Rosenlund G, Froyland L, Lundebye AK (2006) Dietary intake of differentially fed salmon: a preliminary study on contaminants. Eur J Clin Invest 36:193–201

Bordajandi LR, Gómez G, Abad E, Rivera J, Del Mar Fernandez-Baston M, Blasco J, González MJ (2004) Survey of persistent organochlorine contaminants (PCBs, PCDD/Fs, and PAHs), heavy metals (Cu, Cd, Zn, Pb, and Hg), and arsenic in food samples from Huelva (Spain): levels and health implications. J Agric Food Chem 52:992–1001

Bordajandi LR, Martín I, Abad E, Rivera J, Gonzalez MJ (2006) Organochlorine compounds (PCBs, PCDDs and PCDFs) in sea fish and seafood from the Spanish Atlantic Southwest Coast. Chemosphere 64:1450–1457

Bories G (ed) (1988) Tossicologia e sicurezza degli alimenti. Tecniche Nuove, Milan, p 359

Commission Regulation (2006) Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Available from (eurlex.europa.eu/LexUriServ/site/en/oj/2006/l_364/l_36420061220en00050024.pdf)

Cortazar E, Bartolomé L, Arrasate S, Usobiaga A, Raposo JC, Zuloaga O, Etxebarria N (2008) Distribution and bioaccumulation of PAHs in the UNESCO protected natural reserve of Urdaibai, Bay of Biscay. Chemosphere 72:1467–1474

Directiva 2000/60/CE del Parlamento Europeo y del Consejo, de 23 de octubre de 2000, por la que se establece un marco comunitario de actuación en el ámbito de la política de aguas.

DouAbdul AAZ, Heba HMA, Farred KH (1997) Polynuclear aromatic hydrocarbons (PAHs) in fish from the Red Sea Coast of Yemen. Hydrobiologia 352:251–262

EPA (1987) Quality criteria for water. EPA 440/5–86-001. US Environmental Protection Agency, Washington DC

EPA (1993) Provisional guidance for quantitative risk assessment of polycyclic aromatic hydrocarbons. EPA 600/R-93/089. US Environmental Protection Agency, Washington DC

EPA (2000) Guidance for assessing chemical contaminant data for use in fish advisories. EPA. 823-B-00–008. US Environmental Protection Agency, Washington DC

Easton MDL, Luszniak D, Von der Geest E (2002) Preliminary examination of contaminant loadings in farmed salmon, wild salmon and commercial salmon feed. Chemosphere 46:1053–1074

Ferreira M, Moradas-Ferreira P, Reis-Henriques MA (2006) The effect of long-term depuration on phase I and phase II biotransformation in mullets (Mugil cephalus) chronically exposed to pollutants in River Douro Estuary, Portugal. Marine Environ Res 61:326–338

Fontcuberta M, Arqués JF, Martínez M, Suárez A, Villalbí JR, Centrich F, Serrahima E, Duran J, Casas C (2006) Polycyclic aromatic hydrocarbons in food samples collected in Barcelona, Spain. J Food Prot 69:2024–2028

Hellou J, Haya K, Steller S, Burridge L (2005) Presence and distribution of PAHs, PCBs and DDE in feed and sediments under salmon aquaculture cages in the Bay of Fundy, New Brunswick, Canada. Aquat Conserv 15:349–365

Hendricks JD, Meyeres TR, Shelton DW, Casteel JL, Bailey GS (1985) Hepatocarcinogenicity of benzo(a)pyrene to rainbow trout by dietary exposure and intraperitoneal injection. J Natl Cancer Inst 74:839–851

Hites RA, Foran JA, Carpenter DO, Hamilton MC, Knuth BA, Schwager SJ (2004) Assessment of organic contaminants in farmed salmon. Science 303:226–229

IARC, (1987). IARC monographs on the evaluation of carcinogenic risks to humans, supplement 7. Overall evaluations of carcinogenicity: an updating of IARC monographs, vol 1-42., Vol. International Agency for Research on Cancer, Lyon.

Jacobs MN, Santillo D, Johnston PA, Wyatt CL, French MC (1998) Organochlorine residues in fish oil dietary supplements: comparison with industrial grade oils. Chemosphere 37:1709–1721

Kennedy CJ, Higgs D, Tierney K (2004) influence of diet and ration level on benzo[a]pyrene metabolism and excretion in rainbow trout (Oncorhyncus mykiss). Arch Environ Contam Toxicol 47:379–386

Latimer JS, Zheng J (2003) Sources, transport and fate of PAHs in the marine environment. In: Douben PET (ed) PAH: an ecotoxicological perspective. Wiley, Chichester, UK, pp 9–33

Law RJ, Kelly C, Baker K, Jones J, McIntosh AD, Moffat CF (2002) Toxic equivalency factors for PAH and their applicability in shellfish pollution monitoring studies. J Environ Monit 4:383–388

Liang Y, Tse MF, Young L, Wong MH (2007) Distribution patterns of polycyclic aromatic hydrocarbons (PAHs) in the sediments and fish at Mai Po Marshes Nature Reserve, Hong Kong. Water Res 41:1303–1311

Llobet JM, Falcó G, Bocio A, Domingo JL (2006) Exposure to polycyclic aromatic hydrocarbons through consumption of edible marine species in Catalonia, Spain. J Food Prot 69:2493–2499

Loufty N, Fuerhacker M, Tundo P, Raccanelli S, Ahmed MT (2007) Monitoring of polychlorinated dibenzo-p-dioxins and dibenzofurans, dioxin-like PCBs and polycyclic aromatic hydrocarbons in food and feed samples from Ismailia city, Egypt. Chemosphere 66:1962–1970

Martí-Cid R, Bocio A, Llobet JM, Domingo JL (2007) Intake of chemical contaminants through fish and seafood consumption by children of Catalonia, Spain: health risks. Food Chem Toxicol 45:1968–1974

Martí-Cid R, Llobet JM, Castell V, Domingo JL (2008) Evolution of the dietary exposure to polycyclic aromatic hydrocarbons in Catalonia, Spain. Food Chem Toxicol 46:3163–3171

Martin-Skilton R, Saborido-Rey F, Porte C (2008) Endocrine alteration and other biochemical responses in juvenile trubot exposed to the Prestige fuel oil. Sci Total Environ 404:68–76

Maule AG, Gannam AL, Davis JW (2007) Chemical contaminants in fish feeds used in federal salmonid hatcheries in the USA. Chemosphere 67:1308–1315

Moret S, Conte LS (2000) Polycyclic aromatic hydrocarbons in edible fats and oils: occurrence and analytical methods. J Chromatogr A 882:245–253

Moret S, Dudine A, Conte LS (2000) Processing effects on the polyaromatic hydrocarbon content of grapeseed oil. J Am Oil Chem Soc 77:1289–1292

Nácher-Mestre J, Serrano R, Benedito-Palos L, Navarro JC, Lopez FJ, Pérez-Sanchez J (2009a) Effects of fish oil replacement and re-feeding on the bioaccumulation of organochlorine compounds in gilthead sea bream (Sparus aurata L.) of market size. Chemosphere 76:811–817

Nácher-Mestre J, Serrano R, Portolés T, Hernández F, Benedito-Palos L, Pérez-Sanchez J (2009b) A reliable analytical approach based on gas chromatography coupled to triple quadrupole and time of flight analyzers for the determination and confirmation of polycyclic aromatic hydrocarbons in complex matrices from aquaculture activities. Rapid Commun Mass Spectrom 23:2075–2086

Nisbet IC, LaGoy PK (1992) Toxic equivalency factors (TEFs) for polycyclic aromatic hydrocarbons (PAHs). Regul Toxicol Pharm 16:290–300

Oros DR, Ross JRM (2005) Polycyclic aromatic hydrocarbons in bivalves from the San Francisco estuary: spatial distributions, temporal trends, and sources (1993–2001). Marine Environ Res 60:466–488

Oterhals Å, Nygård E (2008) Reduction of persistent organic pollutants in fishmeal: a feasibility study. J Agric Food Chem 56:2012–2020

Oterhals Å, Solvang M, Nortvedt R, Berntssen MHG (2007) Optimization of activated carbon-based decontamination of fish oil by response surface methodology. Eur J Lipid Sci Technol 109:691–705

Perugini M, Visciano P, Giammarino A, Manera M, Di Nardo W, Amorena M (2007) Polycyclic aromatic hydrocarbons in marine organisms from the Adriatic Sea, Italy. Chemosphere 66:1904–1910

Pitarch E, Medina C, Portolés T, Lopéz FJ, Hernández F (2007) Determination of priority organic micro-pollutants in water by gas chromatography coupled to triple quadrupole mass spectrometry. Anal Chim Acta 583:246–258

Saeed T, Al-Yakoob S, Al-Hashash H, Al-Bahloul M (1995) Preliminary exposure assessment for Kuwaiti consumers to polycyclic aromatic hydrocarbons in seafood. Environ Int 21:255–263

Santerre CR, Ingram R, Lewis GW, Davis JT, Lane LG, Grodner RM, Wei CI, Bush PB, Xu DH, Shelton J, Alley EG, Hinshaw JM (2000) Organochlorines, organophosphates, and pyrethroids in channel catfish, rainbow trout, and red swamp crayfish from aquaculture facilities. J Food Sci 65:231–235

Schultz M, Schultz J (1982) Induction of hepatic tumors with 7, 12-dimethyl-benzantracene in two species of viviparous fishes (genus Poecillopsis). Environ Res 27:337–351

Serrano R, Barreda M, Blanes MA (2008a) Investigating the presence of organochlorine pesticides and polychlorinated biphenyls in wild and farmed gilthead sea bream (Sparus aurata) from the Western Mediterranean Sea. Marine Pollut Bull 56:963–972

Serrano R, Blanes MA, López FJ (2008b) Biomagnification of organochlorine pollutants in farmed and wild gilthead sea bream (Sparus aurata) and stable isotope characterization of the trophic chains. Sci Total Environ 389:340–349

Stegeman JJ, Lech JJ (1991) Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environ Health Perspect 90:101–109

Yunker MB, Macdonald RW, Vingarzan R, Mitchell RH, Goyette D, Sylvestre S (2002) PAHs in the Fraser river basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. Org Geochem 33:489–515

6.2.3. Discusión de los resultados.

La selección de los PAHs objeto de estudio en las muestras analizadas se basó en la lista propuesta por la USEPA sobre toxicidad (EPA. 1993; EPA. 2000; ATSDR 1995b). El estudio de los PAHs propuestos nos permite comparar nuestros resultados con trabajos sobre la acuicultura atlántica en salmones. Esta lista de PAHs incluye compuestos con 2, 3, 4, 5 y 6 anillos de benceno con capacidad tóxica. También está incluido en la lista el benzo(a)pireno que está considerado como referencia de toxicidad según la documentación europea (Reglamento 1881/2006/CE). Además, incluye el naftaleno, un PAH muy ubicuo que presenta grandes dificultades analíticas.

En general, las muestras analizadas presentaron numerosos positivos de PAHs (**Tabla 2, 3. Artículo de Investigación 5**.). Los PAHs más frecuentemente identificados en las muestras fueron el fluoreno, fenantreno, fluoranteno y pireno. También son estos los que presentaron mayor concentración. Hay una excepción con el naftaleno, pues presenta concentraciones muy altas en piensos en comparación con los demás PAHs cuantificados. El aceite más contaminado fue, como era de esperar, el aceite de pescado (Σ_{PAHs}= 56.7 µg/Kg). También presentó concentraciones mayor de lo esperadas el aceite de lino (Σ_{PAHs}= 47 µg/Kg) mientras que el aceite de colza y el aceite de palma presentaron Σ_{PAHs}= 12 µg/Kg en ambos casos. La mayor concentración en piensos (FO, 33VO, 66VO) se debió principalmente a la concentración individual del naftaleno, cercana a los 200 µg/Kg en todos los piensos. Sin tener en cuenta el naftaleno, otros trabajos publicados recientemente presentan concentraciones de PAHs mucho mayores en comparación a las encontradas en nuestro trabajo en piensos utilizados en la acuicultura (Tsapakis et al., 2010).

Los PAHs detectados en los piensos no siguen el mismo perfil de las materias primas analizadas, sino que presentan un perfil propio y homogéneo entre los diferentes piensos ensayados. Este hecho puede ser una consecuencia del proceso de manufacturación del pienso, durante el cual, los ingredientes son sometidos a secado mediante aire caliente (Moret et al., 2000). Este proceso puede, por una parte degradar los PAHs con más anillos aromáticos, y por otra parte formar otros PAHs con menor número de anillos como el

naftaleno. Como se ha comentado anteriormente, este último está presente en los piensos a una concentración mucho más elevada que en el resto de muestras analizadas (hasta 215 µg/Kg, peso fresco). Este nivel elevado de naftaleno se apoya también en resultados de otros investigadores que lo consideran como un PAHs muy ubicuo (Liang et al., 2007). Se sospecha que el origen de la alta concentración de naftaleno detectada es la quema de hojarasca que periódicamente tiene lugar en las inmediaciones del Instituto (IATS) donde se realizaron los experimentos de engorde.

Respecto a los PAHs detectados en los filetes de los peces cultivados, en la **Figura 6.1.** se puede observar que la carga total de PAHs decreció considerablemente en los primeros 11 meses de cultivo, a partir de los cuales se produjo una ligera tendencia al alza en todos los grupos sin alcanzar, en ningún caso, concentraciones mayores a 11 µg/Kg.

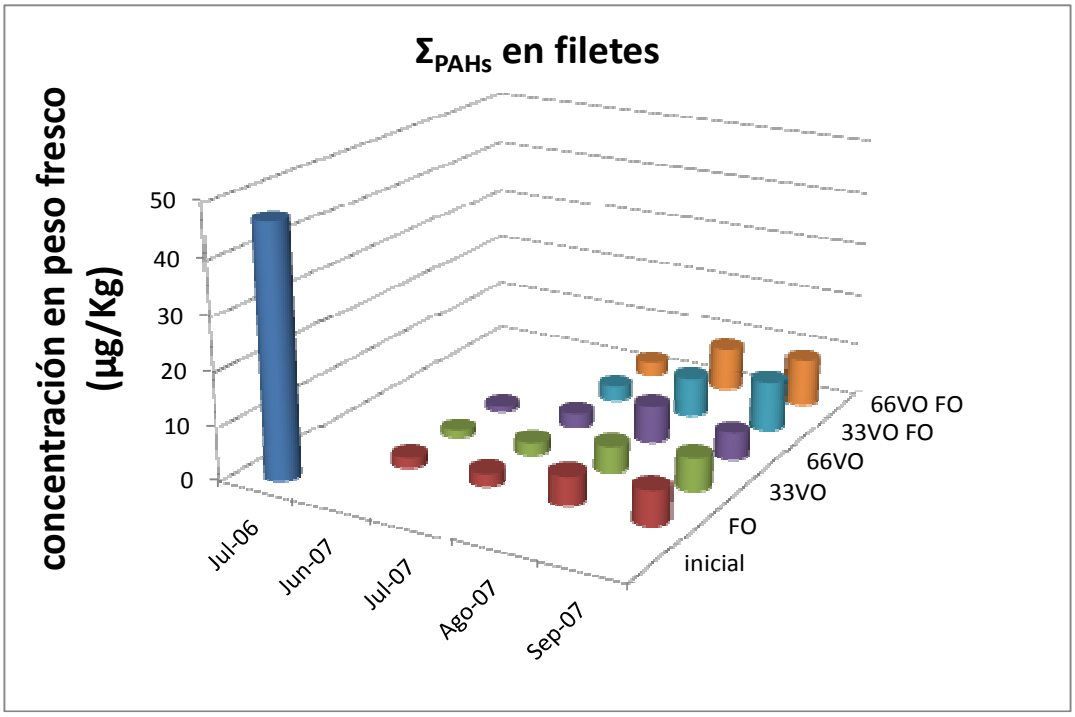

Figura 6.1. Niveles de contaminación de los filetes de dorada expresados como Σ_{PAHs} durante el proceso experimental mediante la ingesta de diferentes piensos experimentales.

Los filetes de dorada no presentaron concentraciones de contaminantes significativas durante los 11 primeros meses del ciclo de crecimiento. Este hecho puede deberse a los

bajos niveles de concentración presentes en las dietas utilizadas. Después de este período de alimentación las concentraciones de PAHs aumentaron ligeramente y este crecimiento se mantuvo estable al final del ciclo de alimentación (últimos tres meses).

Estos resultados demuestran alta calidad del producto obtenido, con una carga de contaminantes muy baja, probablemente como consecuencia de los piensos utilizados, también con una carga baja de contaminantes, y sugiere la capacidad de los peces de metabolizar y eliminar los contaminantes ingeridos en la dieta en las condiciones de cultivo aplicadas en el experimento.

6.2.4. Referencias.

ATSDR (Agency for Toxic Substances and Disease Registry). 1995b. Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs). U.S. Department of Health and Human Services, Public Health Service, Atlanta, GA.EPA. 1987. Quality criteria for water. EPA 440/5–86-001. US Environmental Protection Agency, Washington DC.

EPA. 1993. Provisional guidance for quantitative risk assessment of polycyclic aromatic hydrocarbons. EPA 600/R-93/089. US Environmental Protection Agency, Washington DC.

EPA. 2000. Guidance for assessing chemical contaminant data for use in fish advisories. EPA. 823-B-00–008. US Environmental Protection Agency, Washington DC.

IARC. 1987. IARC monographs on the evaluation of carcinogenic risks to humans, supplement 7. Overall evaluations of carcinogenicity: an updating of IARC monographs, vol 1-42., Vol. International Agency for Research on Cancer, Lyon.

Directiva 2000/60/CE del Parlamento Europeo y del Consejo, de 23 de octubre de 2000, por la que se establece un marco comunitario de actuación en el ámbito de la política de aguas.

Fernández-González, V.; Concha-Graña, E.; Muniategui-Lorenzo, S.; López-Mahía, P.; Prada-Rodríguez, D. 2007. Solid-phase microextraction-gas chromatographic-tandem mass spectrometric analysis of polycyclic aromatic hydrocarbons. Towards the European Union water directive 2006/0129 EC. J. Chromatogr. A. 1176, 48-56.

Latimer J.S.; Zheng J. 2003 Sources, transport and fate of PAHs in the marine environment. In: Douben PET (ed) PAH: an ecotoxicological perspective. Wiley, Chichester, UK, pp 9–33.

Menzie, C.A.; Potocki, B.B.; Santodonato, J. 1992. Ambient concentrations and exposure to carcinogenic PAHs in the environment. Environ. Sci. Technol. 26, 1278-1284.

Nisbet, I.C.; LaGoy, P.K. 1992. Toxic equivalency factors (TEFs) for polycyclic aromatic hydrocarbons (PAHs). Regul Toxicol Pharm 16, 290–300.

Reglamento 1881/2006/CE de la comisión de 19 de diciembre de 2006 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios.

Serrano, R.; Barreda, M.; Pitarch, E. 2003. Determination of Low Concentrations of Organochlorine Pesticides and PCBs in Fish Feed and Fish Tissues from Aquaculture Activities by Gas Chromatography with Tandem Mass Spectrometry. J. Sep. Sci. 26, 75-86.

Tsapakis, M.; Dakanali, E.; Stephanou, E.G.; Karakassis, I. 2010. PAHs and n-alkanes in Mediterranean coastal marine sediments: Aquaculture as a significant point source. J. Environ. Monitor. 12, 958-963.

USEPA. Toxicological profile for polyclyc aromatic hydrocarbons (PAHs). http://www.epa.gov

Van der Oost, R.; Beyer, J.; Vermeulen, N.P.E. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environ. Toxicol. Pharm. 13, 57-149.

6.3. Estudio de la bioacumulación de contaminantes organoclorados en doradas procedentes de la acuicultura.

6.3.1. Introducción.

Compuestos químicos de toxicidad reconocida han sido utilizados en grandes cantidades desde la antigüedad tanto en la agricultura como en actividades industriales (Lacorte et al., 2006). Entre ellos, los PCBs, PCDDs y OCPs como el DDT y sus derivados DDD y DDE son considerados sustancias de riesgo y hoy día, a pesar de estar prohibido su uso desde hace años, es posible encontrar concentraciones de estos compuestos asociados a muestras medioambientales y seres vivos.

Los PCBs se han utilizado comercialmente desde 1930 en numerosas aplicaciones pero principalmente como fluidos dieléctricos e intercambiadores de calor en transformadores y condensadores debido a su baja conductividad eléctrica, elevada conductividad térmica y gran resistencia a la degradación por el calor. En los años 60 la comunidad científica inició una llamada de atención respecto al uso y la peligrosidad de los PCBs, dadas sus características de baja biodegradabilidad y el carácter bioacumulativo del producto. Su producción está hoy prohibida en casi todos los países desarrollados, habiéndose establecido también condiciones especiales para la utilización y destrucción de los aparatos que contenían PCBs (Directiva 96/59/CE de 1996). La directiva 96/59/CE establece la prohibición de su abandono y evacuación incontrolada. También regula su eliminación controlada, regula el procedimiento de autorización de las instalaciones de descontaminación y aplica el principio “quien contamina, paga”. Como sucede con la mayoría de los compuestos OCs, los PCBs son muy persistentes y se acumulan en la cadena alimentaria. A medida que aumenta el número de átomos de cloro se incrementa su estabilidad y liposolubilidad. Los distintos congéneres se identifican con un sistema de numeración propuesto por Ballschmiter y Zell (Ballschmiter and Zell, 1980).

Hasta hace muy pocos años no se disponía comercialmente de patrones analíticos de congéneres individuales y la exposición a estos contaminantes se expresaba como ingesta de PCBs totales, siendo esta aproximación insuficiente por no tener en cuenta la diferente toxicidad de los distintos congéneres. Por otro lado, no existe un valor de referencia

toxicológico generalmente aceptado para la exposición a PCBs totales, comprometiendo todo ello la evaluación de riesgos.

Los congéneres no-orto sustituidos y en menor medida algunos mono-orto sustituidos, pueden adoptar una configuración plana que les confiere características específicas incluyendo respuestas bioquímicas y tóxicas, algunas de las cuales, coinciden con las causadas por la 2,3,7,8-tetraclorobibenzo-p-dioxina (**Figura 6.2**). Los congéneres de PCBs que exhiben una actividad toxicológica homóloga a la de las dioxinas se denominan *Dioxin-like* PCBs (DL-PCBs) o PCBs coplanares (PCB nº: 77, 105, 118, 126, 156, 169). En la **Figura 6.3.** se representan tres ejemplos de DL-PCBs.

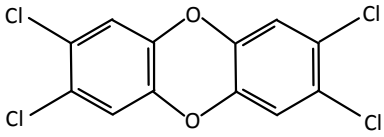

Figura 6.2. 2,3,7,8-tetraclorobibenzo-p-dioxina.

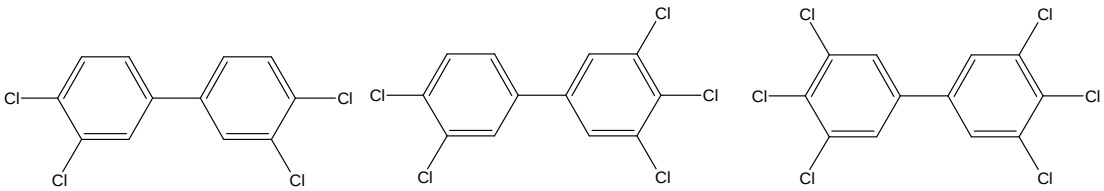

Figura 6.3. Estructura molecular de PCBs coplanares.

Todos los PCBs cumplen una serie de características estructurales que les permiten ser reconocidos por el receptor específico Ah-receptor (receptor para aril-hidrocarbono), que también reconoce a otras moléculas como los PAHs (Safe et al., 1994).

Han sido establecidos TEFs para los PCBs, expresándose la toxicidad de los mismos, al igual que para las dioxinas, en forma de equivalentes tóxicos (Van den Berg et al., 1998). Generalmente la presencia de dioxinas en diferentes muestras analíticas hace que se suela evaluar también la presencia de PCBs. Para evaluar la exposición a los PCBs se suelen incluir tres PCBs no-orto sustituidos (77, 126, 169) y dos mono-orto (105, 118) para los que existen TEFs. Los seis restantes que se suelen incluir representan algunos de los mayoritarios en alimentos (28, 52, 101, 138, 153 y 180) (Bordajandi et al., 2006).

Por otro lado, los OCPs son un grupo de POPs que han sido utilizados en gran medida para evitar plagas y combatir enfermedades tan graves como la malaria o la fiebre amarilla. En muchos casos los OCPs son utilizados para combatir las plagas de insectos, los hongos o mala hierba en superficies de cultivo. Este hecho ha dado lugar a su presencia en el medio ambiente, llegando a la población humana. La estructura de estos compuestos orgánicos puede ser muy diversa en la que átomos de cloro sustituyen a átomos de hidrógeno. Aunque su uso está hoy estrictamente restringido, debido a su persistencia en el medio ambiente, todavía se encuentran concentraciones apreciables en todos los compartimentos del ecosistema. Como ejemplo, el DDT y sus derivados DDD y DDE son frecuentemente detectados en el medio ambiente y asociados al tejido de algunos animales y personas (Chu et al., 2003; Yang et al., 2006).

Tanto los PCBs como los OCPs se consideran potencialmente peligrosos por provocar daño al sistema nervioso central y periférico, desordenes reproductivos, trastornos al sistema inmunológico, malformaciones en el nacimiento y hasta la muerte. Algunos de ellos poseen actividad mutagénica y carcinogénica y es muy extensa su presencia medioambiental y en organismos vivos (Muir et al., 1999; Domingo et al., 2007). En muchos casos son ingeridos alimentos u organismos contaminados por PCBs u OCPs. Una de las características más importantes de los POPs es su capacidad para bioacumularse

en tejidos animales. Además dentro de la cadena trófica, su concentración incrementa por cada nivel trófico, magnificándose su presencia en el hígado de los consumidores. Ha sido comprobado en algunos casos que la acumulación de estos contaminantes en los animales de granja está debida preferentemente, a la ingesta de piensos contaminados. Un ejemplo claro lo representan los peces procedentes de la acuicultura. Estos muestran contaminación por PCBs y algunos OCPs, siendo la alimentación el único factor al que se encuentran expuestos estos animales (Smith et al., 2002; Serrano et al., 2003a). El hombre no está exento de este peligro puesto que el consumo de alimentos procedentes de la acuicultura marina ha crecido mucho en los últimos años y el consumo de organismos contaminados constituye un verdadero riesgo para la salud.

En el presente estudio se determinó la concentración de OCPs y PCBs en materias primas, piensos y filetes de dorada procedentes de un estudio experimental de cultivo de doradas (*Sparus aurata L.*) en el marco del Proyecto AQUAMAX (Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers. www.aquamaxip.eu). La información generada a partir de estos estudios es de gran importancia pues permitirá identificar cuales son las concentraciones de estos compuestos y el BMF de contaminantes en el individuo una vez haya alcanzado su tamaño comercial.

6.3.2. Artículo científico 6:

Effects of fish oil replacement and re-feeding on the bioaccumulation of organochlorine compounds in gilthead sea bream (Sparus aurata L.) of market size.

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Effects of fish oil replacement and re-feeding on the bioaccumulation of organochlorine compounds in gilthead sea bream (*Sparus aurata* L.) of market size

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Abstract

Organochlorine pesticide residues and polychlorinated biphenyls were determined in raw materials, fish feeds and fillets from fish exposed through the productive cycle (14 months) to experimental diets with different percentages of fish oil replacement with vegetable oils. Detectable amounts of organochlorine compounds were found in raw materials derived from fish sources with none being detected in vegetable ingredients. Fish feeds presented trace concentrations of contaminants at the ng/g level, which varied according to the contribution of the different resources used in their manufacture. Contaminants did not accumulate during the first 11 months of exposure, and low concentrations of organochlorine compounds were found both at the start and at the end of this feeding period. Fillets from fish fed the fish oil diet presented the highest concentrations of organochlorine compounds, with these decreasing in proportion to fish oil replacement. Three months of fish oil re-feeding during the finishing phase only produced significant bioaccumulation over the course of the first month. By optimizing fish meal and fish oil replacement with vegetable oils alternative feeds can contribute to significantly reduce the risk of organochlorine uptake by consumers.

Keywords: Organochlorine compounds; Bioaccumulation; Sparus aurata; Fish feed; Marine aquaculture

1. Introduction

Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) are non polar, highly lipophilic, and persistent ubiquitous environmental pollutants. Both are classified as Persistent Organic Pollutants (POP) and are present in the contamination pattern of marine environments worldwide. Despite the use of OCPs being strongly restricted and PCBs production being banned, these compounds are distributed across the marine environmental biota (Hernández et al., 2000; Hoekstra et al., 2003; Bocquene et al., 2005; Yang et al., 2007; Serrano et al., 2008b). Special concern exists about dioxin-like PCB (DL-PCBs), which have been shown to cause toxic responses similar to those induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin, the most potent congener within polychlorinated dibenzo-p-dioxins (Van Den Berg et al., 1998). The characteristics of these compounds lead to high biomagnification in the food chain, and involve a wide range of trophic levels (Borga et al., 2001; Kidd et al., 2001; Hoekstra et al., 2003; Konwick et al., 2006; Serrano et al., 2008a,b; Sagratini et al., 2008). Some studies have shown that food is the major contributor for PCBs accumulation in farmed fish (Serrano et al., 2003a; Antunes and Gil, 2004). Thus, fish and in general seafoods have been considered the most important source of organochlorine compounds (OCs) in the human diet (Johansen et al., 1996; Bjerregaard et al., 2001; Tsukino et al., 2006), these compounds being frequently detected in human tissue lipids and fluids (Hernández et al., 2002a,b,c; Pitarch et al., 2003; De Felip et al., 2004; Muñoz-de-Toro et al., 2006; Tsukino et al., 2006; Lopez-Espinosa et al., 2008; Mueller et al., 2008).

Persistent organic pollutants are concentrating in lipid-rich feed grade fish used for production of fish oils, the major resource of these contaminants in aquaculture diets, and have been detected in fish feed used in aquaculture and in farmed fish by several authors (Santerre et al., 2000; Easton et al., 2002; Hites et al., 2004; Navas et al., 2005; Bordajandi et al., 2006; Maule et al., 2007; Ábalos et al., 2008; Serrano et al., 2008a,b), so dietary fish oil replacement with alternative oils can significantly reduce the load-charge of lipophilic contaminants in aquafeeds and thereby farmed fish (Bell et al., 2005; Berntssen et al., 2005; Bethune et al., 2006). Unfortunately, when fish oil is replaced with vegetable oils, the dietary supply of n-3 polyunsaturated fatty acids (PUFA) is also reduced (Bell et al., 2005; Benedito-Palos et al., 2007, 2008; Drew et al., 2007). This is a major constraining factor for marine fish due to the inability of marine fish to convert C18 PUFA to long-chain PUFA, specially eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3) that become essential nutrients in marine fish aquafeeds (Sargent et al., 1999, 2002). However, among others, recent studies in gilthead sea bream (Sparus aurata L.), indicate that fish oil can be replaced

up to 66% with vegetable oils in plant protein-based diets without signs of growth retardation and histopathological tissue damage in a 8-month trial (Benedito-Palos et al., 2007, 2008). Thus, a large amount of either fish meal or fish oil can be, and is practically replaced in the new sustainable diets for marine fish.

The feasibility of the fish oil replacement has been demonstrated through an entire production cycle, including a three month finishing fish oil diet phase (wash-out period), without negative effects on the growth performance of gilthead sea bream, a high valuable fish for the Mediterranean aquaculture. Also, a kinetic analysis of the fatty acids (FA) demonstrated that changes in the fillet FA profile arise because the existing stores become diluted as fish grow and deposit increasing amounts of dietary derived FAs (Benedito-Palos et al., 2009). The goal of the present study was to analyze, in the same fish, OCPs and PCBs bioaccumulation. Organochlorine compounds were determined in fish fillets, feeds and major raw materials of the diets, providing information on the end-product quality and safety from the consumer point of view.

2. Materials and methods

2.1. Experimental diets

Three isoproteic, isolipidic and isoenergetic plant protein-based diets were formulated with a low inclusion level (20%) of fish meal and fish soluble protein concentrates. Fish oil from the southern hemisphere was the only lipid source in the control diet (FO), which was also used as a finishing diet. The two remaining diets contained a blend of vegetable oils (2.5 rapeseed oil:8.8 linseed oil:3 palm oil), replacing 33% (33VO) and 66% (66VO) of the fish oil. All diets were manufactured using a twin-screw extruder (Clextral, BC 45) at the “Institut Scientifique de Recherche Agronomique” (INRA) experimental research station of Donzacq (Landes, France), dried under hot air, sealed and kept in air-tight bags until use. Ingredients and proximate composition are shown in Table 1.

Samples for pollutant analyses were collected and stored at −20 °C until analysis. Determination of organochlorine residue and polychlorinated biphenyls in each diet was carried out by triplicate.

Table 1.

Ingredients and chemical composition of experimental diets. For more details in diet composition see Benedito-Palos et al., 2009.

Ingredient (%)	*FO*	*33VO*	*66VO*
Fish meal (CP 70%) [a]	15	15	15
CPSP 90 [b]	5	5	5
Corn gluten	40	40	40
Soybean meal	14.3	14.3	14.3
Extruded wheat	4	4	4
Fish oil [c]	15.15	10.15	5.15
Rapeseed oil	0	0.85	1.7
Linseed oil	0	2.9	5.8
Palm oil	0	1.25	2.5
Soya lecithin	1	1	1
Binder	1	1	1
Mineral premix [d]	1	1	1
Vitamin premix [e]	1	1	1
$CaHPO_4.2H_2O$ (18%P)	2	2	2
L-Lys	0.55	0.55	0.55
Proximate composition			
Dry matter (DM, %)	93.13	92.9	92.77
Protein (% DM)	53.2	52.81	52.62
Fat (% DM)	21.09	21	20.99
Ash (% DM)	6.52	6.69	6.57

[a]Fish meal (Scandinavian LT)
[b]Fish soluble protein concentrate (Sopropêche, France)
[c]Fish oil (Sopropêche, France)
[d]Supplied the following (mg · kg /diet, except as noted): calcium carbonate (40% Ca) 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0.3.
[e]Supplied the following (mg · kg /diet): retinyl acetate 2.58, DL-cholecalciferol 0.037, DL-□ tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5, riboflavin 15, pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium pantothenate 2.5, vitamin B_{12} 0.025, ascorbic acid 250, inositol 500, biotin 1.25 and choline chloride 500.

2.2. Bioaccumulation experiment

Gilthead sea bream of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were cultured at the Instituto de Acuicultura de Torre de la Sal (IATS) for 20 days before the start of the study. Fish of around 18 g initial mean body weight were allocated into nine fiberglass tanks (3000 L) in groups of 150 fish per tank. Water flow was 20 L/min and oxygen content of outlet water remained higher than 85% saturation. The growth study was undertaken over 14 months (July 11th, 2006–September 2nd, 2007), and day-length and water temperature (10–26 °C) varied over the course of the trial to match natural sea changes offshore from IATS (40° 5'N; 0° 10'E).

During the first 11 months of the trial, the three diets were randomly allocated to triplicate groups of fish, and feed was offered by hand to apparent visual satiety. During the finishing diet phase (12 weeks, June 6th, 2007–September 2nd, 2007), two tanks of 33VO and two of 66VO groups were fed with FO diet. The names of those groups became 33VO/FO and 66VO/FO, respectively. Fish fed FO diet, and one tank of fish fed 33VO and 66VO diets were maintained on the initial diets until the end of the experiment. This meant that the bioaccumulation study consisted of five treatments: FO, 33VO, 66VO, 33VO/FO and 66VO/FO (Fig. 1).

At the beginning and at regular intervals through the finishing diet phase (0, 330, 360, 390 and 420 days) randomly selected fish (eight fish from all tanks of the same treatment) were sacrificed by a blow on the head prior to tissue sampling. The left-side fillet (with skin and bone removed) was excised and stored at −20 °C until analysis. As reported by Benedito-Palos et al. (2009), body weight and fillet yield were not affected by the dietary treatment over the course of all feeding trial.

Sea water for fish culture was analyzed using the multi-residue method for pesticides described by Hernandez et al. (1993) and OCs were not detected (limit of detection between 0.01 and 0.1 μg/L). Therefore, fish were cultured in sea water free of pesticides without any other known exposure to organochlorines except feed.

2.3. Analytical methodology

Organochlorine compounds, including selected non polar pesticides and derivatives (DDTs-p,p′-DDT, p,p′-DDE, p,p′-DDD–, HCB, lindane, mirex, methoxychlor) and polychlorinated biphenyls IUPAC nos 28, 31, 52, 77, 101, 105, 118, 126, 128, 138, 153, 156, 169, 170, 180, as indicators of the

presence of PCBs in the samples, were analyzed in raw materials, fish feeds and fish fillets following the method described by Serrano et al. (2003b).

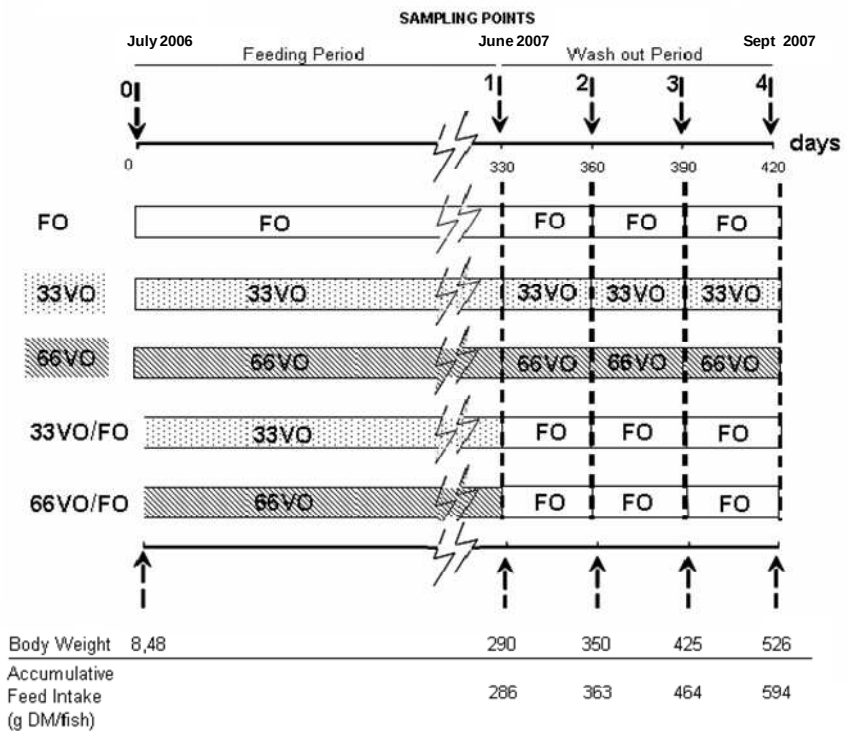

Fig. 1. Schematic representation of the long-term feeding trial over 14 months (for abbreviations see the text).

Eight fillets from each treatment (four from each replicate) were selected randomly to obtain three composite samples and were analyzed independently in triplicate. Fish diets and raw materials were homogenized and analyzed in triplicate. In brief, extraction of fish feed, raw materials and muscle was carried out by refluxing ca. 8 g homogenized fresh sample in n-hexane for 4 h. Clean up of fish tissue was performed by means of normal phase liquid chromatography (NPLC) injecting 1 mL hexanic extract (4 g sample per mL) into a silicagel HPLC column. The liquid chromatography (LC) system used was comprised of a LC Pump Master 305 piston pump, Gilson (Middleton, USA), with a column 150 × 3.9 mm i.d. packed with 4 µm silica Novapack (Waters, Milford, MA, USA); mobile phases were n-hexane and ethyl acetate for column regeneration after a clean up cycle; flow rate was 1 mL/min. For the fish feed and raw materials an additional clean-up step with concentrated

sulphuric acid was necessary prior to normal phase HPLC clean up as a consequence of the high lipid content (from 20% to 100% of fresh weight) (Serrano et al., 2003b).

Analysis of the fat-free LC fractions collected was performed by gas chromatography with tandem mass spectrometry (GC–MS/MS) using a gas chromatograph Varian CP-3800 coupled with an ion trap mass spectrometry detector (Saturn 4000, Varian) operating in the electron impact ionisation mode (EI) to identify and quantify PCBs and OCPs present in the diets and fish fillets. Separation of the analytes was carried out on a 30 m × 0.25 mm DB-5MS (0.25 μm film thickness) Varian capillary column, using helium at 1 mL/min as the carrier gas. The temperature program was as follows: 90 °C for 1 min, increased to 180 °C at a rate of 30 °C/min, increased to 260 °C at a rate of 4 °C/min, and finally increased to 300 °C at a rate of 20 °C/min, with a final isothermal stage of 4 min (total chromatographic analysis time was 30 min). Injection of 1 μL of samples (injection port temperature 250 °C) in the splitless mode was carried out using a Varian 8400 autosampler equipped with a 10 μL syringe. The gas chromatograph was directly interfaced with the Varian 4000 mass-spectrometer (ion trap) in the internal ionization mode with electron impact ionization energy of 70 eV in the positive ion mode. Transfer line temperature was established at 260 °C and ion source and trap temperatures were adjusted to 200 °C (more details in Serrano et al. (2003b) Quantification of samples was carried out by external calibration curve using pp'-DDE-D8 as internal standard. Fifty nanograms of this isotopically labeled standard (100 μL of a 0.5 ng/μL hexanic standard solution) were added before extraction, allowing its use as internal standard and surrogate in order to detect possible analytical errors in every sample analysis. The limit of quantification has been calculated as three times the detection limit (i.e. nine times the background noise in the chromatograms), accepting coefficients of variation less than 30%. Statistical validation of the method included the study of linearity, accuracy, precision, selectivity and limits of quantification and detection. Moreover, the absence of matrix effect in the instrumental determination (GC–MS/MS) was proved by means of the Standard Additions Method (more details Serrano et al., 2003b).

The whole analytical process was carried out in the Good Laboratory Practices certified laboratories of the Research Institute for Pesticides and Water, University Jaume I, Spain.

2.4. Determination of fat

The total fat content in the sample extracts was determined gravimetrically, with evaporation at 95 °C until constant weight.

2.5. Data analysis

PCBs and OCPs concentrations in raw materials and fish feed are expressed as ng/g fresh weight and in fillets also as lipid based concentrations. Biomagnification Factors (BMF) were calculated as the ratio between lipid based concentrations of organochlorine compounds in the fish fillets and in the fish feed at each sampling point. As two different fish feeds were used in 33VO/FO and 66VO/FO groups, calculations of BMF were made taking into account the time of exposure to each fish feed. Thus, the arithmetic mean of organochlorine concentrations in the diet was calculated on both the basis of months of exposure to each fish feed and their concentration in each fish feed. The calculated data expressed as ng/fillet allowed to identify the amount of pollutant present in a whole fish fillet. The t-Student test ($P < 0.05$) was applied to compare data on diets and ingredients. Data for concentrations of PCBs and OCPs in fish fillets, BMF values and amounts of pollutants per fillet were compared by means of ANOVA I and "a posteriori" Scheffe´s test ($P < 0.05$). All data were transformed to Log 10 before statistical analysis to achieve normality. Homoscedasticity of variances was tested by means of Bartlett's test ($P < 0.05$). All the statistical tests were conducted using STATGRAPHICS plus for Windows version 4.1 (Statistical Graphics Corporation).

3. Results and discussion

3.1. PCBs and OCPs content in experimental diets

The analytical method showed excellent sensitivity and selectivity as a consequence of the use of gas chromatography coupled tandem mass spectrometry (GC–MS/MS). It was validated by recovery experiments down to 5 ng/g, with lower spiked levels not being checked due to the presence of DDTs and PCBs in the blank samples (Serrano et al., 2003b). However, the powerful analytical characteristics of GC–MS/MS together with the efficiency of the HPLC clean up has allowed here analyte quantification at concentrations as low as 0.1 ng/g with acceptable precisions (below 30%). Samples analysis were carried out in the Research Institute for Pesticides and Water facilities, whose laboratories are Good Laboratory Practices certified, which support the quality of the analytical data released in the present research.

Table 2. Concentration (ng/g fresh weight) of organochlorine compounds in marine resources used in fish feed manufacture and experimental diets. Pollutants in vegetable resources were no detected.

Compound	Fish oil		Fish meal		Fish soluble protein concentrate		Diet FO		Diet 33VO		Diet 66VO	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
PCB 28+31	1.6	4	<0.1	–	<0.1	–	0.2	9	0.1	9	<0.1	
PCB 52	1.2	14	<0.1	–	<0.1	–	0.2	6	0.2	8	0.1	15
PCB 101	3.6	1	0.7	29	0.5	6	0.5	6	0.5	9	0.3	13
PCB 118	4.7	9	<0.4	–	1	1	0.6	5	0.6	5	0.4	3
PCB 153	6.5	19	2.1	4	3.5	14	1.6	3	1.5	6	0.9	7
PCB 105	3.8	5	<0.2	–	0.3	22	<0.1	3	<0.1	–	<0.1	–
PCB 138	4.9	8	1.4	20	1.9	3	0.8	14	0.7	7	0.5	4
PCB 128	2	7	<0.2	–	0.3	27	<0.1	–	<0.1	–	<0.1	–
PCB 156	1.9	4	<0.4	–	<0.4	–	<0.1	–	<0.1	–	<0.1	–
PCB 180	4.8	14	0.4	14	0.9	12	0.4	8	0.4	37	0.3	11
PCB 170	0.6	31	0.3	11	0.3	18	<0.2	–	<0.1	–	<0.1	–
SUM PCBs	34.8	4	4.7	18	8.7	11	4.4	3	4.1	5	2.8	3
HCB	<0.1	–	<0.1	–	<0.1	–	0.2	18	0.2	30	0.5	4
p,p-DDE	16.8	3	5.5	7	3.6	7	2.5	12	2	10	1.7	5
p,p-DDD	8.8	29	1.4	7	0.6	15	2.3	4	2	1	1.3	3
p,p-DDT	15.9	6	1.5	12	1	11	2.5	10	2.1	2	1.4	4
SUM OCPs	41.6	7	8.4	6	5.2	2	7.6	5	6.2	3	4.8	2
Total load	76.4	4	13.1	5	13.9	4	11.9	3	10.2	2	7.6	2

CV: coefficient of variation. Compounds were quantified with CV < 30.

Concentrations of PCBs and OCPs present in the raw materials used for the manufacture of experimental diets are shown in Table 2. As can be observed raw materials derived from fish contain detectable amounts of organochlorine compounds, up to 76.4 ng/g of total organochlorine charge-load (34.8 ng/g total PCBs and 41.6 ng/g total OCPs). The predominant pesticide was pp'-DDT and its derivatives. For the PCBs investigated, the analytical methodology applied allowed the detection and quantification of 12 congeners from 15 considered as markers of total load. Congener 153 was the most abundant, as is usual in marine samples (Serrano et al., 2008a,b). This pattern is in agreement with the pollution pattern of different marine products (Zabik et al., 1996; Santerre et al., 2000; Johnson et al., 2007; Serrano et al., 2008a, 2008b).

The presence of organochlorine contaminants in fish oil has been reported previously by other authors. Jacobs et al. (1997, 1998) reported concentrations up to 990 µg/L total PCBs and 119 µg/L total DDTs. Hilbert et al. (1998) reported concentrations up to 300 ng/g total PCBs and 60 ng/g total DDTs during the refining process. In all cases, levels reported are higher than those found in the raw materials used in this work. This fact could be related with the northern origin of the raw materials used in the cited papers.

Organochlorine compounds were not detected in any vegetable ingredient used to formulate the experimental diets, which allows the total load of contaminants in diets to be reduced with fish oil replacement. The non detectable levels of organochlorine contaminants in vegetable derivative resources and the low levels present in the marine derivatives used allow production of a new generation of fish feeds almost free of contaminants.

Table 2 also shows the concentrations of OCPs and PCBs present in the experimental diets used during the diet exposure experiment. The sum of OCPs only reached 7.6 ng/g fresh weight in the case of the FO diet, the diet with highest fish oil content. The sum of PCBs analyzed reached 4.4 ng/g fresh weight in the reference fish feed (FO).

The charge-load of contaminants present in the diets is in accordance with the contribution of the different resources used in their manufacture (t-Student, $P < 0.05$), which leads to very low levels of organochlorines. Thus fish oil replacement by vegetable oils without detectable levels of contaminants, decreases the charge of contaminants in correlation with the percentage of fish oil in the different diets ($r = 0.9978$, $P < 0.01$). Similar results have been previously obtained by other authors (Bell et al., 2005; Berntssen et al., 2005).

The concentrations of organochlorines in different fish feeds have been reported by several authors (Easton et al., 2002; Hites et al., 2004; Carubelli et al., 2007; Maule et al., 2007) with loads of contaminants in the different feeds higher or similar than levels found in the experimental diets used in this work. This highlights the quality of the new diets employed in this study in comparison to others. It is interesting to note that feeds for salmon farming present higher levels of contaminants than those used for Mediterranean aquaculture (Hites et al., 2004). This fact could be related to the use of fish oil from northern Atlantic and the higher percentage of fish oil used in salmonid diets (around 25–33% versus 15–20% in Mediterranean aquaculture feeds), or other factors such as the quality of raw materials used in manufacture.

3.2. Fish diet exposure

Table 3 and Table 4 show the concentrations of PCBs and OCPs in fish fillets during the long term diet exposure experiments. Fish fed the FO diet presented the highest concentrations of organochlorine compounds at the end of the experiment (PCBs and OCPs) (Scheffe's test, $P < 0.05$) as would be expected due to the higher content of pollutants quantified in this diet. Following the expected trend, the fish fed 33VO presented lower organochlorine concentrations than those fed FO diet but were higher than 66VO group ($P < 0.05$). For the experiments with finishing diets, 33VO/FO and 66VO/FO, the supply of FO diet during the last three months of the experiment, provokes a tendency towards the increase of the levels of organochlorine compounds in the fish fillets with respect to fish kept without finishing diets (33VO and 66VO), although not reaching the levels of PCBs and OCPs found in the control FO group.

Table 3 and Table 4 also show the trend of PCBs and DDTs levels during the bioaccumulation experiment. During the first 11 months, contaminant concentrations increased very little compared with the last three months of the experiment. In fact contaminants did not accumulate during the first 11 months of exposure to the experimental diets, presenting similar concentrations (referred to both fresh and lipid weight) of organochlorine compounds at the start and finish of this feeding period (fish mean weights were 18 g at the start and 290 g after 11 months of exposure) (sampling times 0 and 1, July 06 and June 07, respectively) ($P > 0.05$). The fact that levels of PCBs, dioxin-like

Table 3. Concentrations of total PCBs and total DL-PCBsa in fillets (mean ± coefficient of variation, n = 3) from fish exposure to the different experimental diets in long-term sea bream feeding trial (see Fig. 1).

Compound	T0	FO		33VO		33VOFO	66VO		66VOFO
	July/06	June/07	Sept/07	June/07	Sept/07	Sept/07	June/07	Sept/07	Sept/07
ng/g fresh weight									
PCB 28+31	<0.1	<0.1	0.2 (19)	<0.1	0.1 (26)	0.2 (19)	<0.1	0.2 (4)	0.2 (18)
PCB 52	<0.1	0.1 (27)	0.3 (15)	<0.1	0.2 (35)	0.2 (35)	<0.1	0.2 (9)	0.2 (21)
PCB 101	<0.1	0.2 (30)	0.6 (31)	0.1 (22)	0.4 (27)	0.4 (31)	<0.1	0.3 (16)	0.5 (15)
PCB 77	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
PCB 118	0.5 (9)	0.5 (13)	1 (16)	0.2 (23)	0.7 (31)	0.8 (16)	0.3 (25)	0.6 (12)	0.8 (2)
PCB 153	2 (26)	0.7 (19)	1.8 (26)	0.4 (25)	1.4 (29)	1.6 (26)	0.5 (28)	1.2 (12)	1.8 (14)
PCB 105	<0.2	0.5 (30)	0.6 (25)	<0.2	0.3 (18)	0.4 (25)	0.2 (19)	0.3 (21)	0.4 (13)
PCB 138	1 (11)	0.6 (8)	1.1 (22)	0.2 (22)	0.8 (29)	0.9 (22)	0.4 (23)	0.7 (16)	1.0 (5)
PCB 126	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
PCB 128	<0.2	0.2 (4)	0.3 (19)	0.1 (20)	0.2 (14)	0.2 (19)	0.1 (24)	0.2 (10)	0.2 (24)
PCB 156	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4
PCB 180	0.5 (26)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
PCB 169	<0.1	0.5 (33)	0.6 (24)	0.2 (30)	0.5 (19)	0.5 (24)	<0.1	0.4 (16)	0.6 (23)
PCB 170	<0.2	0.3 (3)	0.2 (23)	0.1 (6)	0.2 (23)	0.1 (23)	0.1 (4)	0.2 (14)	0.1 (20)
SUM PCBs	4.0 (3)	3.7 (11)	6.5 (5)	1.7 (10)	4.8 (11)	5.4 (11)	1.6 (14)	4.1 (5)	5.7 (6)
SUM DL-PCBs[a]	0.4 (1)	1.2 (0.2)	1.3 (0.1)	0.5 (0.1)	1.0 (0.1)	1.0 (0.3)	0.5 (0.1)	0.8 (0.1)	1.1 (0.2)
ng/g lipid weight									
SUM PCBs	83.5 (4)	47.5 (2)	70.6 (6)	22.6 (2)	48.3 (5)	51.3 (5)	27.9 (4)	40.6 (2)	51.9 (3)
SUM DL-PCBs[a]	9.8 (1)	15.4 (2)	14.6 (2)	7.1 (1)	10.2 (1)	9.7 (2)	6.9 (1)	8.5 (1)	10.1 (2)

[a] See list of PCBs analyzed in Section 2.

Table 4. Concentrations of total OCPs in fillets (mean ± coefficient of variation, n = 3) from fish exposure to the different experimental diets in long-term sea bream feeding trial (see Fig. 1).

Compound	T0	FO		33VO		33VOFO	66VO		66VOFO
	July/06	June/07	Sept/07	June/07	Sept/07	Sept/07	June/07	Sept/07	Sept/07
ng/g fresh weight									
HCB	<0.1	<0.1	0.2 (22)	<0.1	<0.1	0.2 (34)	<0.1	0.4 (2)	<0.1
p,p-DDE	2.2 (5)	2(15)	3.6 (19)	1.0 (8)	2.5 (20)	2.6 (30)	0.7 (18)	2.1 (11)	2.0 (5)
p,p-DDD	0.7 (11)	1.4 (19)	2.3 (15)	0.4 (29)	1.7 (29)	2.1 (28)	0.6 (10)	1.3 (8)	1.8 (4)
p,p-DDT	<0.4	1.2 (24)	2.2 (13)	0.3 (29)	1.4 (19)	1.6 (28)	0.6 (33)	1.0 (19)	1.8 (4)
SUM OCPs	2.9 (5)	4.6 (11)	8.3 (4)	1.7 (11)	5.8 (7)	6.6 (7)	1.8 (12)	4.8 (5)	5.9 (4)
ng/g lipid weight									
SUM OCPs	67.1 (1)	58.9 (6)	90.4 (3)	22.7 (2)	58.5 (4)	62.6 (4)	23.6 (3)	47.8 (2)	54.1 (2)

PCBs and organochlorine pesticides in fish fillets did not increase in fresh weight and lipid based concentrations indicates the no bioaccumulation of these contaminants, suggesting the ability of fish to depurate the pollutants ingested in the period previous to the start of the experiment as well as in the first 11 months. Possibly dilution, excretion or detoxifying systems operate efficiently when toxicant load is low in diets with adequate nutritional composition.

From June 2007 to September 2007 fish ingested almost the same weight of feed as in the previous experimental period (from July 2006 to June 2007) (Fig. 1). An increase in food intake during the finishing diet phase can cause major increases in the concentration of pollutants. Such increases can be seen after June 2007. As consequence of the different feeding behavior during the grow out and finishing periods, a kinetic model study was not considered. Treatments 33VO/FO and 66VO/FO showed a higher change compared to fish always fed FO, 33VO and 66VO diets. The ingestion of FO diet from June 2007 onwards as well as the higher feed intake resulted in a major increase in pollutants charge in the fish fillets. In a short time fish accumulated more pollutants from the diet than could be eliminated by any detoxification route.

Fig. 2 shows the Biomagnification Factors (BMF) at the four sampling points during the last three months of total experimental period. As can be observed, BMF values were close to one at sampling point 1 (July 07) and, fish fed on the diet with no fish oil replacement presented a steady, continuous increase over time. This same trend was observed but was softer in fish fed always 33VO and 66VO diets. Fish submitted to fish oil re-feeding showed stable BMF values (Scheffe's test, $P > 0.05$) the three last sampling points. This observation supports the hypothesis discussed above on the high efficacy of detoxification routes, probably due to low oxidative stress because of the very low levels of contaminants and adequateness of the fish feeds composition supplied.

In September 2007 the total feeding trial finished as fish had reached commercial size. The concentration of the sum of PCBs and OCPs in fish fillets at this time revealed the low charge of contaminants and allowed the contaminant intake per fish by consumers to be checked (Fig. 3). As shown, the charge of pollutants per fillet decreased with reduced percentages of fish oil in their diet. As well, experimental diets that included FO as finishing diets for the three last months did not increase the load of contaminants up to the load present in fish fed FO for the full 14 months (Scheffe's test, $P > 0.05$). Besides, polybrominated biphenyl diethyl ethers (PBDEs) have been analyzed in fish feed and fish fillets studied in this work, showing very low levels of individual PBDEs

around 0.2–0.3 ng/g fresh weight in fish feeds and below 0.1 ng/g fresh weight in fish fillets (data not shown).

Several authors have reported the presence of persistent organic contaminants in cultured fish. Santerre et al. (2000) noticed that farmed channel catfish, rainbow trout and red swamp crayfish from Southern USA sources presented absence or low levels of organochlorine contaminants, which were lower than the concentration levels reported in wild fish. On the other hand, Easton et al. (2002) assessed the risk of the organochlorine contaminants in wild and farmed salmon, indicating higher levels of PCBs and OCPs in cultured fish as a consequence of the presence of these contaminants in fish feed.

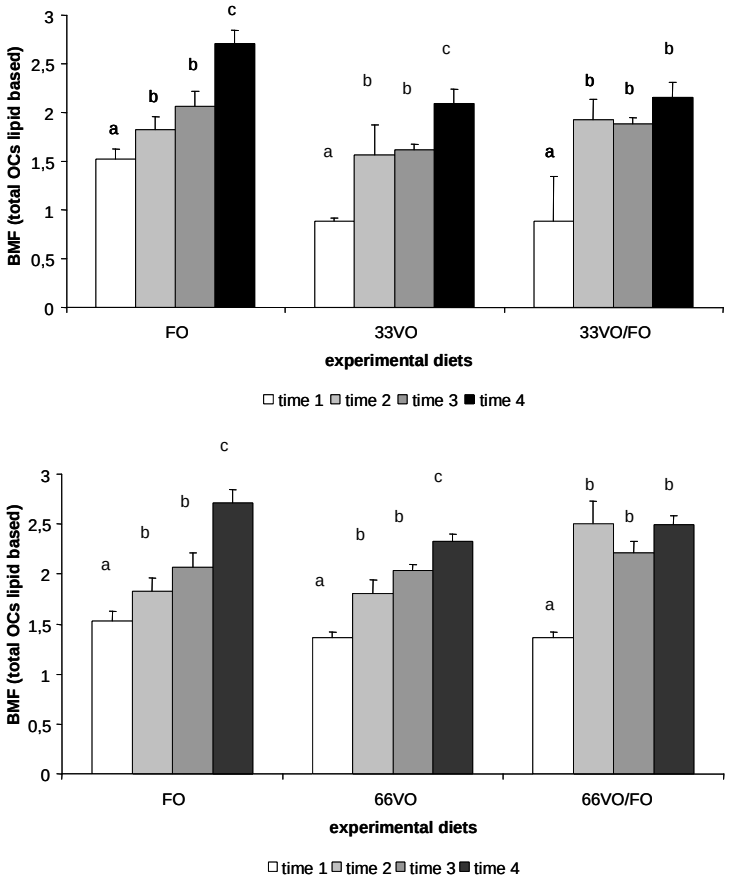

Fig. 2. Biomagnification Factors for total OCs (ng/g lipid based) of fish fed different experimental diets with varying levels of fish oil substitution, at four sampling points (Fig. 1 for details). *Letters*: comparison among BMF at different sampling points for each experimental diet, Scheffe's test, $P < 0.05$. bars: standard deviation.

This fact has been confirmed in a recent study carried out by Hites et al. (2004), also suggesting the fish feed consumed is the cause of the high levels of contaminants in farmed salmon, especially in Northern Europe. In these samples, the levels of organic compounds were higher than those found in farmed fish from North and South America.

In regard to temperate marine aquaculture, (Serrano et al., 2008a) and (Serrano et al., 2008b) reported higher levels of contaminants in wild fish than those found in cultured fish from the

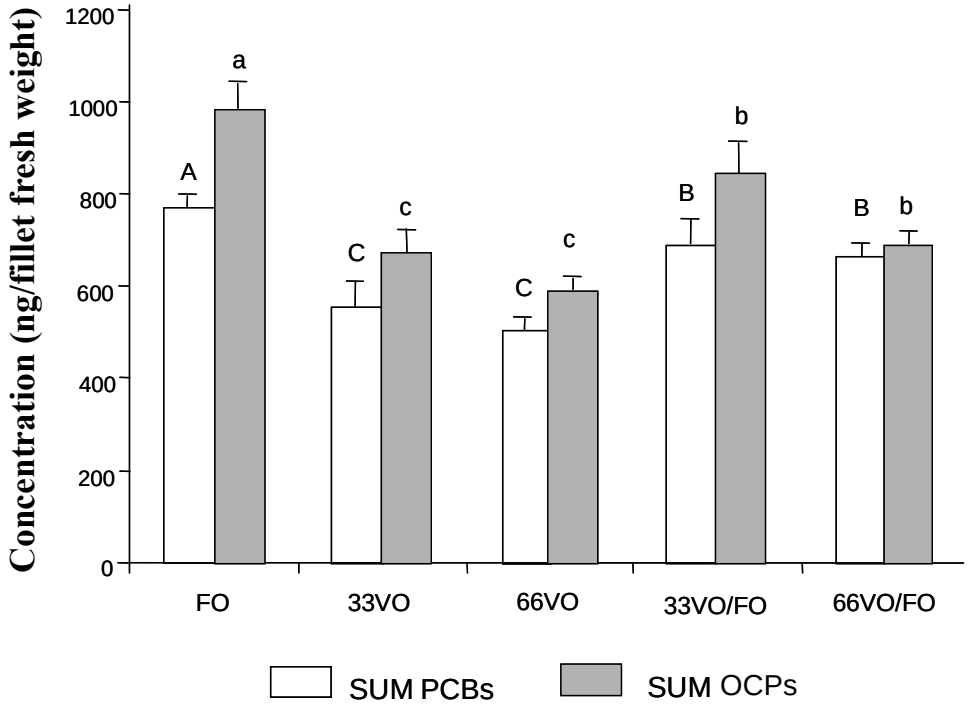

Fig. 3. Concentration (ng/fillet fresh weight) of sum PCBs and OCPs in fillets of fish fed different experimental diets with varying levels of fish oil substitution at the end of the long-term feeding trial (September 2007). Fillet weight was not altered by dietary intervention and ranged between 113 and 126 g (Benedito-Palos et al., 2009). *Letters*: comparison among diets for every category (Capitals: sum PCBs, Lower case: sum OCs). Scheffe's test, $P < 0.05$. bars: standard deviation.

Western Mediterranean region. This could be explained again by of the low contaminants load of fish feeds supplied to the farmed fish (see Table 2). From this point of view and in a food-safety framework, the results of the present work show that there is further margin for improvement in the elaboration of "cleaner" feeds and cultured fish.

Levels of pollutants in initial fish (T0) (Table 3 and Table 4), can be attributed to the nutritional background of the previous diets that influenced the profile of pollutants. These were commercial diets with major levels of pollutants compared to the new generation of diets used in this work. Besides, it has to be taken into account that the maternal transfer of organochlorine compounds in fish has been reported as a possible source of accumulation (Miller, 1993; Russell et al., 1999; Monosson et al., 2003; Oka et al., 2006; Serrano et al., 2008c).

In this study fillets from fish fed FO diet presented trace level concentrations of organochlorine compounds with this decreasing in proportion to fish oil replacement. This fact has made it possible to produce a new generation of aquaculture fish feeds with optimal replacement of fish meal and fish oil with sustainable, alternative feed resources such as vegetable oils and meals. In this way, feeds can be produced that contribute to ensure production of healthy, marketable fish, ensure also that the risk of organochlorine transfer to consumers is reduced, and improve sustainability of marine aquaculture by reducing the use of wild fish sources for feed production.

Acknowledgments

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References

Antunes, P., Gil, O., 2004. PCB and DDT Contamination in Cultivated and Wild Sea Bass from Ria De Aveiro, Portugal. Chemosphere. 54, 1503-1507.

Bell, J.G., McGhee, F., Dick, J.R., 2005. Dioxin and Dioxin-Like Polychlorinated Biphenyls (PCBs) in Scottish Farmed Salmon (Salmo Salar): Effects of Replacement of Dietary Marine Fish Oil with Vegetable Oils. Aquaculture. 243, 305-314.

Benedito-Palos, L., Navarro, J.C., Bermejo-Nogales, A., 2008a. The Time Course of Fish Oil Wash-Out Follows a Simple Dilution Model in Gilthead Sea Bream (Sparus Aurata L.) Fed Graded Levels of Vegetable Oils. Aquaculture.

Benedito-Palos, L., Navarro, J.C., Sitjà-Bobadilla, A., 2008b. High Levels of Vegetable Oils in Plant Protein-Rich Diets Fed to Gilthead Sea Bream (Sparus Aurata L.): Growth Performance, Muscle Fatty Acid Profiles and Histological Alterations of Target Tissues. British J.Nutr. 100, 992-1003.

Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J., 2007. Combined Replacement of Fish Meal and Oil in Practical Diets for Fast Growing Juveniles of Gilthead Sea Bream (Sparus Aurata L.): Networking of Systemic and Local Components of GH/IGF Axis. Aquaculture. 267, 199-212.

Berntssen, M.H.G., Lundebye, A. Torstensen, B.E., 2005. Reducing the Levels of Dioxins and Dioxin-Like PCBs in Farmed Atlantic Salmon by Substitution of Fish Oil with Vegetable Oil in the Feed. Aquacult. Nutr. 11, 219-231.

Bethune, C., Seierstad, S.L., Seljeflot, I., 2006. Dietary Intake of Differently Fed Salmon: A Preliminary Study on Contaminants. Eur. J. Clin. Investig. 36, 193-201.

Bjerregaard, P., Dewailly, E., Ayotte, P., 2001. Exposure of Inuit in Greenland to Organochlorines through the Marine Diet. J. Toxicol. Env. Health. 62, 69-81.

Bocquené, G., Franco, A., 2005. Pesticide Contamination of the Coastline of Martinique. Mar. Pollut. Bull. 51, 612-619.

Bordajandi, L.R., Martín, I., Abad, E., 2006. Organochlorine Compounds (PCBs, PCDDs and PCDFs) in Seafish and Seafood from the Spanish Atlantic Southwest Coast. Chemosphere. 64, 1450-1457.

Borga, K., Gabrielsen, G.W., Skaare, J.U., 2001. Biomagnification of Organochlorines Along a Barents Sea Food Chain. Environ. Pollut. 113, 187-198.

Carubelli, G., Fanelli, R., Mariani, G., 2007. PCB Contamination in Farmed and Wild Sea Bass (Dicentrarchus Labrax L.) from a Coastal Wetland Area in Central Italy. Chemosphere. 68, 1630-1635.

De Felip, E., Di Domenico, A., Miniero, R., 2004. Polychlorobiphenyls and Other Organochlorine Compounds in Human Follicular Fluid. Chemosphere. 54, 1445-1449.

Drew, M.D., Ogunkoya, A.E., Janz, D.M., 2007. Dietary Influence of Replacing Fish Meal and Oil with Canola Protein Concentrate and Vegetable Oils on Growth Performance, Fatty Acid Composition and Organochlorine Residues in Rainbow Trout (Oncorhynchus Mykiss). Aquaculture. 267, 260-268.

Easton, M.D.L., Luszniak, D., Von der Geest, E., 2002. Preliminary Examination of Contaminant Loadings in Farmed Salmon, Wild Salmon and Commercial Salmon Feed. Chemosphere. 46, 1053-1074.

Hernandez, F., Morell, I., Beltran, J., 1993. Multi-Residue Procedure for the Analysis of Pesticides in Groundwater: Appliction to Samples from the Comunidad Valenciana, Spain. Chromatographia. 37, 303-312.

Hernández, F., Pitarch, E., Beltran, J., 2002a. Headspace Solid-Phase Microextraction in Combination with Gas Chromatography and Tandem Mass Spectrometry for the Determination of

Organochlorine and Organophosphorus Pesticides in Whole Human Blood. J. Chromatogr. B. 769, 65-77.

Hernández, F., Pitarch, E., Serrano, R., 2002b. Multiresidue Determination of Endosulfan and Metabolic Derivatives in Human Adipose Tissue using Automated Liquid Chromatographic Cleanup and Gas Chromatographic Analysis. J. Anal. Toxicol. 26, 94-103.

Hernández, F., Pitarch, E., Serrano, R., 2002c. Determination of Organochlorine Compounds in Human Adipose Tissue using Automated Liquid Chromatographic Clean Up and Gas Chromatography - Tandem Mass Spectrometry. Chromatographia , 55, 715-722.

Hernández, F., Serrano, R., Roig-Navarro, A.F., 2000. Persistent Organochlorines and Organophosphorus Compounds and Heavy Elements in Common Whale (Balaenoptera Physalus) from the Western Mediterranean Sea. Mar. Pollut. Bull. 40, 426-433.

Hilbert, G., Lillemark, L., Balchen, S., 1998. Reduction of Organochlorine Contaminants from Fish Oil during Refining. Chemosphere. 37, 1241-1252.

Hites, R.A., Foran, J.A., Carpenter, D.O., 2004. Global Assessment of Organic Contaminants in Farmed Salmon. Science. 303, 226-229.

Hoekstra, P.F., O'Hara, T.M., Fisk, A.T., 2003. Trophic Transfer of Persistent Organochlorine Contaminants (OCs) within an Arctic Marine Food Web from the Southern Beaufort-Chukchi Seas. Environ. Pollut. 124, 509-522.

Jacobs, M.N., Johnston, P.A., Wyatt, C.L., 1997. Organochlorine Pesticide and PCB Residues in Pharmaceutical, Industrial and Food Grade Fish Oils. Int. J. Environ. Pollut. 8, 74-93.

Jacobs, M.N., Santillo, D., Johnston, P.A., 1998. Organochlorine Residues in Fish Oil Dietary Supplements: Comparison with Industrial Grade Oils. Chemosphere. 37, 1709-1721.

Johansen, H.R., Alexander, J., Rossland, O.J., 1996. PCDDs, PCDFs, and PCBs in Human Blood in Relation to Consumption of Crabs from a Contaminated Fjord Area in Norway. Environ. Health Persp. 104, 756-764.

Johnson, L.L., Ylitalo, G.M., Sloan, C.A., 2007. Persistent Organic Pollutants in Outmigrant Juvenile Chinook Salmon from the Lower Columbia Estuary, USA. Sci. Total Environ. 374, 342-366.

Kidd, K.A., Bootsma, H.A., Hesslein, R.H., 2001. Biomagnification of DDT through the Benthic and Pelagic Food Webs of Lake Malawi, East Africa: Importance of Trophic Level and Carbon Source. Environ. Sci. Technol. 35, 14-20.

Konwick, B.J., Garrison, A.W., Black, M.C., 2006. Bioaccumulation, Biotransformation, and Metabolite Formation of Fipronil and Chiral Legacy Pesticides in Rainbow Trout. Environ. Sci. Technol. 40, 2930-2936.

Lopez-Espinosa, M. Lopez-Navarrete, E., Rivas, A., 2008. Organochlorine Pesticide Exposure in Children Living in Southern Spain. Environ. Res. 106, 1-6.

Maule, A.G., Gannam, A.L., Davis, J.W., 2007. Chemical Contaminants in Fish Feeds used in Federal Salmonid Hatcheries in the USA. Chemosphere. 67, 1308-1315.

Miller, M.A., 1993. Maternal Transfer of organochlorine compounds in salmonids to their eggs. Can. J. Fish. Aquat. Sci. 50, 1450-1413.

Monosson, E., Ashley, J.T.F., McElroy, A.E., 2003. PCB Congener Distributions in Muscle, Liver and Gonad of Fundulus Heteroclitus from the Lower Hudson River Estuary and Newark Bay. Chemosphere. 52, 777-787.

Mueller, J.F., Harden, F., Toms, L., 2008. Persistent Organochlorine Pesticides in Human Milk Samples from Australia. Chemosphere. 70, 712-720.

Muñoz-de-Toro, M., Beldoménico, H.R., García, S.R., 2006. Organochlorine Levels in Adipose Tissue of Women from a Littoral Region of Argentina. Environ. Res. 102, 107-112.

Oka, M., Arai, T., Shibata, Y., 2006. Reproductive Transfer of Organochlorines in Viviparous Surfperch, Ditrema Temmincki. Environ. Pollut. 142, 383-387.

Pitarch, E., Serrano, R., López, F.J., 2003. Rapid Multiresidue Determination of Organochlorine and Organophosphorus Compounds in Human Serum by Solid-Phase Extraction and Gas Chromatography Coupled to Tandem Mass Spectrometry. Anal. Bioanal. Chem. 376, 189-197.

Russell, R.W., Gobas, F.A.P.C., Haffner, G.D., 1999. Maternal Transfer and in Ovo Exposure of Organochlorines in Oviparous Organisms: A Model and Field Verification. Environ. Sci. Technol. 33, 416-420.

Sagratini G, Buccioni M, Ciccarelli C, Conti P, Cristalli G, Giardina D, Lambertucci C, Marucci G, Volpini R, Vittori S., 2008b. Levels of polychlorinated biphenyls in fish and shellfish from the Adriatic sea. Food Add. Contam. 1, 69-77.

Santerre, C.R., Ingram, R., Lewis, G.W., 2000. Organochlorines, Organophosphates, and Pyrethroids in Channel Catfish, Rainbow Trout, and Red Swamp Crayfish from Aquaculture Facilities. J. Food Sci. 65, 231-235.

Sargent, J., Tocher, D.R., Bell, J. G. The lipids. In: Halver, J.E. Sargent, J.; Tocher, D. R.; Bell, J. G. the Lipids. in: Halver, J.E.; Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, San Diego, 2002, Pp. 181-257.

Sargent, J., McEvoy, L., Estevez, A., 1999. Lipid Nutrition of Marine Fish during Early Development: Current Status and Future Directions. Aquaculture. 179, 217-229.

Serrano, R., Simal-Julian, A., Pitarch, E., 2003a. Biomagnification Study on Organochlorine Compounds in Marine Aquaculture: The Sea Bass (Dicentrarchus Labrax) as a Model. Env. Sci. Tech. 37, 3375-3381.

Serrano, R., Barreda, M., Pitarch, E., 2003b. Determination of Low Concentrations of Organochlorine Pesticides and PCBs in Fish Feed and Fish Tissues from Aquaculture Activities by Gas Chromatography with Tandem Mass Spectrometry. J. Sep. Sci. 26, 75-86.

Serrano, R., Barreda, M., Blanes, M.A., 2008a. Investigating the Presence of Organochlorine Pesticides and Polychlorinated Biphenyls in Wild and Farmed Gilthead Sea Bream (Sparus Aurata) from the Western Mediterranean Sea. Mar. Pollut. Bull. 56, 963-972.

Serrano, R., Blanes, M.A., López, F.J., 2008b. Biomagnification of Organochlorine Pollutants in Farmed and Wild Gilthead Sea Bream (Sparus Aurata) and Stable Isotope Characterization of the Trophic Chains. Sci. Total Environ. 389, 340-349.

Serrano, R., Blanes, M.A., López, F.J., 2008c. Maternal Transfer of Organochlorine Compounds to Oocytes in Wild and Farmed Gilthead Sea Bream (Sparus Aurata). Chemosphere. 70, 561-566.

Tsukino, H., Hanaoka, T., Sasaki, H., 2006. Fish Intake and Serum Levels of Organochlorines among Japanese Women. Sci. Total Environ. 359, 90-100.

Van Den Berg, M., Birnbaum, L., Bosveld, A.T.C., 1998. Toxic Equivalency Factors (TEFs) for PCBs, PCDDs, PCDFs for Humans and Wildlife. Environ Health Persp. 106, 775-792.

Yang, R., Yao, T., Xu, B., 2007. Accumulation Features of Organochlorine Pesticides and Heavy Metals in Fish from High Mountain Lakes and Lhasa River in the Tibetan Plateau. Environ. Int. 33, 151-156.

Zabik, M.E., Booren, A., Zabik, M.J., 1996. Pesticide Residues, PCBs and PAHs in Baked, Charbroiled, Salt Boiled and Smoked Great Lakes Lake Trout. Food Chem. 55, 231-239.

6.3.3. Discusión de los resultados.

La determinación de PCBs y OCPs se llevó a cabo mediante la metodología analítica descrita por Serrano et al. (2003b) (**Figura 6.4**). Brevemente, la muestra previamente homogeneizada y triturada, es pesada en un matraz erlenmeyer y disgregada en sulfato de sodio anhidro a la cual se añaden 100 mL de hexano. Posteriormente se procede a una extracción sólido-líquido por reflujo de la muestra en hexano durante cuatro horas. El extracto es filtrado y concentrado hasta 2 mL.

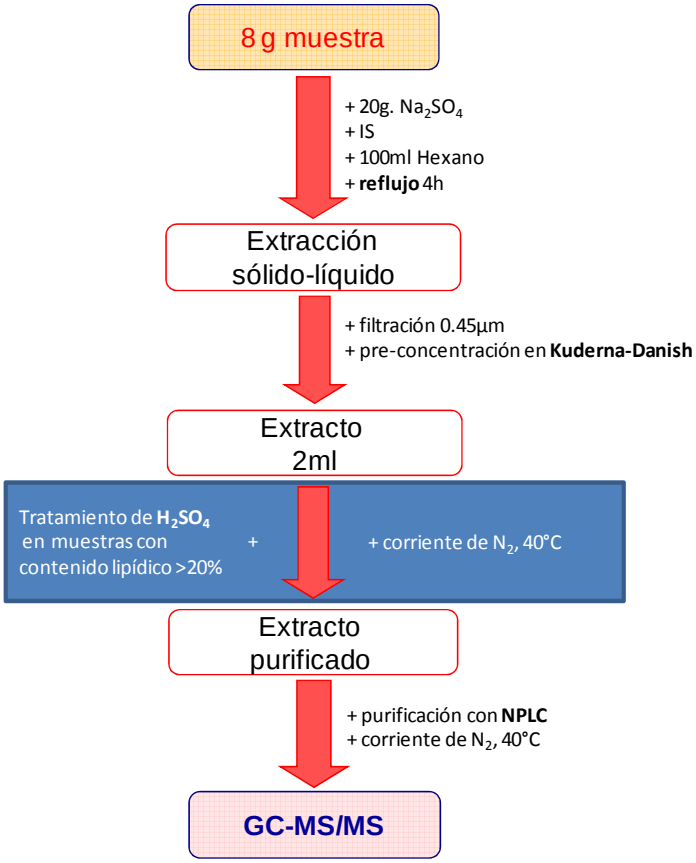

Figura 6.4. Esquema del proceso experimental para el análisis de PCBs y OCPs.

El extracto hexánico obtenido tiene un contenido muy alto de lípidos, que resultan incompatibles con el sistema de GC utilizado para la determinación. Así pues, para poder determinar correctamente los analitos, se deben eliminar en la medida de lo posible los

interferentes del extracto para conseguir una mayor sensibilidad y selectividad en el análisis. Las etapas de extracción, purificación y fraccionamiento de los compuestos son pasos precisos y delicados antes del análisis mediante GC de OCs porque los compuestos co-extraídos, como por ejemplo los lípidos, interfieren en la detección de OCs a niveles bajos. En este sentido, Van Leeuwen et al. (2008) presenta diferentes metodologías atendiendo a las etapas de extracción y purificación de matrices complejas procedentes del medio ambiente marino previo análisis mediante GC-MS.

El procedimiento de purificación aplicado en el presente trabajo se basa en LC con columna de sílica, con carácter semi-preparativo.

Para las matrices con elevado contenido lipídico (contenido graso > 20%) es conveniente la realización de un primer tratamiento con ácido para eliminar la gran carga lipídica de las muestras, puesto que la técnica de NPLC no es capaz de retener tantos lípidos. Este procedimiento supone la eliminación de más del 90% del contenido graso de la muestra (Serrano et al., 2003b). Como los PCBs y los OCPs estudiados no son ácido lábiles éste tratamiento resulta de gran eficacia para la obtención de extractos limpios de interferentes previa purificación con el sistema automatizado de NPLC y posterior análisis mediante GC-MS/MS.

- Contaminantes en materias primas y piensos.

Los resultados obtenidos en el análisis de las muestras del estudio revelan numerosos positivos de PCBs y OCPs. Las concentraciones de PCBs y OCPs estuvieron por encima de 4 ng/g y 5 ng/g respectivamente, alcanzando los valores más altos para los aceites de pescado. Mediante el análisis de muestras de pienso se obtuvieron valores de PCBs y OCPs por encima de 2 ng/g y 4 ng/g respectivamente, siendo el pienso con mayor contenido en aceite de pescado (FO) el pienso que presentó mayor concentración de contaminantes. Hay que destacar que los OCPs cuantificados fueron el DDT, DDD, DDE y el HCB (**Tabla 2. Artículo científico 6**). El PCB 153 y el PCB 138 siempre presentaron mayor concentración con respecto a los demás PCBs encontrados para este tipo de muestras. Respecto a las materias primas de origen vegetal consideradas (aceite de colza, aceite de

palma, aceite de lino, gluten de maíz, trigo, lecitina de soja, y derivados de la soja), no se detectaron concentraciones de OCs por encima del LOD. Este hecho confirma que estas materias primas de origen vegetal permiten disminuir la carga de contaminantes OCs mediante la sustitución del aceite de pescado en la composición de los piensos.

En general, las concentraciones de PCBs y OCPs encontradas en los piensos fueron bajas en comparación con las presentadas en piensos por otros autores. En este sentido, Ying et al. (2009) presentan un intervalo de valores de concentracion para OCPs entre 16.2-7120 ng/g y para PCBs entre 1.93-59.3 ng/g en piensos para pescado. Igualmente, Jacobs et al. (2002), presentan valores de concentración de PCBs en piensos de acuicultura en el intervalo de 75.6-1153 ng/g mientras que recientemente Berntssen et al. (2010) obtienen resultados similares a los de nuestro estudio en piensos para salmones.

En la **Tabla 2** del **artículo científico 6**, se pueden observar valores bajos para la carga total en las dietas 33VO y 66VO, como consecuencia de la sustitución del aceite de pescado por aceites vegetales en su formulación (un 33% y un 66% de sustitución respectivamente).

- Contaminantes PCBs y OCPs en doradas alimentadas con dietas con diferente contenido en aceite de pescado.

Se procedió a estudiar los niveles de PCBs y OCPs en filetes de dorada a lo largo de su ciclo de crecimiento. Teniendo en cuenta los resultados de materias primas comentados anteriormente, un pienso con menor porcentaje de materias primas de origen animal, tendrá menor carga de PCBs y OCPs, y viceversa.

No se observó bioacumulación de compuestos OCs durante los primeros 11 meses del estudio experimental en los tres grupos experimentales (FO, 33VO y 66VO). En todos los casos, al final de los 11 meses (Junio 2007), las concentraciones de PCBs y OCPs eran muy similares en comparación al inicio del estudio (Julio 2006). Este hecho podría revelar un proceso de dilución como consecuencia del crecimiento y/o la capacidad de las doradas de liberar o metabolizar los contaminantes ingeridos mediante rutas de desintoxicación en las condiciones de cultivo experimentadas. Por otra parte, las concentraciones presentes en el pez al inicio del experimento se deberían a los piensos con los que se alimentaron

anteriormente al experimento, ya que los valores en Junio 2007 son más bajos que los analizados en Julio 2006. Otra posibilidad sería la acumulación de contaminantes en un sistema de circuito cerrado con altas densidades de cultivo en las fases de preengorde (2 g-15 g), antes del inicio de la fase experimental en las instalaciones de cultivo del IATS. El estudio de los valores de BMF evidenció que no se produjo bioacumulación de PCBs y OCPs durante esta primera parte del experimento, si no todo lo contrario, ya que los peces disminuyeron los niveles de PCBs y OCPs.

En la segunda parte del experimento (Junio 2007-Septiembre 2007) las doradas ingirieron la misma cantidad de piensos que en la primera etapa de 11 meses (Julio06-Junio07). Este cambio en la ingesta cambia los niveles de BMF en los peces y lleva consigo una ingesta mayor de contaminantes. Durante los meses comprendidos entre Julio 2007-Septiembre 2007 (correspondientes a los meses 11° a 14° del experimento) se pudo observar como aumentaban los niveles de OCs. Los valores de BMF no crecen igual para todos los grupos (**ver Figura 2. Artículo científico 6**). Se observó que los valores de bioacumulación eran menores para aquellos especímenes que se alimentan con piensos con menor contenido de materias primas de origen de pescado. Para el caso de los grupos de peces que pasan a ingerir piensos FO como dieta finalizadora (grupos 33VO/FO y 66VO/FO), los valores de BMF no aumentan tanto, como consecuencia de los niveles bajos de contaminantes y adecuación de la alimentación del pescado.

6.3.4. Referencias.

Ballschmiter, K.; Zell, M. 1980. Analysis of polychlorinated biphenyls (PCB) by glass capillary gas chromatography - Composition of technical aroclor- and Clophen-PCB mixtures. Fresen. Z. Anal. Chem. 302, 20-31.

Berntssen, M.H.G.; Julshamn, K.; Lundebye, A.-K. 2010. Chemical contaminants in aquafeeds and Atlantic salmon (Salmo salar) following the use of traditional- versus alternative feed ingredients. Chemosphere 78, 637-646.

Bordajandi, L.R.; Abad, E.; González, M.J. 2008. Occurrence of PCBs, PCDD/Fs, PBDEs and DDTs in Spanish breast milk: Enantiomeric fraction of chiral PCBs. Chemosphere 70, 567-575.

Chu, S.; Covaci, A.; Schepens, P. 2003. Levels and chiral signatures of persistent organochlorine pollutants in human tissues from Belgium. Environ. Res. 93, 167-176.

Directiva 96/59/CE del Consejo, de 16 de Septiembre de 1996, relativa a la eliminación de los policloro bifenilos y de los policlorotercenilos (PCB/PCT) DOCE 243/L, de 24-09-96.

Domingo, J.L., Bocio, A. 2007. Levels of PCDD/PCDFs and PCBs in edible marine species and human intake: A literature review. Environ. Int. 33, 397-405.

Jacobs, M. N.; Covaci, A.; Schepens, P. 2002. Investigation of selected persistent organic pollutants in farmed Atlantic Salmon (Salmo salar), salmon aquaculture feed, and fish oil components of the feed. Environ. Sci. Technol. 36, 2797–2805.

Lacorte, S.; Raldúa, D.; Martínez, E.; Navarro, A.; Diez, S.; Bayona, J.M.; Barceló, D. 2006. Pilot survey of a broad range of priority pollutants in sediment and fish from the Ebro river basin (NE Spain). Environ. Pollut. 140, 471-482.

Muir, D.; Braune, B.; DeMarch, B.; Norstrom, R.; Wagemann, R.; Lockhart, L.; Hargrave, B.; Reimer, K. 1999. Spatial and temporal trends and effects of contaminants in the Canadian Arctic marine ecosystem: A review. Sci. Total Environ. 230, 83-144.

Saera-Vila, A.; Benedito-Palos, L.; Sitjà-Bobadilla, A.; Nácher-Mestre, J.; Serrano, R.; Kaushik, S.; Pérez-Sánchez, J. 2009. Assessment of the health and antioxidant trade-off in gilthead sea bream (*Sparus aurata L.*) fed alternative diets with low levels of contaminants. Aquaculture 296, 87-95.

Safe, S.H. 1994 Polychlorinated biphenyls (PCBs): Environmental impact, biochemical and toxic responses, and implications for risk assessment. Crit. Rev. Toxicol. 24, 87-149.

Serrano, R.; Simal-Julian, A.; Pitarch, E.; Hernandez, F.; Varó, I.; Navarro, J.C. 2003a. Biomagnification study on organochlorine compounds in marine aquaculture: The sea bass (Dicentrarchus labrax) as a model. Environ. Sci. Technol. 37, 3375-3381.

Serrano, R.; Barreda, M.; Pitarch, E. 2003b. Determination of Low Concentrations of Organochlorine Pesticides and PCBs in Fish Feed and Fish Tissues from Aquaculture Activities by Gas Chromatography with Tandem Mass Spectrometry. J. Sep. Sci. 26, 75-86.

Smith, A. G.; Gangolli, S. D. 2002. Organochlorine chemicals in seafood: occurrence and health concerns. Food Chem. Toxicol. 40, 767-779.

Van Den Berg, M.; Birnbaum, L.; Bosveld, A.T.C.; Brunström, B.; Cook, P.; Feeley, M.; Giesy, J.P.; Zacharewski, T. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ. Health Persp. 106, 775-792

Van der Oost, R.; Beyer, J.; Vermeulen, N.P.E. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environ. Toxicol. Pharm. 13, 57-149.

Van Leeuwen, S.P.J.; de Boer, J. 2008. Advances in the gas chromatographic determination of persistent organic pollutants in the aquatic environment. J. Chromatogr. A 1186, 161-182.

Yang, N.; Matsuda, M.; Kawano, M.; Wakimoto, T. 2006. PCBs and organochlorine pesticides (OCPs) in edible fish and shellfish from China. Chemosphere 63, 1342-1352.

Ying, G.; Huan-Yun, Y.; Bao-Zhong, Z.; Eddy Y.Z. 2009. Persistent halogenated hydrocarbons in fish feeds manufactured in south china. J. Agr. Food Chem. 57, 3674-3680.

CAPÍTULO 7

CONCLUSIONES

La conclusión general surgida a raíz de las investigaciones realizadas en esta Tesis Doctoral sugiere que las técnicas basadas en el acoplamiento GC-MS son muy adecuadas para el estudio de la presencia de contaminantes orgánicos en muestras de matriz compleja. Las múltiples posibilidades de trabajo con analizadores de MS como el triple cuadrupolo y el analizador de tiempo de vuelo, la convierten en una herramienta potente y muy versátil tanto para la determinación como para la confirmación de la identidad de contaminantes seleccionados, excluyendo aquellos de baja volatilidad y elevada polaridad cuya determinación se lleva a cabo preferentemente por acoplamiento LC-MS. Igualmente, se pueden plantear las siguientes conclusiones específicas relacionadas con los diferentes trabajos incluidos en esta Tesis:

1. Se ha desarrollado metodología analítica rápida, sensible y selectiva para la determinación de PAHs en matrices complejas procedentes de la acuicultura marina. El procedimiento desarrollado para la eliminación de interferentes, principalmente grasa, permitió validar y aplicar metodología analítica para la determinación de PAHs en estas muestras mediante GC-(QqQ)MS/MS. Mediante GC-TOF MS ha sido posible conseguir una extraordinaria capacidad de confirmación para los analitos detectados en las muestras mediante GC-(QqQ)MS/MS.

2. Se ha desarrollado metodología analítica sensible para la determinación de PBDEs en matrices complejas procedentes de la acuicultura marina. El tratamiento con ácido junto a la etapa posterior de SPE resultó ser un tratamiento muy eficaz para concentrar los extractos permitiendo alcanzar LOQs bajos, a niveles de µg/Kg. La

metodología permitió la correcta determinación del BDE 209, uno de los PBDEs que presentan mayor dificultad en la determinación.

3. Se ha desarrollado metodología analítica avanzada para el *screening non-target* de contaminantes en muestras de sal y agua de mar mediante GC-TOF MS. Este trabajo ha permitido la investigación de contaminantes orgánicos en muestras de sal marina y agua de mar, de importancia desde el punto de vista de salud pública, expresamente en el ámbito ambiental y de seguridad alimentaria.

4. Se ha desarrollado metodología analítica avanzada para el *screening* de OPEs en muestras medioambientales mediante GC-TOF MS. Gracias a la adquisición de información espectral completa con elevada exactitud de masa que ofrece el TOF se desarrollaron estrategias de *screening target* y *post-target* de contaminantes orgánicos.

5. Mediante la aplicación de metodología desarrollada para la determinación de PAHs, se ha podido realizar el estudio de la bioacumulación de PAHs en doradas (*Sparus aurata L.*) cultivadas a lo largo de un estudio experimental que incluye un ciclo completo de engorde. Los resultados obtenidos demostraron la ausencia de bioacumulación en las condiciones experimentales aplicadas, proporcionando información esencial sobre el comportamiento de los PAHs en productos de la acuicultura y sobre la calidad y seguridad del producto final desde el punto de vista del consumidor.

6. El análisis de compuestos OCs en piensos y filetes de peces cultivados nos ha permitido estudiar la bioacumulación de OCs en doradas (*Sparus aurata L.*) cultivadas a lo largo de un estudio experimental que incluye un ciclo completo de engorde. Se evidenció que la baja bioacumulación de estos compuestos en las condiciones experimentales aplicadas dió como resultado bajas concentraciones de contaminantes organoclorados en las especies cultivadas.

Artículos científicos presentados en la Tesis

1. *A reliable analytical approach based on gas chromatography coupled to triple quadrupole and time of flight analyzers for the determination and confirmation of polycyclic aromatic hydrocarbons in complex matrices from aquaculture activities*
   J. Nácher-Mestre, R. Serrano, T. Portolés, F. Hernández, L. Benedito-Palos, J. Pérez-Sánchez
   Rapid Commun. Mass Spectrom. 23 (**2009**) 2075-2086

2. *Gas chromatography–mass spectrometric determination of polybrominated diphenyl ethers in complex fatty matrices from aquaculture activities*
   J. Nácher-Mestre, R. Serrano, F. Hernández, L. Benedito-Palos, J. Pérez-Sánchez
   Anal. Chim. Acta 664 (**2010**) 190–198

3. *Non-target screening of organic contaminants in marine salts by gas chromatography coupled to high-resolution time-of-flight mass.*
   R. Serrano, J. Nácher-Mestre, T. Portolés, F. Amat, F. Hernández
   Submitted to Talanta

4. *Investigation of OPEs in fresh and sea water, salt and brine samples by GC-TOF MS.*
   J. Nácher-Mestre, R. Serrano, T. Portolés, F. Hernández.
   Submitted to Analytical and Bioanalytical Chemistry

5. *Bioaccumulation of Polycyclic Aromatic Hydrocarbons in Gilthead Sea Bream (Sparus aurata L.) Exposed to Long Term Feeding Trials with Different Experimental Diets.*
   J. Nácher-Mestre, R. Serrano, L. Benedito-Palos, J. C. Navarro, F. J. López, S. Kaushik, J. Pérez-Sánchez
   Arch Environ Contam Toxicol 59 (**2010**) 137–146

6. *Effects of fish oil replacement and re-feeding on the bioaccumulation of organochlorine compounds in gilthead sea bream (Sparus aurata L.) of market size.*

   J. Nácher-Mestre, R. Serrano, L. Benedito-Palos, J. C. Navarro, F. J. López, *J. Pérez-Sánchez*

   Chemosphere 76 (**2009**) 811-817

Artículos científicos relacionados con la Tesis

1. *Assessment of the health and antioxidant trade-off in gilthead sea bream (Sparus aurata L.) fed alternative diets with low levels of contaminants*

   A. Saera-Vila, L. Benedito-Palos, A. Sitjà-Bobadilla, J. Nácher-Mestre, R. Serrano, S. Kaushik, J. Pérez-Sánchez.

   Aquaculture 296 (**2009**) 87–95

2. GC-MS determination and confirmation of PAHs

   Roque Serrano, Jaime Nácher-Mestre, Tania Portolés, Félix Hernández, Laura Benedito-Palos, Jaume Pérez-Sánchez.

   Separation Science, driving analytical chemistry forward. Enviro report. 2009.

3. Sea bream performance and health. Food safety - Nutrient metabolism, Nutrigenomics and Flesh quality

   L. Benedito-Palos, J. Nácher-Mestre, A. Saera-Vila, A. Bermejo-Nogales, I. Estensoro, R. Serrano, J.C. Navarro, A. Sitjà-Bobadilla, J. Pérez-Sánchez.

   Aquamax News. Issue N° 6. March 2010.