UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

Facultad de Química

Departamento de Química Analítica, Nutrición y Bromatología

ESTRATEGIAS DE PREPARACIÓN DE MUESTRA PARA EL ANÁLISIS DE RESIDUOS DE AGENTES QUIMIOTERÁPICOS Y PESTICIDAS EN EL MEDIO MARINO

DIEGO GARCÍA RODRÍGUEZ

Memoria para optar al grado de Doctor en Química

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DÑA. ROSA ANTONIA LORENZO FERREIRA Y **DÑA. ANTONIA MARÍA CARRO DÍAZ**, PROFESORAS TITULARES DEL DEPARTAMENTO DE QUÍMICA ANALÍTICA, NUTRICIÓN Y BROMATOLOGÍA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA,

INFORMAMOS: Que la presente memoria titulada ***"ESTRATEGIAS DE PREPARACIÓN DE MUESTRA PARA EL ANÁLISIS DE RESIDUOS DE AGENTES QUIMIOTERÁPICOS Y PESTICIDAS EN EL MEDIO MARINO"*** ha sido realizada bajo nuestra dirección por el licenciado en química **D. Diego García Rodríguez** en el **Departamento de Química Analítica, Nutrición y Bromatología** y, una vez concluida, autorizamos su presentación para ser juzgada por el tribunal correspondiente.

Para que así conste, expedimos y firmamos el presente informe en Santiago de Compostela, a 5 de noviembre de 2012.

Fdo. Rosa A. Lorenzo Ferreira **Fdo. Antonia Mª Carro Díaz**

Fdo. Diego García Rodríguez

"La vida no es más que un vistazo momentáneo a las maravillas de este asombroso Universo,
y es triste que tantos la estén malgastando soñando con fantasías espirituales"
CARL E. SAGAN

"El aspecto más triste de la vida actual es que la ciencia gana en conocimiento más
rápidamente que la sociedad en sabiduría."
ISAAC ASIMOV

AGRADECIMIENTOS

Esta Tesis Doctoral no habría sido posible sin la ayuda de a mis directoras, Rosa A.Lorenzo Ferreira y Antonia Mª Carro Díaz. Gracias por dedicarme gran parte de vuestro tiempo y por todo el apoyo que me habéis brindado durante estos años.

También me gustaría agradecer a la Xunta de Galicia (Consellería de Innovación e Industria) la beca *"Lucas Labrada"* concedida; así como a la Xunta de Galicia, al Ministerio de Educación, Ciencia e Innovación y Fondos FEDER (proyectos 10TAL209005PR, 10PXIB208089PR, CTQ2006-03334/BQU y CTQ2009-08377MEC) que han financiado este trabajo.

Mi agradecimiento también a todo el departamento de Química Analítica, Nutrición y Bromatología de la USC, especialmente a todos aquellos que conforman el Grupo de Cromatografía y Quimiometría. A todos ellos, muchísimas gracias.

A todos los compañeros del IIAA, sobre todo a Eva, Carmen, Rosa, Brais, Juan, Maite y Mónica por ser mucho más que compañeros de trabajo, por vuestras ideas y aportes, por la hora del café y por las risas que hacen llevadero el día a día en el laboratorio. También a mi familia, especialmente a mi madre, porque siempre han creído en mí y me han apoyado todos estos años. A toda la *"Peña del Victor García"* por todas esas largas partidas de cartas y las noches de vino y rosas.

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I. PLANTEAMIENTO Y OBJETIVOS

I. Planteamiento y objetivos

En los últimos años, la contaminación de los alimentos y del medio ambiente por los pesticidas se ha convertido en objeto de gran interés y preocupación social debido a los posibles efectos adversos de una exposición prolongada a estos compuestos. Por ello el estudio de la contaminación química de los alimentos ha crecido considerablemente en los últimos años convirtiéndose en una parte fundamental para el mantenimiento de la seguridad alimentaria en todo el mundo.

La acuicultura es en la actualidad uno de los sistemas de producción alimentaria de más rápido crecimiento en todo el mundo. En un momento en que se están estancando los rendimientos de la pesca y aumenta la demanda de pescado y otros productos marinos (moluscos, algas, etc.), se han generado grandes expectativas sobre la acuicultura y la posibilidad de incrementar su contribución a la producción mundial de alimentos de origen acuático. La acuicultura comprende una variedad muy amplia de diferentes prácticas piscícolas en lo que se refiere a las especies, entornos y sistemas utilizados.

Además de su uso aplicado a explotaciones acuícolas, los pesticidas también pueden acceder a los sistemas de acuicultura indirectamente mediante procesos naturales y artificiales. Ejemplos de estos procesos incluyen los derivados de la pulverización agroquímica, la escorrentía y la filtración de las tierras agrícolas hacia los reservorios de aguas subterráneas o los emisarios de industrias urbanas (curtidos, productos farmacéuticos) que llegan a los sistemas acuáticos a través de agua dulce o marina.

La normativa de la U.E. establece medidas para el seguimiento de las repercusiones de la acuicultura en el medio ambiente. Estas medidas incluyen evaluaciones del impacto medioambiental producido por la ubicación, diseño y funcionamiento de los centros de acuicultura intensiva. Para llevar a cabo el control de estas explotaciones se limitan los derechos de acceso al agua, se utilizan técnicas de control de los efluentes, se controlan los alimentos y se restringe el uso de fármacos, antibióticos y otros productos químicos.

La Unión Europea es líder mundial en cuanto a producción acuícola de especies como salmón, rodaballo, dorada, mejillón o lubina. Con una producción acuícola de unas 320000 toneladas/año (el 90% de origen marino), España está a la cabeza en la producción europea de mejillones y entre los primeros productores europeos de especies mediterráneas (lubina y dorada). En Galicia, la producción total de la acuicultura alcanza las 200000 toneladas/año, lo que representa, aproximadamente, el 35% del total generado por la U.E

El objetivo de este trabajo es el desarrollo y validación de metodología analítica para la determinación de pesticidas en muestras relacionadas con el medio marino: agua de mar, algas cultivadas y salvajes utilizadas con fines alimentarios, pescados y moluscos. Los compuestos que se determinan son pesticidas pertenecientes a las siguientes familias: organofosforados (azametifos, clorpirifos-metil y clorpirifos-etil); piretroides (empentrina, bioaletrina, resmetrina, tetrametrina, cialotrina, permetrina, ciflutrina cipermetrina, flucitrinato, fenvalerato, deltametrina); carbamatos (carbaril y propoxur); derivados de la benzoilfenilurea (diflubenzurón, teflubenzurón); avermectinas (abamectina, doramectina, ivermectina); piperonil butóxido (PBO) que actúa como sinergista de piretroides, organofosforados y carbamatos, y 2-fenilfenol. Todos ellos son usados, en mayor o menor medida, en el medioambiente y como agentes quimioterápicos en el tratamiento de infecciones parasitarias presentes en las granjas de cultivo.

Este objetivo general se concreta en los siguientes objetivos específicos agrupados según la matriz estudiada:

1. El desarrollo de metodología analítica para la determinación de residuos de pesticidas en agua de mar, basada en el empleo de técnicas de microextracción (microextracción en fase sólida, microextracción líquido-líquido dispersiva) y en la determinación cromatográfica empleando detectores selectivos de captura de electrones y espectrometría de masas en tándem con inyección de grandes volúmenes.

2. El desarrollo de métodos analíticos que permitan la determinación de los residuos de distintos grupos de pesticidas en algas y pescados, utilizando diferentes técnicas de extracción (extracción con disolventes a temperatura y presión elevadas, extracción asisitida por microondas, dispersión de la matriz en una fase sólida) y de determinación por cromatografía de gases o por cromatografía líquida acopladas a espectrometría de masas en tándem.

De forma detallada se pretende:

- El desarrollo y la optimización de los procesos de preparación de muestra en las diferentes matrices, para la separación de los compuestos de interés, haciendo uso de diseños experimentales.
- El estudio de los criterios de validación habitualmente aplicados a un método analítico: rango lineal, sensibilidad, límites de detección, límites de cuantificación, precisión y exactitud.
- Aplicación de los métodos propuestos al análisis de muestras procedentes de zonas de acuicultura.

II. INTRODUCCIÓN

II. Introducción

1. Acuicultura

La acuicultura se define, en un sentido amplio, como la cría en condiciones más o menos controladas de especies que se desarrollan en un medio acuático. Por lo tanto, la acuicultura supone una interacción entre el hombre y el agua, con la finalidad de obtener, ya sea con fines productivos o lúdicos, especies de origen acuático de utilidad para el hombre. Estas especies objeto de la acuicultura pueden clasificarse en algas, moluscos, crustáceos y peces. Es importante tener en cuenta que la acuicultura tiene como objetivo no solo la producción cuantitativa, sino también la mejora cualitativa del producto final [Acuicultura, 2012].

La acuicultura es el sector de producción alimentaria que más ha crecido en los últimos años. En 1996 ya aportó el 20% de la producción pesquera mundial. La mayor parte de esta producción (15.1 millones de toneladas) fue de origen continental, 9.7 millones de toneladas fueron de origen marino y 1.6 toneladas se originaron en aguas salobres. En 1997 la pesca alcanzó un máximo histórico mundial con una producción conjunta de pesca y acuicultura de 122 millones de toneladas de pescado. Este aumento se debió principalmente a la acuicultura, ya que la pesca, propiamente dicha, apenas aumentó. Hoy en día aproximadamente un tercio de los alimentos de origen acuático son de cría [FAO, 2012].

La Unión Europea es líder mundial en cuanto a producción acuícola de especies como salmón, rodaballo, dorada, mejillón o lubina. Con una producción acuícola de unas 320000 toneladas/año (el 90% de origen marino), España está a la cabeza en la producción europea de mejillones y entre los primeros productores europeos de especies mediterráneas (lubina y dorada). En Galicia, la producción total de la acuicultura alcanza las 200000 toneladas/año, lo que representa, aproximadamente, el 35% del total generado por la U.E [Acuicultura, 2012; Carro, 2005; Rodil, 2005]. En las *figuras II.1, II.2* y *II.3* se muestra la evolución de las especies producidas en Galicia en el periodo 2005-2010.

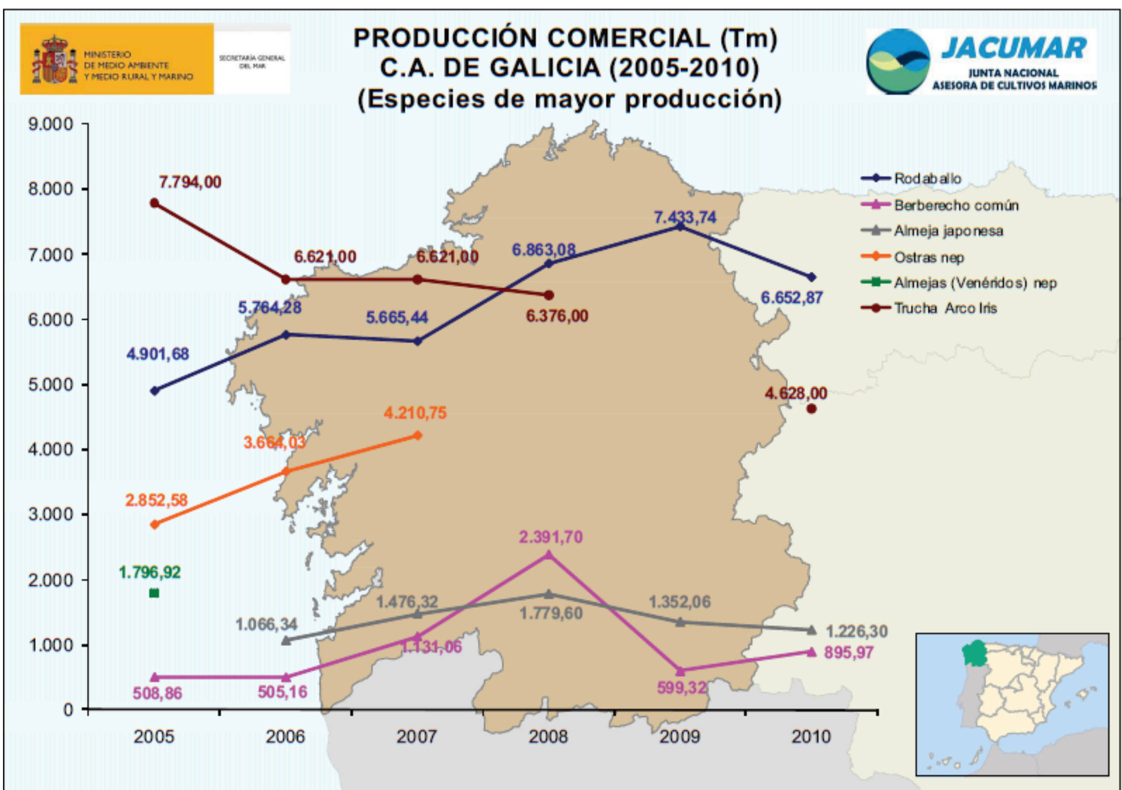

Figura II.1. *Especies de acuicultura de mayor producción en Galicia (2005-2010)[Acuicultura, 2012].*

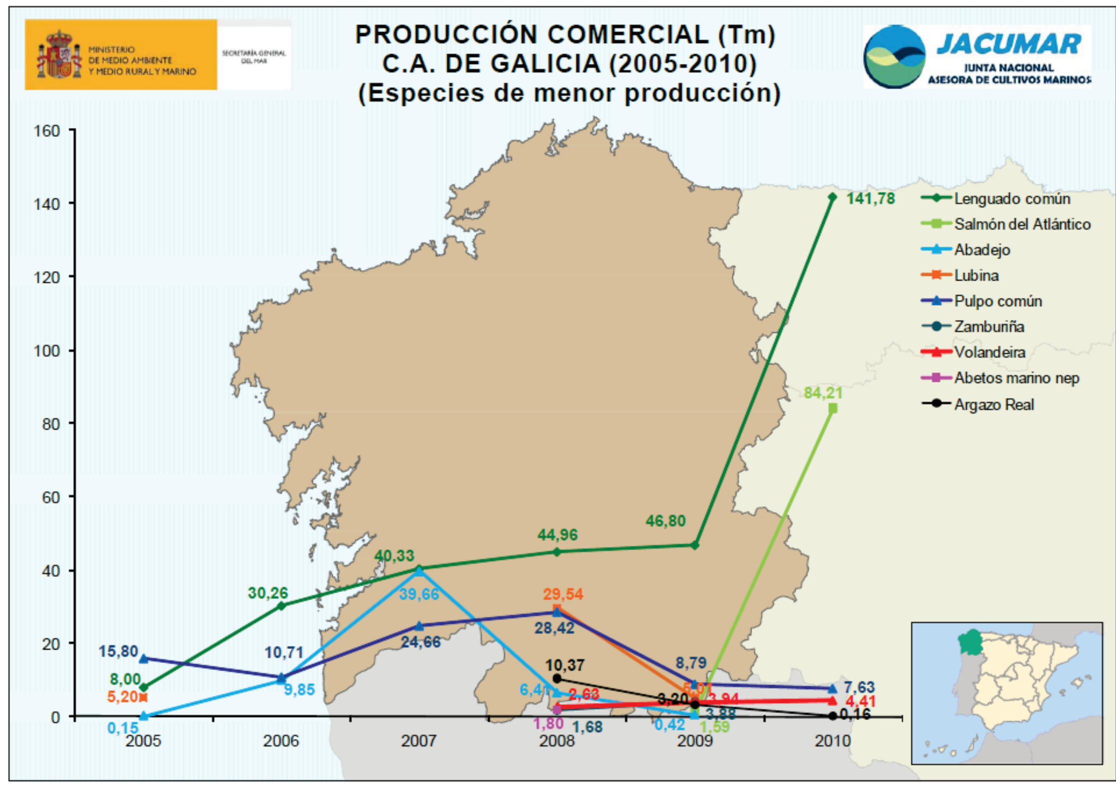

Figura II.2*. Especies de acuicultura de menor producción en Galicia (2005-20010)[Acuicultura, 2012].*

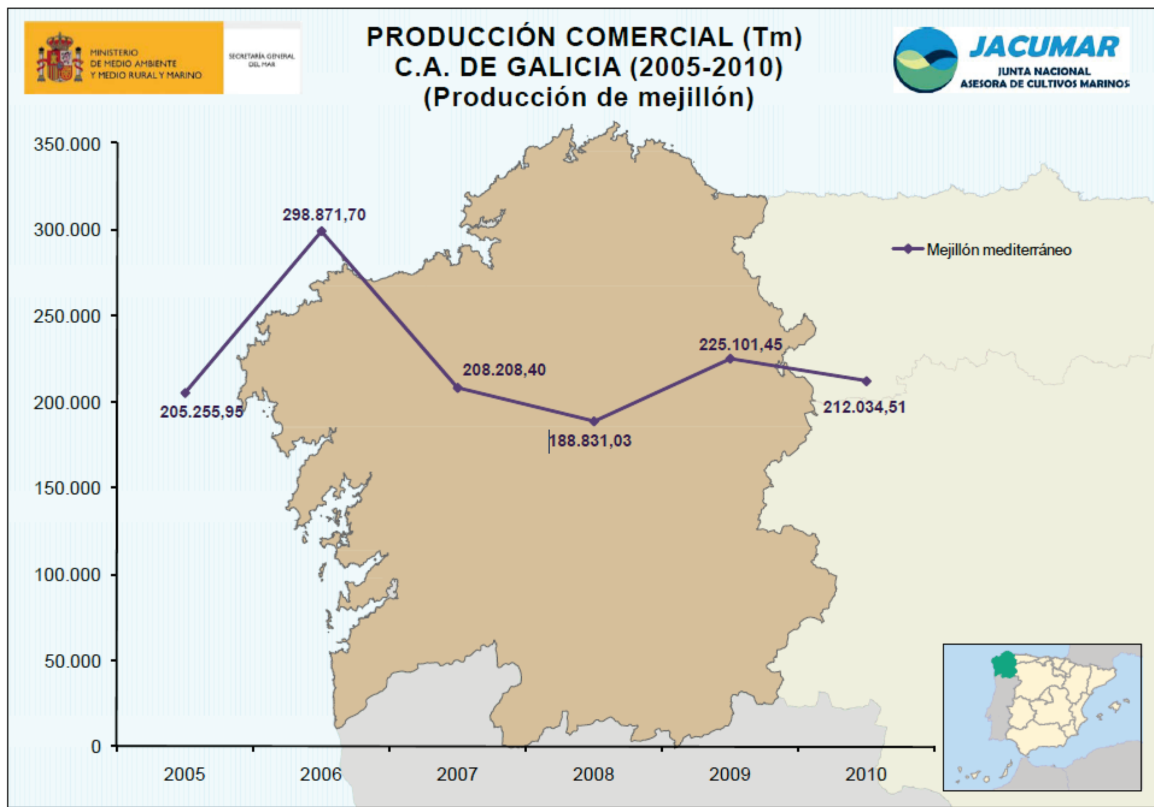

Figura II.3. *Producción de mejillón en Galicia (2005-20010)[Acuicultura, 2012].*

El consumo de algas comestibles ha sido descrito en los siglos IV y VI en Japón y China, respectivamente. En la actualidad, estos dos países, junto a la República de Corea, son los mayores consumidores de estos productos. Hoy en día el consumo de algas comestibles se ha expandido por todo el mundo, creciendo fuertemente en Estados Unidos, América del Sur y Europa. Por otro lado, investigaciones realizadas en los ciclos biológicos de las algas así como en su cultivo, han provocado que en la actualidad más del 90% de las algas vendidas en el mundo sean de cultivo [McHugh, 2003]. En cuanto al cultivo de algas, esta industria factura alrededor de 5 billones de dólares anuales en productos destinados a su consumo directo y cerca de 1 billón de dólares en otros productos entre los que destacan los ficocoloides (agar, alginatos y carragenina), fertilizantes, piensos, etc [McHugh, 2003].

China es el mayor productor de algas comestibles, cultivando alrededor de 5 millones de toneladas anuales de este producto. La mayor parte de la producción se dirige a alga Kombu, a la cual se dedican cientos de hectáreas de algas marrones, *Laminaria japónica*, que se cultivan en cuerdas suspendidas en el océano. La República de Korea prodece sobre 800000 toneladas de tres especies diferentes, dedicando el 50% de la producción al alga Wakame (*Undaria pinnatifalda*) que se cultiva de forma similar a la Kombu. La producción japonesa se situa en unas 600000 toneladas al año y el 75% se dedica a Nori (*Porphyra*), un alga con un elevado precio de mercado [McHugh, 2003].

Distintas algas rojas y marrones se emplean en la producción de tres hidrocoloides: agar, alginato y carragenina. Un hidrocoloide es una sustancia no cristalina con moléculas de alto peso molecular que se disuelven en agua para dar lugar a disoluciones de elevada viscosidad. Estos hidrocoloides se emplean con asiduidad en la industria alimenticia como agentes estabilizantes. El uso de algas como fuente de hidrocoloides de uso alimentario comenzó en 1658 en Japón y se expandió rápidamente por todo el mundo tras la Segunda Guerra Mundial. Actualmente, aproximadamente 1 millón de toneladas de algas son cultivadas y tratadas para la producción de los tres hidrocoloides mencionados anteriormente [McHugh, 2003]. En las *figuras II.4 y II.5* se muestra la escasa producción de algas en Galicia, periodo 1998-2010 [Acuicultura, 2012].

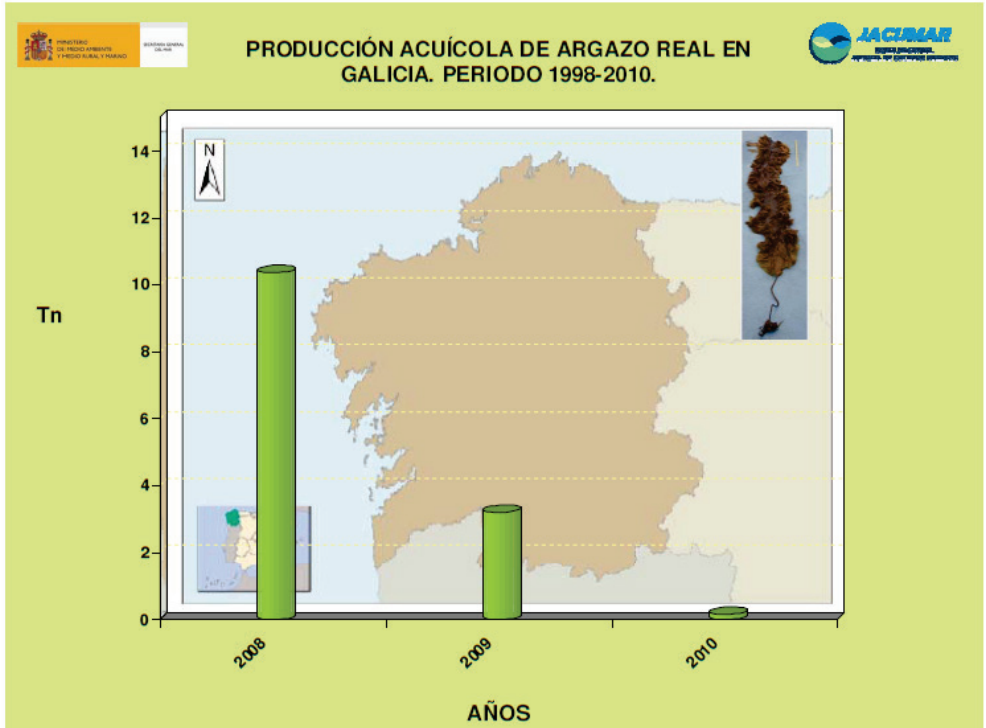

Figura II.4. *Producción de argazo real en Galicia (1998-2010) [Acuicultura, 2012].*

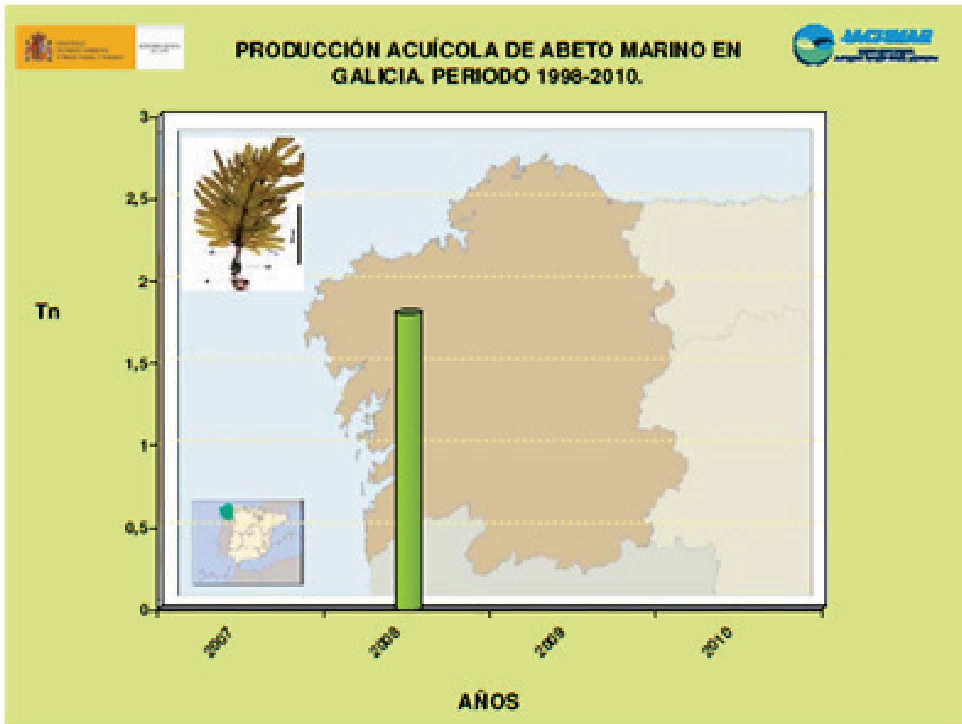

Figura II.5. *Producción de abeto marino en Galicia (1998-2010) [Acuicultura, 2012].*

El impacto medioambiental de una explotación acuícola de cualquier tipo depende en gran medida de la especie cultivada, del volumen de producción, del método de cultivo, del tipo de alimentación y de las condiciones hidrológicas existentes. Los desechos provenientes de estas plantas pueden llegar a causar eutrofización de las aguas, y los productos químicos usados (pesticidas, antibióticos, anestésicos, etc.) pueden contaminar las aguas y las especies cultivadas, pudiendo ser tóxicos para el hombre si se consumen [Acuicultura, 2012]

Toda explotación basada en el desarrollo controlado de seres vivos (ganadería, agricultura, acuicultura, etc.) debe cumplir ciertas premisas: Se debe optimizar al máximo la calidad de las instalaciones, el impacto medioambiental debe ser mínimo, se deben aportar los nutrientes necesarios, se debe evitar la proliferación de enfermedades y debe de optimizarse el crecimiento de la especie que se trate. En el caso de la acuicultura estas premisas también deben cumplirse, aunque las dificultades son mayores dadas las características fisiológicas de los peces y el medio en que éstos viven [Burka, 1997].

Las enfermedades suponen una importante limitación de la producción, desarrollo y expansión de cualquier sistema de producción animal. En la acuicultura el control de las enfermedades es particularmente difícil ya que las diferentes especies se cultivan a menudo en lugares donde la producción depende de las condiciones medioambientales naturales, a diferencia de otras producciones animales intensivas donde los parámetros medioambientales pueden ser estrictamente controlados. Otro problema característico de la acuicultura es la relativa escasez de agentes terapéuticos y de medidas preventivas para

el control de agentes infecciosos. Incluso cuando se dispone de terapias adecuadas, su aplicación en el medio acuático es muy difícil y, en ocasiones, imposible [Acuicultura, 2012].

Un buen ejemplo del tratamiento necesario para el control de enfermedades mediante productos químicos se da en la acuicultura del salmón (*Salmo salar*). Con frecuencia se tienen que combatir plagas parasitarias, como la del piojo de mar (*Lepeophtheirus salmonis* y *Caligus elongatus*), o enfermedades víricas como la anemia infecciosa del salmón. En esta industria es muy común el uso de pesticidas y desinfectantes para evitar todo tipo de plagas. También se hace uso de otros productos químicos, incluyendo aditivos alimentarios, herbicidas, antiadherentes, antibióticos, anestésicos, etc. (ver *tabla II.1*) [Burridge, 2010]. En muchos casos estas sustancias terminan acumulándose en el medio ambiente, lo que puede suponer un impacto negativo en la zona, perjudicando a otros organismos y pudiendo llegar a poner en peligro la salud de las especies cultivadas y del propio ser humano [Haya, 2001; Willis, 2005].

Tabla II.1. *Modo de acción y posible destino medioambiental de algunos agentes químicos usados en acuicultura.*

Tipo de compuesto	Ingrediente activo	Producto comercial	Propiedades	Destino medioambiental
Organofosforado	Diclorvos Azametifos	Aquagard Salmosan	▪ Soluble en agua ▪ Volátil ▪ No bioacumulable	Dilución en el agua de mar
Peróxido de hidrógeno	—	Salartect Paramove	▪ Se degrada rápidamente	Se degrada rápidamente
Piretroides sintéticos	Cipermetrina Deltametrina	Excis,Betamax AlphaMax, Pharmaq	▪ Baja solubilidad en agua ▪ Fuertemente adsorbido en suelos y sedimentos ▪ Poca bioacumulación	Adsorbidos en sedimentos
Avermectina	Ivermectina Emamectina Benzoato	Ivomec Slice	▪ Baja solubilidad ▪ Gran afinidad por lípidos, suelos y materia orgánica	Adsorbido en sedimentos
Benzamida (benzoilfenilurea) Reguladores del crecimiento en insectos (IGR)	Diflubenzuron Teflufenzuron	Lepsidon® Premix Ektobann Calicide®	▪ Baja solubilidad en agua	Adsorbido en sedimentos

El tratamiento con algunos de los pesticidas (agentes quimioterápicos) estudiados en esta memoria, se realiza mediante un baño de inmersión o se administran en los piensos. En el baño de inmersión, en primer lugar, se levantan las jaulas que contienen las especies a tratar para reducir el volumen de agua que contienen. A continuación, la jaula se rodea con una lona y se aplica el tratamiento necesario. Se deja el baño durante cierto tiempo (variable en función del pesticida usado y la especie a tratar) antes de quitar la lona y bajar la jaula. De esta forma se libera la disolución del pesticida al agua, donde se dispersa en función de las corrientes de la zona. Cada jaula se trata por separado y el tratamiento completo de una planta piscícola puede tardar varios días, lo que resulta en múltiples vertidos de los

pesticidas empleados al medioambiente [Willis, 2005]. Otra opción es la administración del producto en el pienso, en forma de mezcla medicamentosa preparada ya por el fabricante como ocurre con Calicide (cuyo principio activo es teflubenzurón), y Slice (benzoato de emamectina). En el caso de los pesticidas esta opción es menos viable dada su alta toxicidad. De todos modos esta forma de tratamiento tiene la ventaja de que es mucho menos costoso que el tratamiento en el agua. La principal limitación de esta forma de administración es que el pez que ha de ser tratado debe ser capaz de ingerir el pienso, por lo que no es de utilidad para el tratamiento de huevos y está muy limitada en el caso de peces recién nacidos. También hay que tener en cuenta que casi todas las enfermedades que afectan a los peces producen en éstos un descenso en el consumo de pienso. Esto implica que la utilización de piensos medicados es más útil cuando se trata de prevenir enfermedades en ejemplares de más edad [Acuicultura, 2012; Little, 2008].

Figura II.6. *Diagrama que representa las vías de exposición y los posibles efectos del uso de pesticidas sobre los organismos acuáticos [Steeger, 2010].*

Además, en el hábitat acuático, la exposición a los pesticidas está relacionada con su aplicación y transporte a través de la escorrentía, la descarga a las aguas subterráneas y la deposición atmosférica. Los posibles mecanismos de transporte se representan en la *figura II.6*. Este modelo también muestra las rutas de exposición para los receptores biológicos de interés y los potenciales cambios relacionados con la reducción de la supervivencia, crecimiento y reproducción debido a la exposición a los pesticidas. Este esquema ofrece por tanto una amplia visión de cómo los organismos acuáticos pueden potencialmente estar expuestos a los pesticidas [Steeger, 2010; Little, 2008].

En el presente trabajo se ha estudiado la determinación de pesticidas en el hábitat de los organismos acuáticos. Se han seleccionado las aguas, algas, pescados y moluscos como matrices más representativas para el desarrollo de nueva metodología analítica.

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2. Propiedades y mecanismos de acción de los compuestos

Los pesticidas son sustancias o mezclas de sustancias que se usan para prevenir, destruir, repeler o mitigar cualquier tipo de plaga. Aunque muchas veces se utiliza el término pesticida/plaguicida como equivalente a insecticida, la palabra pesticida también se puede aplicar a herbicidas, fungicidas y, en general, a cualquier sustancia utilizada en el control de pestes/plagas. La clasificación química de los pesticidas puede hacerse en función del grupo funcional responsable de la actividad biológica. En la *tabla II.2* se puede observar una clasificación de los distintos tipos de pesticidas en función de su estructura química, su modo de acción y las técnicas analíticas más usadas para su determinación. La división en organoclorados, organofosforados y organonitrogenados se realizó en función de los distintos métodos de análisis utilizados en el pasado, aunque actualmente los sistemas de detección basados en espectrometría de masas y captura electrónica son, con mucho, los más usados.

Tabla II.2. *Clasificación de pesticidas en función de su estructura, modo de acción y técnicas usadas para su determinación.*

Tipo de pesticida	Actividad biológica	Número de pesticidas en el grupo	Método de análisis
Compuestos inorgánicos	Fungicida	7	LC
Compuestos orgánicos de estaño	Fungicida, antiadherente	5	GC-FPD
Compuestos organofosforados	Insecticida, acaricida	76	GC-FPD
Otros	Insecticida, acaricida	4	LC
	Compuestos organonitrogenados		
N-metilcarbamatos	Insecticida, acaricida	12	LC
Dinitrocompuestos	Herbicida, funguicida	6	LC
Ditiocarbamatos	Funguicida	9	LC
Derivados del benzoimidazol	Funguicida	4	LC
Derivados de aminas aromáticas	Herbicida	22	LC
Triacinas	Herbicida	9	GC-NPD
Quats	Regulador del crecimiento, herbicida	6	LC
Otros	Herbicida, funguicida	91	GC-NPD y LC
	Compuestos organohalogenados		
Piretroides	Insecticida	12	GC-ECD
Ácidos carboxílicos	Herbicida	9	LC
Otros	Insecticida, funguicida	44	GC-ECD
Compuestos orgánicos de azufre	Herbicida, acaricida	2	GC-FPD
Otros	Regulador del crecimiento, insecticida	11	LC
Fumigantes	Insecticida, nematicida	10	GC-ECD

Hoy en día, el análisis traza de pesticidas en cualquier tipo de matriz engloba aproximadamente 500 compuestos, de los cuales unos 300 pueden ser separados mediante cromatografía de gases, la técnica más

usada para el análisis de residuos de pesticidas en cantidades traza [Hoff, 1999; Ratola, 2006; LeDoux, 2011].

El desarrollo y uso de pesticidas ha jugado un papel muy importante en el incremento de la producción agrícola y de otras explotaciones como la acuicultura. La mayor parte de estas sustancias se aplican directamente al suelo, se mezclan con el agua o se fumigan sobre la zona a tratar. Esto hace que los pesticidas sean una fuente importante de contaminación en las zonas en las que su uso está extendido. Además, el transporte de estas sustancias fuera de su área de utilización provoca que se haga patente la presencia de pesticidas en zonas ajenas a su uso. Se han encontrado cantidades apreciables de pesticidas en el mar, lagos, agua potable, agua de lluvia e incluso en el hielo de regiones polares [Vidal, 2000].

2.1 Pesticidas organofosforados

El estudio de los compuestos organofosforados (*OPs*) comenzó a realizarse durante la Segunda Guerra Mundial dado su potencial como agentes neurotóxicos para la guerra química y, más adelante, comenzaron a usarse como insecticidas. Los organofosforados están entre los pesticidas cuyo uso está más extendido, lo que justifica que hayan sido detectados en muestras de aguas subterráneas, aguas superficiales, agua potable, suelos, vegetales, etc [Vidal, 2000; Lambropoulou, 2002]. Dado su menor precio y mayor efectividad, estos pesticidas se convirtieron en una alternativa a los organoclorados (*OCPs*). Por otro lado, se considera que los pesticidas organofosforados tienen menor persistencia en el medio ambiente que sus homólogos *OCPs* [Carro, 2001; Zhao, 2006].

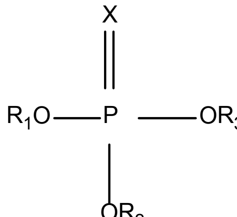

Figura II.7: Fórmula general de los pesticidas organofosforados.

Los *OPs* son ésteres orgánicos de ácidos fosfóricos que corresponden a la fórmula general de la *figura 6.* Los radicales R_1 y R_2 suelen ser metilo (Me) o etilo (Et), mientras que R_3 es el que caracteriza el tipo de pesticida organofosforado. Si el grupo X es un oxígeno se denominará fosfato mientras que si es azufre el compuesto que se obtendría será un tiofosfato. Además, si se elimina el grupo OR_2 y se sustituye por un ligando orgánico R unido directamente al fósforo, el compuesto obtenido será un fosfonato [Walker, 2001].

La mayoría de *OPs* son líquidos con carácter lipofílico y cierta volatilidad, mientras que tan solo unos pocos son sólidos. En general, son menos estables que los *OCPs* y son fácilmente degradados por agentes químicos y biológicos presentes en el medioambiente, lo que explica su corto tiempo de vida. Es la

poca persistencia de los *OPs* lo que hace que los peligros derivados del uso de estos pesticidas suelan estar, aunque no siempre, relacionados con períodos de toxicidad aguda a corto plazo. En general, los *OPs* son más polares y solubles que los pesticidas organoclorados aunque esta solubilidad es muy variable. Así, encontramos *OPs* con muy alta solubilidad en agua (*dimetoato*) y otros con solubilidad baja (*azametifos*). De todos modos, las formas activas de la mayoría de *OPs* tienen la suficiente solubilidad como para ser efectivos en su labor plaguicida [Walker, 2001].

La formulación de los *OPs* es importante ya que determinará los efectos medioambientales que puedan provocar. Muchos se comercializan en forma de emulsiones concentradas para ser utilizadas en forma de aerosol aunque también se presentan en forma de granulado. El granulado es más seguro que las emulsiones y es la forma en la que se suelen presentar los *OPs* más tóxicos. En el granulado, el pesticida queda "encerrado" dentro de las partículas y se va liberando paulatinamente al medio en que se aplique. A pesar de los peligros que conlleva, en algunos países todavía se aplican tratamientos con *OPs* mediante aerosol en campos de cultivo, explotaciones ganaderas, etc. También se usan para el control de pestes como la langosta y el mosquito [Walker, 2001]. En la acuicultura, su mayor aplicación está en el tratamiento de parásitos del salmón y los pesticidas organofosforados más usados en este campo son clorpirifos-metil, clorpirifos-etil y azametifos (ver *tabla II.3*) [Burridge, 2010].

Tabla II.3. *Identidad química de los pesticidas organofosforados estudiados*

Nombre común [n^o CAS]	NombreIUPAC	Peso molecular (g mol^{-1})	Estructura química
Clorpirifos-etil [2921-88-2]	*O,O*-diethyl O -3,5,6-trichloropyridin-2-il phosphorothioate	350.6	S, Cl, N, O, P, OEt, OEt, Cl, Cl
Clorpirifos-metil [5598-13-0]	*O,O*-dimethyl*O* -3,5,6-trichloro-2-pyridyl phosphorothioate	322.6	S, Cl, N, O, P, OMe, OMe, Cl, Cl
Azametifos [6915-15-7]	S-[(6-chloro-2-oxooxazolo[4,5-b]pyridin-3(2H)-yl)methyl] *O,O*-dimethylphosphorothioate	324.7	Cl, O, C=O, N, N, S, P(OCH$_3$)$_2$, O

La toxicidad de los pesticidas organofosforados es elevada, de ahí su gran efectividad, y se basa en la inhibición de la acetilcolinesterasa (*AchE*) [Walker, 2001; Sudakin, 2011]. Esta inhibición se debe principalmente a la fosforilación de la enzima *AchE* en las terminaciones nerviosas con la consecuente pérdida de funcionalidad enzimática [Abgrall, 2000]. Esto causa una acumulación del neurotransmisor acetilcolina en la terminación nerviosa, lo que provoca una respuesta excesiva por parte del nervio. La

enzima *AchE* juega un papel crítico en el control de la transmisión de impulsos nerviosos desde los nervios a músculos, glándulas, ganglios y al sistema nervioso central. Los efectos tóxicos de estos compuestos solo se ponen de manifiesto cuando la acumulación de acetilcolina alcanza niveles altos. El envenenamiento por organofosforados provoca síntomas como contracción muscular, espasmos, pérdida de la coordinación, alteraciones en las funciones motoras y fallo respiratorio [NPIC, 2012]. El grupo activo del azametifos, muy inusual en los pesticidas organofosforados, ha demostrado tener una capacidad alquilante bastante alta. Este poder alquilante del azametifos lo convierte en un agente mutágeno, lo que explica que este pesticida sea más efectivo en especies resistentes a otros *OPs* y que su uso provoque cambios en el comportamiento de crustáceos como la langosta [Abgrall, 2000; Zitko, 2001, Burridge, 2008].

2.2 Pesticidas del grupo piretroides

Las piretrinas son insecticidas naturales que se encuentran en las flores de algunas plantas como el crisantemo (*Chrysantemum compositae*) o el pelitre (*Chrysanthemum cinerariaefolium*). Estos insecticidas naturales se usaron como modelo para la síntesis de los pesticidas conocidos como piretroides. Los piretroides sintéticos son, en general, más estables química y bioquímicamente que las piretrinas naturales [Mueller-Beilschmidt, 1990]. Estos compuestos son sólidos con baja solubilidad en agua que actúan como neurotoxinas de forma similar al *DDT* [Soderlund, 2012]. Los piretroides (*figura II.8*) son ésteres formados entre un ácido orgánico (normalmente ácido crisantémico) y una base orgánica.

Figura II.8. Ejemplo de la estructura de un piretroide, la permetrina.

Aunque los piretroides son más estables que las piretrinas, éstos son fácilmente degradables por microorganismos y por la luz y no tienen, por lo tanto, tiempos de vida muy elevados en el medioambiente. Es importante tener en cuenta que estos compuestos se pueden fijar a partículas del suelo y sedimentos, presentando cierta persistencia a largo plazo cuando se encuentran en estas condiciones [Mueller-Beilschmidt, 1990]. Dada su baja solubilidad, no presentan propiedades sistémicas y no pueden ser empleados como insecticidas de uso general. Los peligros derivados del uso de estos compuestos son principalmente de toxicidad a corto plazo (aguda). De todos modos hay que tener en cuenta que presentan una gran selectividad por insectos y crustáceos, mientras que su actividad hacia mamíferos y pájaros es relativamente baja [Mueller-Beilschmidt, 1990]. Aun así, la presencia de residuos de estos pesticidas en el medioambiente puede causar exposición humana a los piretroides, ya sea por ingestión, inhalación, adsorción cutánea o a través del tracto gastrointestinal [Todd, 2003]. Los principales problemas medioambientales derivados de su uso en la acuicultura se refieren a la toxicidad en peces e invertebrados

distintos a la plaga que se pretende combatir [Rawn, 2010]. En los últimos años se comercializan como mezclas de dos o más piretroides para aumentar el espectro de utilización y la efectividad del insecticida, o mezclados con otros compuestos como el butóxido de piperonilo que actúa como sinergista incrementando la efectividad del insecticida [Todd, 2003].

Tabla II.4. *Identidad química de los pesticidas piretroides estudiados*

Nombre común [nº CAS]	Nombre IUPAC	Peso molecular (g mol^{-1})	Estructura química
Empentrina [54406-48-3]	*E*-(RS)-1-etinil-2-metilpent-2-enil (1RS,3RS;1RS,3SR)-2,2-dimetil-3-(2-metilprop-1-enil)ciclopropancarboxilato	271.0	
Bioaletrina [584-79-2]	(*RS*)-3-alil-2-metil-4-oxociclopent-2-enil (1*RS*,3*RS*; 1*RS*,3*SR*)-2-2-dimetil-3-(2-metilprop-1-enil) ciclopropanecarboxilato	302.4	
Resmetrina [10453-86-8]	i5-bencil-3-furylmetil (1RS$_1$3RS;1RS$_1$3SR)-2$_1$2-dimetil-3-(2-metilpropil-enil) ciclopropanecarboxilato	338.4	
Tetrametrina [7696-12-0]	Ciclohex-1-en-1,2-dicarboximidometil (1*RS*,3*RS*;1*RS*,3*SR*)-2,2-dimetil-3-(2-metilprop-1-enil) ciclopropancarboxilato	331.4	
Cialotrina [68085-85-8]	3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethyl-cyano(3-phenoxyphenyl)methyl cyclopropanecarboxylate	449.9	
Permetrina [5264553-1]	3-Phenoxybenzyl(1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate	391.3	
Ciflutrina [68359-37-5]	[(R)-ciano-[4-fluoro-3-(fenoxi)fenil]metil] (1R,3R)-3-(2,2-dicloroetenil)-2,2-dimetilciclopropano-1-carboxilato	434.3	
Cipermetrina [52315-07-8]	(1RS)-cis,trans-3-(2,2-diclorovinil)-2,2-dimetilciclopropano carboxilato de (RS)-ciano-3-Fenoxibencilo	416.3	
Flucitrinato [70124-77-5]	(*RS*)-α-cyano-3-phenoxybenzyl (*S*)-2-(4-difluoromethoxy phenyl)-3-methylbutyrate	451.4	
Fenvalerato [51630-58-1]	(RS)-*alpha*-Cyano-3-phenoxybenzyl (RS)-2-(4-chlorophenyl)-3-methylbutyrate	419.9	
Deltametrina [52918-63-5]	[(*S*)-cyano-(3-phenoxyphenyl)-methyl] (1*R*,3*R*)-3-(2,2-dibromoethenyl)-2,2-dimethyl-cyclopropane-1-carboxylate	505.2	

En la acuicultura se usan habitualmente tres piretroides para combatir las plagas de piojo de mar: *permetrina*, *cipermetrina* y *deltametrina* (ver *tabla II.4*) [Shafer, 2005]. Estos piretroides son muy tóxicos

para invertebrados acuáticos como crustáceos, zooplancton, organismos bénticos, etc. Los piretroides también son altamente tóxicos para la mayoría de peces, siendo la deltametrina uno de los más tóxicos [Sánchez-Fortún, 2005; Pérez-Fernández, 2010]. La neurotoxicidad de estos compuestos en mamíferos está bastante estudiada y se descubrió que los piretroides provocan distintos síntomas en función de su estructura. Así, estos compuestos pueden dividirse en dos tipos según su toxicidad. La diferencia estructural entre los piretroides de tipo I y los de tipo II es la ausencia o presencia, respectivamente, del grupo CN en el carbono en posición alfa respecto al éster [Shafer, 2005].

El principal mecanismo de la neurotoxicidad de los piretroides, tanto en insectos como en mamíferos, es la perturbación de los canales de sodio sensibles al voltaje (*VSSC*) presentes en las membranas neuronales [Soderlund, 2012]. Esta interacción de los piretroides con los *VSSC* es estereoespecífica y los piretroides con mayor poder insecticida son aquellos estereoisómeros que más fuertemente interaccionan con los *VSSC*. Los piretroides disminuyen el tiempo de activación y desactivación (abertura y cierre, respectivamente) de estos canales. Como consecuencia, los *VSSC* se abren a potenciales mayores y permanecen abiertos durante más tiempo de lo normal. Esto permite que un exceso de iones sodio atraviese la membrana neuronal. Generalmente los piretroides del tipo II mantienen los *VSSC* abiertos durante más tiempo que los del tipo I. Esta diferencia en el tiempo que se retarda la inactivación de los canales de sodio puede ser la clave que explique las diferencias observadas en la toxicidad de ambos tipos de piretroides [Shafer, 2005, Soderlund, 2012].

2.3 Pesticidas del grupo carbamatos

Este grupo presenta un gran interés en el campo de los plaguicidas por su gran actividad biológica. El grupo de los carbamatos corresponde en su mayor parte a derivados del ácido N-metil–carbámico, son ésteres de ácido carbámico. Actúan como inhibidores de la acetilcolinesterasa, enzima responsable de la inactivación del neurotransmisor acetilcolina alterando por consiguiente la transmisión del impulso nervioso.

Figura II.9: Estructura genérica de un carbamato

Su estructura general se puede observar en la *figura II.9*, donde R_2 es mayoritariamente un grupo alifiático o aromático. Se pueden clasificar en tres grandes familias de compuestos dependiendo del grupo R_1:

(a) Carbamatos insecticidas; R_1 es un grupo metilo
(b) Carbamatos herbicidas; R_1 es un grupo aromático
(c) Carbamatos fungicidas; R_1 es un benzimidazol.

En la *tabla II.5* se muestran las estructuras químicas de los carbamatos estudiados en esta memoria. Su actividad biológica se descubrió en el 1923, y a día de hoy se conocen más de 50 derivados del ácido carbámico. En ellos se sustituye un grupo alcohólico por un grupo amino. Son de fácil acción sistémica, su persistencia en el medioambiente y su toxicidad es intermedia entre los organoclorados y los organofosforados. De acuerdo con su composición, sus derivados pueden tener propiedades insecticidas, fungicidas o herbicidas. Penetran en los mamíferos a través de la piel, conjuntiva, vía respiratoria y vía digestiva. Aunque presentan grupos químicos diferentes a los organofosforados el mecanismo por el que producen toxicidad es similar: provocan la inhibición de la acetil colinesterasa (ACE) [Oliveira, 2007]. Son inhibidores reversibles pues en poco tiempo dejan la enzima libre. No se ha demostrado aun neurotoxicidad retardada hasta el presente con ningún carbamato. No son bioacumulables, a pesar de ser liposolubles y en su mayoría son de mediana y baja toxicidad [EPA, 2011].

Tabla II.5. *Identidad química de los pesticidas carbamatos estudiados*

Nombre común [nº CAS]	Nombre IUPAC	Peso molecular (g mol^{-1})	Estructura química
Carbaril [63-25-2]	1-naphthyl methylcarbamate	201.2	
Propoxur [114-26-1]	2-isopropoxyphenyl *N*-methylcarbamate	209.2	

2.4 Pesticidas del grupo benzoilfenilurea

Son ureas sustituidas, cuyas estructuras se muestran en la *tabla II.6*, que actúan como insecticidas por inhibir la síntesis de quitina, principal constituyente del exoesqueleto de los insectos [Matsumura, 2010]. Las dosis que se suelen utilizar de estos insecticidas no suelen presentar efectos tóxicos sobre vertebrados y plantas (ya que no poseen quitina) por lo que son seguros para los humanos y perjudiciales para otros artrópodos. Sus residuos pueden alcanzar a la población a través de la cadena alimentaria, causando exposición crónica y efectos tóxicos a largo plazo [Valenzuela, 2000; Gil-García, 2001; Martínez-Galera, 2001].

Tabla II.6. *Identidad química de los pesticidas del grupo benzoilfenilureas*

Nombre común [nº CAS]	Nombre IUPAC	Peso molecular (g mol^{-1})	Estructura química
Teflubenzurón [83121-18-0]	1-(3,5-dichloro-2,4-difluorophenyl)-3-(2,6-difluorobenzoyl)urea	381.1	
Diflubenzurón [35367-38-5]	1-(4-Clorofenil)-3-(2,6-difluorobenzoil) urea	310.7	

Su toxicidad más conocida deriva de su efecto irritante sobre la piel y mucosas. Su metabolismo consiste en la rotura del puente fenilurea, dando lugar a benzoato y un residuo de anilina. Éste puede unirse a la hemoglobina y la albúmina, formando aductos que pueden utilizarse para evaluar la exposición a estos insecticidas. Sobre los eritrocitos, los residuos de anilina pueden producir metahemoglobinemia y, en algunos casos, anemia.

Estos insecticidas se han usado en Escocia en las plantas de cultivo de salmón para el tratamiento del piojo de mar. Su uso está en decadencia ya que no es eficaz contra los piojos en estado adulto [Campbell, 2006].

2.5 Pesticidas del grupo de las avermectinas

La introducción de las primeras avermectinas revolucionó el tratamiento farmacológico veterinario, por su amplio espectro de actividad sobre parásitos internos y externos [Botana, 2002]. Las avermectinas son antibióticos producidos por fermentación del microorganismo *Streptomycesa vermitilis*, del cual se obtiene un anillo lactonamacrocíclico que muestra efectos antibióticos, antinematódicos y marcada toxicidad contra insectos [Campbell, 1983; Seelanan, 2006]. Se conocen cuatro componentes principales (A1a, A2a, B1a y B2a) y entre éstos, el más conocido es la avermectina B1a debido a su potente actividad. Las estructuras de los distintos compuestos de este grupo estudiados en esta memoria se muestran en la *tabla II.7*.

Tabla II.7. *Identidad química de los pesticidas del grupo de las avermectinas*

Nombre común [nº CAS]	Nombre IUPAC	Peso molecular (g mol^{-1})	Estructura química
Abamectina [71751-41-2]	Mezcla de 80%avermectina B1a* y menos del 20% avermectin B1b**	866.6	
Doramectina [117704-25-3]	(1'*R*,2*S*,4'*S*,5*S*,6*R*,8'*R*,10'*E*,12'*R*,13'*S*,14'*E*,20'*R*,21'*R*,24'*S*)-6-cyclohexyl-21',24'-dihydroxy-12'-{[(2*R*,4*S*,5*S*,6*S*)-5-{[(2*S*,4*S*,5*S*,6*S*)-5-hydroxy-4-methoxy-6-methyloxan-2-yl]oxy}-4-methoxy-6-methyloxan-2-yl]oxy}-5,11',13',22'-tetramethyl-5,6-dihydro-3',7',19'-trioxaspiro[pyran-2,6'-tetracyclo[15.6.1.$1^{4,8}$.$0^{20,24}$]pentacosane]-10',14',16',22'-tetraen-2'-one	899.1	
Ivermectina [70288-86-7]	22,23-dihydroavermectin B_{1a} + 22,23-dihydroavermectin B_{1b}	861.1	

***** (10E,14E,16E)-(1R,4S,5’S,6S,6’R,8R,12S,13S,20R,21R,24S)-6’-[(S)-sec-butyl]-21,24-dihydroxy-5’,11,13,22-tetramethyl-2-oxo-(3,7,19-trioxatetracyclo[15.6.1.14,8.020,24]pentacosa-10,14,16,22-tetraene)-6-spiro-2’-(5’,6’-dihydro-2’H-pyran)-12-yl-2,6-dideoxy-4-O-(2,6-dideoxy-3-O-methyl-α-L-arabino-hexopyranosyl)-3-O-methyl-α-L-arabino-hexopyranoside

****** (10E,14E,16E)-(1R,4S,5’S,6S,6’R,8R,12S,13S,20R,21R,24S)-21,22-dihydroxy-6’-isopropyl-5’,11,13,22-tetramethyl-2-oxo-(3,7,19-trioxatetracyclo[15.6.1.14,8.020,24]pentacosa-10,14,16,22-tetraene)-6-spiro-2’-(5’,6’-dihydro-2’H-pyran)-12-yl 2,6-dideoxy-4-O-(2,6-dideoxy-3-O-methyl-α-L-arabino-hexopyranosyl)-3-O-methyl-α-L-arabino-hexopyranoside

Estos compuestos actúan interrumpiendo la transmisión de señales en el sistema nervioso, interactuando con distintos canales de cloruro ligando-dependientes, los cuales aumentan la permeabilidad del cloruro hacia el interior de la célula, resultando en una disfunción del sistema nervioso [Martin, 2002].

Son tóxicos para los organismos acuáticos aunque poco persistentes. Además de tener actividad de amplio espectro, son efectivos a muy bajas concentraciones [Adams, 2001; Fortt, 2007]. La ivermectina, la abamectina y otras lactonas macrocíclicas se emplean también en la agricultura para el control de insectos, ácaros y gusanos parásitos de numerosos cultivos.

La abamectina, mezcla de dos avermectinas (avermectina B1a y avermectina B1b en una relación molar de al menos 4:1) se considera una lactona macrocíclica igual que los demás derivados de las avermectinas. Ha sido desarrollada como un insecticida y antihelmíntico [Bravo, 2005]. Se considera un acaricida-insecticida de origen natural cuyo movimiento es translaminar, actuando por ingestión y por contacto directo. Se usa para tratamientos de prevención y/o curativos. Controla ácaros e insectos (estados adultos e inmaduros).

La doramectina, derivado semi-sintético, presenta efecto muy similar al de las otras avermectinas, tanto en espectro farmacocinético, como por la absorción. Su acción se localiza a nivel de terminaciones nerviosas o en la zona de contacto entre una fibra nerviosa y una fibra muscular. Las vías de aplicación son la subcutánea, intramuscular y oral. Es una substancia muy liposoluble. Se fija a los receptores que aumentan la permeabilidad de las membranas al ión cloruro, estimulando la liberación masiva, a este nivel, de un compuesto químico, el ácido gamma aminobutírico (GABA), el cual cumple con la función de neurotransmisor. La presencia de grandes cantidades de GABA a nivel sináptico conduce a un bloqueo total de los receptores específicos localizados en las terminaciones nerviosas, abre el canal de cloro e hiperpolariza la neurona, lo que produce la interrupción de los impulsos nerviosos del parásito y en consecuencia se produce la muerte por parálisis flácida y eliminación del parásito. El uso de este agente quimioterápico es el control de parásitos gastrointestinales. Las dosis usadas son similares a las otras ivermectinas en las distintas especies. Su efecto residual permanece hasta 30 días en relación con las dosis recomendadas.

La ivermectina es un antiparásitario introducido como fármaco en 1981, pertenece al grupo de las avermectinas con el mismo mecanismo de acción. Se considera un derivado que posee una actividad de amplio espectro contra una gran variedad de artrópodos y nematodos de animales domésticos y del ser humano. Está formado por una mezcla de componentes entre los que destaca la 22,23 -dihidroavermectina B1a. Posee un grupo hidroxilo unido a C-5 lo que le confiere una mayor actividad contra nematodos [Michael, 2001]. Es altamente lipofílico e hidrofóbico, por lo que es soluble en la mayoría

de disolventes orgánicos. Es estable a temperatura ambiente en soluciones no ácidas aunque se degrada al exponerse a luz ultravioleta (UV). La ivermectina es activa contra las diversas etapas de las especies de crustáceos *Caligus elongatus, Lepeophtheirus salmonis* y contra algunos endoparásitos. El metabolismo de la ivermectina incluye transformaciones en hígado y tejido graso. Se metaboliza más rápido en el músculo que en la piel del salmón aunque su eliminación es muy lenta [Bravo, 2005].

2.6 Otros compuestos

El ***piperonil butóxido (PBO)*** es un compuesto que por sí solo no tiene propiedades pesticidas, pero cuando se le añade a insecticidas del grupo de piretroides, organofosforados y carbamatos, actúa como sinergista y su potencia es incrementada considerablemente [EPA, 2010, FAO, 2011].

Tabla II.8. *Identidad química de Piperonil Butóxido*

Nombre común [nº CAS]	Nombre IUPAC	Peso molecular (g mol^{-1})	Estructura química
Piperonil butóxido [51-03-6]	5-[2-(2-butoxietoxi)-etoximetil] -6-propil-1,3-benzodioxol	338.4	n-Pr, n-BuO, O, O, O, O

Los efectos del PBO en la salud humana y en el medioambiente dependen de la cantidad presente y de la duración y frecuencia de la exposición y/o de ciertos factores medioambientales. Su toxicidad es muy baja cuando es ingerido por mamíferos y cuando es inhalado por ratas. La EPA ha clasificado a este sinergista como posible carcinógeno para humanos basándose en la limitada evidencia de cáncer en los animales de laboratorio [EPA, 2010].

En lo que se refiere a su distribución en el medioambiente, los estudios que evaluaron la desaparición del PBO en suelo y agua concluyeron que se trata de un compuesto poco persistente. Además, el PBO tiene poca tendencia a contaminar aguas subterráneas. Cuando se libera hacia el aire en estado líquido termina asentándose en el suelo, mientras que si se libera en fase gaseosa se degrada rápidamente en el aire [EPA, 2010].

El ***2-fenilfenol*** (*tabla II.9*) se emplea en la industria alimentaria como fungicida de amplio espectro, posee además funciones de desinfectante de carácter preventivo en la industria de recolección de la fruta. Por su acción antioxidante se usa como aditivo alimentario, para prevenir enranciamiento, debido a su bajo coste, alto rendimiento y amplia disponibilidad. A pesar de que su uso es muy abundante, un exceso de antioxidantes puede producir efectos tóxicos o mutagénicos, y por tanto poner en peligro la salud de las personas [EPA, 2006; Xiu-Qin, 2009].

Tabla II.9. *Identidad química del fenil fenol*

Nombre común [n° CAS]	Nombre IUPAC	Peso molecular (g mol^{-1})	Estructura química
2-fenilfenol [90-43-7]	Bifenil-2-ol	170,21	OH / Ph

2.7 Legislación

La legislación existente en España acerca de estos grupos de compuestos y su relación con la acuicultura, se encuentra recogida en diversos reales decretos.

REAL DECRETO 1882/1994, de 16 de septiembre, por el que se establecen las condiciones de sanidad animal aplicables a la puesta en el mercado de animales y productos de la acuicultura [BOE, 1994] (vigente hasta el 8 de octubre de 2008)**.**

"El presente Real Decreto establece las normas de sanidad animal que regulan la puesta en el mercado de animales y productos de la acuicultura. El presenta real decreto, se aplicará sin perjuicio de las disposiciones comunitarias o nacionales en materia de protección o de conservación de especies, así como de aquellas otras que regulen en particular las condiciones higiénico-sanitarias de determinadas especies o productos."

En el *artículo 2* de este Real Decreto se establecen las siguientes definiciones:

- *Animales de acuicultura:* Peces, crustáceos o moluscos vivos procedentes de una explotación, incluidos los de origen salvaje destinados a una explotación.
- *Productos de la acuicultura:* Productos derivados de los animales de acuicultura, tanto si están destinados a la cría, tales como los huevos y gametos, como si están destinados al consumo humano.

REAL DECRETO 1749/1998, **de 31 de julio, por el que se establecen las medidas de control aplicables a determinadas sustancias y sus residuos en los animales vivos y sus productos** [BOE, 1998].

"El presente Real Decreto regula aspectos relacionados con el control de determinadas sustancias o sus metabolitos y grupos de residuos, como normativa básica estatal, que pueden ser administrados a los animales, para su detección en cualquier fase, tanto en la elaboración de los productos a administrar a los animales vivos, como en cualquier fase de la obtención o transformación de los productos obtenidos de los mismos. Contiene disposiciones aplicables a las importaciones de terceros países, que deben considerarse de aplicación plena por incidir en el comercio y sanidad exteriores."

En los diferentes capítulos de este real decreto, se tratan los ámbitos de aplicación y definiciones involucradas en ellos, plantea los planes de vigilancia para la detección de residuos o sustancias que deban llevarse a cabo, fija el tipo de autocontrol y corresponsabilidad de los operadores, diseña los controles oficiales que deban realizarse, establece las medidas que deberán tomarse en caso de infracción, regula las

importaciones procedentes de terceros países y decreta aplicación de tasas. En este Real Decreto también se especifican los compuestos químicos a controlar (entre otros, pesticidas organofosforados y piretroides), las estrategias, niveles y frecuencia de muestreo, laboratorios comunitarios de referencia, etc.

REAL DECRETO 1597/2004, de 2 de julio, por el que se modifica el Real Decreto 1882/1994, de 16 de septiembre, por el que se establecen las condiciones de sanidad animal aplicables a la puesta en el mercado de animales y productos de la acuicultura [BOE, 2004].

Se modifica el anexo F del Real Decreto 1882/1994 del 16 de septiembre por el que se cambian los modelos de documentos de transporte para el comercio de productos procedentes de acuicultura.

La normativa sobre los niveles máximos de residuos se inició en Europa en los años 60 y 70, y ha sufrido numerosas modificaciones posteriores. Las primeras normativas de la Comunidad Europea, que regulan la el uso de los agentes quimioterápicos como antibióticos y pesticidas, no aparecieron hasta 1990. Estas normativas pretenden establecer un marco legislativo para los distintos productos químicos utilizados en las explotaciones acuícolas.

A continuación se expone la relación de los Reglamentos[*] y de las Directivas Comunitarias[**] más relevantes publicadas en el Diario Oficial de la Unión Europea hasta Diciembre de 2011, relativas a la fijación de los contenidos máximos de residuos de plaguicidas y agentes quimioterápicos en alimentos de origen marino.

Estas normativas son:

REGLAMENTO (CEE) No 2377/90 DEL CONSEJO de 26 de junio de 1990 por el que se establece un procedimiento comunitario de fijación de los límites máximos de residuos de medicamentos veterinarios en los alimentos de origen animal [DOCE, 1990]:

- *"Los estados miembros sólo pueden autorizar la puesta en el mercado de los medicamentos veterinarios destinados a animales de consumo humano cuyos principios activos tienen un LMR fijado."* Donde el LMR es el nivel de residuos de un producto medicamentoso que, si es consumido, no causará ningún riesgo para la salud humana. Esta normativa también determina la inclusión de todos los medicamentos de uso veterinario en 4 anexos:
    - *Anexo I:* Incluye las sustancias farmacológicamente activas para las que hay un LMR establecido (oxitetraciclina, amoxicilina, ampicilina, sulfonamidas, ác.oxolínico…).
    - *Anexo II:* Sustancias para las que no es necesario establecer un LMR (H_2O_2, formalina, iodóforos, sal, glutaraldehido, ácido acético…).
    - *Anexo III:* Sustancias para las que se han establecido LMR provisionales (fenbendazol, azametifos, neomicina, estreptomicina, flumequina,...).

- *Anexo IV:* Sustancias para las que no se puede establecer un LMR y por ende queda prohibida su administración (furazolidona, nitrofuranos, cloranfenicol, dimetridazol, verde malaquita…).

Directiva 96/23/EC DEL CONSEJO de 29 de abril de 1996 relativa a las medidas de control aplicables respecto de determinadas sustancias y sus residuos en los animales vivos y sus productos [DOUE, 1996].

"Todos los estados miembros deben introducir programas de monitorización para detectar y prevenir la presencia de residuos ilegales o en concentraciones superiores al LMR."

REGLAMENTO (CE) NO 396/2005 DEL PARLAMENTO EUROPEO Y DEL CONSEJO de 23 de febrero de 2005 relativo a los límites máximos de residuos de plaguicidas en alimentos y piensos de origen vegetal y animal y que modifica la Directiva 91/414/CEE del Consejo [DOUE, 2005].

El Reglamento fija las cantidades máximas autorizadas de residuos de plaguicidas que pueden encontrarse en los productos de origen animal o vegetal destinados al consumo humano o animal. Dichos límites máximos de residuos (LMR) comprenden, por una parte, LMR específicos para ciertos alimentos destinados a las personas o los animales y, por otra, un límite general aplicable cuando no se haya fijado ningún LMR.

El contenido máximo de residuos de plaguicidas en los alimentos se sitúa en **0,01 mg kg^{-1}**. Este límite general es aplicable *"por defecto"*, es decir, en todos los casos en que no se haya fijado un LMR de forma específica para un producto o un tipo de producto.

DIRECTIVA 2006/11/CE DEL PARLAMENTO EUROPEO Y DEL CONSEJO de 15 de febrero de 2006 relativa a la contaminación causada por determinadas sustancias peligrosas vertidas en el medio acuático de la Comunidad [DOUE, 2006].

Algunos de los artículos que recoge son los siguientes:

Artículo 1: […] la presente Directiva se aplicará:

a) a las aguas interiores superficiales;

b) a las aguas de mar territoriales;

c) a las aguas interiores del litoral.

* *El Reglamento es una norma jurídica de Derecho comunitario con alcance general y eficacia directa Esto implica que es directamente aplicable en todos los Estados de la Unión por cualquier autoridad o particular, sin que sea precisa ninguna norma jurídica de origen interno o nacional que la transponga para completar su eficacia plena.*

** *La Directiva es una disposición normativa de Derecho comunitario que vincula a los Estados de la Unión o, en su caso, al Estado destinatario en la consecución de resultados u objetivos concretos en un plazo determinado, dejando, sin embargo, a las autoridades internas competentes la debida elección de la forma y los medios adecuados a tal fin.*

Artículo 3

Los Estados miembros adoptarán las medidas apropiadas para eliminar la contaminación de las aguas indicadas en el artículo 1 causada por las sustancias peligrosas incluidas en la categorías y grupos de sustancias enumerados en la lista I del anexo I, denominadas en lo sucesivo «sustancias de la lista I», así como para reducir la contaminación de dichas aguas ocasionada por las sustancias peligrosas incluidas en las categorías y grupos de sustancias enumerados en la lista II del anexo I, denominadas en lo sucesivo «sustancias de la lista II», de conformidad con la presente Directiva.

DIRECTIVA 2006/88/CE DEL CONSEJO de 24 de octubre de 2006 relativa a los requisitos zoosanitarios de los animales y de los productos de la acuicultura, y a la prevención y el control de determinadas enfermedades de los animales acuáticos [DOUE, 2006].

1. La presente Directiva establece:

a) los requisitos zoosanitarios aplicables a la puesta en el mercado de animales, la importación y el tránsito de animales y de productos de la acuicultura;

b) las medidas preventivas mínimas destinadas a aumentar la sensibilización y la preparación de los organismos competentes, los agentes económicos de la producción acuícola y demás agentes relacionados con dicho sector, en relación con las enfermedades de los animales acuáticos;

c) las medidas mínimas de control que deberán aplicarse en caso de sospecha o de aparición de un foco de determinadas enfermedades en animales acuáticos.

REGLAMENTO (CE) No 1213/2008 DE LA COMISIÓN de 5 de diciembre de 2008 relativo a un programa comunitario plurianual coordinado de control para 2009, 2010 y 2011 destinado a garantizar el respeto de los límites máximos de residuos de plaguicidas en los alimentos de origen vegetal y animal o sobre los mismos, así como a evaluar el grado de exposición de los consumidores a estos residuos [DOUE, 2008].

Algunos de los artículos que recoge son los siguientes:

Artículo 1

Durante los años 2009, 2010 y 2011, los Estados miembros tomarán muestras de las combinaciones de productos y residuos de plaguicidas indicadas en el anexo I y las analizarán.

Artículo 3

1. Los Estados miembros presentarán los resultados de los análisis de las muestras efectuados en 2009, 2010 y 2011 antes del 31 de agosto de 2010, 2011 y 2012 respectivamente.

Además de estos resultados, los Estados miembros comunicarán la siguiente información:

a) los métodos analíticos utilizados y los niveles de notificación alcanzados, de acuerdo con los procedimientos de control de calidad fijados en el documento Method Validation and Quality Control Procedures for Pesticide Residue Analysis in food and feed;

b) el límite de determinación aplicado en el programa de control comunitario y en los programas de control nacionales;

c) los datos sobre la acreditación de los laboratorios de análisis que participan en los controles;

d) cuando lo permita la legislación nacional, información detallada sobre las medidas de ejecución adoptadas;

e) en caso de que se superen los LMR, una exposición de los posibles motivos por los que se hayan superado los LMR, junto con las observaciones pertinentes acerca de las opciones en materia de gestión de riesgos.

REGLAMENTO (UE) Nº 915/2010 DE LA COMISIÓN de 12 de octubre de 2010 relativo a un programa plurianual coordinado de control de la Unión para 2011, 2012 y 2013 destinado a garantizar el respeto de los límites máximos de residuos de plaguicidas en los alimentos de origen vegetal y animal o sobre los mismos y a evaluar el grado de exposición de los consumidores a estos residuos [DOUE, 2010].

Mediante los Reglamentos (CE) nº1213/2008 y (CE) nº 901/2009 de la Comisión, se estableció un programa comunitario plurianual coordinado de control para 2010, 2011 y 2012 destinado a garantizar el respeto de los límites máximos de residuos de plaguicidas en los alimentos de origen vegetal y animal o sobre los mismos, así como a evaluar el grado de exposición de los consumidores a estos residuos.

Artículo 1

Este artículo establece que durante los años 2011, 2012 y 2013, los Estados miembros tomarán muestras de las combinaciones de productos y residuos de plaguicidas indicadas en el anexo I del reglamento y las analizarán.

Teniendo en cuenta la legislación presentada, tanto española como de la Unión Europea, se ha elaborado la relación de los LMRs establecidos para los agentes quimioterápicos y plaguicidas estudiados en los diferentes alimentos abordados en esta memoria y que se exponen a continuación en la *tabla II.10*.

Tabla II.10. *Relación de sustancias farmacológicamente activas cuyos límites máximos de residuos se han fijado en pescados*

Principio activo	Residuo marcador	Especie animal	LMR	Tejidos diana	Disposición
Cipermetrina	Cipermetrina (Suma de los isómeros)	Salmónidos	50 µg Kg^{-1}	Músculo y piel en proporciones normales	Reglamento (CE) nº1646/2004
Deltametrina	Deltametrina	pescado	10 µg Kg^{-1}	Músculo y piel en proporciones normales	Reglamento (CE) nº 2377/90 EMEA 2001
Diflubenzuron	Diflubenzuron	Salmónidos	1 000 µg Kg^{-1}	Músculo y piel en proporciones normales	Reglamento (CE) nº 2593/1999
Teflubenzuron	Teflubenzuron	Salmónidos	500 µg Kg^{-1}	Músculo y piel en proporciones normales	Reglamento (CE) nº 804/1999
Emamectina	Emamectina B1a	Pescado	100 µg Kg^{-1}	Músculo y piel en proporciones normales	Reglamento (CE) nº 1490/2003
Azametifos	Azametifos	Salmónidos	100 µg Kg^{-1}	Músculo y piel en proporciones normales	Reglamento (CE) nº 508/1999

En la siguiente tabla se muestran los límites de algunos de los compuestos que van a ser analizados en las muestras de algas objeto de estudio y otras matrices similares.

Tabla II.11. *Contenido máximo de residuos de los pesticidas.*

COMPUESTO	*Contenido máximo de residuos (MRLs, mg Kg^{-1})*			
	LECHUGA	**ESPINACAS**	**ACELGAS**	**ALGAS**
2-Fenilfenol	0.05	0.05	0.05	0.05
Bioaletrina	NE	NE	NE	NE
Carbaril	0.05	0.05	0.05	NE
Clorpirifos-etil	0.05	0.05	0.05	NE
Clorpirifos-metil	0.05	0.05	0.05	NE
Ciflutrina	1.0	0.02	0.02	0.02
Cialetrina	NE	NE	NE	NE
Cipermetrina	2.0	0.7	0.7	0.05
Deltametrina	0.5	0.05	0.05	0.05
Empentrina	NE	NE	NE	NE
Fenvalerato	0.02	0.02	0.02	0.02
Flucitrinato	0.05	0.05	0.05	0.05
Permetrina	0.05	0.05	0.05	0.05
Azametifos	NE	NE	NE	NE
Propoxur	0.05	0.05	0.05	NE
Teflubenzurón	0.05	0.05	0.05	0.05
Diflubenzurón	0.05	0.05	0.05	0.05
Abamectina	0.1	0.01	0.01	0.01
Doramectina	NE	NE	NE	NE
Ivermectina	NE	NE	NE	NE

[a] Reglamento EU nº 600/2010; REGLAMENTO (UE) nº 893/2010; REGLAMENTO (CE) NO 396/2005
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3. Metodologías analíticas para la determinación de pesticidas en el medio marino. Muestras líquidas.

3.1 Introducción

Debido a la preocupación social y científica sobre la contaminación del medio ambiente, en los últimos años se han introducido tecnologías respetuosas con el medio ambiente en diferentes áreas de la sociedad, especialmente de la investigación. Los principales objetivos de la química analítica verde (GAC) son obtener nuevas tecnologías analíticas o modificar métodos ya desarrollados incorporando procedimientos que utilicen sustancias químicas menos peligrosas o menores cantidades de disolventes. Malissa presentó por primera vez la base de GAC en París, y, hace 15 años, de la Guardia y col., [de la Guardia, 1995] introdujeron por primera vez la química analítica ambiental como un nuevo enfoque de la práctica analítica. Desde entonces, este concepto ha ido ganando interés [Armenta, 2008], pero fue en esta última década cuando se ha hecho un gran esfuerzo para obtener tecnologías analíticas capaces de hacer un análisis directo, utilizando miniaturización de equipos, cantidades menores de disolventes y reducción de energía, costes y residuos.

En el proceso analítico, el pre-tratamiento de la muestra puede considerarse como la etapa más contaminante porque se parte de la muestra, en la que los analitos pueden existir en pequeñas concentraciones en la matriz, por lo que, a menudo, se requiere el uso de disolventes orgánicos para extraer y concentrar selectivamente los compuestos objetivo y eliminar las interferencias de la matriz. La tendencia actual es evitar el pre-tratamiento de la muestra, sin embargo, en la mayoría de los casos, esto no es factible, por lo que se han desarrollado nuevas técnicas de preparación de muestra cuyo objetivo es reducir el uso de disolventes [Farré, 2010].

Las técnicas de preparación de muestra para la determinación de contaminantes en el medio acuático se pueden clasificar de acuerdo con el tipo de matriz a analizar, muestras sólidas y muestras líquidas. En este contexto, algunas de estas técnicas como la microextracción en fase sólida (SPME), y la microextracción líquido-líquido dispersiva (DLLME) se van a proponer y desarrollar en esta memoria para la extracción de pesticidas a niveles traza en aguas.

Los métodos convencionales para extraer pesticidas de muestras líquidas se han basado principalmente en la extracción líquido-líquido (LLE) [Oudou 2002; Makebi, 2008] y extracción en fase sólida (SPE) [Xue, 2005; Gil-García 2006]. La extracción líquido-líquido (LLE, *liquid-liquid extraction*) se basa en la distribución o reparto de los analitos entre dos fases líquidas inmiscibles, regidas por la constante de distribución K_D, que se define según la ecuación siguiente:

$$K_D = [A]_2/[A]_1$$

donde $[A]_2$ y $[A]_1$ son las concentraciones de analito en la fase orgánica y en la muestra, respectivamente. La constante de distribución es característica de cada analito y depende de la temperatura [Cela, 2002].

La eficiencia del proceso depende de varios factores como son la afinidad del analito por el disolvente y el número de extracciones sucesivas que se lleven a cabo. El uso de mezclas de disolventes, la adición de sales o el cambio de pH pueden mejorar el rendimiento de la extracción.

La extracción en fase sólida (SPE) es un método de extracción alternativo en el que se reduce significativamente el tiempo de análisis y el consumo de disolventes. La extracción de piretroides ha sido realizada en cartuchos OASIS HLB [Xue, 2005] y C18 [Gil-García 2006]. El principal inconveniente de la SPE es la necesidad de volúmenes de muestra grandes (> 500 ml). Por esta razón, en esta memoria se han desarrollado nuevos métodos miniaturizados (SPME y DLLME), que son simples y utilizan pequeños volúmenes de disolventes. Además, con estos métodos se aumenta la sensibilidad porque la fracción extraída puede ser introducida cuantitativamente en el sistema cromatográfico.

A continuación, se describen las técnicas empleadas en esta memoria para extraer pesticidas de muestras de acuosas relacionadas con el medio marino

3.2 Microextracción en fase sólida (SPME)

La microextracción en fase sólida (*Solid-Phase Micro Extraction,* SPME) fue introducida por Belardi y Pawliszyn en 1989 y, en los últimos años, ha sido objeto de un gran desarrollo [Belardi, 1989; Pawliszyn, 1997; Cela, 2002; Pawliszyn, 2010]. La SPME permite la extracción simultánea y la preconcentración de analitos de una muestra de forma mucho más rápida que la LLE y SPE y, al contrario que en estas otras técnicas, no es necesario el uso de disolventes. La SPME se ha convertido en una técnica de extracción muy usada en distintas áreas de la química tales como el análisis de muestras alimentarias, medioambientales y biológicas.

Hoy en día sigue usándose la configuración ideada por Belardi y Pawliszyn en la que la fase sólida es un polímero que recubre una fibra de sílice fundida que, a su vez, forma parte de un dispositivo porta fibra que tiene un émbolo que permite exponerla a la muestra (*figura II.9*). La fibra de sílice fundida es químicamente inerte, estable a altas temperaturas y está recubierta por un polímero adsorbente. Esta fibra está montada dentro de una aguja de acero inoxidable que protege la fase sólida cuando se manipula el portafibras o cuando se introduce a través del *septum* de un vial o del inyector de un equipo [O'Reilly, 2005].

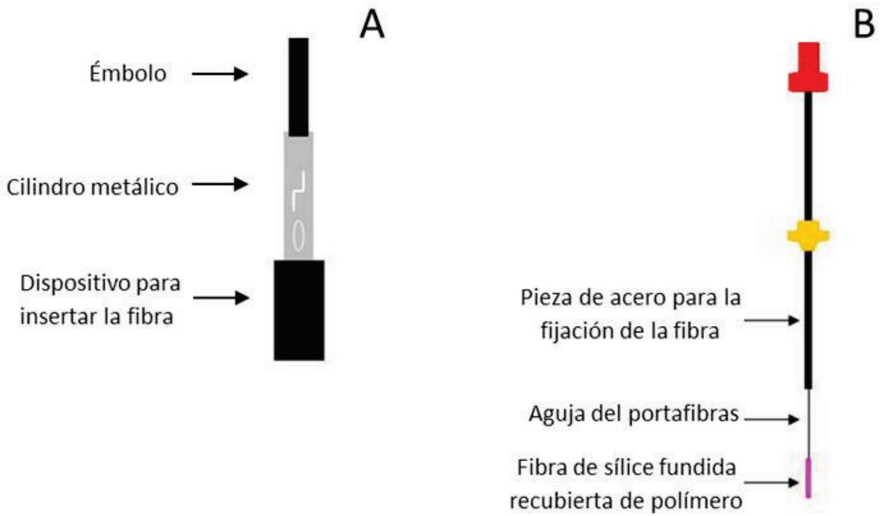

Figura II.9. *Diagrama de un dispositivo portafibra (A) y su aguja con la fibra (B).*

La microextracción en fase sólida consta de dos etapas: *Extracción* y *desorción*. En la etapa de extracción o muestreo, la fibra se expone a la muestra contenida en un vial, de manera que los analitos se reparten entre la muestra y el recubrimiento de la fibra (*figura II.10*).

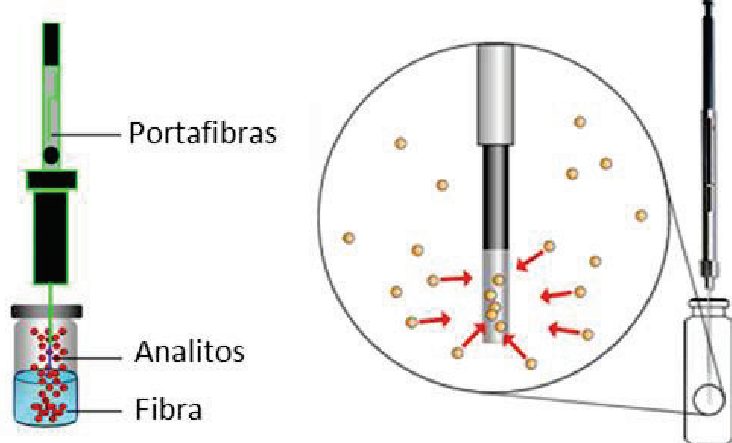

Figura II.10. *Etapa de extracción de los analitos.*

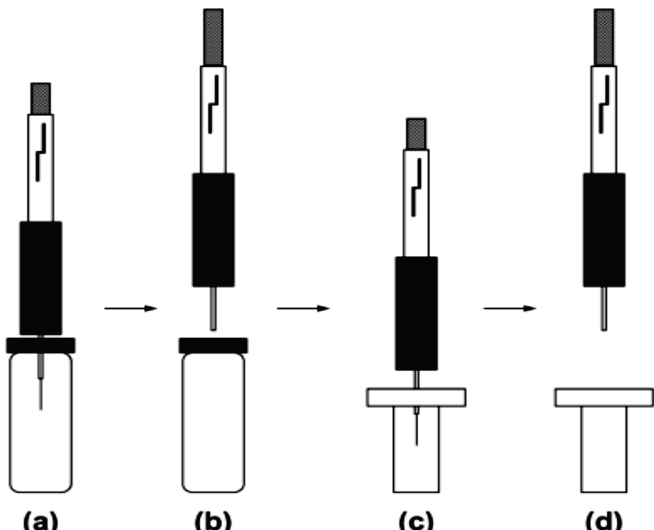

Figura II.11. *Etapas de la microextracción en fase sólida. (a) Agujerear el septum y exponer la fibra. (b) Una vez terminada la extracción, retraer la fibra y quitar el portafibras. (c) Insertar el portafibras en el inyector del equipo usado y exponer la fibra el tiempo necesario. (d) Quitar la fibra y pasar a la siguiente muestra.*

Después de un tiempo adecuado, la fibra se retrae, se retira el portafibra del vial y se pasa a la etapa de desorción. En esta segunda etapa, la jeringuilla portafibra se introduce en el inyector de un instrumento analítico (*figura II.11C*).

El hecho de que el dispositivo portafibras tenga forma de jeringuilla permite que pueda ser usado en equipos de GC y HPLC sin grandes dificultades y es en el inyector del equipo cromatográfico donde los analitos se desorben térmicamente (GC) o por disolución en la fase móvil (HPLC) [O'Reilly, 2005; Aulakh, 2005].

3.2.1 Fundamento

El transporte de los analitos desde la muestra situada en el vial hasta el recubrimiento de la fibra tiene lugar cuando la fibra se pone en contacto con la muestra y se completa cuando la concentración del analito ha alcanzado el equilibrio de distribución entre la matriz de la muestra y el recubrimiento de la fibra. En la práctica esto significa que, una vez que se ha alcanzado el equilibrio, la cantidad extraída permanece constante dentro de los límites del error experimental y es independiente de un incremento del tiempo de extracción. Las condiciones de equilibrio se pueden describir mediante la ecuación:

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

n, cantidad de analito extraída por el recubrimiento,
Kfs, coeficiente de distribución entre la fibra y la muestra,
Vf, volumen del recubrimiento de la fibra,
Vs, volumen de muestra,
C_0, concentración inicial del analito en la muestra analizada.

Esta ecuación asume que la matriz de la muestra puede considerarse como una fase homogénea simple y que no hay espacio de cabeza en el sistema, se puede modificar si se tiene en cuenta la existencia de otros componentes en la matriz, considerando el volumen de las fases individuales y las constantes de distribución adecuadas.

La SPME es una técnica de equilibrio y por esta razón no se consigue una extracción exhaustiva. No obstante, de la ecuación anterior se deduce que la concentración del analito en la muestra es directamente proporcional a la cantidad extraída, lo que permite obtener resultados cuantitativos [Pawliszyn, 1997].

Esta ecuación establece que la cantidad de analito extraída no depende de la localización de la fibra en el sistema. La fibra puede situarse tanto en la matriz de la muestra como en el espacio de cabeza, siempre y cuando los volúmenes se mantengan constantes, En función de la posición dela fibra se pueden considerar tres tipos básicos de microextracción en fase sólida [Cela, 2002; Eisert, 1997]**:**

- *Extracción directa (DI-SPME):* La fibra se introduce directamente en la muestra líquida y los analitos son transportados desde la matriz de la muestra al recubrimiento polimérico de la fibra. Este método está indicado para la separación de compuestos poco volátiles en muestras limpias y es el que se ha usado en este trabajo.
- *Extracción en el espacio de cabeza (HS-SPME):* La fibra se expone al espacio de cabeza existente sobre la muestra. Los analitos se transportan, en una primera etapa, desde la matriz de la muestra al espacio de cabeza donde serán adsorbidos en la fibra en una segunda etapa. Usando esta técnica se protege la fibra de la matriz de la muestra, donde se podrían adsorber otros compuestos no volátiles. Además, permite modificaciones en la muestra como cambios de pH sin que la fibra se deteriore.

- *Extracción indirecta a través de una membrana:* La fibra se protege con una membrana para evitar su deterioro cuando se extraen muestras con matrices complejas. La extracción indirecta es más lenta que las anteriores, ya que los analitos deben difundir a través de la membrana antes de adsorberse sobre la fase extractante.

3.2.2 Parámetros que afectan a la SPME

La termodinámica predice los efectos de distintos parámetros que pueden afectar a la microextracción en fase sólida. Estos parámetros son el recubrimiento polimérico de la fibra, la temperatura y tiempo de extracción, el efecto salino, el pH de la muestra, el volumen de la muestra y del espacio de cabeza, la agitación de la muestra y la forma del vial, la adición de un disolvente y la derivatización [Cela, 2002].

El *recubrimiento polimérico de la fibra* es una variable muy importante en la SPME ya que la eficacia del proceso de extracción depende de la constante de distribución entre la matriz y la fibra (K_{fs}). Este parámetro determinará la selectividad de la fibra por el analito frente a otros compuestos de la matriz. El volumen del recubrimiento determina la sensibilidad del método, pero si se usan fibras con demasiado espesor de fase, la cinética del proceso se hará muy lenta y los tiempos de extracción serán muy altos [Cela, 2002]. Por otro lado, la naturaleza química del analito será lo que determine el tipo de fase que se ha de utilizar en la extracción. En la actualidad, están disponibles en el mercado varios tipos de fases estacionarias con diferentes espesores y polaridades que, por lo tanto, muestran afinidad por distintas familias de analitos. Algunos de estos recubrimientos disponibles en el mercado se muestran en la *tabla II.12* junto sus características y aplicaciones [Cela, 2002; Lambropoulou, 2002].

Tabla II.12. *Distintos recubrimientos poliméricos disponibles comercialmente y algunas de sus características.*

Fase estacionaria	Espesor de fase	Tª máxima	Tª desorción	Características	Aplicaciones
Polidimetil-siloxano (*PDMS*)	100 µm	220ºC	200ºC	No enlazada	Compuestos volátiles de bajo punto de ebullición y no polares
	30 µm	220ºC	200ºC	No enlazada	Semivolátiles de mayor peso molecular
	7 µm	340ºC	220-320ºC	Enlazada	Semivolátiles de alto punto de ebullición y no polares
Polidimetilsiloxano-divinilbenceno (*PDMS/DVB*)	65 µm	270ºC	200-250ºC	Parcialmente reticulada	Volátiles polares
	60 µm	270ºC	—	Parcialmente reticulada	Aminas y compuestos polares (HPLC)
Poliacrilato (*PA*)	85 µm	310ºC	220-300ºC	Parcialmente enlazada	Semivolátiles polares
Carbowax/templated resina (*CW-TPR*)	50 µm	260ºC	—	Parcialmente reticulada	Tensioactivos (HPLC)
Carbowax/divinil-benceno (*CW/DVB*)	65 µm	260ºC	220-250ºC	Parcialmente reticulada	Alcoholes y compuestos polares
Carboxen/Polidimetil-siloxano (*Carboxen-PDMS*)	75 µm	340ºC	310-320ºC	Parcialmente reticulada	Gases y compuestos de bajo peso molecular

La *temperatura* también es un parámetro muy a tener en cuenta en la optimización de la SPME ya que afecta a la cinética de la extracción y, por lo tanto, influye de manera apreciable en la sensibilidad y

selectividad del proceso. La temperatura de extracción tiene dos efectos opuestos: Por un lado aumenta la difusión de los analitos hacia la fibra y por otro lado hace disminuir la constante de distribución entre la fibra y la matriz, disminuyendo la sensibilidad cuando se trabaja cerca del equilibrio.

El *tiempo de extracción* debe ser suficiente para que se alcance el equilibrio de distribución, ya que cuando esto ocurra se extraerá la cantidad máxima de analito. La agitación reduce el tiempo necesario para alcanzar el equilibrio al favorecer la migración de los analitos hacia la fibra. A efectos prácticos se puede trabajar con tiempos de extracción menores (sin alcanzar el equilibrio) siempre y cuando se haga un control del mismo para que los resultados sean reproducibles [Pawliszyn, 1997].

El ***efecto salino*** provoca un aumento de la fuerza iónica de la matriz forzando a las moléculas de agua a solvatar los iones presentes en la muestra y no a las moléculas neutras de los analitos (siempre que estén sin disociar). Este efecto provoca que disminuya la solubilidad de los analitos en el agua, favoreciendo su paso a otras fases del sistema como la fibra, lo que termina originando un incremento en la sensibilidad [Pawliszyn, 1997].

El ***pH de la muestra*** puede afectar a equilibrios de disociación de los analitos en medio acuoso. La extracción es más eficaz si los analitos están sin disociar como ocurre en otras técnicas como la extracción líquido-líquido. Asumiendo que solamente se adsorberán en la fibra las especies no disociadas de los analitos (ácidos o básicos), el ajuste del pH puede cambiar la distribución de estas especies. De este modo, tamponando el pH de la muestra al valor en el que la especie mayoritaria es el analito no disociado, se puede aumentar la efectividad de la extracción, aunque hay que tener en cuenta que pH extremos pueden dañar la fibra [Pawliszyn, 1997].

El ***volumen de la muestra*** así como el *volumen del espacio de cabeza* también deben ser optimizados ya que ambos afectan a las constantes de distribución y, por lo tanto, a la cinética de la SPME. A medida que aumenta el volumen de muestra también lo hará la cantidad de analito extraída, hasta que se llegue a un punto en el que el volumen de la muestra será tan grande respecto al volumen de la fibra que se puede considerar constante y la sensibilidad no aumentará más. Al contrario, el volumen del espacio de cabeza debe ser pequeño, para que los analitos se concentren antes de su difusión hacia el recubrimiento de la fibra [Pawliszyn, 1997].

La ***agitación*** de la muestra favorece la extracción ya que facilita el transporte de los analitos hacia la fibra, acelerando la cinética del proceso y reduciendo los tiempos de extracción. Normalmente se usan agitadores magnéticos, aunque también se pueden usar sistemas de agitación más efectivos como los ultrasonidos o la vibración de la propia fibra. Siempre que se tenga agitación, la fibra debe introducirse

descentrada en el vial, para que no coincida con el vórtice y se favorezca el contacto fibra-disolución. La *forma del vial* también es importante, ya que debe favorecer el contacto entre fases y permitir que la agitación genere suficiente convección [Pawliszyn, 1997].

La ***adición de un disolvente orgánico*** a muestras acuosas produce una reducción en la cantidad de analito extraído. Por otra parte, la adición de agua o de disolventes orgánicos a muestras de suelos y lodos resulta muy eficaz para facilitar la liberación de analitos desde la matriz y mejorar su difusión a la fase sólida [Pawliszyn, 1997].

La ***derivatización*** favorece la extracción de compuestos polares en matrices biológicas y medioambientales, compuestos que normalmente son problemáticos en separaciones cromatográficas. La derivatización se puede hacer de tres modos: En el primero se realiza la adición directa del reactivo derivatizante a la muestra, se produce la reacción y los productos se extraen con la fibra de SPME. En segundo lugar, se puede unir el reactivo derivatizante al recubrimiento de la fibra, produciéndose la derivatización y la extracción simultáneamente, o bien la extracción y posterior derivatización. En último lugar, se puede realizar la derivatización en el inyector del cromatógrafo de gases cuando los analitos y el reactivo derivatizante presentes en la fibra se someten a altas temperaturas. Hay que tener en cuenta que la derivatización realizada antes o durante la extracción incrementa la sensibilidad y selectividad del proceso de determinación, mientras que si se realiza después de la extracción solo se mejora la separación cromatográfica [Pawliszyn, 1997].

3.2.3 Análisis pesticidas mediante SPME.

En la actualidad el uso de la SPME está muy extendido y se ha utilizado para la extracción de pesticidas organofosforados y piretroides en multitud de matrices. En la *tabla II.13* se recogen algunos trabajos realizados en los últimos años en los que se emplea la SPME como técnica de extracción y la GC como técnica de separación.

***Tabla II.13.** Estudios que hacen uso de la SPME en la determinación de residuos de pesticidas en distintas matrices.*

Matriz	Grupo de Pesticidas	Método de determinación	LOD	Referencia
Aguas naturales	OPs	GC-FTD / GC-MS	20 ng L^{-1} / 30 ng L^{-1}	Lambropoulou, 2002
Aguas naturales	OPs	GC-FTD / GC-MS	20 ng L^{-1} / 30 ng L^{-1}	Lambropoulou, 2001
Agua de río, zumo y vino tinto	OPs	GC-ECD / GC-ICP-MS	241 ng L^{-1} / 30 ng L^{-1}	Used, 2006
Aguas	OPs	GC-ECD	60 ng L^{-1}	Li, 2003
Agua potable	Piretroides	GC-ECD	0,1 ng L^{-1}	Barrionuevo, 2000
Agua de río	Piretroides	GC-ECD	—	Barrionuevo, 2002
Agua potable	Piretroides	GC-ECD	0.1 ng L^{-1}	Barrionuevo, 2000
Agua de río	Piretroides	GC-ECD	—	Barrionuevo, 2002
Agua de zona urbana	Piretroides	GC-FID / GC-MS	70 µg L-1 / 20 µg L^{-1}	Fuh, 2003
Agua potable y de río	Piretroides y OPs	GC-ECD	27 ng L^{-1}	Gonçálves, 2002
Agua subterránea agricola	Piretroides	MA-HS-SPME GC-ECD	0,2 to 2,6 ng L^{-1}	Li, 2009
Agua de mar Agua superficial	Piretroides	(MWCNTs/Ppy) GC-ECD	0.12–0.43 ng mL^{-1}	Chen,2011
Aguas naturales	Piretroides	GC-µECD	2.2 ng L^{-1}	Casas, 2006
Agua de lago	OPs	GC-ECD	13 ng L^{-1}	Dong, 2005
Agua de lluvia	OPs	GC-ITD-MS/MS	5 ng L^{-1}	Scheyer, 2006
Sedimentos zona residencial	Piretroides	GC-ITD-MS	0.6 ng g^{-1}	Weston, 2005
Fresas	Piretroides	GC-MS	10 µg Kg^{-1}	Sanusi, 2004
Muestras vegetales	Piretroides	GC-MS	0.1 µg L^{-1}	50 Beltran, 2003
Aceite de oliva	OPs	GC-FTD	10 µg Kg^{-1}	Tsoutsi, 2006
Sangre	OPs	GC-EI-MS	1 µg g^{-1}	Aprea, 2002

OPs: Pesticidas organofosforados
MA-HS-SPME: Microwave-assisted headspace solid-phase microextraction
MWCNTs/Ppy: Multiwalled carbon nanotubes/polypyrrole

3.3 Microextracción líquido-líquido dispersiva (DLLME)

La microextracción líquido-líquido dispersiva (Dispersive Liquid-Liquid Microextraction, DLLME) es un tipo de extracción miniaturizada desarrollada recientemente por Rezaee y col [Rezaee, 2006] que ha sido aplicada a diferentes matrices tales como alimentos, fluidos biológicos y muestras sólidas [Rezaee, 2010]. Esta técnica integra en una única etapa la extracción y concentración de los analitos.

3.3.1 Fundamento

La DLLME se basa en la adición a una muestra líquida de una mezcla binaria compuesta por un disolvente con densidad superior a 1 g mL^{-1} (extractante) y un disolvente polar y miscible con agua (dispersante). Tras la adición, normalmente mediante inyección rápida de esta mezcla a la muestra, se forma una dispersión de pequeñísimas gotas de extractante que asegura una enorme superficie de contacto entre la muestra y la fase extractante. Esto favorece y acelera el proceso de transferencia de masa y hace que se alcance el equilibrio muy rápidamente [Rezaee, 2006; Zang, 2009; Bosch, 2009]. Tras la centrifugación de esta dispersión, las microgotas de extractante se agregan y se depositan en forma de gota en el fondo del tubo en el que se ha llevado a cabo el proceso. Por razones prácticas, se utilizan tubos de vidrio de forma cónica para facilitar así la recuperación de la gota mediante una microjeringa (*figura II.12*).

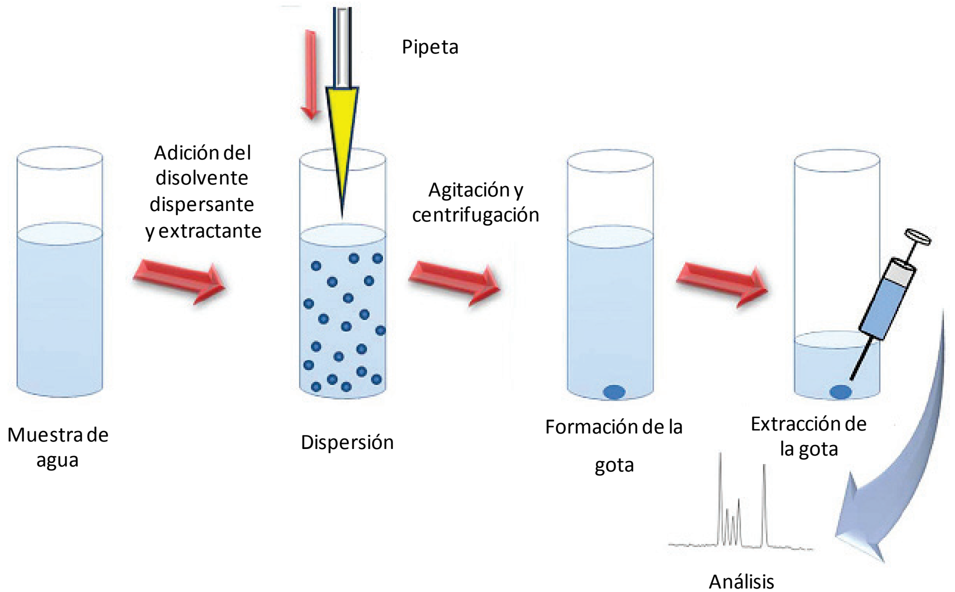

Figura II.12. *Etapas de la microextracción líquido-líquido dispersiva.*

Algunas de las ventajas más destacadas de la técnica son su simplicidad, rapidez, bajo coste, consumo reducido de muestra y disolventes orgánicos, versatilidad, buenas recuperaciones y factores de concentración altos. Comparada con otras técnicas de microextracción como SDME, SPME o LPME ofrece normalmente mayor precisión y tiempos de extracción más cortos [Li, 2008; Yanyan, 2008; Ru, 2009].

Como desventajas cabe destacar la necesidad de una cierta destreza para la manipulación y recuperación de la gota y, sobre todo, la dificultad de automatización.

Cálculo de recuperaciones y factores de enriquecimiento

En el desarrollo de los métodos de DLLME se puede evaluar la influencia de las diferentes

variables, en términos de recuperaciones y factores de enriquecimiento, a partir de las siguientes expresiones:

$$EF = C_{sed}/C_o$$

$$R\ (\%) = 100\ (C_{sed}\ V_{sed})/(C_o\ V_m)$$

EF es el factor de enriquecimiento, $\boldsymbol{C_o}$ y $\boldsymbol{C_{sed}}$ son las concentraciones de analito en la muestra y en la fase sedimentada, respectivamente. ***R*** es la recuperación (en %) y $\mathbf{V_{sed}}$ y $\mathbf{V_m}$ son los volúmenes de la gota sedimentada y de la muestra, respectivamente. El volumen de gota sedimentada varía en función de las condiciones de extracción. Por ello, es necesaria la utilización de ambos parámetros, recuperaciones y factores de concentración, en la optimización del método. En el desarrollo y optimización del método de DLLME se busca que la recuperación y factor de enriquecimientos sean máximos.

3.3.2 Variables que afectan a la eficacia de extracción

Algunos de los factores que afectan a la eficacia de DLLME son el *tipo y el volumen de disolventes extractante y dispersante, la fuerza iónica, los tiempos de extracción y centrifugación, el control del pH y la adición de reactivos* para llevar a cabo la extracción de los compuestos de interés. A continuación se comentan algunos de estos factores.

La selección del ***disolvente extractante*** es un factor clave en la eficacia de DLLME, y ha de reunir algunas características:

- Densidad superior a la del agua.
- Baja solubilidad en agua.
- Capacidad de formar una dispersión al añadirlo.
- Capacidad para extraer los compuestos de interés.
- Buen comportamiento cromatográfico.

En esta memoria se ha probado algunos hidrocarburos halogenados tales como el tetracloruro de carbono, clorobenceno, cloroformo, diclorometano, tricloroetano, tetracloruro de etileno y diclorometano [Zang, 2009; Bosch, 2009].

El ***disolvente dispersante*** actúa de puente entre el extractante y la muestra y, por ello, debe ser miscible en ambos. Tiene como objetivo la reducción de la tensión superficial del extractante para conseguir la formación de minúsculas gotas, que garantizan una gran superficie de contacto entre extractante y muestra, responsable de la rapidez de las extracciones. Pueden utilizarse como dispersantes la acetona [Rezaee, 2006], metanol [Yazdi, 2008], etanol [Birjandi, 2008], acetonitrilo [Li, 2008] y tetrahidrofurano [Melwanski, 2008].

Las variaciones en el ***volumen de extractante*** no producen grandes cambios en las recuperaciones del método. Esto se debe a que las constantes de distribución de los analitos son altas y las recuperaciones ya cuantitativas para pequeños volúmenes. Por otro lado, el aumento de disolvente extractante conlleva un incremento del volumen de fase sedimentada y, por consiguiente, una disminución del factor de preconcentración, ya que el extracto final está más diluido. Por tanto, cuanto menor sea el volumen de extractante utilizado menores serán los límites de cuantificación del método [Farajzadeh, 2007; Montes, 2009]. La obtención de recuperaciones y factores de preconcentración altos es lo que condiciona el volumen de extractante óptimo. Normalmente suelen usarse cantidades entre 20 y 200 µL.

El ***volumen de dispersante*** afecta directamente a la formación de la emulsión y, por tanto, a la eficacia de extracción. En la mayoría de las publicaciones en las que se estudia la influencia de este parámetro se varía también simultáneamente el volumen de extractante [Rezaee, 2006], de tal forma que el volumen de la gota obtenida se mantenga constante al variar el volumen de dispersante empleado. Suelen seleccionarse volúmenes comprendidos entre 0.5 y 1.5 mL para muestras de 10 mL.

El aumento de la ***fuerza iónica*** de la muestra produce una disminución de la solubilidad tanto de los analitos como del agente extractante, lo que resulta favorable para las recuperaciones. Esta disminución implica un aumento del volumen de la fase sedimentada obtenida y, en consecuencia, una dilución de los analitos o lo que es lo mismo, una disminución del factor de concentración [Zang, 2009].

El ***tiempo de extracción*** se define como el intervalo que transcurre entre la adición de la mezcla binaria a la muestra y la centrifugación [Rezaee, 2006]. Esta variable no ejerce casi ninguna influencia en la eficacia de extracción. La razón es la rapidez con la que se alcanza el equilibrio lo que garantiza una transferencia rápida de los analitos al extractante [Zang, 2009]. La centrifugación de la mezcla es necesaria para la separación de fases y la obtención dela gota sedimentada. Tiempos cortos, no superiores a 5 minutos, son suficientes para tal fin.

La ***variación del pH*** es especialmente importante cuando se trata de analitos con características ácidas o básicas, ya que ajustando el pH puede desplazarse el equilibrio de los mismos hacia su forma neutra, consiguiendo la extracción de especies que, a priori, no podrían ser extraídas.

3.3.3 Análisis de pesticidas mediante DLLME.

En la determinación de pesticidas, se buscan metodologías analíticas de preparación de muestra que sean rápidas, simples, exactas, precisas, sensibles, de bajo coste y que generen pocos residuos al medio ambiente. En DLLME se ha conseguido la miniaturización de la extracción líquido-líquido tradicional mediante la reducción de la relación fase aceptora-fase donadora lo que ha dado lugar a la publicación de

numerosos artículos relativos a la extracción de analitos orgánicos e inorgánicos de diferentes matrices [Boch, 2011; Asensio-Ramos, 2011; Zgota-Grzéskowiak, 2011].

H. Yan *y col* [Yan, 2010a] han aplicado la DLLME a la extracción de nueve pesticidas piretroides de aguas residuales domésticas. El proceso fue asistido por ultrasonidos para acelerar la formación de la emulsión usando un pequeño volumen de disolvente dispersante, incrementando así la eficacia de la extracción, reduciendo el tiempo de equilibrio. En las condiciones óptimas se alcanzaron factores de enriquecimiento desde 728 a 1725 veces. Estos mismos autores han extraído piretroides de aguas de rio utilizando tetraclorometano como extractante y acetona como dispersante, alcanzando límites de detección del orden de 0.1-0.3 µg L^{-1}[Yan, 2010b]. Además, Zacharisa *y col* [Zacharisa, 2010] han propuesto una modificación de la DLLME, utilizado un segundo disolvente que actúa rompiendo la emulsión, favoreciendo la separación de fases sin centrifugación. Este método fue aplicado a la extracción de pesticidas organoclorados de aguas alcanzando LODs de 2-50 ng L^{-1}.

Tabla II.14. *Estudios que hacen uso de la DLLME en la determinación de residuos de pesticidas en aguas.*

Matriz	Grupo de Pesticidas	Método de determinación	LOD	Referencia
Aguas residuales domesticas	Piretroides	GC-FID	0.2-0.7 µg L^{-1}	Yan, 2010
Aguas de rio	Piretroides	HPLC-UV-VIS (220 nm)	0.1-0.3 µg L^{-1}	Yan, 2010
Aguas	Organoclorados	GC-MS	2-50 ng L^{-1}	Zacharis, 2010
Aguas	Organofosforados	GC-FPD	0.01-0.05 µg L^{-1}.	Yang, 2011
Aguas	Organofosforados	GC-FPD	3 - 20 ng L^{-1}	Berijani, 2006
Aguas	Organoclorados	GC-ECD	0.011-0.11 µg L^{-1}	Leong, 2009
Aguas	Organofosforados	GC-FID GC-MS	3- 4 µg L^{-1} 0.003 µg L^{-1}	Farajzadeh, 2009
Aguas grifo	Herbicidas Ácido fenoxiacético	HPLC-UV	0.16 µg L^{-1}	Farhadi, 2009
Aguas	Organofosforados	HPLC-UV	0.1-5.0 µg L^{-1}	He, 2009
Aguas	Organofosforados	HPLC-UV	10-50 ng L^{-1}	He, 2010
Aguas	Benzoil urea	HPLC-UV	0.21-0.45 µg L^{-1}	Zhou, 2010
Aguas	Triazol	GC-FID	0.53-24.0 µg L^{-1}	Farajzadeh, 2010

GC-FPD: Gas chromatography-flame photometric detection

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4. Metodología analítica para la determinación de pesticidas en el medio marino. Muestras sólidas.

Los métodos tradicionales para el análisis de muestras sólidas incluyen extracciones con disolventes orgánicos, fraccionamiento en columnas, etc. Estos métodos suelen ser laboriosos, lentos y requieren demasiada manipulación de la muestra. Uno de los métodos clásicos más empleados en la extracción de compuestos orgánicos de matrices sólidas o semi-sólidas es la extracción *Soxhlet*, que presenta las desventajas de ser una técnica que requiere largos tiempos de extracción (normalmente alrededor de 24 horas) y que emplea grandes volúmenes de disolventes orgánicos tóxicos [Cela, 2002; Schantz, 2006]. Además, los extractos orgánicos obtenidos tienen que ser concentrados y sometidos a procesos de purificación con el fin de obtener los bajos límites de detección requeridos en el análisis de pesticidas. Todo esto ha llevado al desarrollo de nuevas técnicas de extracción para la preparación de las muestras y su posterior análisis que sean limpias, rápidas, selectivas, y cuya automatización sea posible.

A continuación se describen estas nuevas técnicas de extracción utilizadas en la preparación de muestras para el análisis de residuos de pesticidas.

4.1 Extracción con disolventes a temperatura y presión elevadas (PLE)

La *extracción con disolventes a alta presión y temperatura* (Pressurized Liquid Extraction, PLE) se introdujo por primera vez en 1996 [Ritcher, 1996] aunque existen evidencias de que se empleó un sistema de extracción que utilizaba disolventes bajo presión en el siglo XIX [Fournier, 2005]. Desde entonces se han publicado numerosos trabajos en los que se describe la PLE como técnica de preparación de muestra para análisis ambiental, alimentario y biológico [Sun, 2012; LeDoux, 2011; Mendiola, 2007; Carabias-Martínez, 2005; Ramos 2002].

4.1.1 Fundamento

En PLE (también denominada *Pressurized Fluid Extraction, PFE*; o por su nombre comercial *Acelerated Solvent Extraction*, ASE) la muestra sólida o semi-sólida, normalmente dispersada en tierra de diatomeas, sulfato sódico anhidro, arena, etc., se introduce en una celda cerrada. A continuación se introduce el disolvente orgánico elegido (hexano, acetato de etilo, acetonitrilo, etc.) para la extracción estática de la muestra a elevada temperatura (50-200 ºC) y presión (500-3000 psi) durante períodos relativamente cortos de tiempo (5-10 min). Se emplea un gas comprimido, normalmente nitrógeno, para purgar el extracto de la celda a un vial sellado con un *septum.*

El uso de disolventes a elevada temperatura y presión da lugar a extracciones con eficacias superiores comparadas con las realizadas a temperatura y presión ambiente. Esto obedece principalmente a dos razones: El efecto de la *solubilidad y la transferencia de masa* y la *ruptura de los equilibrios en la interfase sólido-líquido* [Ritcher, 1996].

- ***Efecto de la solubilidad y transferencia de masa:*** El uso de disolventes orgánicos a temperaturas elevadas aumenta su capacidad para solubilizar los distintos compuestos extraídos de la matriz. Además, el aumento de la temperatura origina mayores velocidades de difusión de acuerdo con la primera ley de difusión de Fick [Ritcher, 1996].
- ***Ruptura de equilibrios en la interfase sólido-líquido:*** La temperatura elevada permite la ruptura de las interacciones matriz-analito causadas por fuerzas de van der Waals, enlaces de hidrógeno y atracciones dipolo-dipolo. El aumento de temperatura origina la disminución de la viscosidad del disolvente orgánico, lo que permite que una mayor penetración de éste en las partículas de la matriz. Por otro lado, la presión elevada fuerza el paso de disolvente hacia el interior de poros presentes en la muestra aumentando así la eficacia de la extracción [Ritcher, 1996].

4.1.2 Parámetros que afectan a la PLE

La extracción con disolventes a elevada temperatura y presión es una forma más eficiente de la extracción clásica con disolventes orgánicos. Como en todas las técnicas de extracción con disolventes orgánicos la preparación de la muestra es un paso esencial en PLE. Al igual que en Soxhlet, la matriz ideal es aquella que se encuentra completamente seca y finamente dividida, por lo que cualquier pre-tratamiento realizado para que la muestra a analizar cumpla estas premisas tendrá un impacto positivo en la extracción mediante PLE, con la ventaja de la reducción del tiempo de extracción y empleo de menor cantidad de disolventes orgánicos. En general, hay que considerar el *tamaño de partícula, dispersión de la muestra y secado* [Dionex, 2004; Sporring, 2004]. En PLE se deben optimizar una serie de parámetros de extracción como el *disolvente* empleado, *temperatura*, *presión*, *número de ciclos* y *tiempo* de extracción [Dionex, 2004; Sporring, 2004].

- ***Disolvente:*** El disolvente usado debe tener una polaridad similar a la de los compuestos a extraer para favorecer su solubilidad y no dañar a la matriz. Por norma general en PLE se utilizan los mismos disolventes que usan en técnicas de extracción convencionales. La cantidad usada puede ser de un 95% menor que el empleado en otros métodos de extracción.
- ***Temperatura de extracción*:** Es el parámetro más importante en PLE. Al aumentar la temperatura disminuye la viscosidad del disolvente y con ello la capacidad de solubilizar a los analitos, además aumenta la cinética de desorción de los compuestos de la matriz. La mayoría de aplicaciones publicadas

con esta técnica emplean temperaturas dentro del rango de 75-125ºC, siendo 100ºC la temperatura más usada.

- ***Presión de extracción:*** La función de la presión en PLE es mantener los disolventes en estado líquido durante el proceso de extracción (a temperaturas por encima de sus puntos de ebullición en condiciones normales) además de ayudar a recoger el extracto en el vial de recolección. Por otro lado, los cambios en la presión de trabajo no suelen influir en cuanto a recuperación de analitos, por lo que no se considera un factor crítico a la hora de optimizar esta metodología analítica. En la mayoría de aplicaciones publicadas, las presiones empleadas están en el rango de 1000 a 2000 psi [Dionex, 2004; Sporring, 2004].
- ***Número de ciclos:*** El número de ciclos está directamente relacionado con la porción de disolvente *"fresco"* que se introduce durante el proceso de extracción, lo que permite mantener un equilibrio de extracción favorable. Se define un volumen de *"flush"* como un porcentaje del volumen total de la celda. El uso de varios ciclos estáticos ha demostrado ser útil en muestras con concentraciones altas de analito así como en situaciones en las que el disolvente encuentra dificultades para penetrar en la matriz.
- ***Tiempo de extracción:*** El tiempo de extracción es un factor que debe ser optimizado de forma conjunta con el número de ciclos para conseguir una extracción cuantitativa en el menor tiempo posible [Sporring, 2004].
- ***Limpieza de los extractos:*** En esta técnica es posible realizar una etapa de *"clean-up"* simultáneamente a la extracción, se consigue así reducir el tiempo de análisis y la reducción de las etapas de preparación de muestra. De este modo se han desarrollado técnicas de extracción selectivas como la publicada por Dionex en su *"Application Note 322"* en la que se incluía una capa de alúmina ácida en celda de extracción con el objetivo de retener las grasas en la extracción de tejido de pescado [Dionex, 2004a].

4.1.3 Etapas de extracción.

La extracción se puede realizar en modo dinámico (que presenta el inconveniente de obtener extractos con un volumen de disolvente relativamente elevado) o bien en modo estático, que es el más usado. En la *figura II.13* se muestra un esquema de un sistema ASE [Schantz, 2006; Richter, 1996; Mendiola, 2007].

En modo estático se diferencian las siguientes etapas:

1. La celda de acero con la muestra se introduce en el horno.
2. El disolvente, a través de una bomba, es introducido en la celda de extracción.
3. Se aplica la presión y temperatura fijadas.

4. La celda que está en el horno se calienta a la temperatura fijada, manteniendo la presión constante durante un tiempo predeterminado.
5. Se realiza la extracción estática.
6. El disolvente contenido en la celda se transfiere al vial colector.
7. Se hace pasar un volumen de disolvente, el cual viene expresado como porcentaje del volumen de celda (% de *flush*) para arrastrar posibles trazas de los analitos que pudiesen quedar en la celda. Ese volumen, en el caso de que se produzcan dos o más ciclos de extracción, es dividido entre el número de los mismos.
8. Una vez terminado el proceso de extracción se purga la celda con nitrógeno presurizado y los restos se transfieren automáticamente al mismo vial.

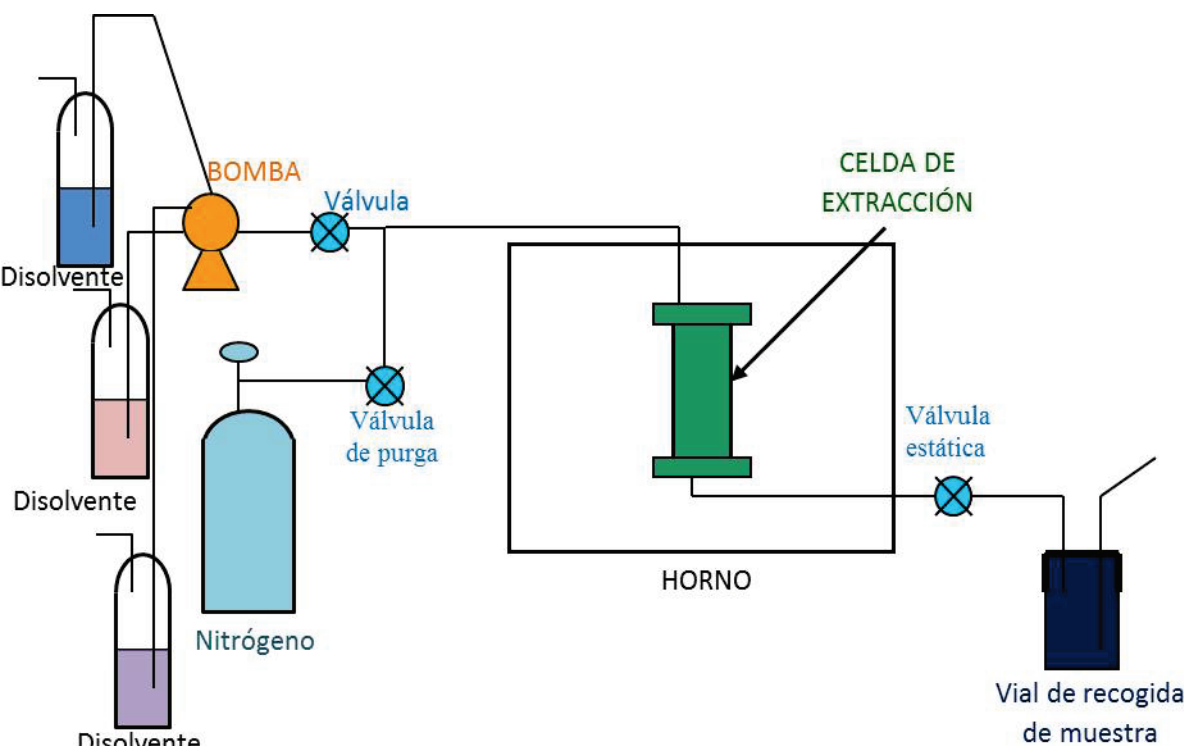

Figura II.13. *Esquema de un sistema de extracción con fluidos presurizados*

4.1.4 Ventajas y desventajas de la técnica

Ventajas

- Es una técnica rápida, el tiempo de extracción se reduce considerablemente al trabajar a temperaturas y presiones elevadas.
- El consumo de disolventes es bajo.
- Permite la introducción de materiales adsorbentes, directamente en la celda de extracción. De este modo la extracción y purificación de los extractos se realiza en una sola etapa. Los extractos son filtrados automáticamente con lo que pueden ser inyectados directamente.
- El dispositivo comercial incorpora sensores seguridad para la temperatura y presión, así como para el control de pérdidas de disolvente.
- Más eficaz independientemente de la matriz.
- Permite desarrollar métodos de forma sencilla, ya que el número de parámetros experimentales que hay que optimizar es pequeño.
- Es automatizable y puede extraer 24 muestras secuencialmente.

Desventajas

- Extracciones más completas pero menos selectivas.
- Requiere el empleo de temperaturas elevadas.
- El coste inicial del equipo es elevado.

4.1.5 Análisis de pesticidas mediante PLE.

Los pesticidas en general, y los organofosforados, piretroides, carbamatos y avermectinas en particular, son ampliamente usados en la agricultura, ganadería, acuicultura, y a nivel doméstico. El análisis de estos compuestos en frutas y vegetales, en productos de origen animal y procedentes de la acuicultura, requiere métodos sensibles y selectivos para su determinación en la cadena alimentaria [Hoff, 1999].

En las últimas décadas, los métodos para la determinación de pesticidas a niveles traza han cambiado considerablemente. A principios de la década de los setenta la mayoría de análisis de pesticidas se realizaba por cromatografía de gases utilizando detectores como ECD, NPD y FPD. Hoy en día, utilizando GC-MS la detección y confirmación de residuos de pesticidas puede realizarse mediante un solo análisis con una sensibilidad similar a la de los detectores clásicos empleados en cromatografía de gases. Otra ventaja del acoplamiento CG-MS es la selectividad de la técnica, que puede ajustarse seleccionando los fragmentos

moleculares apropiados evitándose así interferencias de matriz [Alder, 2006]. De todos modos, detectores como el de micro-captura electrónica, con gran sensibilidad hacia compuestos halogenados, siguen siendo ampliamente usados en el análisis rutinario de muestras medioambientales, recurriéndose al sistema GC-MS para la confirmación de positivos.

Los métodos basados en cromatografía líquida se aplicaron en menor medida en el pasado, ya que con los detectores ultravioleta tradicionales (DAD) no se conseguía la sensibilidad ni la selectividad necesaria. En los últimos años, la aparición de equipos que permiten la ionización a presión atmosférica supuso un cambio importante. Comparados con los detectores tradicionales, el electrospray (ESI) y la ionización química a presión atmosférica (APCI) en combinación con espectrómetros de masas han incrementado enormemente la sensibilidad de esta técnica cromatográfica [Alder, 2006]. Existen numerosas aplicaciones de la PLE a la extracción de pesticidas en las distintas matrices, algunas se recogen en la *tabla II.15*.

Tabla II.15. *Estudios que hacen uso de la PLE para la determinación de residuos de pesticidas en distintas matrices.*

Matriz	Grupo de Pesticidas	Método de determinación	LOD	Referencia
Cereales	405 Pesticidas	GC-MS / LC-MS	0.5-300 μg Kg^{-1}	Pang, 2006
Aguas y suelos agrícolas	OPPs	GC-MS	0.57-5.37 ng mL^{-1}	Hildebrand, 2007
Leche	Pesticidas	GC-MS/MS	0.01-2.6 μg Kg-^{1}	Mezcua, 2007
Alimentos	OPPs	GC-FPD	–	Obama, 1997
Semillas de colza	Pesticidas	GC-(TSD/PFPD/ECD)	–	Pihlström, 2002
Pescado	OCPs y PCBs	HRGC-ECD	–	Suchan, 2004
Pescados	OCPs, PCBs y PBDEs	GC-MS	–	Ghosh, 2011
Carne	OCPs y OPPs	GC-MS/MS	0.02-1.50 μg Kg^{-1}	Frenich, 2006
Pescados, moluscos	OCPs y PCBs	GC–MS-NCI	0.06-7.48 ng g^{-1}	Murad, 2012
Mejillones	Pesticidas	U-HPLC-MS/MS	1-10 ng g^{-1}	Wille, 2011
Productos alimentarios	Pesticidas	GC-MS/MS LC-MS/MS	0.001-0.100* mg Kg^{-1}	Garrido Frenich, 2005
Frutas	Pesticidas	LC-MS	0.025-0.25 mg Kg^{-1}	Blasco, 2005
Vegetales	Pesticidas	LP-GC-MS/MS	0.01-2.50 ng g^{-1}	Moreno, 2006
Vegetales	Pesticidas	GC-MS	3-8 μg Kg-1	Tanaka, 2007

Tabla II.15. *Continúa*

Matriz	Grupo de Pesticidas	Método de determinación	LOD	Referencia
Vegetales	Pesticidas	GC-MS	3-8 μg Kg^{-1}	Tanaka, 2007
Suelos	OPPs	GC-MS	3-31 ng g^{-1}	Díaz-Cruz, 2006
Suelos	OCPs	GC-MS	1.6-14.7 ng mL^{-1}	Hussen, 2007
Suelos	Piretroides	GC-MS/MS	0.26-0.87 ng g^{-1}	Luo, 2010
Agua y sedimentos	Avermectinas	LC-MS/MS	0.5-2.5 μg Kg^{-1}	Krogha, 2008
Lodos	Pesticidas	LC-MS/MS	10.3 μg Kg^{-1}	Baugros, 2009
Sedimentos marinos	OCPs, PCBs y PBDEs	GC-MS/MS	0.001-0.210 ng g^{-1}	Camino-Sánchez, 2011
Alimentos	OPPs y Piretroides	GC-MS	–	Chuang, 2011
Tabaco	Pesticidas	GC-MS/MS	0.003-0.150 μg g^{-1}	Haib, 2003
Productos marinos	Dioxinas y PBDEs	GC-MS	0.01-0.2 pg g^{-1}	Ashizuka, 2005

*LOQ: Limit of Quantification; OCPs: pesticidas organoclorados; PCBs: Bifenilos policlorados; OPPs: Pesticidas organofosforados; PBDEs: Difenil éteres polibromados

4.2 Extracción asistida por microondas (MAE)

La extracción asistida por microondas (MAE, microwave assisted extraction) ha surgido como una alternativa a la extracción Soxhlet ya que se mediante el calentamiento rápido y eficiente del disolvente/muestra se obtienen recuperaciones mejores o similares a las obtenidas en procedimientos de extracción clásicos. Desde el punto de vista de la química verde, ofrece ventajas sobre las técnicas convencionales como menor consumo de energía, menores volúmenes de disolventes, reducción de la cantidad de muestra y menor tiempo de extracción. Este último efecto es debido a la diferencia entre el calentamiento convencional, en el que el calor se transfiere a la disolución después de ser calentado el recipiente contenedor, y el calentamiento por microondas, que permite un calentamiento directo de las disoluciones [Lambropoulou, 2007; Tobiszewski, 2009; Sanchez-Prado, 2010].

4.2.1 Fundamento

Las microondas son ondas electromagnéticas cuya frecuencia oscila en el intervalo 300 MHz y 300 GHz y su longitud de onda (λ) entre 1 m y 1 cm. En este intervalo la radiación interacciona con la materia provocando el desplazamiento de iones y tránsitos rotacionales, pero no cambios estructurales. Para que se genere calor, el material debe tener propiedades dieléctricas. La capacidad de un material para absorber

energía de microondas y transformarla en calor depende del factor de disipación *"tan δ"*, que indica cuanta de esa energía se convierte en calor.

$$\text{Tan}\ \delta = \varepsilon''/\varepsilon'$$

siendo ε" la pérdida dieléctrica, es decir, la eficacia para convertir la energía electromagnética en calor; ε' la constante dieléctrica del material, que expresa la capacidad del material para absorber radiación (medida de la polarizabilidad de una molécula en un campo eléctrico).

Una característica de los materiales dieléctricos es su capacidad para almacenar energía eléctrica, que se lleva a cabo por el desplazamiento de cargas positivas y negativas bajo el efecto del campo eléctrico aplicado y en contra de las fuerzas de atracción molecular y atómica. La energía se disipa mediante dos mecanismos: la conducción iónica y la rotación dipolar. La conducción iónica es la migración de los iones disueltos cuando se aplica un campo eléctrico. La resistencia de la disolución a este movimiento origina fricción, calentando la disolución. La rotación dipolar se refiere al alineamiento de los dipolos con el campo aplicado. Cuando se elimina el campo eléctrico, se vuelve al desorden molecular causado por la agitación térmica, liberándose o disipándose la energía acumulada en forma de calor. El tiempo que se tarda en volver al desorden se llama tiempo de relajación dieléctrica (τ). La eficacia del calentamiento por rotación dipolar depende del tiempo de relajación dieléctrica, que a su vez depende de la viscosidad y temperatura de la muestra [Kington, 1998]. La máxima capacidad de calentamiento es característica de cada material y disminuye cuando se produce una disminución de la pérdida dieléctrica. El calentamiento por microondas es específico, no todos los materiales absorben microondas, los no conductores son transparentes, los conductores las reflejan y los dieléctricos las absorben.

La extracción se lleva a cabo en recipientes transparentes a la radiación y puede ocurrir por tres mecanismos dependiendo de si se utiliza:

- Un disolvente o mezcla de disolventes con coeficientes de pérdida dieléctrica altos.
- Una mezcla de disolventes con alta y baja pérdida dieléctrica.
- La muestra con pérdidas dieléctricas altas en un disolvente con pérdidas bajas.

Los disolventes polares tienen altos coeficientes de pérdida dieléctrica que producen elevadas temperaturas, así estas temperaturas altas aumentan la solubilidad del analito y aceleran la cinética de desorción del analito desde la matriz [Cela, 2001].

4.2.2. Instrumentación MAE

La instrumentación de microondas utilizada para el tratamiento de muestras se clasifica, en función de cómo se aplica la energía a la muestra, en *sistemas multimodo* y *sistemas de enfoque* [Luque, 2002]:

Sistemas multimodo, en los que la radiación de microondas se dispersa en una cavidad en la que se encuentra la muestra. Utiliza vasos cerrados. El disolvente puede calentarse a temperaturas superiores a su punto de ebullición a presión atmosférica; esto proporciona rapidez de extracción y eficiencia. Este sistema permite controlar la temperatura del proceso de extracción. Además permite varias extracciones simultáneas si se usan sendos recipientes. Como el campo eléctrico no es homogéneo en la cavidad, los recipientes se colocan en un soporte giratorio [Camel, 2000].

Sistemas de enfoque, en los que la radiación de microondas incide sólo sobre la zona en la que se sitúa la muestra, sometida a un campo electromagnético más intenso que en el sistema multimodo. Generalmente utiliza recipientes abiertos, a presión atmosférica. Como consecuencia la máxima temperatura posible viene determinada por el punto de ebullición del disolvente a esa presión. El sistema utiliza microondas enfocadas, siendo el calentamiento de la muestra muy homogéneo y eficaz.

El sistema comercial multimodo utilizado en esta memoria se muestra en la *figura II.14A*. Este sistema ha sido diseñado para trabajar con disolventes orgánicos y permite que hasta 12 vasos de extracción sean irradiados simultáneamente hasta alcanzar 1000 W de energía de microondas al 100 % de potencia. El sistema está formado por los siguientes componentes:

- Vasos o bombas de extracción (*figura II.14B*): son vasos de teflón con una capacidad de 100 mL, que resisten temperaturas máximas de trabajo de 260 ºC y una presión máxima de 35 bar (500 psi).
- Sistema de control de temperatura mediante una sonda colocada en el interior del vaso de referencia.

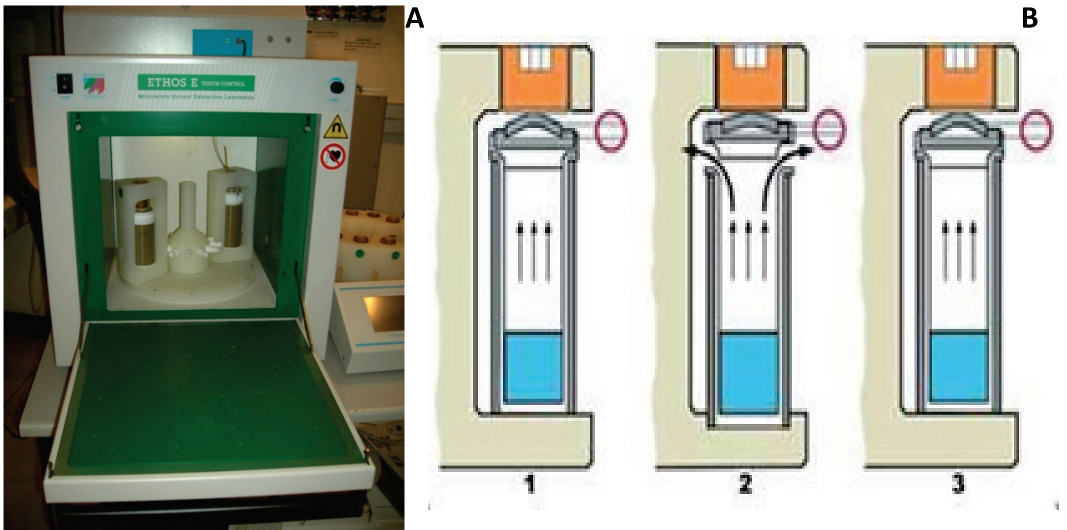

Figura II.14. *A, sistema de extracción asistida por microondas Milestone®. B, Venteo-sellado de los vasos.*

4.2.3 Parámetros que afectan a la eficacia de la extracción

La potencia del microondas y el tiempo de exposición, temperatura y presión, tipo de disolvente y naturaleza de la muestra, son los parámetros más estudiados.

- La ***potencia del microondas*** y el ***tiempo de exposición*** tienen efectos contrarios; para un proceso dado, una potencia elevada de microondas proporciona un tiempo de exposición bajo y viceversa.
- La ***temperatura*** es una variable clave ya que afecta a los procesos de extracción, la degradación de especies termolábiles y la solubilidad de algunas sustancias. Si se trabaja a una temperatura superior al punto de ebullición del disolvente la eficacia de la extracción aumenta porque aumenta la desorción de analitos de la matriz y la capacidad de solubilización de los disolventes, mientras que disminuye la tensión superficial y la viscosidad del disolvente (Eskilsson, 2000).
- ***Disolvente:*** La selección del disolvente adecuado es fundamental para conseguir una extracción eficaz. Para su elección debe considerarse su comportamiento ante la radiación de microondas, su interacción con la matriz y la solubilidad de los analitos en él. Los disolventes polares absorben la energía de microondas fácilmente y se calientan mientras están sometidos a la radiación (mayor constante dieléctrica ε, mayor interacción). La extracción de compuestos no polares requiere una mezcla de disolventes de diferente polaridad, como por ejemplo hexano-acetona.
- El ***volumen del disolvente*** también es importante para una extracción eficaz; debe ser suficiente para asegurar que cubra la totalidad de la muestra y normalmente está en el rango de 10-30 mL [Eskilsson, 2000].
- ***Naturaleza de la matriz:*** el contenido en agua de la matriz es de gran importancia porque las moléculas de agua tienen un elevado momento dipolar, así que absorben la radiación microondas

fuertemente, produciendo un calentamiento más efectivo de la muestra. Como consecuencia obtener resultados reproducibles requiere el control del contenido en agua de la matriz [Camel, 2000; Luque, 2002].

4.2.4 Ventajas y desventajas de la técnica

Las principales ventajas de la extracción asistida por microondas son: la rapidez (tiempos de extracción cortos), la posibilidad de utilizar diferentes temperaturas, bajo consumo de disolventes, alta eficacia de la extracción, posibilidad de automatización y de extracción simultanea de diferentes muestras. La principal desventaja de MAE es la falta de selectividad en comparación con otras técnicas, como la SFE, extracción con fluidos supercríticos, siendo necesaria una etapa adicional de limpieza para la eliminación de componentes de la matriz que son co-extraídos con los analitos. También antes de su determinación los extractos deben ser centrifugados o filtrados. Además, puede considerarse como desventaja la escasa eficacia de las microondas cuando los compuestos de interés o los disolventes son no polares.

4.2.5 Análisis de pesticidas mediante MAE

La extracción asistida por microondas (MAE), también ha sido utilizada para la extracción de pesticidas de diversas muestras [Le Doux, 2011; Albaseer, 2010; Weichbrodt, 2000]. La aplicación de energía de microondas como fuente de calor produce el calentamiento selectivo de la matriz con el extractante. La temperatura muy localizada y la presión alcanzada causan la migración selectiva de los compuestos de interés desde la matriz al disolvente circundante más rápidamente y con recuperaciones similares a las obtenidas con los métodos de extracción convencionales [Paré, 1997]. A continuación, en la *tabla II.16*, se presentan diferentes procedimientos basados en la extracción asistida por microondas que han sido aplicados para la extracción de pesticidas de matrices complejas.

Tabla II.16. *Estudios que hacen uso de la MAE para la determinación de residuos de pesticidas en distintas matrices.*

Matriz	Grupo de Pesticidas	Método de determinación	LOD	Referencia
Vegetales	7 OCPs	GC-MS	0.15-0.54 μg Kg^{-1}	Zhao, 2012
Vegetales	Thiamethoxam	HPLC-DAD	0.03 μg g^{-1}	Karmakar, 2012
Plantas	21 OPPs	GC-ECD	–	Barriada-Pereira, 2003
Vegetales	OCPs	GC-ECD	0.2-2 ng g^{-1}	Barriada-Pereira, 2007
Frutas y vegetales	72 pesticidas MAE-d-SPE	GC-MS	0.002-0.02 mg Kg^{-1}	Satpathy, 2011
Plantas	27 pesticidas OPPs,OCPs,PYR, carbamatos	GC-MS	0.0002-0.01 mg Kg^{-1}	Mao, 2012
Plantas	16 OPPs MAE-d-SPE	GC-MS	0.001-0.009 mg Kg^{-1}	Wan, 2010
Naranjas	Atrazina y 4 OPPs	GC-NPD	–	Bouaid, 2000
Uvas	8 Fungicidas	GC-MS	0.7-1.7 mg Kg^{-1}	Lagunas-Allué, 2011
Aguas	OCPs (DDT y metabolitos)	GC-ECD	20-30 ng L^{-1}	Kumar, 2011
Suelos	PYRs	GC-MS	1-200 μg L^{-1} ECD 0.3-2 μg L^{-1} NCI-MS	Albaseer, 2010
Suelos	PYRs	GC-MS	1-200 μg L^{-1} ECD 0.3-2 μg L^{-1} NCI-MS	Albaseer, 2010

OPPs: organophosphorated pesticides; OCPs: organoclorinated pesticides; MAE-d-SPE: microwave-assisted extraction -dispersive solid-phase extraction

En la *tabla II.17* se muestran otras aplicaciones de MAE a diferentes matrices para la extracción-determinación de otros compuestos organohalogenados.

Tabla II.17. *Extracción de compuestos organohalogenados de diferentes matrices mediante MAE.*

Matriz	Analitos estudiados	Disolvente extracción	Condic. Extracción	Limpieza	Método de determinación	Ref.
Suelo Pescado	PCBs PBDEs	30 ml hexano/acetona (1:1 v/v)	115ºC 10 min	10 g silica gel ácida+alumina	HRGC/HRMS	Wang, 2010
Cenizas	PCBs CBzs	Agua	120ºC 15 min	Columna sílica, sílica ácida y Na_2SO_4anh.	GC-MS	Sun, 2005
Cenizas	PCBs	30 ml hexano/acetona (1:1; v/v), tolueno o diclorometano	90-110ºC 10 min	Columna Na_2SO_4anh., florisil y alúmina	GC-ECD	Ramil, 2003
Tejidos biológicos marinos	PBDEs	25 ml pentano/DCM (1:1; v:v)	115ºC 10 min	1.Columna sílica ácida 2. GPC.	GC-MS	Bayen, 2004
Sedimentos de río	PCBs	Hexano	2 min 80 kPa	Columna sílica gel y Na_2SO_4.	GC-ECD	Gfrerer, 2005
Suelo	PCBs	25 ml hexano/acetona (1:1; v/v)	110ºC 10 min 1200W	Columna Na_2SO_4anh., sílica	GC-ECD	Sporring, 2005
Membranas de SPME	OCPs PCBs PAHs	33 ml distintos dtes.	750 W 3 ciclos	GPC	HPLC y GC	Yusà, 2005
Tejido adiposo materno	PBDEs	25 ml hexano/DCM (1:1; v:v)	115ºC 10 min.	Columna sílica ácida	GC-MS	Li, 2005
Productos marinos	PBDEs	Hexano	100ºC 10 min 1500 psi	Columna florisil	HRGC/HRMS	Ashizuka, 2005
CMR de sedimentos	PCBs OCPs	Hexano: Acetona	145ºC 20 min	SPE sílica	HPLC	Numata, 2007

HRGC/HRMS: high-resolution gas chromatography coupled with high-resolution mass spectrometry.

En la extraction asistida por microondas es frecuente que los constituyentes de la matriz sean co-extraidos y por tanto interfieran en la identificación y cuantificación de los analitos. Además, los compuestos co-extraidos, especialmente los lípidos, tienden a quedar retenidos en el inyector y en cabeza de columna del sistema cromatográfico, afectando a la resolución cromatogáfica [Hong, 2004]. Una etapa adicional de purificación de los extractos minimiza el efecto matriz, mejora la sensibilidad, la reproducibilidad y se alarga la vida de las columnas cromatografícas. En la bibliografía aparecen descritos diferentes procedimientos de *clean-up* para eliminar las interferencias co-extraidas en el análisis de

pesticidas en matrices complejas: la centrifigación a bajas temperaturas [Hong, 2004], etapa adicional de extracción líquido-líquido, cromatografía de exclusión en gel (GPC) [Suchan, 2004], extracción en fase sólida [Doong, 1999; LeDoux, 2011], extracción en fase sólida dispersiva (d-SPE) [Mao, 2012] y microextracción en fase sólida [Carro, 2007].

4.3 Dispersión de la matriz en una fase sólida (MSPD)

La dispersión de la matriz en fase sólida (MSPD, *matrix solid-phase dispersion*), es una técnica de preparación de muestra que fue introducida por Barker en 1989 [Barker, 1989] y se ha demostrado que es una técnica eficiente para el aislamiento de un amplio rango de drogas, pesticidas y otros componentes procedentes de una gran variedad de muestras vegetales y animales [Bogialli, 2007; Barker, 2007]. Este método es aplicable como un proceso analítico para la preparación, extracción y fraccionamiento de muestras sólidas, semi-sólidas y líquidos viscosos. Su simplicidad y flexibilidad han contribuido a que, en muchas ocasiones, sea escogida sobre otros métodos similares. MSPD se basa en principios simples que involucran fuerzas aplicadas a la muestra por mezclado mecánico para producir la completa dispersión y las máximas interacciones de la muestra con un soporte sólido [Bogialli, 2007, Barker, 2007].

La MSPD presenta características únicas como método de preparación de muestra. El uso de condiciones de trabajo suaves (temperatura ambiente y presión atmosférica) con una combinación apropiada de dipersante y disolvente de elución, proporciona buenas recuperaciones y selectividades. Una ventaja adicional que presenta es el bajo coste por extracción, ya que no se necesita instrumentación excesivamente cara ni se consumen demasiados reactivos. En su concepción original, la mezcla de una fase sólida con una muestra biológica, actúa de dos maneras. Por un lado, como un abrasivo al cortar y moler induciendo la ruptura de la muestra y por otro lado como un disolvente de unión que logra la dispersión completa de la muestra [Barker, 2007].

4.3.1 Fundamento

En el proceso de dispersión de la matriz en fase sólida, la muestra (fruta, algas…), se sitúa en un mortero de vidrio o ágata que contenga el dispersante apropiado y se homogeniza manualmente. Este material homogeneizado se transfiere a un cartucho apropiado para su posterior elución. El proceso se muestra en la *figura II.15* [Barker, 2007]. De manera más detallada, la técnica de MSPD consta de los siguientes pasos [Kristenson, 2006]:

- Una muestra líquida, viscosa, semi-sólida o sólida, se introduce en un mortero de vidrio y se mezca con un dispersante sólido para obtener la ruptura y dispersión total de la muestra en el dispersante. La

cantidad de dispersante a utilizar depende del tipo de muestra y las proporciones más comunes muestra: dispersante están entre 1:1 y 1:4.

- Cuando la mezcla es completa, la muestra debe ser introducida en una columna apropiada que contenga en su interior la fase sólida y la co-columna que se utiliza para limpieza de los extractos. La columna suele ser una jeringuilla o un cartucho vacío o que contiene en su interior una frita de polipropileno o acero inoxidable o un filtro de celulosa en el fondo. Una segunda frita es situada en la parte superior de la muestra antes de la compresión con un émbolo.

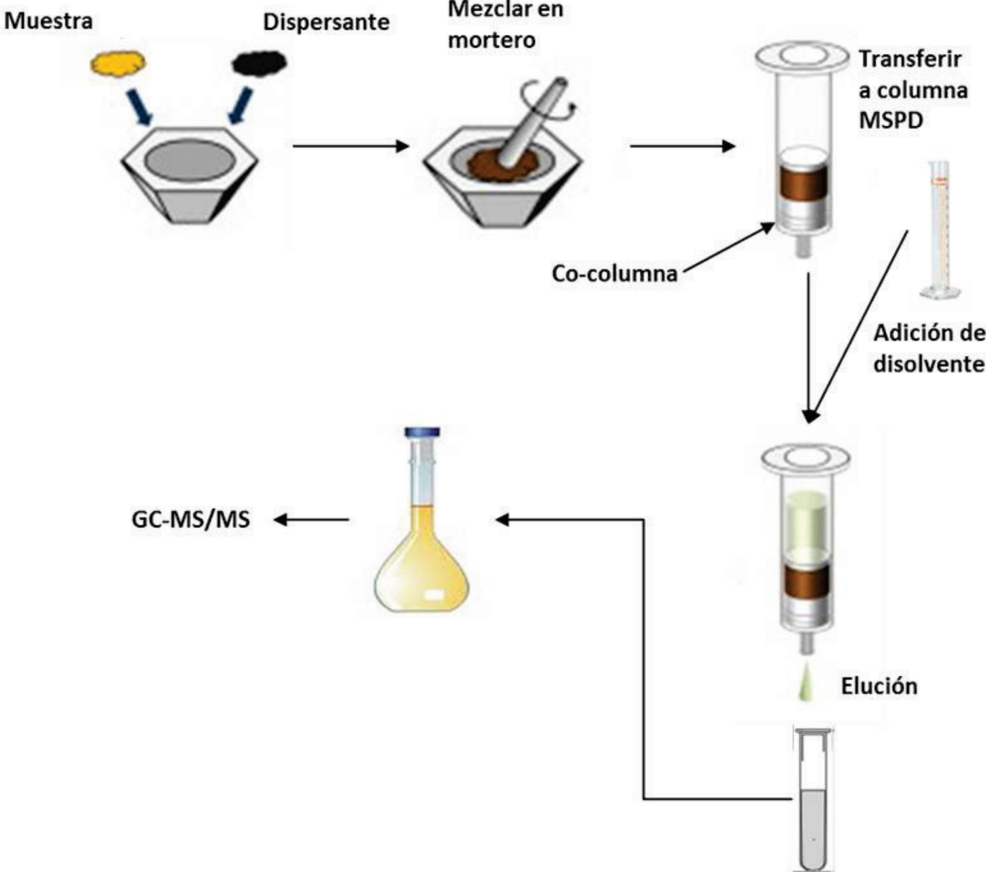

Figura II.15. *Esquema del proceso de MSPD.*

- En lo que respecta a la elución, existen dos posibilidades:

  - Los componentes interferentes de la matriz son eluidos en una primera etapa mientras que los analitos, son retenidos en la columna y serán extraídos posteriormente con un eluyente distinto.
  - Las interferencias son retenidas selectivamente en la columna y los analitos son extraídos directamente.

- Finalmente, se realiza una limpieza adicional. En ocasiones, el reactivo que se utiliza para limpieza se puede empaquetar en el mismo cartucho.

4.3.2 Factores que afectan a la extracción mediante MSPD

Existen diversos factores que han de ser examinados cuando se desarrolla un procedimiento de dispersión de la matriz en fase sólida. Entre ellos, se incluyen [Barker, 2007]:

1. ***Efecto del diámetro medio de partícula:*** Si se trabaja con tamaños de partícula muy pequeños (3-10 µm de diámetro), tienden a aumentar los tiempos de elución, así como la necesidad de mayores presiones o incluso vacío para obtener un flujo adecuado. Se puede utiliza una mezcla de sílices que posee un rango de tamaños de partícula de 40-100 µm.
2. ***El carácter del dispersante escogido:*** Distintas polaridades pueden tener distintos efectos.
3. ***La cantidad adecuada de soporte sólido:*** Varía mucho dependiendo de cada proceso.
4. ***Modificaciones químicas que puedan realizarse:*** La adición de agentes quelatantes, ácidos o bases, pueden afectar a la elución de los analitos de interés.
5. ***Elección del eluyente:*** El eluyente aísla los analitos de las sustancias interferentes, pero cantidades distintas de distintos tipos de eluyentes, pueden actuar de manera diferente.
6. ***Volumen del eluyente:*** Se ha observado que usando 8 mL de elución para 2 gramos de mezcla (con 0.5 g) los analitos eluyen en los primeros 4 mL, pero este dato varía en función de cada experiencia. Este parámetro debe estudiarse en cada proceso de exteacción, para reducir el uso excesivo de disolvente de elución y evitar que se extraigan interferencias.

Los eluatos obtenidos en MSPD pueden ser llevados directamente al instrumento de medida si se encuentran suficientemente limpios (para ello puede usarse la co-columna referida anteriormente).

4.3.3 Análisis de pesticidas mediante MSPD

Actualmente, existe un gran número de aplicaciones en las que se utiliza dispersión de la matriz en fase sólida. A continuación, se muestran en la *tabla II.18* los resultados obtenidos de la utilización de esta técnica para la extracción de pesticidas en diferentes matrices [Picó, 2007].

Tabla II.18. *Estudios que hacen uso de la MSPD para la determinación de residuos de pesticidas en distintas matrices.*

Matriz	Grupo de Pesticidas	Método de determinación	LOD	Recuperación (%)	Referencia
			Muestras sólidas		
Pescados	16 OCPs 7 PCBs	GC-ECD GC-MS	<MRL	–	Papadopoulos, 2011
Pescados	16 OCPs	GC-MS GC-ECD	0.008-0.05 ng g^{-1}	91.0-104.1	Shen, 2011
Pescados	6 OCPs	GC-ECD	0.4-1.2 ng g^{-1}	39.1-81.5	Rezaei, 2011
Frutas, vegatales	4 Atrazine	MEKC	12.9-31.5 ng g^{-1}	53.5 – 98.4	Wen, 2012
Piensos	OCPs, PYR	GC-MS	0.03-1.5 ng g^{-1}	73-125	Fernandez-Alvarez, 2009
Hígado bovino	9 OPPs	HPLC-DAD	0.04-0.25 µg g^{-1}	91-101	Gutiérrez-Valencia, 2011
Vegetales, alimentos	3 PHU	EC	0.1-0.2 µg L^{-1}	78.1- 93.8	Wang, 2011
Agua coco liofilizada	2 BzPU 1 PYR	HPLC-UV	0.04-0.05 mg kg^{-1}	74-116	Santana-Santos, 2012
			Muestras líquidas		
Zumos de frutas	Pesticidas	LC-MS/MS	0.07-0.9 ng L^{-1}	77-102	Perret, 2002
Zumo de frutas	OPPs[a]	GC-NPD	0.1-0.6 ng L^{-1}	70-110	Albero, 2003
Leche bovina	Pesticidas	HPLC-ESI-MS/MS	5-360 ng L^{-1}	> 75	Dagnac, 2009
			Muestras semi-sólidas (no grasas)		
Miel	OPPs	GC-NPD	6-15 ng g^{-1}	81-100	Sanchez-Brunete, 2002
Frutas y vegetales	Fungicidas Insecticidas	GC-ECD/MS	5-50 ng g^{-1}	70-105	Torres, 1997
Naranjas	OPPs y OCPs	GC-ECD	2-170 ng g^{-1}	67-102	Torres, 1996

Tabla II.18. *Continúa.*

Matriz	Grupo de Pesticidas	Método de determinación	LOD	Recuperación (%)	Referencia
		Muestras semi-sólidas (grasas)			
Aceites semillas	27 OPPs 23 OCPs, 11 PYR, 7 carbamatos	GCxGC-TOF/MS	< MRL	–	Wang, 2012
Insectos	OPPs y PYRs	GC-MS	5-80 ng g^{-1}	52-94	Kristenson, 2004
Grasa de ternera	OCPs	GC-ECD	<31.3 ng g^{-1}	85-102	Long, 1991
Huevos	Avermectinas	UHPLC-MS-MS	5 ng g^{-1}	2-32	Garrido-Frenich , 2010

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III SECCIÓN EXPERIMENTAL
RESULTADOS Y DISCUSIÓN

III. Sección experimental. Resultados y discusión

1. Desarrollo de métodos de análisis para la determinación de pesticidas en agua de mar

Relación de trabajos publicados en este capítulo:

A.M. Carro, S. Fernández, I. Racamonde, D. García-Rodríguez, P. González, R.A. Lorenzo *"Dispersive liquid–liquid microextraction coupled with programmed temperature vaporization-large volume injection-gas chromatography–tandem mass spectrometry for multiclass pesticides in water"* **J. Chromatogr. A (2012) 1253, 134-143**

Diego García-Rodríguez, Antonia M. Carro, Rosa A. Lorenzo, Fátima Fernández, Rafael Cela *"Determination of trace levels of aquaculture chemotherapeutants in seawater samples by SPMEGC-MS/MS"* **J. Sep. Sci. (2008) 31, 2882-2890**

Póster: ***"Analysis of 18 pesticides in water samples using a microdispersive technique (DLLME) and programmed temperature vaporization-based large volume injection-gas chromatography-tandem mass spectrometry (PTV-LVI-GC-MS/MS)"*** 13as Jornadas de Analisis Instrumental, 14-17 Noviembre 2011, Barcelona (España)

Póster: ***"Determination of aquaculture chemotherapeutants in seawater by DI-SPME-GC-MS/MS"*** 32nd International Symposium on Capillary Chromatography and 5th GCxGC Symposium, 26-30 Mayo 2008, Riva de Garda (Italia).

1.1. Determinación de niveles traza de agentes quimioterapúticos usados en acuicultura en muestras de agua de mar mediante microextracción en fase sólida (SPME) y cromatografía de gases y espectrometría de masas-masas (SPME-GC-MS/MS)

El objetivo de este trabajo ha sido la puesta a punto de un método basado en la microextracción en fase sólida para la determinación de dos pesticidas organofosforados (diclorvos y clorpirifos) y tres piretroides (permetrina, α-cipermetrina y deltametrina) en muestras de agua de mar procedente de áreas próximas a zonas de cultivos marinos. Se eligieron estos pesticidas por su uso como agentes quimioterápicos en el tratamiento de infecciones parasitarias debidas al piojo de mar (copépodos) y otras enfermedades presentes en las granjas de cultivo de salmónidos. Estas sustancias aparecen frecuentemente como los ingredientes activos de varias formulaciones comerciales: Lorsban® (clorpirifos), Excis® (α-cipermethrin), Permanone® (permetrina) y Alphamax® (deltametrina).

La SPME permite la extracción simultánea y la preconcentración de analitos de una muestra de forma relativamente rápida y sin uso de disolventes. En la extracción directa, utilizada en este estudio, la fibra se introduce directamente en la muestra líquida y los analitos son transportados desde la matriz de la muestra al recubrimiento polimérico de la fibra.

La optimización del proceso de extracción se realizó empleando la cromatografía de gases con un detector de microcaptura electrónica (µECD) y la confirmación de la identidad de dichos residuos se llevó a cabo por cromatografía de gases acoplada a la espectrometría de masas en tándem. La determinación cromatográfica se optimizó utilizando como patrones de cuantificación extractos de agua de mar a los que se les adicionó la mezcla de compuestos. Se había observado un efecto matriz en los extractos de muestras de control, enriquecidos con los pesticidas, en comparación con los patrones preparados con disolventes. La existencia del efecto matriz era de esperar dada la alta concentración salina del agua de mar. En todos los casos, excepto para el diclorvos, la sensibilidad (pendiente de la recta) disminuye cuando las medidas se realizan en agua de mar. Paralelamente se ha llevado a cabo la medida de la relación de área de picos con relación a los patrones internos ((dietil-D_{10})-clorpirifos para los pesticidas fosforados y (fenoxi-$^{13}C_6$)-cis-permetrina para los piretroides).

La optimización del proceso de SPME se realizó utilizando un diseño central compuesto de dos factores (temperatura y volumen de muestra). Para la extracción simultánea de los piretroides y organofosforados se buscaron unas condiciones de compromiso calculadas mediante una función de deseabilidad global. Estas condiciones óptimas han sido 40ºC y 20 mL para el sistema GC-µECD y 75ºC y 20 mL para GC-MS/MS. Para definir los tiempos de extracción que se usarán en la SPME de los distintos analitos, se estudiaron las cinéticas de extracción para las distintas familias de pesticidas y para el conjunto

global. Se seleccionaron los menores tiempos posibles ya que, aunque el proceso de extracción es menos eficiente, el sistema de detección es suficientemente sensible.

El método SPME-GC-µECD desarrollado permite la determinación de residuos de los analitos elegidos a niveles traza del orden de pg ml^{-1}. La combinación de SPME con GC-MS/MS es una herramienta de gran poder para la identificación y confirmación de los analitos de interés en muestras de agua de mar relacionadas con cultivos marinos.

Se analizaron muestras de agua de mar fortificadas con los pesticidas estudiados, a falta de materiales de referencia certificados. En ningún blanco de muestra se han detectado concentraciones de estos residuos.

DETERMINATION OF TRACE LEVELS OF AQUACULTURE CHEMOTHERAPEUTANTS IN SEAWATER SAMPLES BY SPME-GC-MS/MS

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Abstract

A sensitive and efficient solid-phase microextraction (SPME) method for the determination of organophosphorous (OPPs) and pyrethroid pesticides (Pyrs) in aquaculture-seawater samples using gas chromatography with tandem mass spectrometry (GC-MS/MS) was developed. Dichlorvos and chlorpyrifos (OPPs); permethrin, alpha-cypermethrin and deltamethrin (Pyrs) were selected according to their use as chemotherapeutants in the aquaculture industry. Different parameters affecting extraction efficiency such as fibre coating, agitation, pH and extraction time profiles were investigated. An experimental central composite design (α=1) and desirability functions were used for the simultaneous optimization of extraction temperature and sample volume. Finally, a method based on direct SPME in 40 min at 75 ºC using 100-µm-thick polydimethylsiloxane (PDMS) fibre and 20 mL of sample volume is proposed. The method was validated, exhibiting good linearity, precision and accuracy parameters with pg mL^{-1} limits of detection. The proposed methodology was applied to determine the ultra-trace levels of OPPs and Pyrs in aquaculture-seawater samples by the standard addition approach, which proved to be reliable and sensitive, in addition to requiring only small amounts of sample.

Keywords: Aquaculture chemotherapeutants; pyrethroid and organophosphorous pesticides; seawater; solid-phase microextraction; desirability function; gas chromatography-mass spectrometry.

1. Introduction

The regulation and control of the chemotherapeutants used in aquaculture has become a matter of prime importance [1, 2]. Sea lice infestations represent the most significant disease problem currently affecting the aquaculture of salmon, turbot and trout [3]. Organophosphorus pesticides (OPPs) and synthetic pyrethroids (Pyrs) are active ingredients commonly used in commercial formulations (Table 1) to control different infestations in farmed fish [4, 5]. When fish are immersed in a bath containing chemotherapeutic agents, pesticide residues are released directly into the marine environment. After several treatments, these substances can accumulate in the environment, having an impact on cultured species, wild aquatic organisms and humans through the food chain. [1, 6-8].

OPPs are cholinesterase inhibitors and affect both the host and the parasite. Pyrs have become an important alternative to the highly toxic OPPs in recent years. Nevertheless, synthetic Pyrs are more toxic (neurotoxic) and persistent than natural pyrethrins; some of the newest Pyrs persist in the environment for months [9, 10]. OPPs and Pyrs have been found in varying concentrations in natural and drinking water [9-14], vegetables [15-18], sediments [19, 20], fish [21, 22] and vegetable oils [23, 24]. The determination of pesticides in seawater is a difficult task because of the salinity matrix effect [25]. This is especially true in seawater, where concentrations of organic contaminants are low and a preconcentration step is required. One of the drawbacks of this procedure is that it concentrates other co-extracted substances. For this reason, a strategy for reducing the matrix effect through standard addition or isotope dilution was evaluated [26].

Currently, electron capture detection (ECD) and mass spectrometry (MS) are the detection methods of choice in pesticide trace analysis [27]. Lower detection limits for many trace analytes can be obtained using MS, but ECD is still a powerful detection system, especially for screening environmental samples [14]. Tandem mass spectrometry (MS/MS) featuring excellent selectivity and sensitivity, allows the quantification of trace levels of pesticides due to background reduction [28, 29]. To quantify the extremely low concentration levels of contaminants in all types of water samples, efficient preconcentration and cleanup procedures are needed prior to the chromatographic separation stage. Solid-phase extraction (SPE) is the most widely used method for the enrichment of pesticides in water because it combines the advantages of convenience, low cost and a minimal consumption of organic solvents [10, 12, 22]. Solid-phase microextraction (SPME) allows the simultaneous extraction and preconcentration of pesticides from aqueous samples onto a fibre, removing them from the interfering matrix components before the chromatographic analysis is conducted [30, 31].

There is no record of previous investigations of OPPs (dichlorvos and chlorpyrifos) and Pyrs (permethrin, α-cypermethrin and deltamethrin) in aquaculture-seawater samples. Therefore the present study represents the first multi–residue method targeting their determination based on the extraction by fast direct immersion-SPME of the selected compounds onto a poly(dimethyl)siloxane (PDMS) fibre and subsequent determination by GC–MS/MS. SPME conditions were optimized by means of a multicriteria strategy which implements an experimental design and a total desirability function. The performance of the optimized methodology was also characterized in terms of accuracy, precision, linearity and limits of detection. Finally, the analytical method was applied to several real aquaculture seawater samples.

2. Experimental

2.1 Chemicals and reagents

Pestanal quality analytical standards of α-cypermethrin (99.8%), chlorpyrifos (99.2%), dichlorvos (99.7%) and permethrin (99.3%) were purchased from Riedel-de Haën (Seelze, Germany). Pestanal quality standard of deltamethrin was supplied by Chem Service Inc. (West Chester, PA, USA). Heptachlor (1000 µg mL^{-1} in methanol) was obtained from Supelco (Bellefonte, PA, USA). (Diethyl-D_{10})-chlorpyrifos (100 µg mL^{-1} in nonane) and (phenoxi-$^{13}C_6$)-*cis*-permethrin (50 µg mL^{-1} in nonane) were purchased from Cambridge Isotope Laboratories (Cambridge, UK). Ethyl acetate (Chromanorm), acetone (Pestinorm) and *n*-hexane (Pestinorm) were supplied by VWR-Prolabo (Mollet del Vallés, Barcelona, Spain). Methanol (gradient HPLC grade) was obtained from Merck (Darmstadt, Germany).

Individual stock solutions of Pyrs were prepared in acetone. Individual stock solutions of chlorpyrifos, dichlorvos and heptachlor were prepared in methanol. One stock solution containing the six pesticides was prepared in acetone. All solutions were stored at -18 ºC until use.

Table 1. *Chemoterapeutants currently used in aquaculture, structures of their active compounds and octanol-water partition coefficients.*

Commercial formulation	Active compound	Chemical structure	Log K_{ow}
Aquardard®, Nuvan®	Dichlorvos		1.47
Lorsban®	Chlorpyrifos		4.96
Permanone®	Permethrin		6.50
BetaMax®, Excis®	Cypermethrin		6.00
AlphaMax®	Deltamethrin		6.20

Table 2. *Specific MS/MS conditions for each pesticide and IS.*

Pesticide	Segment	*m/z* range	Parent ion (*m/z*)	Exc. Amp. (V)	Exc. Stg. Lvl. (*m/z*)	Quant. Ions *(m/z)*
Dichlorvos	2	100-250	186	0.75	81.4	109+131
Chlorpyrifos	3	100-350	314	84.0	138.5	286+258
Permethrin	4	100-250	183	77.0	80.5	168+153
α-Cypermethrin	5	100-250	181	89.0	79.7	152
Deltamethrin	6	100-300	253	76.0	111.5	172
(D_{10})-chlorpyrifos (I.S.)	3	100-350	324	84.0	142.9	292+260
($^{13}C_6$)-*cis*-permethrin (I.S.)	4	100-350	189	77.0	83.2	174+159

2.2 Instrumentation

Commercially available 100 µm polydimethylsiloxane (PDMS), 65 µm polydimethylsiloxane-divinylbenzene (PDMS-DVB) and 85 µm polyacrylate (PA) fibres and fibre holders for manual sampling were purchased from Supelco. The fibres were conditioned before use by heating in a gas chromatograph injection port for 30 min at 250 ºC.

Agilent 6890N GC (Avondale, PA, USA) equipped with a micro electron-capture detector was employed for the GC-µECD determination of the analytes using a 30 m × 0.32 mm i.d. HP-5 (5% polydiphenylsiloxane; Agilent Technologies) fused-silica column (0.25-µm film thickness). Injections with SPME were made in splitless mode (3 min). Split flow was set at 30 mL/min. Initial oven temperature was

set at 60 ºC, held for 3 min; ramped at 20 ºC min^{-1} up to 280 ºC and held for 8 min. Helium (purity 99.999%; Carburos Metálicos, A Coruña, Spain) was employed as the carrier gas with a constant pressure of 13 psi. Nitrogen (purity 99.999%; Carburos Metálicos) was used as the make-up gas. Quantification was accomplished by relative areas versus heptachlor used as internal standard (IS), which was added just before the SPME process.

GC-MS/MS analyses were performed in a Varian 3900 GC (Walnut Creek, CA, USA) coupled to an ion trap mass spectrometer Varian Saturn 2100T. Separation was carried out on a HP-5MS (5% polydiphenylsiloxane; Agilent Technologies) fused-silica column (30 m × 0.25 mm i.d. x 0.25-µm film thickness) with helium (purity 99.999%) as carrier gas with at a constant flow of 1.2 mL min^{-1}. Split flow was set at 50 mL min^{-1}. Injection conditions as well as oven temperature program were similar to the GC-µECD conditions described above. The mass spectrometer was operated in electron impact mode at 70 eV. The trap, manifold and transfer line temperatures were maintained at 220, 100 and 280 ºC, respectively. The analyses were performed with a filament-multiplier delay of 6 min. General parameters were as follows: multiplier offset +100 V and AGC target value 4000 counts. Specific conditions for each analyte are listed in table 2. Quantification was accomplished by relative areas versus (phenoxi-$^{13}C_6$)-*cis*-permethrin and (diethyl-D_{10})-chlorpyrifos used as ISs. Ultrapure water was obtained using a Milli-Q® water purification system (Millipore, Bedford, MA, USA).

2.3 Sample preparation

Seawater samples 1 to 5 were collected in the vicinity of different aquaculture factories located on the coast of Galicia (NW Spain). Sample 6 was collected from a turbot farm in Galicia. All samples were filtered using cellulose ester membrane filters (HAWP, 47 mm, 0.45 µm; Millipore) and stored at -18 ºC until analysis. Several parameters affecting extraction efficiency and selectivity were evaluated, as explained in the results and discussion section. The optimal SPME conditions were: 20 mL-aliquots of the samples extracted onto 100-µm-thick PDMS fibre by direct immersion for 20 min at 40 ºC in the SPME-GC-µECD method and 40 min at 75 ºC in the SPME-GC-MS/MS analytical approach. Samples were stirred magnetically at 400 rpm with PTFE-coated stir bars (3 mm in diameter and 7 mm long; Supelco) during the extraction procedure using an Agimatic-E laboratory hotplate/stirrer (Selecta, Barcelona, Spain).

3. Results and discussion

3.1 Influence of the fibre coating

Different relevant SPME parameters were evaluated. The OPPs and Pyrs studied have a wide range of polarities (log K_{ow}, Table 1) and physicochemical properties. Thus, three types of commercial fibres coated with different phases (PDMS, PA and PDMS-DVB) were tested and their extraction efficiencies for the target compounds were evaluated. Seawater samples were spiked at ng mL^{-1} level for the target compounds. DI-SPME was carried out at 60 ºC, 20 mL of sample volume, 40 min of extraction time, 400 rpm and 280 ºC on the chromatograph injection port. The extraction efficiency of the different fibres was evaluated using the GC-µECD system. The PDMS phase provided the best overall performance for Pyrs and chlopyrifos, whereas dichlorvos and the internal standard heptachlor were better extracted with PDMS-DVB fibre (Fig. 1). Therefore, the PDMS fibre was selected for further experiments. These results are in agreement with the published data [9, 32] indicating that PDMS is a more versatile coating than PDMS-DVB for the extraction of pesticide residues, exhibiting good reproducibility and linearity. Following our own experience and literature, desorption time and temperature were fixed at 5 min and 280 ºC [32, 33].

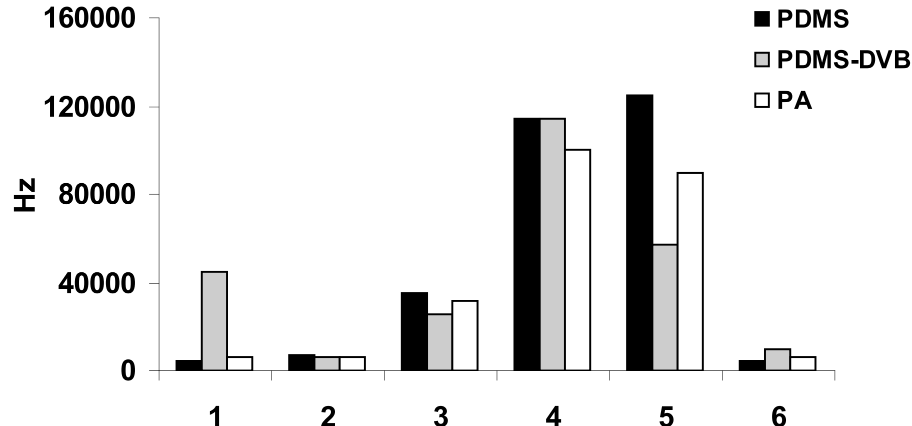

Figure 1. *Extraction efficiency of three different SPME fibre coatings. Compounds numbered as: 1, dichlorvos; 2, chlorpyrifos; 3, permethrin; 4, a-cypermethrin; 5, deltamethrin; 6, heptachlor (IS).*

3.2 Optimization of the SPME procedure by chemometric strategy

Regarding the extraction of non-ionic and non-polar species, such as OPPs and Pyrs, an increase in the ionic strength of the sample is expected to improve the efficiency of the extraction at the expense of slowing down its kinetics [11]. The relatively high ionic strength of seawater samples makes the addition of more ions unnecessary. Additionally, pH does not significantly affect the extraction of predominantly apolar and non-ionic pesticides [11, 31]. Thus, no salt addition or pH modifications were needed. On the other hand, sample agitation was confirmed to be essential for the optimum performance of the SPME, speeding up the equilibrium processes and the response obtained with similar extraction times [9, 31].

Extraction temperature and sample volume were the factors included in the optimization of the experimental design. Three levels of extraction temperature (25, 50 and 75 ºC) and sample volume (10, 15 and 20 mL) were selected to optimize the responses (analyte/IS peak-area ratios) using a composite design with three central points (α=1) [34]. The experimental design was generated and all analytical calculations were supported by Nemrod©W software [35]. Seawater samples were spiked at ng mL^{-1} level of Pyrs and OPPs compounds. All experiments were carried out using general conditions: 100-µm-thick PDMS fibre, 50 min of extraction time, sample agitation at 400 rpm and a temperature of 280 ºC on the GC-µECD injection port, where the fibre was desorbed for 5 min. As was expected, the ANOVA analysis showed that temperature has a statistically significant effect on all the compounds. The Pyrs extraction efficiency was favoured by high temperatures while OPPs extraction had a better efficiency at lower temperature values. In general, PDMS shows excellent selectivity for non-polar compounds such as Pyrs and the extraction, limited basically by mass transfer, is more efficient at higher temperatures [32]. The sample volume was only statistically significant for dichlorvos. High sample volumes showed a negative effect in the response of dichlorvos. The volume factor effect was not significant in Pyrs and chlorpyrifos responses. An analysis of the response surfaces led to the conclusion that the experimental conditions which maximize the analyte responses are the exact opposite for the two families of pesticides (Fig. 2). The maximum response for all Pyrs was achieved at high temperature (T = 75ºC) levels regardless of the volume (V) level (Fig. 2.A), while for chlorpyrifos, medium-low temperatures (T = 40ºC) and high volumes (V = 20 mL) are needed (Fig. 2.B). The maximum response of dichlorvos requires low levels of both factors (Fig. 2.C).

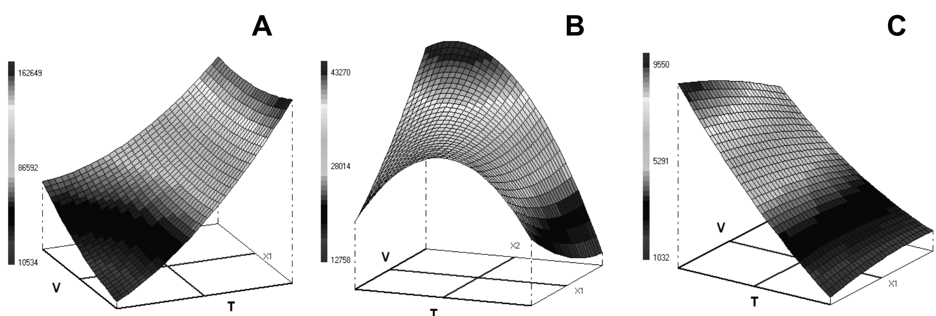

Figure 2. *Response surfaces for permethrin (A), chlorpyrifos (B) and dichlorvos (C). Response surfaces for a-cypermethrin and deltamethrin are similar to A.*

Thus, the individual analysis of the different responses does not lead to common experimental conditions. In order to obtain good compromise experimental conditions that fulfil the expectations of multiresidue analytical procedures, multicriteria methodology is often required [34]. This methodology is based on the construction of a desirability function for each individual response. Each individual desirability function (d_i) is a continuous function chosen from among a family of linear or exponential functions, which varies from zero (undesirable response) to 1 (optimal response). In this case, non-linear partial desirability

functions were easy to build. The global desirability function (*D*) was estimated as the geometric mean of d_i, but no additional experiments were required. Nemrod©W software incorporates an algorithm in the optimization process that is able to find the maximum of the overall desirability function.

The selectivity and sensitivity of the chromatographic systems were observed to be critical factors in the separation and determination of target pesticides when using SPME. These chromatographic responses are directly related to the parameters that define the quality of the extraction. They affect the separation that will allow for the correct identification and quantification of the compounds in the matrix studied. The optimal extraction conditions for OPPs and Pyrs in seawater were 40 ºC and 20 mL (Fig. 3.A) for the analysis of samples with GC-µECD. Under these conditions the *D* function achieved its maximum value of 0.69 and the predicted values of d_i were higher than 0.8 for all analytes, with the exception of permethrin (d_i = 0.30). With high temperatures (75 ºC) a greater amount of interfering compounds were extracted from the seawater matrix than with moderate values and a poor sensitivity for OPPs was obtained in the GC-µECD chromatogram. The maximum *D* obtained when using GC-MS/MS was 0.78 for 75ºC and 20 mL (Fig. 3.B). In this case, the predicted values of d_i ranged between 0.7 and 1, except for dichlorvos (d_i = 0.56). GC-MS/MS chromatograms showed, in general, low peak responses when the extraction temperature was set at 40 ºC. Our aim was to find the trade-off situation where all the response factors are optimized, considering the values that afford the best chromatographic quality. The optimization of an analytical procedure usually implies solving a conflict between the resolution of peaks, signal size and efficiency. Therefore, the optimal conditions needed to perform the SPME and the subsequent chromatographic determination of OPPs and Pyrs in seawater were: 40 ºC and 20 mL for the analysis of samples by GC-µECD and 75 ºC and 20 mL when GC-MS/MS is used.

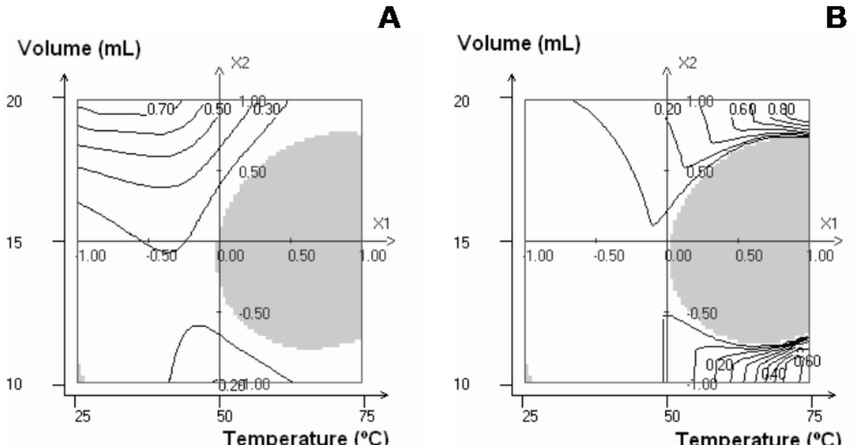

Figure 3. *Desirability (D) study: the isodesirability curves in the plane of sample volume and extraction temperature, for GC-µECD (A) and GC-MS/MS (B). The regions in grey correspond to null values for desirability and thus factor levels are not suitable for selection.*

Once the temperature and volume were set at the most favourable conditions, the extraction time profiles for each compound were studied. The exposure times for the PDMS fibre were studied between 10 and 80 min using the optimal conditions obtained above (Fig. 4). For most of the compounds investigated, the GC-µECD responses augmented, when the extraction time was increased until equilibrium was reached. Reaching equilibrium contributes to improved precision. SPME kinetic processes were slower for dichlorvos (Fig. 4.A). Chlorpyrifos, heptachlor (IS) and Pyrs showed two pseudo-equilibrium steps; the first, between 20 and 40 min and the second, between 60 and 80 min (Fig. 4.A and 4.B). Considering that the responses obtained after extraction for 40 min were very close to those obtained after 20 min for the majority of the compounds and that the goal was to develop a procedure that is fast, yet sufficiently sensitive (SPME-GC-µECD), the sampling time was set at 20 min.

When SPME conditions for GC-MS/MS (75 ºC and 20 mL) were applied to all target analytes and the kinetic study was performed under these conditions, equilibrium was reached more slowly (Fig. 4.C and 4.D) and 40 min were required to obtain a good instrumental response for all the compounds studied.

3.2 SPME-GC-µECD and SPME-GC-MS/MS performance

Chromatograms obtained by SPME-GC-µECD showed that the baseline separation among the compound peaks was appropriate to perform accurate calibrations. The quantification of Pyrs was done by adding up the peak areas of the isomers present in the chromatogram. Linearity for the different pesticides was studied in triplicate at four concentration levels by measuring the area ratio relative to the corresponding IS. Standard addition calibrations in seawater were carried out to avoid the matrix effect Analytical figures of merit obtained using SPME-GC-µECD for seawater samples are shown in table 3. Detection (LOD) and quantification (LOQ) limits of the analytical procedure were calculated for signal-to-noise ratios (S/N) of 3 and 10, respectively, with most of the pesticides studied being in the low part-per-trillion level. Repeatability was tested by seven consecutive micro-extractions of a spiked seawater sample carried out by the same analyst on the same day. Within-day relative standard deviations (%RSD) ranging from 6.1 to 16.5 were obtained. Reproducibility was tested on six replicates of fortified seawater samples carried out by the same analyst over three consecutive days. Between-day relative standard deviations obtained ranged from 8.4 to 12.9. In both cases all experiments were undertaken at concentration levels close to the quantification limits.

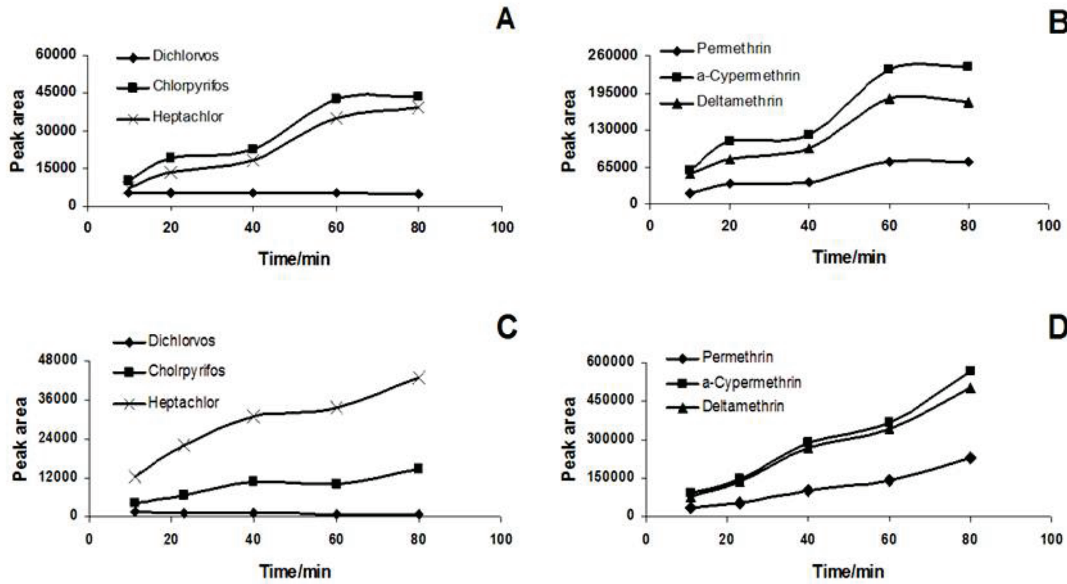

Figure 4. *Extraction time profiles for the target compounds at 40ºC (A and B) and at 75ºC (C and D).*

Table 3: *Figures of merit for the SPME-GC-μECD method.*

Pesticide	Linear ranges (pg mL^{-1})	R^2	Repeatability (%RSD; *n=7*)	Reproducibility (%RSD; *n=6*)	LOD (pg mL^{-1})	LOQ (pg mL^{-1})
Dichlorvos	60-300[a]	0.9989	16.5	12.9	15.6[a]	50.8[a]
Chlorpyrifos	5-500	0.9991	6.1	8.4	0.8	2.8
Permethrin	50-1000	0.9991	10.4	9.3	11.1	37.0
α-Cypermethrin	50-1000	0.9991	12.4	10.6	11.6	38.8
Deltamethrin	50-1000	0.9996	10.2	9.1	3.2	10.8

[a] Concentration given in ng mL^{-1}

Although the μECD is regarded as a sensitive and selective detection system in GC analysis, its selectivity may not be precise enough to analyse complex environmental samples like highly contaminated seawater samples. Using GC-MS/MS, the pesticides were separated and eluted in a short time under the chromatographic conditions described in the instrumentation section. In the MS/MS mode, a parent ion was chosen for each compound by taking into account their *m/z* and their relative abundance (both as high as possible) in order to increase sensitivity. A non-resonant waveform was selected for all compounds except for dichlorvos which needed more cleavage energy to obtain good quality secondary spectra. The optimization of the excitation amplitude voltage for each pesticide was carried out using the automated method development (AMD) option included in the MS/MS software tool kit [36]. The optimum value for this parameter was reached when the secondary spectrum showed multiple and intense product ions while the parent ion intensity remained at around 10%. Multiple reactions monitoring (MRM) were employed to separate overlapping signals of permethrin and chlorpyrifos from their isotopically labelled internal standards. The EI-MS/MS spectra of the pesticides under experimental conditions were stored in our own

laboratory-made library. Quantification ions are shown in table 2. The target analytes were identified by retention times and EI-MS/MS libraries (a fit better than 800 required) characteristic of standards.

Linearity was studied in triplicate at five concentration levels by measuring the peak-area ratios relative to internal standards, ((diethyl-D_{10})-chlorpyrifos for OPPs and (phenoxi-$^{13}C_6$)-*cis*-permethrin for Pyrs). Correlation coefficients obtained were all above 0.9991 (Table 4). Quantification was based on the standard addition method with hair samples spiked with a multistandard mixture of known concentration. The detection (S/N= 3) and quantification (S/N = 10) limits, calculated at pg mL^{-1} level for most analytes, are summarized in table 4.

Repeatability was tested by six consecutive micro-extractions of spiked seawater samples carried out by the same analyst. Within-day relative standard deviations (%RSD) ranged from 8.2 to 11.8. Reproducibility was tested on seven replicates of spiked seawater samples evaluated by the same analyst over three consecutive days. Between-day relative standard deviations obtained ranged from 7.1 to 13.8 (Table 4).

When certified reference materials are not available, the standard addition method can be accepted as a valid approach to evaluate the accuracy and precision of the analytical procedures [37, 38]. Thus, the accuracy of the SPME-GC-MS/MS method was evaluated by means of recovery tests undertaken on six blank controlled aquaculture seawater samples fortified at four concentration levels from 10 to 40 ng mL^{-1} for dichlorvos, from 0.1 to 1.2 ng mL^{-1} for chlorpyrifos, permethrin and α-cypermethrin; and from 2.5 to 8.0 ng mL^{-1} for deltamethrin. A SPME-GC-MS/MS chromatogram of a laboratory-fortified aquaculture seawater sample and the corresponding spectra for each pesticide analysed are shown in figure 5. The recoveries obtained ranged from 81 to 120.0% with relative standard deviations below 12% in all cases (Table 5).

Table 4: *Performance of SPME-GC-MS/MS method.*

Pesticide	Linear ranges (ng mL^{-1})	R^2	Repeatability (%RSD; *n=6*)	Reproducibility (%RSD; *n=7*)	LOD (pg mL^{-1})	LOQ (pg mL^{-1})
Dichlorvos	10.0-1500.0	0.9994	8.2	13.8	600.0	2.0[a]
Chlorpyrifos	0.1-20.0	0.9991	8.4	9.5	1.0	5.0
Permethrin	0.2-25.0	0.9990	11.8	7.1	24.0	80.0
α-Cypermethrin	0.5-50.0	1.0000	11.3	11.7	92.0	308.0
Deltamethrin	2.0-300.0	0.9990	8.2	13.8	571.0	1.9[a]

[a] Concentration given in ng mL^{-1}

Table 5: *Extraction recoveries in aquaculture seawater samples by SPME-GC-MS/MS using standard addition method*

Pesticide	%Recovery ± RSD *(n=4)*					
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**
Dichlorvos	120.0 ± 11.2	94.7 ± 4.8	95.4 ± 4.2	102.8 ± 4.1	92.2 ± 4.8	86.6 ± 2.8
Chlorpyrifos	100.7 ± 3.7	94.0 ± 4.5	91.0 ± 4.4	97.7 ± 6.7	93.6 ± 4.5	80.9 ± 4.6
Permethrin	112.4 ± 8.4	104.3 ± 4.0	86.3 ± 3.5	95.0 ± 4.3	101.9 ± 4.0	88.8 ± 7.5
α-Cypermethrin	101.2 ± 4.2	106.5 ± 1.9	92.3 ± 2.3	104.8 ± 1.9	104.8 ± 1.9	110.3 ± 5.9
Deltamethrin	99.9 ± 4.3	97.5 ± 2.6	102.5 ± 5.5	103.2 ± 7.5	95.0 ± 2.6	91.5 ± 3.1

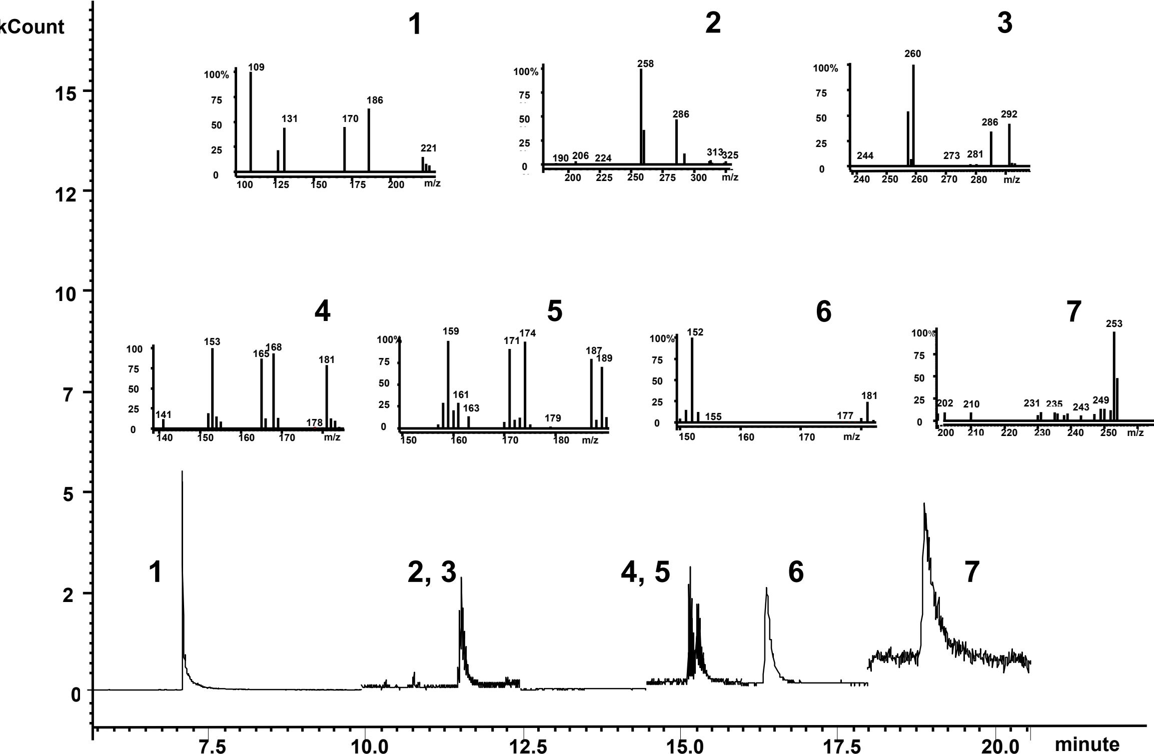

Figure 5. *SPME-GC-MS/MS chromatogram and refragmentation spectra of a fortified seawater sample. Peaks: 1, dichlorvos; 2, chlorpirifos; 3, (diethyl-D10)-chlorpyrifos (IS); 4, permethrin; 5, (phenoxy-13C6)-cis-permethrin (IS); 6, a-cypermethrin; 7, deltamethrin. MRM was employed to separate the overlapping signals of 2 and 4 from their isotopically labeled homologues.*

4. Conclusions

The combination of SPME with GC-MS/MS is a powerful tool for the determination of different OPPs and Pyrs in aquaculture seawater samples. The method developed in this work is fast, sensitive, and simple enough to be applied in routine analysis. PDMS microextraction fibres proved to be very efficient in the simultaneous extraction of all the pesticides studied. The overall conditions that represented the best compromise between SPME extraction yield and chromatographic response were obtained using an experimental design strategy implementing desirability functions for optimization. Good linearity, precision and low detection limits were obtained with this method. In addition, the use of gas chromatography

coupled with tandem mass spectrometry offers the possibility of the unequivocal identification of target compounds due to the high selectivity of the detection system. Standard addition calibrations were used to compensate the matrix effect present in seawater samples. Moreover, the use of SPME as an extraction and preconcentration technique provides freedom from the use of toxic organic solvents and has low sample volume requirements.

Acknowledgments
This research was supported by the Project CTQ2006-03334/BQU MEC (Plan Nacional I+D, SPAIN). D. García-Rodríguez is grateful to the Xunta de Galicia (Consellería de Innovación e Industria) for the financial support of his PhD.

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1.2. Determinación de pesticidas multi-residuo en muestras de agua de mar mediante microextracción líquido-líquido dispersiva (DLLME) y cromatografía de gases acoplada a espectrometría de masas-masas (DLLME-GC-MS/MS) con inyección de grandes volúmenes-vaporización con temperatura programada (PTV-LVI)

El objetivo de este proyecto es el desarrollo y la validación de un método de análisis para la determinación de pesticidas en agua de mar procedente de áreas próximas a zonas de cultivos marinos. Los compuestos que se determinan se han ampliado a diecisiete pesticidas de la familia de los organofosforados, piretroides y carbamatos. Todos ellos pueden ser usados como agentes quimioterápicos en el tratamiento de infecciones parasitarias debidas al piojo de mar y otras enfermedades presentes en las granjas de cultivo de especies acuicolas.

La microextracción líquido-líquido dispersiva es un tipo de extracción miniaturizada que integra en una única etapa la extracción y concentración de los analitos. Algunas de las ventajas más destacadas de la técnica son su simplicidad, rapidez, bajo coste, consumo reducido de muestra y disolventes orgánicos, versatilidad, buenas recuperaciones y factores de concentración altos.

En estudios preliminares se han estudiado los siguientes factores que influyen en el proceso de extracción: efecto salino, la elección del disolvente dispersante y extractante y el tipo de agitación. No se ha añadido ningún tipo de sal a las muestras reales, se ha demostrado que el acetonitrilo, como dispersante, el tricloroetano, como extractante y la agitación manual resultan más eficaces para la DLLME. El volumen de dispersante, extractante y el tiempo de agitación han sido los tres parámetros optimizados mediante un diseño de superficie de respuesta, tipo Doehlert. Las condiciones óptimas obtenidas son: 1.9 mL de acetonitrilo, 178 µL de tricloroetano y agitación durante 3 minutos.

El acoplamiento GC-MS/MS proporciona un método muy sensible y selectivo para le determinación de los compuestos estudiados.

El método propuesto ha sido aplicado, con éxito, a varias muestras de agua de mar, algunas procedentes de zonas relacionadas con industrias de procesado de pescado, en las que han sido detectados/cuantificados algunos de los compuestos estudiados. Se ha utilizado la técnica de inyección de grandes volúmenes con vaporización de temperatura programada (PTV-LVI) para mejorar la sensibilidad y la selectividad de las medidas. Las condiciones cromatográficas han sido optimizadas en un estudio desarrollado en el capítulo III.2 de esta Tesis. La robustez del método se ha demostrado con la aplicación exitosa a varias muestras de aguas de distinta procedencia, incluidos el influente y efluente de plantas purificadoras de aguas residuales.

DISPERSIVE LIQUID-LIQUID MICROEXTRACTION COUPLED WITH PROGRAMMED TEMPERATURE VAPORIZATION-LARGE VOLUME INJECTION-GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR MULTICLASS PESTICIDES IN WATER

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Abstract

A simple solvent-less procedure for the determination of seventeen pesticides and related compounds in environmental water and wastewater using dispersive liquid-liquid microextraction (DLLME) coupled with gas chromatography-mass spectrometry in tandem (GC-MS/MS) with large-volume injection, having a programmed temperature vaporizer (PTV-LVI), is described. The parameters affecting the extraction efficiency of the target analytes from water samples were systematically investigated. A response surface Doehlert design was used. The best extraction conditions involved a rapid injection of a mixture of 1.9 mL of acetonitrile (as a dispersant) and 178 µL of trichloroethane (as an extractant) into 10 mL of water placed in a conical bottom glass tube. After manually shaken for 3.0 min and centrifugation at 3600 rpm (5 min), 50 µL of the sedimented phase was directly injected into the PTV-LVI-GC-MS/MS system. The limits of quantitation (LOQs) ranged from 0.5 to 18 ng L^{-1} for all pesticides, except empentrin (132 ng L^{-1}). The relative standard deviations (RSDs) for the analytes ranged between 0.8 and 14.6 % for both intraday and interday precision. Accuracy, expressed as the mean extraction recovery, was between 70 to 130 %. Using the internal standard method and surrogate deuterated standards the total concentration of pesticides was in the range from 2.7 to 440 ng L^{-1} in seawater, river water and sewage water.

Keywords: Multiresidue pesticides; environmental water; microdispersive liquid liquid extraction; gas chromatography-mass spectrometry.

1. Introduction

Aquaculture is an emergent food production sector with remarkable expectatives of growth in the coming years [1]. Aquaculture is in a constant search for ways and means to improve the production practices, namely the efficiency of the cultures and the quality of the products [1]. This activity requires the use of chemical products that may become part of the marine environment, namely organophosphorus (OPs) carbamates (CARs) and synthetic pyrethroid pesticides (PYRs). Chlorpyrifos ethyl (Lorsban®), carbaryl (Sevin 80SP®), permethrin (Permanone®), cypermethrin (BetaMax®, Excis®), deltamethrin (AlphaMax®) are active ingredients commonly used as veterinary drugs to combat numerous infestations in farmed fish and crustacean [2-4]. Furthermore, pesticides are introduced directly to the marine environment as the result of widely agricultural and urban activities, being present at home in sprays and shampoos [5-7]. Their large volumes of production and continuous widespread use make some of these compounds to be persistent substances in the environment [8-9]. These compounds cause a variety of neurotoxic and endocrine disruptor effects. WHO and various national governmental institutions have established residue limits and published guidelines for quantification of pesticide residues in waters [10]. This increasing public concern prompted us to develop a simple and reliable method for determining pesticides in aquatic environments. Several multi-residue methodologies based on gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) detectors have been described to determine different types of pesticides in environmental waters [11-14]. The extraction from aqueous samples is commonly performed using liquid-liquid extraction (LLE) [15, 16] and solid-phase extraction (SPE) [17, 18]. Development of dispersive liquid-liquid microextraction (DLLME) has greatly contributed to the miniaturization, the simplification and the automation of the whole analytical procedure, especially enabling the speed up of sample treatment, which is currently the bottleneck of analysis [19-21]. DLLME has been successfully applied to the determination of various pollutants such as organophosphorus [20, 22-24], organochlorine [20, 25, 26], carbamate [20, 27, 28] and pyrethroid [20, 29-31] pesticides in various environmental waters. However, it is desirable to extend the DLLME technique to the simultaneous extraction of several pesticide families and to more complex matrices such as seawater and wastewater.

The aim of the present work was to employ DLLME coupled with PTV-LVI-GC-MS/MS for quantification of fifteen multiresidue pesticides of different families and two related compounds, a preservative (2-phenylphenol) and a synergist (piperonyl butoxide) in samples of riverwater, wastewater and seawater. To the best of our knowledge, this is the first time that DLLME is combined with PTV-LVI-GC-MS/MS. Although the two related analytes included in the study are not pesticides in fact, they were also considered because of their concomitant presence with typical pesticides [32]. The influence of operational parameters of the DLLME, such as types of extractant and dispersant, type of shaking, pH and ionic

strength, on the extraction efficiency of these analytes, was systematically investigated. Time of shaking, volume of dispersant and extractant were also evaluated with a response surface Doehlert design [33]. The performance of the method was elucidated, and its suitability for the determination of trace levels of analytes in various water samples was demonstrated.

2. Experimental

2.1. Reagents and materials

Pestanal® quality analytical standards of 2-phenylphenol, propoxur, carbaryl, chlorpyrifos-methyl, chlorpyrifos-ethyl, piperonyl butoxide, empenthrin, bioallethrin, resmethrin, tetramethrin, λ-cyhalotrhin, permethrin, cyfluthrin, α-cypermethrin, flucythrinate and fenvalerate were from Riedel-de-Haën (Seelze, Germany). Pestanal® quality deltamethrin was from Chem Service Inc. (West Chester, PA, USA). Internal standards (diethyl-D_{10})-chlorpyrifos and (phenoxi-$^{13}C_6$)-*cis*-permethrin were from Cambridge Isotope Laboratories (Cambridge, UK). Ultra pure water was obtained using a Milli-Q® purification system (Millipore, Spain). Dichloromethane and acetone pestinorm grade solvent were from VWR-Prolabo (Mollet del Vallés, Barcelona, Spain). Acetonitrile, chloroform and methanol were from Merk (Darmstadt,Germany). Trace analysis grade carbon tetrachloride, ethylene tetrachloride, 1, 1, 1-trichloroethane, and chlorobenzene were from Sigma Aldrich (Milwaukee, WI, USA).

Individual standard stock solutions of 5 mg mL^{-1} were prepared in acetone. A stock mixture solution of all target analytes at 100 μg mL^{-1} was obtained by appropriate dilution of the individual standard solutions in acetone. Working solutions were prepared by convenient dilution of the stock mixture solution in acetone. All solutions were stored in amber-colored vials at -20 °C until use.

Glass tubes (12 mL volume) with a conical bottom and a screw cap, furnished with a polytetrafluoroethylene (PTFE) - lined septum, were from Afora (Barcelona, Spain).

Abbreviations of pesticides: 2-Phenylphenol (2-PP); Chlorpyrifos-methyl (CLP-M); Chlorpyrifos-ethyl (CLP-E); Carbaryl (CARB); Bioallethrin (BIOAL); Tetramethrin (TETRA); Cyhalothrin (CYHAL); Permethrin (PERM); Cypermethrin (CYPER); Deltamethrin (DELTA); Resmethrin (RESME); Flucythrinate(FLUCY); Piperonyl butoxide (PBO); Cyfluthrin (CYFLU); Empenthrin (EMPEN); Fenvalerate (FENVA); Propoxur (PROP).

2.2. Samples

Seawater samples were collected near to a shellfishing area and in the influent and in the efluent of a water treatment plant (WTP) of a cannery, at the coast of Galicia (NW Spain). Wastewater samples were taken from the influent and the effluent of two urban sewages collected at the WTP of Galicia (Spain).

Besides river water samples were obtained from urban and rural collection sites in Galicia. Samples were collected in 250-mL amber-colored glass bottles previously rinsed with sample water, and transported immediately to the laboratory. Then, they were passed through cellulose acetate membranes (0.45 µm pore size; Millipore, Billerica, MA, USA) and stored at 4ºC in the dark, until the analysis. The water samples were spiked with different amounts of working standard solutions in order to prepare the samples used for the different studies.

2.3. Extraction procedure

Aliquots of 10 mL water were placed in a 15 mL screw capped glass tube with a conical bottom. Under optimized conditions, the binary extraction mixture consisting in 1.9 mL of acetonitrile (as a disperser) containing 178 µL of trichloroethane (as extraction solvent) was rapidly injected into the water sample. The tubes were closed, shaken manually for 3 min. The formed emulsion contained fine droplets of the extraction solvent dispersed in the aqueous phase. The cloudy solution was then separated applying centrifugation (3600 rpm, 5 min) and the sedimented phase (around 100 µL) was collected and an aliquot (50 µL) was injected in the GC-MS system. Optimization of DLLME conditions was performed with ultrapure water spiked with standard solutions containing the target analytes at 50 ng mL^{-1}.

2.4. PTV-LVI-GC-MS/MS analysis

Analyses were performed using a Varian 450GC gas chromatograph (Walnut Creek, CA, USA) coupled to an ion-trap mass spectrometer Varian 240MS operating in external configuration, and equipped with a Varian CP-8400 autosampler. A Varian Factor Four VF-5MS capillary column (30 m x 0.25 mm, 0.25 µm film thickness) was used for the separation. Initial oven temperature was set at 70 °C and held for 3.5 min; ramped at 25 °C·min^{-1} up to 180 °Cand held for 3 min; and finally ramped at 10 °Cmin^{-1} up to 300 °C and held for 5 min. Helium (purity 99.999%, Carburos Metálicos, A Coruña, Spain) was employed as carrier gas with a constant flow of 1.0 mL min^{-1}. This instrument is equipped with a 1079 PTV injector and a cryogenic CO_2 cooling. The PTV-LVI mode parameters were optimized in a previous work [34]. PTV injections were performed in four steps: injection, solvent evaporation, analyte transfer, and cleaning. In the injection step, the split valve was open at 20 mL min^{-1}, and 50 µL of sample were introduced into a Siltek deactivated liner with frit (Restek, Bellafonte, PA, USA) at 70 °C. During the evaporation step, the temperature was raised to 85 °C at a rate of 30 °C·min^{-1} for 30 s in order to eliminate the solvent, which was vented through the split valve at a flow of 36 mL min^{-1}. In the transfer step the split valve was closed and the temperature increased to 300 °C at a rate of 75 °C min^{-1} in splitless mode for 3 min. Finally, the injector was kept at 300 °C with a purge flow of 50 mL min^{-1} until the end of the run for cleaning purposes. The mass spectrometer was operated in EI mode at 70 eV. The temperatures of ion source, trap, manifold, and

transfer line were maintained at 150, 150, 40 and 285 °C, respectively. Analyses were performed with a filament-multiplier delay of 6.5 min. General parameters were as follows: multiplier offset, +200 V; AGC target value, 5,000 counts; damping gas flow, 2.5 $mLmin^{-1}$ and emission current, 90 µA. Specific conditions for each analyte were previously described [32]. A parent ion was chosen for each compound by taking the m/z and relative abundance of parent ions as high as possible in order to increase sensitivity. Good quality secondary spectra for every compound were obtained selecting a non-resonant waveform. The optimization of the excitation amplitude voltage for each pesticide was achieved using the automated method development option included in the MS/MS software toolkit (Varian MS Workstation ver. 6.91, Varian 2008). This value was considered optimum when the secondary spectra showed multiple and intense product ions, while the parent ion intensity remained around 10%. The excitation amplitude ranged between 38 V to bioallethrin and 99 V for chlorpyrifos-methyl, while the excitation storage level ranged between 41.9 m/z for propoxur and 123.5 m/z for (diethyl-D10)-chlorpyrifos.

Quantification was accomplished by relative areas versus (phenoxi-13C6)-cis-permethrin used as internal standard (IS) for PYR while (diethyl-D10)-chlorpyrifos was used as IS for the remaining analytes.

3. Results and discussion

3.1. Optimization of DLLME

Optimized extraction conditions and high extraction efficiency was accomplished by means of the evaluation of several factors including the type of extraction and dispersion solvents, ionic strength, type of shaking, and pH [19]. The effects of the volume of extractant and dispersant and the extraction time were studied through an adequate experimental design. The extraction efficiency of the proposed DLLME method was characterized by the product of volume of the sedimented phase and peak area.

3.1.1. Effect of the extraction solvent

The extraction solvent should have a high density, a low solubility in water, good chromatographic behavior and a high extraction capability for the target compounds [21]. Six commonly used extraction solvents: carbon tetrachloride, tetrachloroethylene, trichloroethane, chloroform, dichloromethane and chlorobenzene [19, 21, 35, 36] were investigated, by mixing spiked artificial seawater (10 mL of ultrapure water containing 1.5 g of sodium chloride) with 100 µL of each extraction solvent and 1 mL of acetonitrile as disperser in order to achieve the appropriate amount for the sedimented phase at the bottom of conical tube. The choice of solvent was based on extraction efficiency. Chlorobenzene and dichloromethane did not lead to droplet formation. If the extraction solvent is miscible with the dispersant+water medium, no

droplets will be formed. That is clearly the case of dichloromethane (solubility in water 1.6%) in the conditions of our study. Also chlorobenzene has a solubility around 1% in acetonitrile:water 1:10 weight ratio medium. As shown Fig. 1a, trichloroethane, followed by carbon tetrachloride, tetrachloroethylene and chloroform provided the highest extraction efficiency. Polarity of these solvents is similar and the results obtained are in agreement with those obtained in the extraction of seven fungicides from wine [37]. Then, trichloroethane was selected for subsequent experiments.

3.1.2. Effect of the disperser solvent

To enable the formation of a cloudy state when the organic extraction solvent is injected into the water sample, the dispersant should be well-miscible with the organic extraction solvent and the aqueous sample. Three commonly used dispersers, namely methanol, acetone and acetonitrile [19, 21, 35, 36], were introduced not only as dispersers but also as demulsifiers to break up the O/W emulsions [20, 28]. The effect of these solvents was investigated by mixing 1.9 mL of each dispersant with 100 µL of carbon trichloroethane, and then rapidly injecting them into 10 mL of artificial sea water sample (spiked with 50 ng mL^{-1}). Fig. 1b shows the best results achieved when acetonitrile was used as the disperser for the most of the seventeen analytes, which is explained by the higher solubility for the target analytes. Acetonitrile was non-optimal disperser for empenthrin, bioallethrin and tetramethrin. However, acetonitrile was chosen as compromise solution for subsequent experiments.

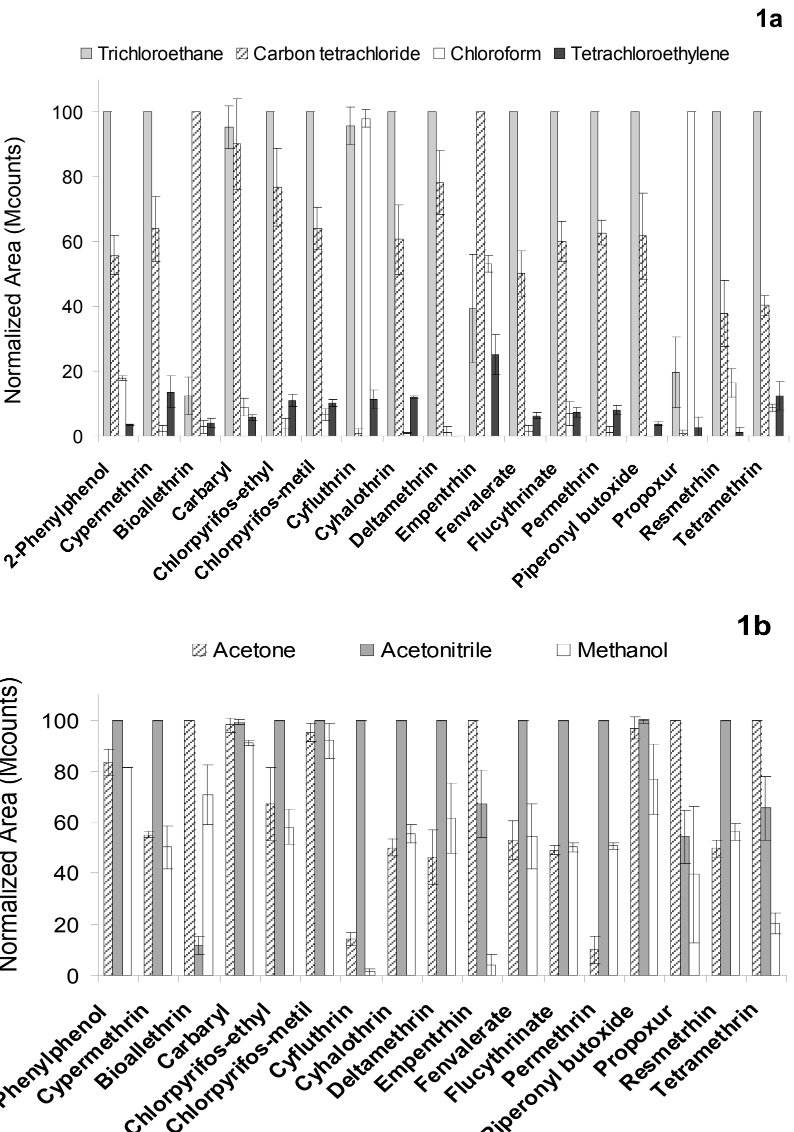
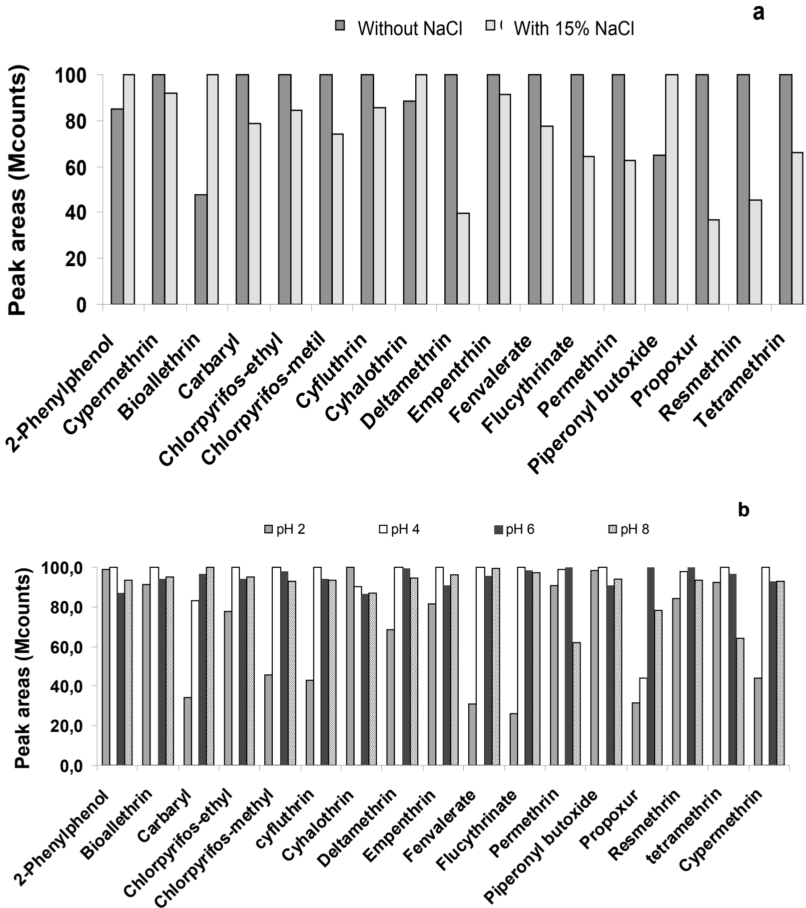


Figure 1. *Areas of the chromatography peaks of the target analytes when trichloroethane, carbon tetrachloride, chloroform, and tetrachloroethylene were used as extractant solvents (a) when acetone, acetonitrile or methanol were used as disperser solvents (b) in the presence of trichloroethane as extractant solvent (n = 2 replicates). Concentration of mixture standard solution: 50 ng mL^{-1}; volume of water samples: 10 mL; volume of extractant: 100 µL; volume of dispersive solvent: 1 mL; extraction time: 1 min; 1.5 g of sodium chloride added.*

3.1.3. Effect of the ionic strength and pH

The addition of sodium chloride to water samples has been used to improve the extraction efficiency [21, 38]. Preliminary tests were performed starting from Milli-Q water and the influence of ionic strength on the performance of DLLME was investigated by adding different amounts of NaCl (0- 15%, w/v). The results demonstrated that the salt had not significant effect on the extraction recoveries, as observed in other studies [22, 25, 39-42]. Moreover, the extraction efficiency decreased, especially for pyrethroids and carbamates, when 15% w/v NaCl concentration was used (data provided in electronic supporting information, file 1a). These results can be explained by: i) the dissolution of NaCl in water might change the physical properties of the Nernst diffusion film and reduce the rate of diffusion of the target analytes into the extraction solvent; and ii) the addition of salt could also affect the phase ratio [43]. Therefore, no salt was added in all the subsequent experiments.The results for seawater (3.5% w/v) are expected to be quite close to those obtained for 0% of NaCl, and the method can be still considered suitable for seawater samples.

The pH of the water sample is expected to have a significant impact on the extraction [21]. Thus, the effect of pH varying from 2.0 to 8.0 was investigated on extraction efficiency. The pH value above 8.0 was not tested since degradation of many pesticides may occur under this high alkaline condition [28, 42, 44]. No significant difference in analytical response was observed when pH varied in the tested range (data provided in electronic supporting information, file 1b); in spite of the pesticides tend to form neutral molecules at low pH, which may have higher affinity for the non-polar solvent. . These results prompted us to use the original water samples (pH 6.0) without changing their pH.



Electronic supporting information, file 1: *Effect of the salt addition (a) and sample pH (b) on extraction efficiency. Concentration of mixture standard solution: 50 ng mL^{-1}, volume of water samples: 10 mL; volume of extractant (trichloroethane): 100 µL; volume of dispersive solvent (acetonitrile): 1 mL; extraction time: 1 min.*

3.1.4. Effect of shaking mode

According to previous studies [21], transfer of analytes from the sample aqueous phase to the extraction phase is fast due to the extremely large interfacial area [19, 28, 30, 31]. Equilibrium can be achieved quickly and consequently extraction times can be very short. In this work, manual and ultrasonic shaking during 5 min, were evaluated. Compared to the non-shaken situation and the ultrasound-mediated shaken, the extraction efficiency was slightly higher when the manual shaking was chosen. This can be due to the fact that the mechanical and thermal effects of ultrasound could result in the volatile loss of analytes and extraction solvent [45]. Centrifugation was a relevant key factor to achieve the separation of sediment phase from the aqueous phase. Usually, the peak areas of the compounds gradually increased as the centrifugation time increased from 0 to 5 min. Centrifugation times beyond 5.0 min did not lead to a further increase in the peak areas [30]. Therefore, 5 min at 3600 rpm was selected as an adequate centrifugation time to achieve the phase separation and to obtain good extraction efficiency [30, 31, 44, 46].

3.1.5. DLLME optimization using an experimental design

Once the optima dispersive and extraction solvents, the ionic strength, the pH and the shaking mode were found, the effect of disperser, the extraction solvent volumes and the manual shaking time were also evaluated. DLLME efficiency was examined for the target analytes according to a response surface Doehlert design, chosing as factors the dispersive solvent (0.5, 0.8, 1, 1.3, 1.6, 1.8, 2.1 mL), extraction solvent (60, 95, 130, 165, 200 μL) volumes and the time of manual shaking (1, 3, 5 min) [33].The design involved 16 experiments randomly performed to provide protection against the effects of lurking variables, including four replicates at the central point of the experimental domain. The response, namely extraction efficiency, calculated as the mathematical product of the peak area by the volume of the sedimented phase [47], was fitted by a multiple regression equation (1), including curvature and interaction terms:

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} \sum_{j=1}^{n} b_{ij} x_i x_j \qquad (1)$$

Where, x_i is the coded value of the factors studied and y is the response function obtained for each analyte. The *b* values are the estimated polynomial coefficients: b_0 is the intercept term, the b_i coefficients represent the main effect for each variable, the b_{ij} coefficients in the quadratic terms are responsible for the curvature effects, and the $b_{ij(i \neq j)}$ coefficients describe the interaction effects. The results obtained were evaluated using NemrodW® software [48]. The ANOVA of the quadratic regression model indicated a high significance (p<0.05-0.01) for twelve of the compounds. The regression was not statistically significant for 4 pyrethroids (cyfluthrin, empentrin, flucythrinate, and permethrin) and for PBO. The coefficients of the model for each response are shown in Table 1. The main effect of the three factors, b_1: extraction solvent volume, b_2: disperser volume and b_3: shaking time, were statistically significant for propoxur, α-cypermethrin and cyhalothrin. The main effect of two factors was statistically significant for seven compounds: b_1 and b_2 for 2-phenylphenol, b_1 and b_3 to tetramethrin, bioallethrin and chlorpyrifos-ethyl, and b_2 and b_3 for resmethrin, deltamethrin and fenvalerate. The main effect of a single factor was statistically significant for carbaryl (b_2), chlorpyrifos-methyl (b_1) and cyfluthrin (b_3). The effects of some of the quadratic terms of the model were also statistically significant for eight compounds: 2-phenylphenol, bioallethrin, chlorpyrifos-ethyl, chlorpyrifos-methyl, cyfluthrin, propoxur, α-cypermethrin and tetramethrin. In the most cases, negative quadratic coefficients of the variables indicated an antagonistic effect between them. Similarly, the effects of the interactions between two factors were statistically significant in some cases: 2-phenylphenol and chlorpyrifos-ethyl (b_{12}), bioallethrin, tetramethrin, cypermethrin, and propoxur-α (b_{12} and b_{23}), resmethrin (b_{12} and b_{13}) cyhalothrin (b_{13}), PBO (b_{23}). The

application of surface response methodology (SRM) revealed an increase of the DLLME efficiency when medium-to-high levels of the three factors were used. At a low volume of acetonitrile, a cloudy state was not completely developed, thus giving a low recovery; at a higher volume of acetonitrile, the solubility of the analytes in water was increased, leading to a decreased extraction efficiency because of a decrease of the distribution coefficient. The equilibrium state can be achieved relatively quickly in DLLME and, consequently, the extraction time required is not long. To find the best-compromise conditions, a multicriteria optimization approach based on desirability functions was applied without additional experimentation [33]. Two-dimensional plots of the isodesirability are shown in Fig. 2. The regions in grey correspond to null values for desirability when the factors levels are not suitable to be chosen. Optimal compromise conditions resulted in 178 μL of trichloroethane (extraction solvent), 1.9 mL of acetonitrile (dispersion solvent) and manual shaking for 3 min.

3.2. Method validation

The linearity of the DLLME-PTV-LVI-GC-MS/MS method was evaluated using a series of samples at concentration levels of 0.1, 0.4, 0.8, 4, 8, 40 µg L^{-1}. The determination coefficients (r^2) ranging from 0.9989 to 0.9993 indicated an excellent linearity for all species. The precision of the method (n=6) was evaluated by measuring six replicate samples in one day (intra-day precision) and in three consecutive days (inter-day precision), expressed as relative standard deviation (%RSD) by treating seawater samples at two addition levels of 0.5 µg L^{-1} and 10 µg L^{-1}. The method showed good intra-day and inter-day precision with RSD values between 2.0 to 11.0% and 2.0% to 12.9% at 0.5 µg L^{-1}, respectively and between 2.2 to 8.6 % and 0.8% to 14.6% at 10 µg L^{-1}, respectively. Two-way ANOVA was used to evaluate differences between precision of two factors: concentration level (0.5 and 10 μg L^{-1}) and precision type (intra-day and inter-day). The F-test in the ANOVA table and the p-values (0.3465 and 0.070) higher than 0.05, shown that these factors did not have statistically significant effect on precision at the 95 % confidence level. The analytical characteristics of the optimized DLLME method in terms of limit of detection (LOD) and quantification (LOQ), precision, enrichment factor and linear range were calculated to gain an insight into the efficiency and the feasibility of application of the method for the analysis of seawater, river water and sewage water samples, as summarized in Table 2. LOD and LOQ were experimentally estimated from the analysis of real samples as the concentration of analyte giving a signal-to noise ratio of 3 and 10, respectively. Two ions (except only one for carbary, permetrhrin and deltametrhin) were used to estimate LODs, as is described in a previous work [34]. The LOD for each compound ranged from 0.1 to 8.8 ng L^{-1}, except for empenthrin that was 50 ng L^{-1}. The limits of quantification (LOQs), calculated as ten times of the signal-to-noise ratio, ranged for all target analytes from 0.3 to 26.4 ng L^{-1} except for empenthrin that was 151 ng L^{-1}. Theses LOQs are lower than the maximum contaminant level (MCL) permitted in the UE for individual pesticide (0.1 μg L^{-1})

and for total pesticides (0.5μg L^{-1}) in water [49]. Furthermore, the values reported in Table 2 were similar or even better than those obtained for other applications of DLLME [22, 27, 29-31, 39, 46, 50]. The LOQ chromatograms obtained in seawater are provided in electronic supporting information (file 2). Table 3 shows details of the chromatographic methods published regarding related DLLME applications.

Table 1: *Estimates of the model coefficients for the responses. Bold numbers indicate significant effects (5%-1%).*

Analyte	b_0	b_1	b_2	b_3	b_{11}	b_{22}	b_{33}
2-PP	**83531.275**	**27197.563**	**6618.255**	471.046	**−13843.125**	5411.314	−5339.060
CLP-M,	**24234.125**	**5306.250**	1417.472	1173.984	**−3482.875**	−656.962	−1039.488
CLP-E	**88385.025**	**15564.587**	2060.248	**4642.545**	**−11874.875**	384.710	−2972.325
CARB	**21778.375**	**11376.825**	**3273.033**	845.227	−1291.175	3.377.186	−936.199
BIOAL	**25242.725**	**4407.850**	360.928	**1137.966**	**−7674.875**	**1947.441**	−411.779
TETRA	**44037041.300**	**81985.700**	−626.737	**9804.412**	**−43947293.00**	**−41600206.91**	**−29334797.93**
CYHAL	**256313.250**	**26288.550**	**34146.366**	**35128.303**	−24367.600	18206.332	−8155.081
PERME	**121454.525**	15683.038	38846.285	53300.982	19274.875	42345.669	−14864.137
CYPER	**49113.500**	**−19281.163**	**6930.373**	**6705.020**	**53532.250**	**−10410.477**	**−4791.482**
DELTA	**113310.700**	13358.688	**16595.923**	**16798.820**	−15376.300	8388.119	468.191
RESME	**104141.650**	5345.850	**9737.030**	**11533.486**	2330.000	4781.500	−4925.210
FLUCY	**174.600**	−19.325	14.400	23.250	−24.800	91.916	−13.000
PBO	**348.550**	**−311.850**	−223.791	63.372	414.600	−103.054	57.837
CYFLU	**29051.050**	−969.613	4663.034	**4550.812**	**−9398.900**	3931.664	−633.908
EMPEN	**1716.000**	636.338	6.307	161.016	288.550	299.811	−46.388
FENVA	**4866.500**	399.063	**722.862**	**834.327**	−724.900	354.185	−110.743
PROP	**139.325**	**141.912**	**146.285**	**49.232**	**158.625**	**163.100**	**−50.804**

Table 1: *Continued.*

Analyte	b_{12}	b_{13}	b_{23}
2-PP	**12.967.425**	−4221.725	5.325.703
CLP-M,	**4.738.669**	486.394	−167.992
CLP-E	**13.661.831**	−1838.844	−324.381
CARB	5.848.256	−734.069	2.808.751
BIOAL	**4.119.019**	−1435.156	**3.090.345**
TETRA	**16.453.856**	6.246.832	**3.042.244.752**
CYHAL	46.827.224	**−54914.774**	−13466.236
PERME	25.371.000	−16240.350	−67558.519
CYPER	**7.275.712**	−6247.287	**−20243.057**
DELTA	16.557.694	−20213.031	−4267.139
RESME	**24.295.556**	**−23194.568**	−2029.479
FLUCY	41.231	5.206	29.285
PBO	356.456	−26.569	**−877.535**
CYFLU	4.866.694	−3159.131	274.444
EMPEN	597.656	−406.419	−2773.986
FENVA	18.506	−887.269	−425.208
PROP	**386.325**	−0.625	**−129.672**

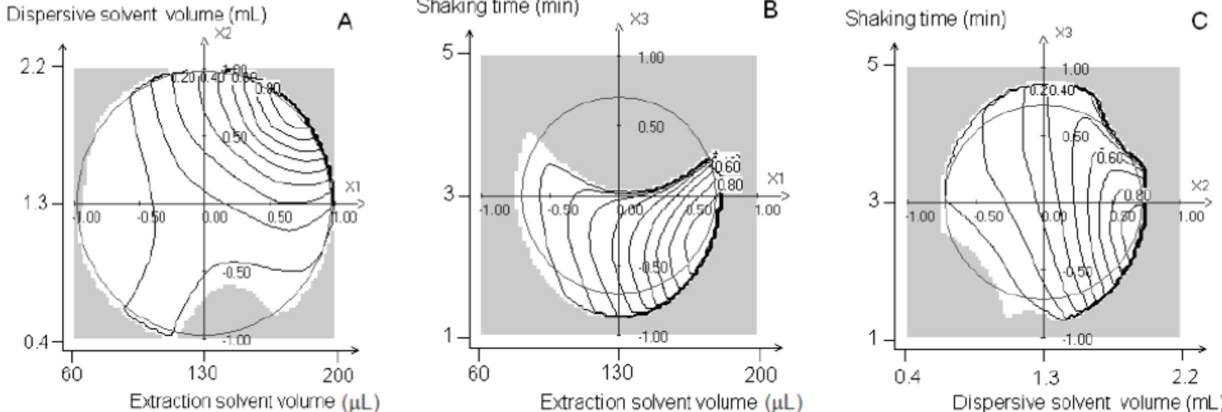


Figure 2. *Response surfaces of the global desirability obtained using a Doehlert design for the target analytes, obtained for dispersive solvent volume versus extraction solvent volume (A), shaking time versus extraction solvent volume (B), and shaking time versus dispersive solvent volume (C).*

The evaluation of the DLLME efficiency was accomplished by means of the enrichment factor (EF) and the extraction recovery (% R). EF is defined as the ratio between the concentration of analyte in the sediment phase (C_{sed}) and the initial concentration of analyte (C_i) in the sample [31, 38, 50].

The extraction recovery was calculated as follows:

$$\%R = \frac{V_{sed}C_{sed}}{V_iC_i} \times 100 = \frac{V_{sed}}{V_i} \times EF$$

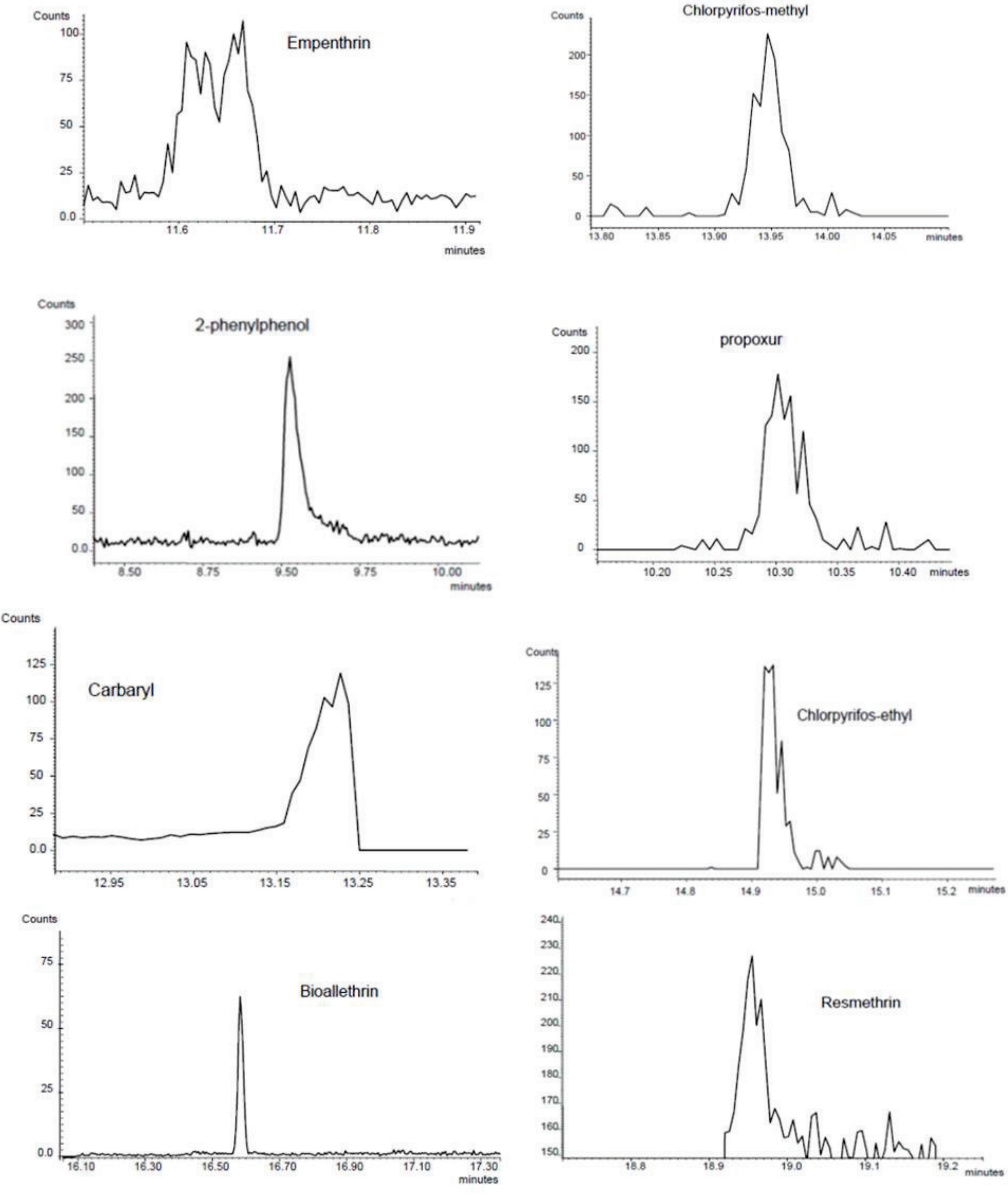
where, V_{sed} and V_i are the volume of the sediment phase and the initially aqueous samples, respectively. C_{sed} was calculated from the direct injection of the standard solutions with concentrations in the range of 0.5-10 µg L^{-1}. The extraction efficiency (the peak area x the volume of the sedimented phase) is inversely

proportional to EF. Under the optimal extraction conditions, EF for the analytes was in the 46 to 72 range. Drop volumes greater than 50 µL, required to apply the PTV-LVI- GC-MS/MS method, are most likely the reason to obtain relatively low EF values. The combination of the proposed sample preparation approach (DLLME) with a sensitive and selective determination technique, e.g. PTV-LVI-GC-MS/MS, provided detection and quantification limits very low and good selectivity. These facts proved that the enrichment method is robust. Residues-free samples were used as blanks for matrix-matched standard calibrations. The internal standard (IS) method involved the use of deuterated analogues as surrogate standards. Recoveries were evaluated in water samples such as seawater, river water and sewage water, after fortification at concentration of 0.5 µg L^{-1} and 10 µg L^{-1}, to test accuracy of the DLLME-PTV-LVI-GC-MS/MS method.

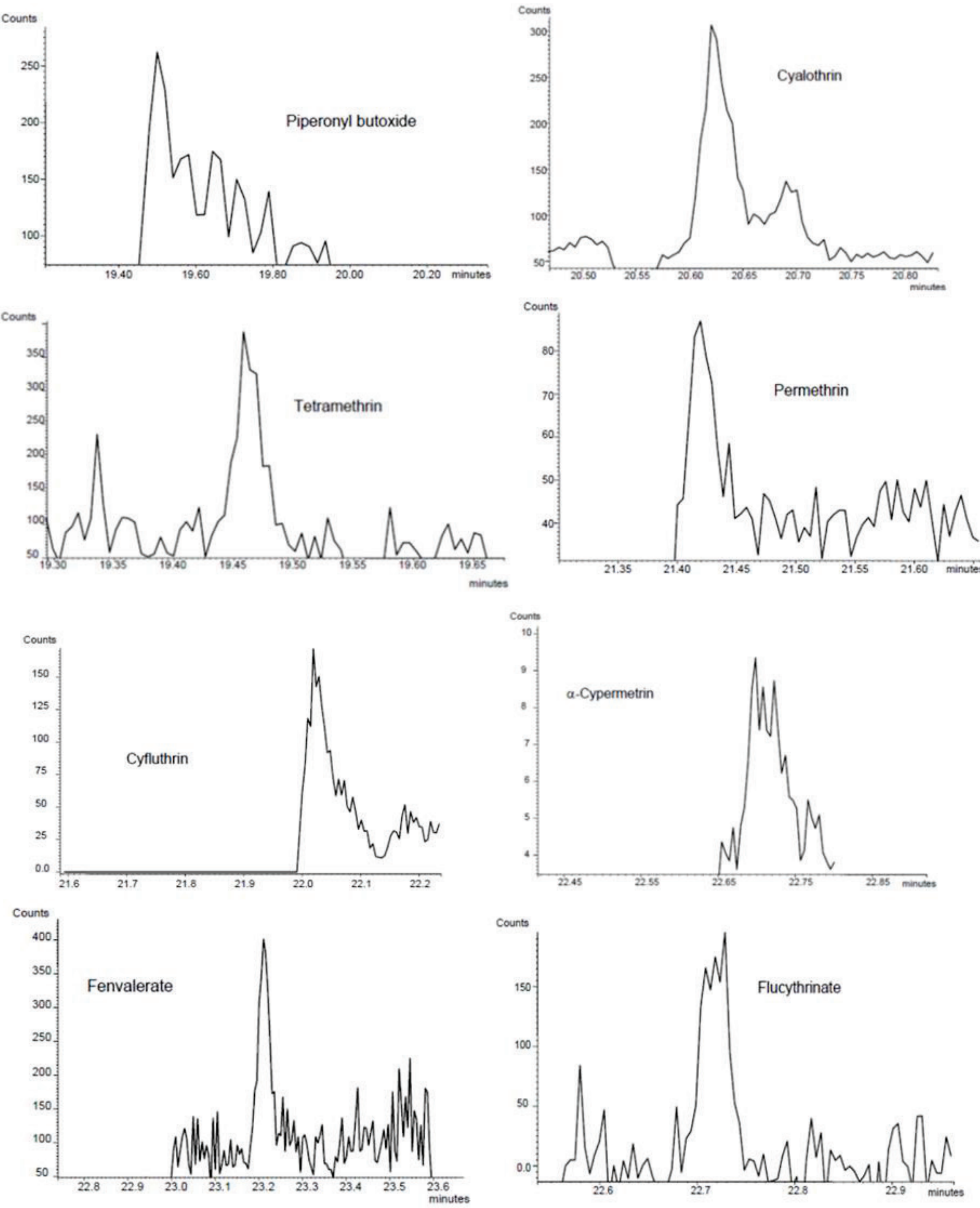
Table 2: *Analytical performance of DLLME procedure optimized for determination of seventeen residues in seawater samples (n=6).*

Comp.	R2	E.F.[a]	LOD[b]			LOQ[b]			Intra-day precision (%RSD)		Inter-day precision (%RSD)	
			River water	Sewage water	Sea water	River water	Sewage water	Sea water	0.5 µg L^{-1}	10 µg L^{-1}	0.5 µg L^{-1}	10 µg L^{-1}
2-PP	0.9990	66	0.5	2.0	5.0	1.8	7.0	18.0	8.2	3.3	3.0	3.5
CLP-M	0.9990	70	0.1	0.3	0.7	0.3	0.9	2.3	5.2	4.7	8.1	4.2
CLP-E	0.9992	71	0.3	0.7	0.5	1.0	2.2	1.8	8.1	2.5	1.8	2.2
CARB	0.9989	52	2.0	3.1	1.0	7.0	9.3	4.0	7.3	8.0	12.2	8.3
BIOAL	0.9993	72	2.3	3.0	1.9	7.4	9.9	6.5	3.9	5.6	2.0	5.8
TETRA	0.9993	66	2.7	2.7	1.1	9.0	9.0	3.7	8.0	2.7	5.0	0.8
CYHAL	0.9991	65	0.2	0.7	0.7	0.7	2.3	2.5	5.5	3.6	12.9	3.7
PERM	0.9990	68	1.1	2.9	1.2	3.8	9.5	4.1	6.5	2.2	12.3	1.8
CYPER	0.9991	66	1.2	2.0	0.7	4.1	6.7	2.2	5.5	4.7	6.8	4.9
DELTA	0.9990	65	7.0	8.8	5.0	22.3	26.4	17.5	2.0	5.0	4.6	3.5
RESME	0.9993	69	1.4	1.0	0.5	4.6	3.2	1.7	6.8	5.5	6.1	11.9
FLUCY	0.9993	64	0.7	0.6	0.8	2.4	2.1	2.5	9.0	5.8	12.4	14.6
PBO	0.9981	67	2.9	3.1	0.7	9.4	9.7	2.5	7.0	4.0	2.0	4.2
CYFLU	0.9993	69	4.0	7.0	1.0	12.6	20.3	3.0	7.0	5.1	8.7	5.2
EMPEN	0.9990	58	31.0	50.0	40.0	93.0	151.0	132.0	8.6	5.1	1.9	9.6
FENVA	0.9990	64	4.8	3.2	3.3	14.9	10.5	10.8	2.5	5.6	9.3	5.7
PROP	0.9991	46	2.0	3.0	2.0	8.0	9.0	7.0	11.0	8.6	8.8	10.7

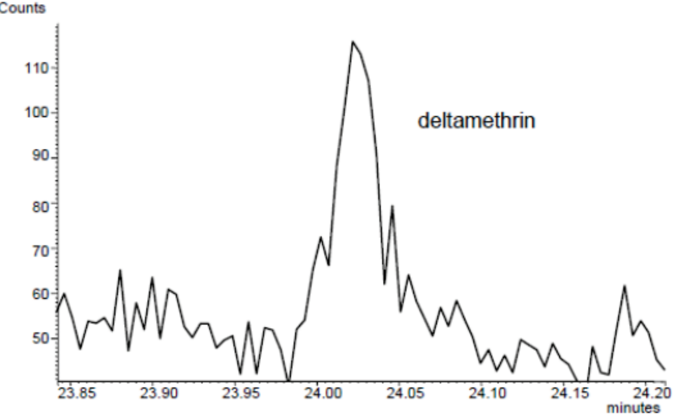
[a] Enrichment factor, [b] Expressed as ng L^{-1}



Electronic supporting information, File 2: *LOQs chromatograms obtained in seawater sample.*



Electronic supporting information, File 2: *continued*



Electronic supporting information, File 2: *continued*

The recoveries, calculated in sextuplicate, of the seventeen target analytes in the real samples at the different concentration levels are summarized in Table 4. The recovery was 102 ± 7% for all compounds except fenvalerate (82%) and cyhalothrin (118%) for river water; 98 ± 13% for all compounds except flucythrinate (70 %) and chlorpyrifos-ethyl (126 %) for sewage water; and 103 ± 11% for all compounds except empentrhin (65%) and 2-phenylphenol (129%) for seawater. These results demonstrate that the DLLME can be applied as a simple, rapid and low cost method for the simultaneous determination of multiclass pesticide traces in environmental water samples with suitable accuracy and precision.

Table 3: *Optimal conditions and analytical characteristics of chromatographic methods, based on DLLME procedure, for related compounds.*

Analyte	Disperser solvent volume	Extraction solvent volume	Tech.	Linear range	E.F.	LOD	RSD %	App.	Ref.
TETRA **CYPER**	1 mL acetone	20 µL tetrachloroethane	HPLC-UV	0.0006-1.52 mg L^{-1}	846 777	0.12 µg L^{-1} 0.30 µg L^{-1}	2.7 2.7	River water	[30]
CYHAL **FENVA**	0.4 mL acetone	8 µL dodecan-1-ol	GC-ECD	10-2000 ng L^{-1} 50-2000 ng L^{-1}	681 409	4 ng L^{-1} 16.3 ng L^{-1}	13.5 18.4	Peach juices	[39]
CARB	1 mL ACN	15 µL tetrachloroethane	HPLC-FLD	0.1-1000ng mL^{-1}	87.3	12.3 ng L^{-1}	2.7	Fruit juice, water	[27]
CLP-M **CLP-E**	1 mL 2-propanol	50 µL chloroform	GC-FID	0.8-100 µg L^{-1}	257 235	1.5 ng L^{-1} 8.2 ng L^{-1}	9.9 9.7	Tap water	[22]
TETRA **CYPER** **PERME**	0.3 mL acetone	15 µL chlorobenzene	GC-FID	0.8-100 µg L^{-1}	768 803 748	0.6 µg L^{-1} 0.6 µg L^{-1} 0.7 µg L^{-1}	4.2 6.8 2.9	Domestic wastewater	[31]
PERME **CYHAL**	500 µL methanol	8 µL dodecan-1-ol	GC-ECD	10-2000 ng L^{-1} 5-2000 ng L^{-1}	475 790	1.4 ng L^{-1} 1.5 ng L^{-1}	4.3 4.2	Tap and lake water	[29]
CARB	1.5 mL ACN	126 µL chloroform	HPLC-UV	0.001-10 µg L^{-1}	-	0.1 µg L^{-1}	4.5	Tap, sea, river and mineral water	[50]
CLP-M **CLP-E**	2 mL acetone	15 µL carbon tetrachloride	GC-MS	10-100 µg L^{-1}	40000	120 pg L^{-1} 330 pg L^{-1}	8.6 9.0	Tap, irrigation and well water	[46]
PYRs **CARBs** **OPPs** **2-PP** **PBO**	1.9 mL ACN	178 µL trichloroethane	PTV-LVI-GC-MS/MS	0.08-40 µg L^{-1}	46.2-71.6	0.5-5 ng L^{-1}	0.8-14.6	Sea, river and wastewater	This work

Table 4: Mean recoveries of multiclass pesticides in water samples. Six replicate analyses at 0.5 and 10 µg L^{-1}spiking level.

Compund	River water (Recovery±SD, %)		Seawage water (Recovery±SD, %)		Seawater (Recovery±SD, %)
	0.5 µg L^{-1}	10 µg L^{-1}	0.5 µg L^{-1}	10 µg L^{-1}	0.5 µg L^{-1}
2-PP	85±4	99±2	90±3	102±4	129±7
CLP-M	98±8	116±12	95±8	124±5	104±4
CLP-E	101±7	100±3	108±2	126±3	100±8
CARB	112±7	101±4	103±13	98±8	127±2
BIOAL	107±6	101±3	71±1	107±6	102±4
TETRA	114±6	101±3	86±4	118±1	91±5
CYHAL	118±7	91±3	73±8	105±4	97±8
PERME	101±6	101±5	102±12	120±2	93±6
CYPER	92±6	100±5	74±5	96±5	91±6
DELTA	112±6	100±5	96±4	104±4	128±3
RESME	99±13	98±14	91±6	90±11	101±4
FLUCY	97±6	100±4	103±13	70±10	101±5
PBO	110±7	100±4	95±2	97±4	109±7
CYFLU	105±7	101±8	95±8	102±5	104±3
EMPEN	93±2	115±14	103±2	102±10	65±3
FENVA	82±10	104±7	92±9	115±6	103±9
PROP	108±10	109±8	80±7	93±10	102±3

3.3. Application of the method

The optimized methodology was applied to the determination of the target pesticides in seawater, river water and wastewater (WTP) samples in triplicate using the matrix-matched standard and IS calibration curves. The results for each set of experiments are summarized in Table 5. No signals for cyfluthrin, empenthrin, fenvalerate and propoxur were observed in any tested matrix. Chlorpyrifos-ethyl and carbaryl were the most ubiquitous analytes detected in real samples. The maximum concentrations of several analytes were higher than the MCL (0.1 µg L^{-1}) of the individual pesticides [49]. The highest concentration levels corresponded to carbaryl in seawater samples related to aquaculture, with concentrations up to 400 ng L^{-1}, and to bioallethrin and resmethrin in rural river water and influent WTP 3 sample. 2-Phenylphenol, cypermethrin and piperonyl butoxide were also found in WTP samples in concentrations up to 100 ng L^{-1}. Seven of the investigated compounds were detected in river water samples, with concentrations up to 200 ng L-1 for bioallethrin. Traces of six pesticides were quantified in the WTP samples. Deltamethrin was detected and quantified in only one seawater sample from a shellfishing area. Average concentration of total pesticides found in the cannery WTP2 was 517.3 ng L^{-1}, which is higher than the MCL (0.5 µg L^{-1}) for total pesticides. Concentrations of chlorpyrifos-ethyl, carbaryl and bioallethrin in effluent WTP 3 samples were slightly higher than in inffluent WTP 3 samples. Nevertheless, it is important to note that although the concentration for total pesticides (435 ng L^{-1}) are lower than the MCL (0.5 µg L^{-1}) [49], the individual concentrations may still represent a risk due to due to the partial effectiveness of the WTP operation.

The chromatograms obtained for different water samples (WTP1 inffluent, aquaculture seawater 1, rural river water and cannery WTP2) working in the optimal conditions described are shown in Fig. 3. Chromatograms from a residues-free water sample are also shown for comparison.

Table 5: *Found concentration (ng L^{-1}) of multiclass pesticides in seawater, river water and wastewater samples (average $\pm$ SD) in triplicate. Number of positive samples (Freq.).*

	2-PP	CLP-M	CLP-E	CARB	BIOAL	TETRA	CYHAL
Freq	7/13	3/13	13/13	13/13	7/13	6/13	5/13
Seawater[a]	nd	<LOQ	116.0±0.1	<LOQ	nd	<LOQ	<LOQ
Seawater[b]	nd	nd	9.0±0.2	420±0.4	nd	<LOQ	<LOQ
Effluent WTP 1[c]	94±4.9	nd	<LOQ	21.0±0.1	90±7	10.0±0.1	nd
Effluent WTP 2[c]	nd	nd	49.0±0.3	360±0.1	nd	nd	<LOQ
Aquaculture seawater 1	nd	nd	5.3±0.1	440.0±0.3	14.0±0.1	nd	<LOQ
Aquaculture seawater 2	nd	<LOQ	15.4±0.1	<LOQ	nd	nd	nd
Aquaculture seawater 2	nd	<LOQ	36.0±0.4	<LOQ	nd	<LOQ	<LOQ
Urban river water	34.6±3	-	3.9±0.4	17±1	nd	24±2	nd
Rural river water	16±2	-	24±2	<LOQ	210±7	<LOQ	nd
Inffluent WTP 3[d]	113±11	-	<LOQ	13±1	79±8	nd	nd
Inffluent WTP 3[d]	78±7	-	2.7±0.1	22±3	92±11	nd	nd
Inffluent WTP 4[d]	135±7	-	<LOQ	13±2	73±7	nd	nd
Inffluent WTP 4[d]	40.6±3	-	<LOQ	10±1	69±3	nd	nd

Table 5: *Continued.*

	PERME	CYPER	DELTA	RESME	FLUCY	PBO
Freq	4/13	5/13	2/13	5/13	2/13	6/13
Seawater[a]	nd	nd	nd	nd	nd	nd
Seawater[b]	<LOQ	<LOQ	20.0±0.2	nd	<LOQ	nd
Effluent WTP 1[c]	36±3	9.3±3	nd	nd	nd	nd
Effluent WTP 2[c]	nd	108.3±0.1	<LOQ	nd	<LOQ	nd
Aquaculture seawater 1	<LOQ	<LOQ	nd	nd	nd	nd
Aquaculture seawater 2	nd	nd	nd	nd	nd	nd
Aquaculture seawater 2	nd	nd	nd	nd	nd	nd
Urban river water	<LOQ	<LOQ	nd	nd	nd	40±6
Rural river water	nd	nd	nd	130±12	nd	10.0±0.2
Inffluent WTP 3[d]	nd	nd	nd	110±6	nd	120±11
Inffluent WTP 3[d]	nd	nd	nd	80±12	nd	60±4
Inffluent WTP 4[d]	nd	nd	nd	60±6	nd	50±6
Inffluent WTP 4[d]	nd	nd	nd	40±1	nd	10±1

[a] Seawater from a no shellfishing area; [b] Seawater from a shellfishing area; [c] cannery water treatment plant; [d] wastewater treatment plant; nd: not detected

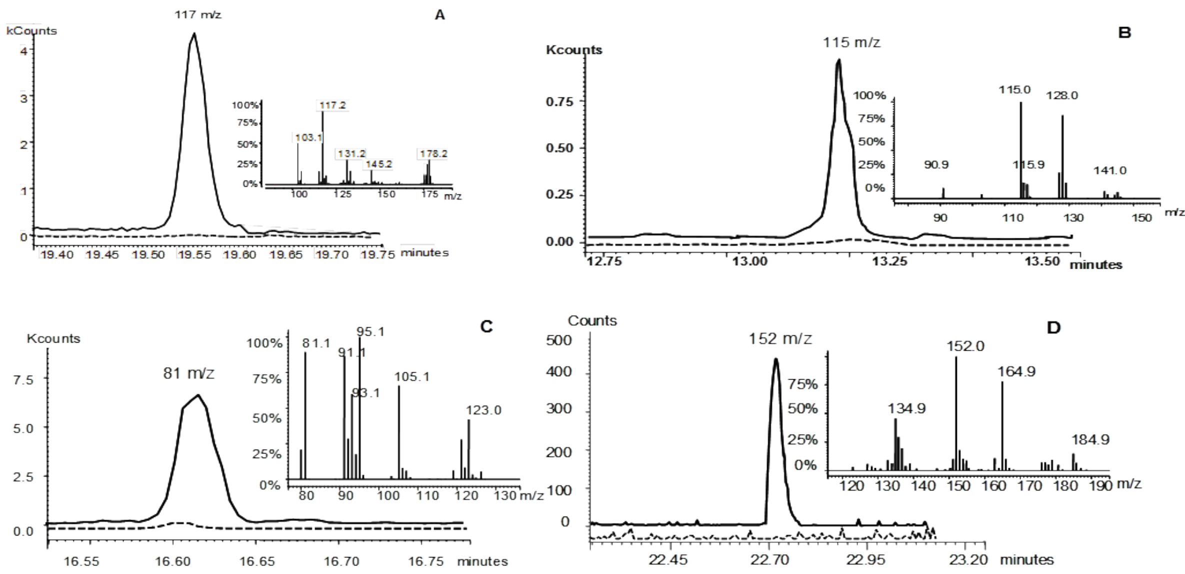


Figure 3. *Selected ion chromatograms (PTV-LVI-GC-MS/MS) and mass spectra of WTP 3 influent extract containing piperonyl butoxide (A), of aquaculture seawater 1 extract containing carbaryl (B), of rural river water extract containing bioallethrin (C) and of cannery WTP 2 extract containing cypermethrin (D) in measured concentrations of 120, 440, 210 and 108 ng L^{-1}, respectively. Chromatograms from a residues-free water sample are shown in each case as dash lines. Selected ion chromatogram of a standard solution spiked with 50 μg L^{-1} of target analytes.*

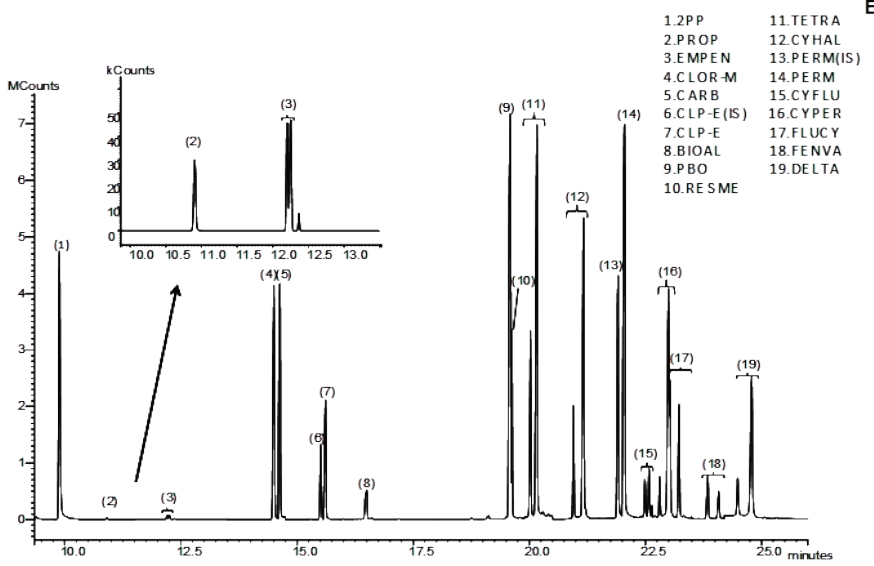


Figure 3. *Continued. Selected ion chromatogram of a standard solution spiked with 50 μg L^{-1} of target analytes (E).*

4. Conclusions

A new DLLME combined with PTV-LVI-GC-MS/MS method was developed for determination of trace levels of multiclass pesticide residues in water. Extraction parameters were optimized applying an experimental design. The extractant and dispersant solvent volumes are the variables with the highest influence on the efficiency extraction. Remarkable advantages of the developed method can be

highlighted: i) very simple and quick sample pretreatment, which not required pH adjustment or salt addition, ii) high efficiency with enrichment factors and LOQs in the low nanogram per liter range, and iii) DLLME can be used as a clean-up procedure, to change the solvent or to reverse the polarity of the solvent. Moreover, DLLME allows carrying out a derivatization reaction simultaneously with the extraction process. The method present recoveries appropriate for the pesticide residue analysis and, when applied to real samples, revealed the presence of eleven target pesticides in seawater at areas of aquaculture production, and nine of them in river and wastewater. The proposed DLLME method contributes to detect pesticide levels at ng·mL^{-1} levels. Wide application of this method for monitoring target compounds and their metabolites is foreseen.

Aknowledgments

This research was supported by the Dirección Xeral de I+D of Xunta de Galicia (Project 10TAL209005PR (SPAIN).

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2. Desarrollo de métodos de análisis para la determinación de pesticidas en algas

Relación de trabajos publicados en este capítulo:

Diego García-Rodríguez, Rafael Cela-Torrijos, Rosa A Lorenzo-Ferreira, Antonia M Carro-Diaz *“Analysis of pesticide residues in seaweeds using matrix solid-phase dispersion and gas chromatography mass spectrometry detection”* **Food Chem. (2012) 135, 259-267**

D. García-Rodríguez, A. M. Carro, R. A. Lorenzo, R. Cela *“Determination of pesticides in seaweeds by pressurized liquid extraction and programmed temperature vaporization-based large volume injection-gas-chromatography-tandem mass spectrometry”* **J. Chromatograph. A (2010) 1217, 2940-2949**

Diego García-Rodríguez, Antonia M. Carro, Rafael Cela, Rosa A. Lorenzo *“Microwave-assisted extraction and large-volume injection gas chromatography tandem mass spectrometry determination of multiresidue pesticides in edible seaweed”* **Anal. Bioanal. Chem. (2010) 398, 1005-1016**

Póster: ***“Optimization of a matrix solid-phase extraction method for the determination of pyrethroid, organophosphorus and carbamate pesticides in eddible seaweed samples”*** 36th International Symposium on Environmental Analytical Chemistry, 05-09 Octubre 2010, Roma (Italia)

Póster: ***“Application of statistical designs of experiment with desirability function for the extraction of multiresidue pesticides from seaweed samples”*** VII Colloquium Chemiometricum Mediterraneum, 21-24, Junio 2010, Granada (España)

Póster: ***“Optimisation of PTV-LV injection for determination of pyrethroid, organophosphorus and carbamate pesticides in edible seaweed samples using gas chromatography-mass spectrometry”*** XV Reunión SEQA, 19-29 Julio 2009, San Sebastian (España)

Póster: ***“Optimization of a pressurized liquid extraction method for the determination of pyrethroid and organophosphorus pesticidas in marine samples”*** 12as Jornanadas de Análisis Instrumental, 21-23 Octubre 2008, Barcelona (España)

2.1. Determinación de pesticidas en algas mediante extracción con líquidos a presión e inyección de grandes volúmenes con vaporización de temperatura programada-cromatografía de gases con espectrometría de masas en tándem.

En la actualidad los productos derivados del mar son muy demandados. Entre ellos las algas, usadas tanto en el sector de la alimentación por sus importantes aportes nutricionales y el agrícola por su empleo como fertilizantes. Las algas comestibles se han incorporado a la dieta por su bajo aporte calórico, con una alta concentración en minerales, vitaminas y proteínas y bajo contenido lipídico. Son una excelente fuente de vitaminas A, B1, B12, C, D y E, riboflavina, niacina y ácidos pantoténico y fólico, y minerales como Ca, P, Na y K. El objetivo de este trabajo es la validación de un método analítico para la determinación de pesticidas en algas cultivadas con fines alimentarios. Los compuestos que se determinan son: Dos pesticidas organofosforados (clorpirifos-metil y clorpirifos-etil), tres pesticidas pertenecientes al grupo de los piretroides (permetrina, α-cypermetrina y deltametrina) y un carbamato (carbaril). Para el tratamiento de parásitos del salmón se utilizan pesticidas estudiados en este trabajo (organofosforados); para combatir las plagas de piojo de mar (piretroides); y en el control de enfermedades en el cultivo de camarones (carbaril) se realiza normalmente mediante un baño de inmersión.

Inicialmente, el método analítico desarrollado para la determinación de estos compuestos en algas comestibles se basa en la extracción con disolventes a temperatura y presión elevadas (PLE) seguida de la separación y cuantificación mediante cromatografía de gases con detección de micro-captura electrónica (GC-µECD). El desarrollo y la optimización del proceso de PLE en algas para la extracción de los compuestos de interés se han llevado a cabo haciendo uso de diseños experimentales. Un diseño de superficie de respuesta (Doehlert) ha sido usado para la optimización de las cantidades de dos adsorbentes de limpieza añadidos en la celda de PLE (Florisil y carbón grafitizado, GCB). Las condiciones de PLE, incluyendo % de hexano en la mezcla de disolvente de extracción con acetato de etilo, temperatura, tiempo de extracción estática, % *flush* (volumen de disolvente fresco añadido para la extracción) y número de ciclos, han sido evaluadas mediante un diseño de *screening* o cribado.

La inyección en PTV se lleva a cabo en cuatro pasos: inyección, evaporación del disolvente, la transferencia de analito, y la limpieza. La introducción de grandes volúmenes (LVI) usando PTV puede mejorar los límites de detección del sistema cromatográfico y se minimizan fenómenos de degradación térmica y descomposición en el inyector. Se han considerados diversos volúmenes de inyección. También se ha evaluado el efecto sobre la sensibilidad del sistema de varias rampas de temperatura partiendo de un valor inicial de 70 ºC. La influencia en la eficacia de la inyección, del tiempo de splitless y el flujo de split han sido estudiados mediante un diseño Doehlert.

El método desarrollado permite la extracción y la purificación de las muestras simultáneamente, por lo que es un procedimiento sencillo y rápido siendo, por tanto, una alternativa a los métodos clásicos de extracción. Además, se pueden cuantificar residuos de estos compuestos a niveles muy bajos gracias a los excelentes límites de cuantificación logrados. Se ha validado el método de PLE-PTV-LVI-GC-MS/MS de forma satisfactoria y ha sido aplicado, con éxito, a varias muestras de algas salvajes y comerciales, para consumo humano, en las que han sido detectados/cuantificados algunos de los compuestos estudiados.

DERMINATION OF PESTICIDES IN SEAWEEDS BY PRESSURIZED LIQUID EXTRACTION AND PROGRAMMED TEMPERATURE VAPORIZATION-BASED LARGE VOLUME INJECTION -GAS-CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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Abstract

A rapid method for the simultaneous identification and quantification of pesticide residues in edible seaweed has been developed. Target analytes were three pyrethroid, a carbamate and two organophosphorus pesticides. The procedure consists of a pressurized liquid extraction (PLE) with integrated clean-up, followed by gas chromatography coupled to tandem mass spectrometry. Five PLE parameters were investigated using a screening design: temperature, static extraction time, number of cycles, percent of flush volume and quantitative composition of the n-hexane/ethyl acetate extraction solvent. The effect of the in-cell clean-up with Florisil® and graphitized carbon black adsorbents was investigated using a Doehlert response surface design. Large-volumes of sample extracts were injected using a programmed-temperature vaporizer (PTV-LVI) to improve both sensitivity and selectivity of measurements. Quantification was carried by the internal standard method with surrogate deuterated standards. The method showed excellent linearity (R^2 > 0.999) and precision (Relative Standard Deviation, RSD ≤ 8%) for all compounds, with detection limits ranging from 0.3 pg g^{-1} for chlorpiryfos-ethyl, to 3.0 pg g^{-1} for carbary (23.1 pg g^{-1} for deltamethrin). Recoveries in real seaweed samples were within the range 82-108 %. The method was satisfactory validated for the analysis of wild and cultivated edible seaweeds. The presence of pyrethroid and organophosphorus pesticides in some of the samples was evidenced.

Keywords: Pressurized liquid extraction; large volume injection-programmed temperature vaporisation; GC-MS/MS; pyretroid; organophosphorus pesticides; carbaryl; seaweed.

1. Introduction

The extensive use of pyrethroid (PYR), organophosphorus (OP) and carbamate (CAR) pesticides may lead to their discharge into surfacewater, groundwater, and soil. Since they are employed as chemotherapeutants in aquaculture, residues of these pollutants and their degradation products can also remain in the marine environment [1]. OP and CAR insecticides interfere with acetylcholine-mediated synaptic transmission in the nervous systems of fish and other aquatic animals via the inhibition of AChE (acetylcholinesterase) enzyme activity [2]. CAR pesticides cause metabolic and behavioural alteration in fish [3]. Most of the PYR compounds are considered endocrine disruptors and affect oestrogens and progesterone hormones [4]. PYR, OP and CAR pesticides are relatively hydrophobic and tend to accumulate on the solid matter of the seawater.

The seaweed industry uses 7.5–8 million metric tonnes of wet seaweeds annually, either from the wild or from cultivated crop. Seaweeds have gained importance as foodstuffs in Western countries and most recently as components of functional foods because of their high dietary fiber, mineral, vitamin, phytochemical content, low energy levels, and high concentrations of certain polyunsaturated fatty acids [5]. The multipurpose uses of seaweed phycocolloids (emulsifiers in dairy products, pharmaceutical industries, food additives commonly used in fast food, etc.) have an immense value. The association of pesticides to the seaweed can produce a bioaccumulative effect along the food chain with the consequent risk for human health [6-9]. Therefore, some aspects of food security and risk assessment studies should be considered before we go ahead for any commercial seaweed exploitation [9].

The extraction of non-polar and semi-polar organic compounds (e.g. PYR, OP and CAR pesticides) from environmental matrices was classically undertaken by Soxhlet, sonication extraction [10, 11] or microwave-assisted extraction (MAE) [10, 12, 13]. The matrix components co-extracted when this procedures are used, are generally removed in successive clean-up steps prior to the chromatographic analysis [14]. Matrix solid-phase dispersion (MSPD) [15] and supercritical fluid extraction (SFE) [16] have been successfully applied for the analysis of trace organic pollutants from biota samples. Both techniques offer the advantage of the simultaneous clean-up of the extracts. *Table 1* summarizes analytical procedures that have been used to determine PYR, OP and CAR pesticides in different sample matrices.

Pressurized liquid extraction (PLE) has been successfully applied for the extraction of persistent organic pollutants from different matrices as soil, compost, vegetables or fish with off-line clean-up or integrated clean-up [17-24]. Integration of the PLE and cleanup operations has also been achieved by loading a matrix retainer (Florisil®, H_2SO_4/silica, alumina or carbon) at the bottom of the PLE extraction cell [19, 20, 23]. In this work, a one-step extraction and cleanup PLE procedure for PYR, OP and CAR pesticides

is evaluated using a mixture of Florisil® and graphitized carbon black (GCB). The main advantages are a substantial reduction of the extraction time (2 min), the solvent volume and the small amount of sample and adsorbents required (11 mL cells).

Tandem mass spectrometry (MS/MS) using a low-resolution ion-trap mass spectrometer is a very selective technique which is widely employed for pollutant analysis in food. There are two common ways for increasing sensitivity of chromatography determinations: to increase of the sample size and to inject large volumes of sample into the gas chromatography column. In both cases an extensive extract cleanup is required. Large-volume injection with a programmed-temperature vaporizer (PTV-LVI) combined with GC coupled to MS or MS/MS has been previously employed for the determination of pesticide residues in fruits, vegetables [25] and water [26] or flame retardants in urban dust [45]. Also, it has been used HPLC methodologies to determine PYR in soil [27]. In this way the sensitivity was considerably increased, compared to the use of conventional split/splitless injectors. So, PTV-LVI coupled to GC−MS/MS appears to be a good alternative to more sensitive high resolution mass spectrometry (HRMS) equipments, offering a positive balance between sensitivity, versatility, and cost. To the best of our knowledge there are no reports in the literature concerning the application of PLE-PTV-LVI-GC-MS/MS for the multiresidue extraction of OP, PYR and CAR pesticides in seaweed samples.

The aim of this paper is to describe a selective PLE method with in-cell clean-up for the extraction of aquaculture pesticides from edible seaweed and their subsequent analysis by GC-MS/MS. The target analytes were three pyrethroid (permethrin, α-cypermethrin and deltamethrin), a carbamate (carbaryl) and two organophosphorus pesticides (chlorpiryfos-ethyl and chlorpiryifos-methyl). Extraction conditions were optimized by means of experimental designs involving desirability functions. Moreover, a practical and efficient analysis method for OP, PYR and CAR pesticides have been developed based on GC-MS/MS operating in electron impact (EI) mode with a programmable temperature inlet and large volume injection. The developed method was also compared with other methodologies based on traditional GC-MS/MS and GC-µECD and its performance was characterized in terms of accuracy, precision, linearity and LODs. Finally, the method was applied to the analysis of real seaweed samples.

Table 1: Determination of OP, PYR and CAR pesticides in different matrices.

Matrix	Pesticide residues	Extraction procedure	Analytical Technique	LOD	Ref.
Water	PYR	SBSE	LD-LVI-GC-MS	0.5 µg L^{-1}	[26]
Water	OP	SPE	GC-ECD / GC-MS/MS	0.1 µg L^{-1} 6.0ng L^{-1}	[28]
Water	OP	SPE	GC-MIP-AED	17.1-170.3 ng mL^{-1}	[29]
River water	PYR	—	HPLC-PIF	0.02 µg L^{-1}	[41]
Seawater	OP, PYR	SPME	GC-MS/MS	11 pg mL^{-1}	[1]
Ground, sea water	PYR	SPE	LC-ESI-MS	0.5 ng L^{-1}	[31]
Water, vegetables	OP	SPE	HPLC-FD	0.01 ng mL^{-1}	[33]
Vegetables	OP, PYR	PLE	GC-MS	3-8 µg Kg^{-1}	[18]
Vegetables	OP	Solvent extraction (DCM)	GC-PFPD	2.0 µg L^{-1}	[35]
Vegetales	PYR	Solvent extraction (DCM)	LC-ESI-MS	3.0 ng g^{-1}	[39]
Vegetables	PYR	Solvent extraction (DCM)	HPLC-CL	0.04 µg mL^{-1}	[40]
Fruits, vegatables	OP, PYR, CAR	Solvent extraction (acetone) + RP-SPE	PTV-LVI-GC-MS/MS	—	[25]
Fruits, vegetables	OP, PYR	SFE	GC-ECD	0.01 mg Kg^{-1}	[38]
Fruits, vegetables	CAR	PHWE	GC-FE	1 µg mL^{-1}	[42]
Avocado	OP, PYR	PLE-GPC	LP-GC-MS/MS	0.01-2.50 µg Kg^{-1}	[43]
Juice	OP	SDME	GC-FPD	1.0 µg L^{-1}	[30]
Meat	OP	GPC	GC-MS/MS	10 ng mL^{-1}	[36]
Urine, plasma	CAR	SPE	GC-MS	34 µg mL^{-1}	[8]
Urine	OP, PYR	SPE	HPLC-TIS-MS/MS / HPLC-APCI-MS/MS	0.5 ng mL^{-1}	[34]
Urine	OP	SPE	GC-MS/MS	0.1 ng mL^{-1}	[37]
Soil	PYR	FTE	LVI-HPLC (UV)	0.3 mg Kg^{-1}	[27]
Soil	OP	PLE	GC-MS	4.6 µg Kg^{-1}	[44]
Sediment	OP, PYR	UAE	GC-ECD	0.6 µg Kg^{-1}	[32]
Compost	OP, PYR, CAR	PLE +LLE	GC-MS	0-02-0.03 µg g^{-1}	[22]

2. Experimental

2.1 Standards and materials

Pestanal quality analytical standards of α-cypermethrin (cyclopropanecarboxylic acid, 3-(2,2-dichloroethenyl)-2,2-dimethyl-(R)-cyano(3-phenoxyphenyl)methylester) (99.8%), chropiryfos-ethyl (phosphorothioic acid, O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) ester) (99.2%), chropiryfos-methyl

(phosphorothioic acid, O,O-dimethyl O-(3,5,6-trichloro-2-pyridinyl) ester) (99.7%), carbaryl (1-naphthyl-N-methylcarbamate) (99.8%) and permethrin (cyclopropanecarboxylic acid, 3-(2,2-dichloroethenyl)-2,2-dimethyl-, (3-phenoxyphenyl)methyl ester) (99.3%) were from Riedel-de Haën (Seelze, Germany). Deltamethrin (cyclopropanecarboxylic acid, 3-(2,2-dibromoethenyl)-2,2-dimethyl-(S)-cyano(3-phenoxyphenyl)methyl ester) (99.0%) was from Chem Service Inc. (West Chester, PA, USA). (Diethyl-D_{10})-chropiryfos (100 µg mL^{-1} in nonane) and (phenoxi-$^{13}C_6$)-*cis*-permethrin (50 µg mL^{-1} in nonane) were from Cambridge Isotope Laboratories (Cambridge, UK). Ethyl acetate (Chromanorm), acetone (Pestinorm), *n*-hexane (Pestinorm) and dichloromethane (Pestinorm) were from VWR-Prolabo (Mollet del Vallés, Barcelona, Spain). Methanol (gradient HPLC grade) was from Merk (Darmstadt, Germany).

Stock solutions of each individual pesticide at 5000 µg mL^{-1} and of the six studied pesticides together were prepared in acetone and were stored at -18 ºC.

Sodium sulphate anhydrous was from Panreac (Barcelona, Spain). Florisil® (60-100 mesh), sea sand (50–70 mesh) and aluminium oxide activated neutral (150 mesh) were from Aldrich (Madrid, Spain). Silica gel 60 Å (0.040-0.063 mm) was obtained from Merck (Darmstadt, Germany). GCB (bulk packing) was from Supelco (Bellefonte, PA, USA). Syringe filters (Millex GV, 13 mm, and 0.22 µm) were from Millipore (Billerica, MA, USA). Cellulose filters (20 mm diameter) for PLE cell were from Restek (Bellefonte, PA, USA). Metallic sieve (300 µm mesh width) was obtained from Filtra (Barcelona, Spain).

2.2 Samples

All dried edible seaweed samples were purchased in a local market. Wakame (*Undaria pinnatifida*), Nori (*Porphyra umbilicalis*), Irish Moss (*Chondrus crispus*), Sea Spaghetti (*Himanthalia elongata*) and a commercial mixture of Wakame (30%), Sea Lettuce (*Ulva lactuca*, 19%), Kombu (*Laminaria ochroleuca*, 18%), Dulse (*Palmaria*, 18%) and Nori (15%) were grinded by means of an electric mill and stored in sealed vessels until analysis. Wild seaweed samples: *Ascophyllum nodosum*, *Himanthalia elongate, Ulva lactuca, Fucus vesiculosus, Leathesia difformis* and *Chondrus crispus* were collected in the vicinity of seaport and beaches located on the coast of Galicia (NW Spain). The seaweed samples were stored at -18 ºC before processing. The frozen samples were dried in an oven at 50 º C for 24 h, triturated and homogenized in an electric mill until use. All samples were sieved to obtain a particle size under 300 µm.

Spiked Wakame seaweeds were used as the matrix to carry out the optimization study. Approximately 25 g of sample were placed in a beaker with a broad base and covered with 50 mL of acetone spiked with the pesticides (1.5 mL of the stock standard solution of 5-6 µg mL^{-1}, containing the six pesticides) to obtain a final concentration in the food of 0.08-0.30 µg g^{-1} in each analyte. The sample was

allowed to air-dry in the dark for two days and stored at 4ºC before extraction, in order to simulate the normal interaction between the seaweed and the pesticide compounds. Other types of edible seaweed were similarly subjected to the spiking process, in order to demonstrate the suitability of the proposed method for the extraction of the target compounds from real samples.

2.3 Extraction procedure

Extractions were accomplished using a pressurized liquid extractor ASE 200 from Dionex (Sunnyvale, CA, USA), fitted with 11 mL capacity stainless-steel cells. Two cellulose filters followed by layers of adsorbents were placed at the bottom of each cell. After loading the corresponding amount of clean-up adsorbent, the dispersed sample was introduced in the cell and the remaining volume was filled with sea sand. Then, another cellulose filter was placed on the top. The effect of the different extraction parameters, regarding recoveries and selectivity, was investigated using an experimental design methodology. Nemrod®W 2000 software package [46] was used to generate the matrix of experiments and to determine the effect of each factor on the efficiency of the extraction.

Under final conditions, 0.2 g of sample were dispersed in 1 g of anhydrous sodium sulphate using mortar and a pestle. Two cellulose filters were positioned at each end of the thimble; 1.6 g of Florisil® and 0.4 g of GCB, as clean-up adsorbents, were transferred to the cell in two layers, followed by the introduction of the dispersed sample. Finally the empty space above the mixture was filled with sea sand. The PLE process was undertaken in only one step. Thus, analytes were recovered with a mixture of n-hexane/ethyl acetate (80:20) in one static extraction cycle of 2 min, at 100 °C and 1500 psi. The total flush volume and the cell purge time were 7.7 mL (70% of its capacity) and 90 seconds, respectively. The PLE extract (ca. 19 mL) was evaporated using a gentle stream of nitrogen in a Turbo Vap II concentrator (Zymark, Hopkinton, MA, USA) and adjusted to a final volume of 1 mL. Finally, the extract was filtered by means of a syringe filter with a pore size of 0.22 µm before GC injection.

2.4 Gas-chromatographic determination

Analyte separation was determined by GC–MS/MS using a Varian 450 GC gas chromatograph (Walnut Creek, CA, USA) coupled to an ion-trap mass spectrometer Varian 240MS operating in external configuration. Separations were carried out in a Varian Factor Four VF-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Initial oven temperature was set at 70 ºC, held for 3.5 min; ramped at 25 ºC min^{-1} up to 180 ºC, held for 3 min; ramped at 10 ºC min^{-1} up to 300 ºC and held for 5 min. Helium (purity 99.999%; Carburos Metálicos, A Coruña, Spain) was employed as carrier gas with a constant flow of 1.0 mL min^{-1}. LVI mode was carried out by using a 1079 PTV injector, equipped with cryogenic CO_2

cooling. PTV injections were carried out in four steps: injection, solvent evaporation, analyte transfer, and cleaning. In the injection step, the split valve was open at 20 mL min^{-1}, and 50 µL sample were introduced into a Siltek deactivated liner with frit (Restek) at 70 °C. During the evaporation step, the temperature was raised to 85 °C at a rate of 30 °C min^{-1} for 30 s to eliminate the solvent, which was vented through the split valve at a flow of 36 mL min^{-1}. In the transfer step, the split valve was closed, and the temperature increased to 300 °C at a rate of 75 °C min^{-1} in splitless mode for 3 min. The injector was kept at 300 °C with a purge flow of 50 mL min^{-1} for cleaning purposes until the end of the run. The mass spectrometer was operated in EI mode at 70 eV. The ion source, trap, manifold and transfer line temperatures were maintained at 150, 150, 40 and 285 ºC, respectively. The analyses were performed with a filament-multiplier delay of 6.5 min. General parameters were as follows: multiplier offset +200 V, AGC target value 5000 counts, damping gas 2.5 mL min^{-1} and emission current 90 µA. Specific conditions for each analyte are listed in *table 2*. A parent ion was chosen for each compound by taking the *m/z* and relative abundance of parent ions as high as possible in order to increase sensitivity. Good quality secondary spectra for every compound were obtained selecting a non-resonant waveform. The optimization of the excitation amplitude voltage for each pesticide was achieved using the automated method development (AMD) option included in the MS/MS software tool kit [47]. This value was considered optimum when the secondary spectra showed multiple and intense product ions while the parent ion intensity remained around 10%. Quantification was accomplished by relative areas versus (phenoxi-$^{13}C_6$)-*cis*-permethrin used as IS for PYR and (diethyl-D_{10})- chlorpiryfos used as internal standard (IS) for OP and CAR.

Preliminary PLE experiments and comparative analyses were performed in a Agilent 6890N GC (Avondale, PA, USA) fitted with a micro electron-capture detector (µ-ECD) using an Agilent fused-silica column HP-5 (30 m x 0.32 mm i.d., 0.25 µm film thickness). 1 µL of extract was injected in splitless mode (2 min) using an autosampler. Injection port temperature was set at 280 ºC and split flow was adjusted at 50 mL min^{-1}. Initial oven temperature was set at 60 ºC, held for 2 min; ramped at 20 ºC min^{-1} up to 290 ºC and held for 6 min. Helium was employed as carrier gas with a constant flow of 1.5 mL min^{-1}. Nitrogen (purity 99.999%; Carburos Metálicos) was used as make-up gas at a constant flow of 40 mL min^{-1}. Also, a Varian 3900 GC (Walnut Creek, CA, USA) coupled to an IT mass spectrometer Varian Saturn 2100T was used. Separation was carried out on an Agilent HP-5MS fused-silica column (30 m x 0.25 mm i.d. x 0.25µm film thickness) with helium (purity 99.999%) as the carrier gas with a constant flow of 1.2 mL min^{-1}. Split flow was set at 50 mL min^{-1}. Injection conditions as well as oven temperature program were similar to the GC-µECD conditions described above. The mass spectrometer was operated in EI mode at 70 eV. The trap, manifold and transfer line temperatures were maintained at 220, 100 and 280 º C, respectively. The analyses were performed with a filament-multiplier delay of 6 min. General parameters were as follows:

multiplier offset +100 V and AGC target value 4000 counts. Specific conditions for each analyte are listed in *table 2*.

Table 2: *Specific MS/MS conditions for each pesticide and IS.*

Pesticide	$logK_{ow}$	*m/z* range	Parent ion (*m/z*)	Excitation Amplitude (V)		Excitation Storage Level (*m/z*)		Quant. ions[a] (*m/z*)
				240MS	Saturn 2100T	240MS	Saturn 2100T	
CLP-M	3.7	125-325	286.0	85.0	0.9	100.0	126.0	208 (243)
CARB	2.4	80-154	144.0	51.0	63.0	55.0	62.0	116
CLP-E	4.8	120-324	314.0	73.0	84.0	120.0	138.0	258 (286)
PERM	7.1	140-193	183.0	66.0	0.6	75.0	80.0	165 (153)
CYPER	6.3	130-191	181.0	77.0	0.4	75.0	72.0	152
DELTA	6.2	130-191	181.0	78.0	0.6	75.0	111.0	152
(D_{10})-CLP-E (IS)	—	125-334	324.0	77.0	84.0	125.0	138.0	260 (292)
($^{13}C_6$)-PERM (IS)	—	130-199	189.0	67.0	0.6	75.0	83.0	171 (159)

[a] Confirmation ions between brackets.

3. Results and discussion

3.1. Optimization of LVI using PTV-GC/MS technique

The programmed temperature vaporization technique was used to introduce large sample volumes into capillary GC columns at a temperature slightly below the boiling point of the solvent. The PTV inlet has the same basic functions as the split/splitless inlet except that it is temperature programmable (using CO_2 cooling). LVI using PTV can improve GC system detection limits by one to two orders of magnitude over conventional split/splitless inlet [48]. LVI with PTV is ideal for trace analysis of later eluting solutes with boiling points approximately 100 ºC higher than ethyl acetate solvent and for samples with a complex matrix. However, due to the complicated PTV injection process, many factors can affect the performance and efficiency of this system, such as liner type, injection volume, initial inlet temperature and temperature ramp rate [48].

In this work, a Siltek deactivated liner with frit was selected because Siltek treatment proved to be efficient in preventing degradation of pesticides in liners [25]. Using a special split vent program and an injector temperature ramp, it was found that with the increase of injection volume (7, 10, 15, 20 and 50 µL), the response intensities progressively increased. When the injection volume was over 50 µL the chromatogram background increased, and no significant signal enhancement was achieved. Therefore, in the current study, the optimal injection volume was set at 50µL.

One of the advantages of the PTV inlet is that the sample can be introduced in an initial cool inlet. Thus, the evaporation, decomposition and the thermal degradation during the injection process is minimised [25]. In this study, it was found that the initial cool inlet (70ºC) used in PTV-LVI mode could result

in a 35-2700-fold increase of peak area over that of a traditional hot inlet (280 ºC). For most of the PTV application, a slow ramp rate can be applied to minimise the thermal decomposition of the labile analytes. Ramp rates of 75, 100 and 200 ºC min^{-1} were studied to evaluate their effect to the system sensitivity. The inlet ramp rate set at 75 ºC min^{-1} showed the best sensitivity for all the analytes. When the inlet ramp rate was 200 ºC min^{-1}, the analytes could be partially degraded, as proven by the decreased response intensity.

The inlet vent flow is another factor which can affect the system sensitivity [49]. If the vent flow is too low, some solvent will remain in the liner. If the vent flow is too high, some analytes will be swept out of the liner. Thus, the splitless time (min) and the split flow (mL min^{-1}) have been evaluated in the injection efficiency by using a response surface Doehlert design [50]. This design involves 8 experiments randomly performed to provide protection against the effects of lurking variables. Two replicates were made at the central point of the experimental domain. For all the experiments, the injection volume was fixed at 50 µL, the initial inlet temperature was 70 ºC and the inlet ramp was 75 ºC min^{-1}. In the experimental domain, five different splitless times (0.1, 0.3, 0.5, 0.7 and 0.9 min) and three split flows (12, 25 and 38 mL min^{-1}) were considered. The responses that were expressed as peak areas were fitted by a multiple regression equation, including curvature and interaction terms. Splitless time and its interaction with split flow were statistically significant for OP pesticides. The behaviour of PYR and OP was similar, and the better responses were obtained at medium-high level of both factors. However, CARB shows a different behaviour and better responses were acquired at high splitless time and low split flow. *Figures 1A and 1B* show, as examples, the response surfaces obtained with CLP-E and CARB. In order to find the best simultaneous conditions, multicriteria decision-making strategies using desirability function optimization were applied without additional experimentation. These desirability functions were built as partial Derringer functions for each analyte response using the Nemrod®W 2000 software. The responses were transformed using a dimensionless desirability (d_i) scale, which ranged between d=0 for a completely undesirable response to d=1 for a fully desired response. Non-linear left unilateral desirability functions were required to maximize each response efficiency. In a second step, a global desirability function *(D)*, which represents the global quality of the common optimum, was calculated by combining single desirability functions, usually as the geometric mean [50]. The maximum D obtained was 1.0 for 0.6 min of splitless and 34 mL min^{-1} of split flow (*Figure 2C*). Under these conditions, the predicted values of d_i were 1.0 for all analytes.

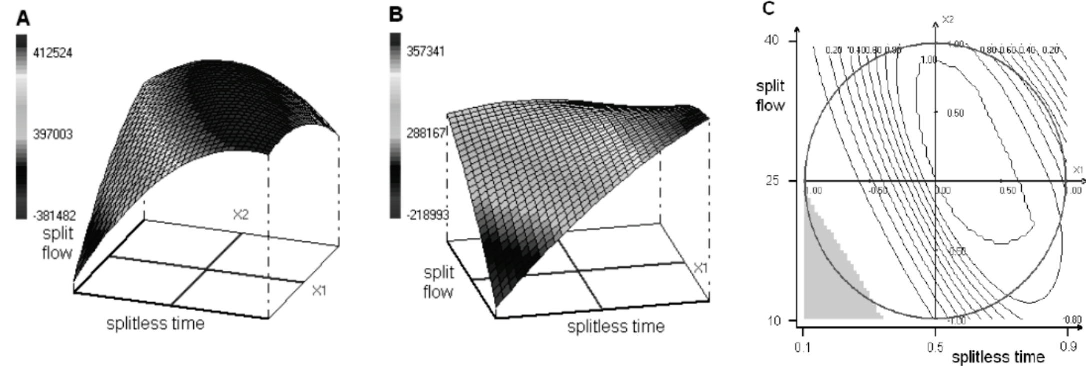

Fig. 1. *Surface response for A, CLP-E; and B, CARB versus amounts of split flow (mLmin−1) and splitless time (min). C, overall desirability function for the target analytes.*

3.2. Pressurized liquid extraction (PLE) procedure

3.2.1. Preliminary assays

PLE was performed on fortified seaweed using the following general conditions: two cycles of 5 minutes at 100 ºC, 1500 psi, solvent flush of 60%, 90 seconds of purge time [51]. Initially, anhydrous sodium sulphate was employed as dispersant and a mixture of n-hexane:ethyl acetate as solvent extraction.

The representative portion size of the spiked sample was evaluated (triplicate portions of 0.1–0.5 g were tested). The best results (RSD 5-8%) were obtained with 0.2 g of sample. Higher amounts gave rise to the co-extraction of matrix pigments that interfere with the signal of the ion selected for quantification, while lower amounts showed poor precision values. In sum, 0.2 g of spiked sample was considered to be homogeneous and chosen as the optimum sample size for subsequent experiments, in order to prevent the variability between sample portions which could mask the influence of the experimental variables.

The aggregation of sample particles may influence on the extraction efficiency and the background chromatogram; therefore the samples are dispersed in an inert material. Four dispersant agents, anhydrous sodium sulphate, neutral alumina, Florisil® and diatomaceous earth were evaluated [18]. 0.2 g of fortified seaweed were mixed in a mortar with 1 g of dispersant agent and the mixture was added directly to the PLE vessel previously loaded with 3 g of Florisil®. The extracts showed an intense green colour, due to pigments of seaweeds, and an additional clean-up step consisting on solid phase extraction (SPE) with ENVI™-Carb cartridges was required [21]. Similar background chromatograms were obtained with all the adsorbents. Therefore anhydrous sodium sulphate was selected to be used as dispersant and drying material.

In general, physico-chemical properties such as boiling point, polarity and density (which may influence the penetration into the sample matrix) and toxicity are aspects to be considered when choosing an extraction solvent [10]. Solvents of different polarities: n-hexane, dichloromethane (DCM), acetone and

ethyl acetate, and mixtures were tested for the PLE extraction of selected analytes from seaweed samples. PLE was carried out in general conditions described above. Extractions made with n-hexane, DCM and mixtures of both solvents yielded transparent and visually clean extracts, but the recovery of spiked analytes were low (*Figure 2*). The mixtures n-hexane/acetone yielded recoveries in the range 50-60%. When mixtures of n-hexane and ethyl acetate were used, better recoveries were obtained for all studied compounds. In both cases, clean-up of PLE extracts was performed by SPE- ENVI™-Carb packing cartridges. As shown in *Figure 2*, the best results were obtained when the mixture n-hexane/ethyl acetate (80:20) was used. Consequently, this solvent mixture was selected for further experiments.

3.2.2. In-cell clean-up optimization

The effect of three adsorbents; neutral alumina, Florisil® and silica gel, was investigated using spiked seaweed samples. For that, 3 g of clean-up adsorbent were loaded into the extraction cell prior to the PLE. Also an additional clean-up step of the PLE extracts based on SPE (ENVI™-Carb cartridges) was performed. Florisil® gave the best recovery results and less complex chromatograms (data not shown). Furthermore, the coloration of the extracts was much less intense. In order to completely remove all co-extracted pigments in one step, bulk GCB was evaluated as an in-cell clean-up adsorbent [18]. Three cleanup systems were loaded in the extraction vessel to be evaluated in a comparative study: 3g of Florisil®, 3g of GCB and a mixture of 1 g of GCB and 3 g of Florisil®. The mixture GCB and Florisil® provided colourless extracts and the best recoveries. Also, a significant reduction on the chromatogram background was achieved. The amounts of adsorbents were optimized using a Doehlert design [50]. Values assigned to the high and low levels for each factor were: Florisil® (1.2 – 2.8 g) studied at five levels and GCB (0.2 – 0.8 g) studied at three levels. The application of Surface Response Methodology (SRM) revealed a similar tendency for all compounds (response surfaces for CLP-M and PER are shown in *Figure 3A and 3B*).

The better PLE efficiency was achieved with medium levels of GCB and low amounts of Florisil®. The amount of Florisil® was a statistically significant factor for CLP-M, CARB and all PYR, while amounts of GCB were not significant for any of the studied compounds. The interaction GCB-Florisil® was statistically significant for OPP. To find the best-compromise conditions, a multicriteria optimization approach, based on desirability functions, was followed [50]. The composite desirability (*D*) that combines the individual desirability (*d*) of all the response variables into a single measure was maximized. A two-dimensional plot of the isodesirability is shown in *Figure 3C*. The regions in grey correspond to null values for desirability when level factors are not suitable to be chosen. Optimal numerical conditions resulted in 0.4 g of GCB and 1.6 g of Florisil®.

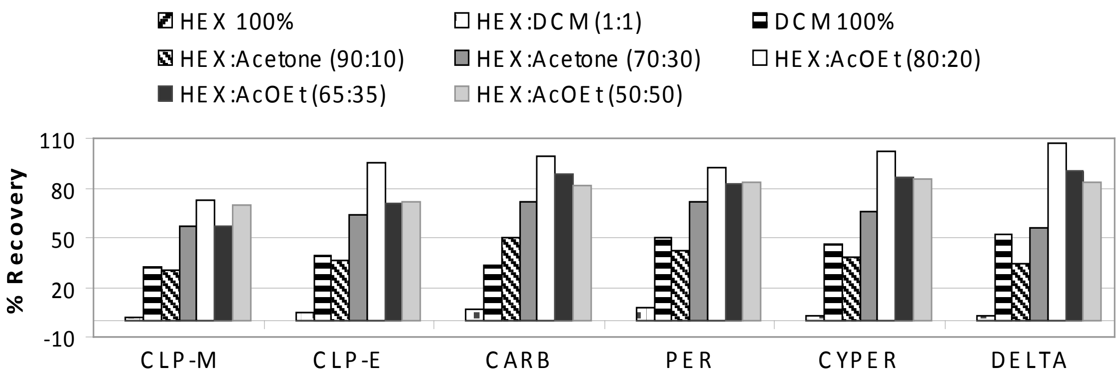

Fig. 2. *Recoveries obtained with tested extraction solvents. Pesticides were not recovered when 100% n-hexane was used as extraction solvent.*

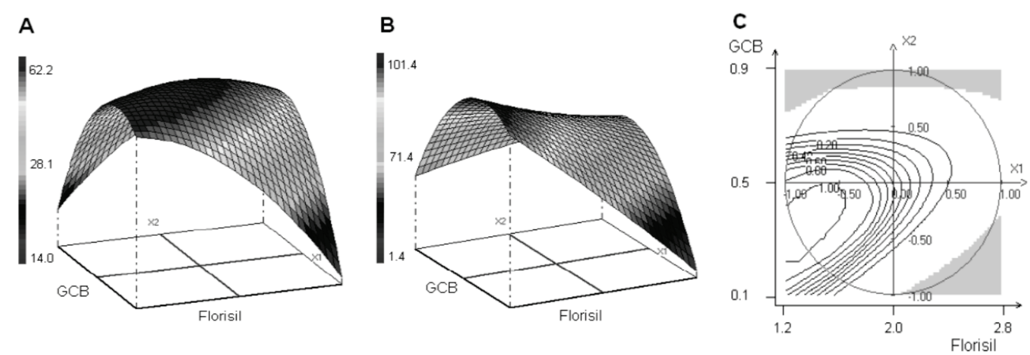

Fig. 3. *Estimated response surfaces for A, CLP-M; and B, PER versus amounts of GCB and Florisil®. C, overall desirability function for all studied compounds.*

3.2.3. Optimization of PLE conditions

Extraction solvent, temperature, static time, total flush volume and number of cycles are important parameters that must be optimized in order to achieve an efficient extraction [18].

Pressure does not have much influence on analyte recoveries in PLE. Nevertheless, an elevated pressure maintains the extraction solvent in a liquid state at high temperatures and may help the solvent to penetrate small pores in the matrices. It also forces flow through the sample and filter during the short dynamic mode. Taking in to account these aspects and considering a safety issue with the equipment, a pressure of 1500 psi was selected and used throughout the study [17].

Temperature (60, 100 and 150 ºC), static extraction time (2, 4 and 6 min), number of cycles (1, 2 and 3), % solvent flush (40, 70 and 100%) and percentage of hexane in the solvent mixture with ethyl acetate (50, 70 and 80%) were the selected factors and levels studied in a three level screening factorial design [50]. The responses (% of recovery) were evaluated using ANOVA, and all the studied factors presented statistical significance for at least one of the studied compounds, and a similar behaviour was

observed for almost all pesticides (*Table 3*). The results obtained were examined with the aid of graphic tools supplied by Nemrod®W 2000 software [46]. *Figure 4* shows, as examples, the statistical significance on the response for CLP-E and CYPER.

***Table 3:** Favourable levels and statistic significance obtained for the screening factorial design for PLE evaluation.*

Factors	Favourable level					
b1: Temperature (ºC)	100*	100*	150	100*	100	100
b2: Static time (min)	2*	2*	6	2*	2*	2
b3: Cycles	1	1	2	1	1	1*
b4: Flush (%)	70*	70	70	70	70	70
b5: Hexane (%)	70	80*	50	50	70	70

* Statistically significant factors at the 95% confidence level.

Figures 4A and *4C* are delta weight plots which allowed the relative effects of a level change in a variable on the response to be compared. The effects are shown as bars, the length of which is proportional to the relative magnitude of the effect, being negative for bars going leftwards and positive for those going rightwards. The dotted lines represent the statistical significance levels as determined using the method of Lenth [50]. When the effect bars surpass the line, the effect of the factor level concerned on the response is statistically significant at a 95% confidence interval. If a coefficient is negative, the response decreases when the factor moves from the low to the high level; the contrary is obtained if the coefficient is positive. The sign and value of the coefficients allow directing the choice of the best level for each factor. *Figures 4B* and *4D* represent the total effects graphs, in which the bars are proportional in length to the effect of each factor level on the analytical response. One cycle of 2 minutes at 100 ºC and a flush of 7.7 mL (70%) were selected as the better common extraction conditions on the basis of these results. The mixture n-hexane:ethyl acetate (80:20) was statistically significant for CLP-E, while no significant differences were observed between the mixture (80:20) and (70:30) for almost all other pesticides (*Figure 4B* and *4D*). The content of pigments and impurities in PLE extracts could be affected by the polarity of the extraction solvent mixture. In this case, the co-extracted pigments by PLE using n-hexane:ethyl acetate (80:20) were lower content in comparison with n-hexane/ethyl acetate (70:30). In this way, the mixture (80:20) was chosen because it leads to cleaner extracts.

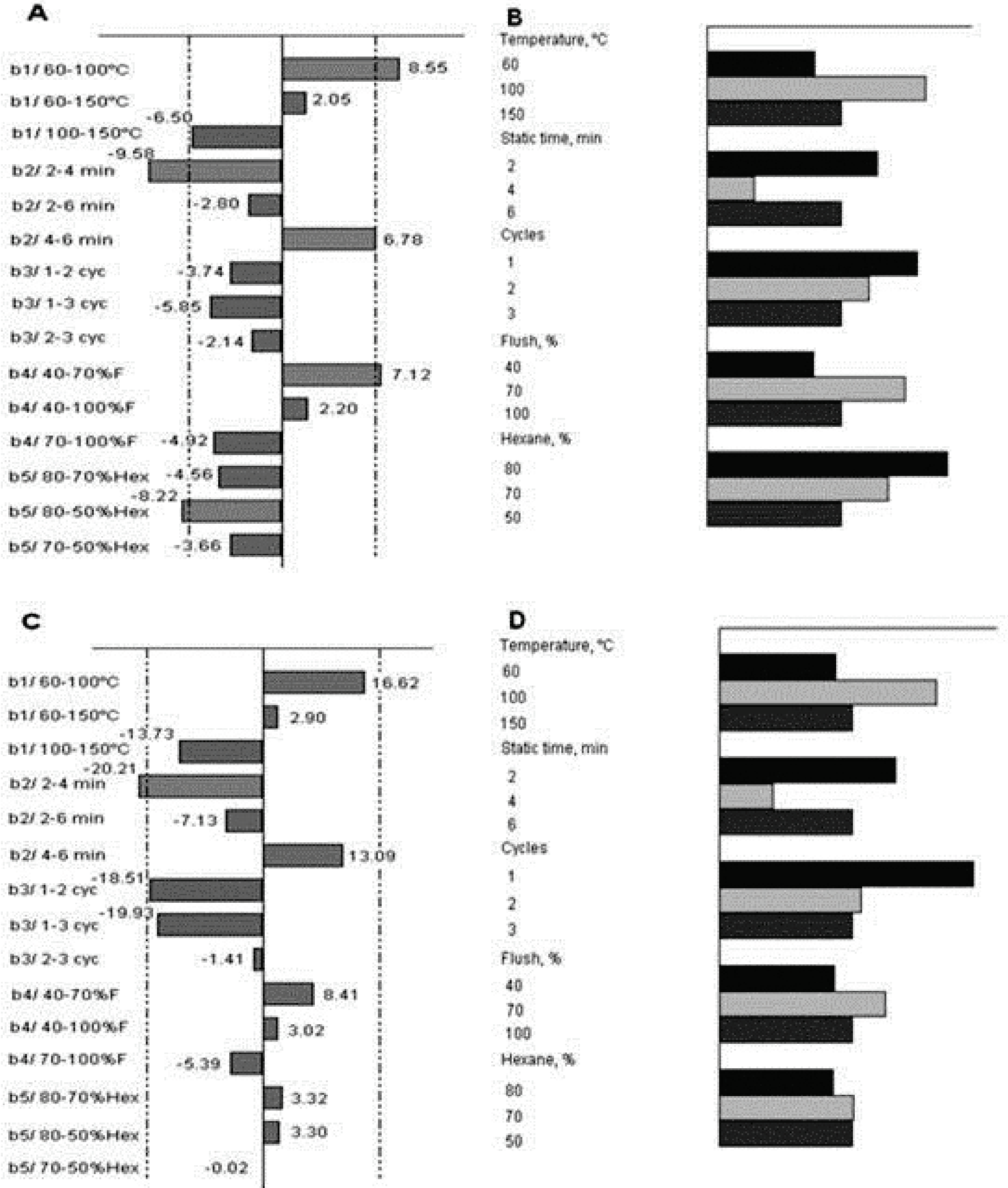

Fig. 4. *Delta weight plots for CLP-E (A) and CYPER (C); and total effect graphics for CLP-E (B) and CYPER (D). Bar units are chromatographic peak areas.*

3.4. Evaluation of the method performance and validation

Target compounds were identified in the seaweed matrix by their retention time and their mass spectrum. A GC-MS/MS chromatogram of a 5 ng mL^{-1} standard mixture (*Figure 5*) shows that the multi-class compounds are simultaneously determined with the proposed methodology.

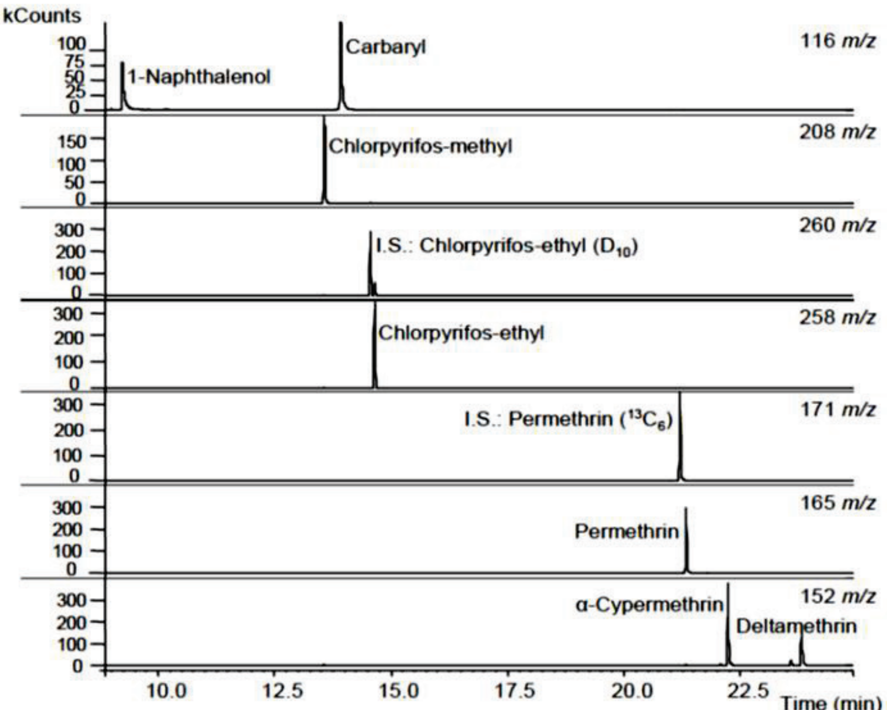

Fig. 5. *PLE–PTV-LVI–GC–MS/MS extracted chromatograms of a 5 ngmL−1 standard mixture of all studied compounds and IS.*

The carbamates physicochemical properties can be summarized by a high polarity, a strong solubility in aqueous media, and an important thermolability. These properties limit the use of GC and some authors proposed derivatization methods or the HPLC use. However, it seems that direct determination can be achieved at very low concentration levels [52, 53]. The thermal instability of CARB provides its transformation in 1-naphthalenol. The chromatogram of *Figure 5* shows the peaks of both compounds, despite that PTV device minimizes decomposition of CARB in the GC system [53]. Only CARB has been quantified.

The optimized PLE conditions with integrated cell clean-up were used to validate the PTV-LVI-GC-MS/MS method by verifying its linearity, accuracy, limits of detection and quantification (LOD and LOQ) and precision (*Table 4*). Quantification, based on peak areas, was performed by the internal standard method, using isotopically labelled analogues (and in their absence, the most similar in terms of structure) as surrogate standards. The linearity of the method was evaluated by constructing seven-point calibration curves with concentration ranging from LOQ to 60 ng mL^{-1}, except for DELTA with 100 ng mL^{-1} as the upper calibration limit. The concentration of the IS along the calibration curve was maintained constant at 100 pg mL^{-1}. Determination coefficients (R^2) were always higher than 0.999 (*Table 4*).

LOD and LOQ were experimentally estimated from the analysis of real samples as the concentration of analyte giving a signal-to-noise ratio of 3 and 10, respectively [54]. Results achieved with different GC

systems are summarized in *Table 4*. GC-MS/MS in 240MS using PTV-LVI mode, presented the best LOD and LOQ values. With the present method (50 µL injected by PTV-GC-MS/MS) the target compounds presented LOD and LOQ in the pg g^{-1} range between 0.3 pg g^{-1} (CLP-E) and 3.0 pg g^{-1} (CARB) and from 0.9 to 10.0 pg g^{-1}, respectively; except for DELTA with LOD of 23.1 pg g^{-1} and LOQ of 76.9 pg g^{-1}. When Varian Saturn 2100T and 240MS instruments were used at split/splitless mode (1µL of extract containing the target analytes was injected at the running program described in section 2.4 and 280 ºC initial inlet temperature) similar LOD and LOQ were obtained. Lower LOD and LOQ for CARB were obtained in 240MS, possible due to its external ion-trap configuration. GC-µECD presented lower LOD and LOQ than GC-MS/MS using split/splitless mode, but CARB was not detected by GC-µECD and MS is mandatory for the confirmation of possible positive results from GC analysis.

Method precision was also evaluated by determining reproducibility (inter-day assays) as relative standard deviation (RSD%). Eight extractions of spiked Wakame sample were analyzed using the proposed described method and three concentration levels for each pesticide (50 pg g^{-1}, 25 and 50 ng g^{-1}) were studied. The RSDs obtained were less than 12, 7 and 8 % in all cases for 50 pg g^{-1}, 25 and 50 ng g^{-1}, respectively (see Table 4). Seaweed samples spiked with a multistandard mixture of a known concentration were used to evaluate the accuracy and applicability of the method. Influence of the type of sample and concentration level on the efficiency of the PLE-PTV-LVI-GC-MS/MS method were evaluated by the extraction and analysis of six replicates of representative market samples (Wakame, Nori, Sea Spaghetti, Irish Moss and Commercial edible seaweed) spiked with each pesticide at low level of 50 pg g^{-1} and high level of 25 ng g^{-1} (data are included as electronic supporting information). In all cases clean and colourless extracts were obtained and recoveries were equivalent and ranged between 86 to 111 % for for 50 pg g^{-1} and from 82 to 108 % for 25 ng g^{-1}. RSDs lower than 12 and 10% in all cases for 50 pg g^{-1} and 25 ng g^{-1}, respectively, were achieved. No value exceeded the 70-120% recovery rule edited by SANCO recommendations [54]. The compounds were quantified versus the corresponding isotopically labelled analogues or those structurally more related, as surrogate standards.

***Table 4**: Comparison of limits of detection and quantification using different gas chromatographic systems (GC-µECD and GC-MS/MS). Figures of merit for the PLE-PTV-LVI-GC-MS/MS method.*

Analyte	GC-µECD[a] ($ng\ g^{-1}$)		Saturn 2100T[a] ($ng\ g^{-1}$)		240MS[a] ($ng\ g^{-1}$)		240MS PTV-LVI (7µL, $pg\ g^{-1}$)		240MS PTV-LVI (50µL, $pg\ g^{-1}$)						
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	Linear range $ng\ mL^{-1}$	R^2	Precision (RSD%) $ng\ g^{-1}$ (n=8) 0.05	25	50
CLP-M	0.2	0.59	0.5	1.7	0.2	0.8	2.1	7.2	0.7	2.3	0.017-60	0.9990	7	7	4
CLP-E	0.1	0.30	0.8	2.7	0.2	0.7	60.3	201.0	0.3	0.9	0.019-60	0.9996	2	7	8
CARB	n.d.	n.d.	550.3	1834.3	6.3	21.0	2.3	7.7	3.0	10.0	0.015-60	0.9991	9	5	7
PER	0.3	1.1	7.6	25.4	1.7	5.8	41.2	137.3	2.4	8.0	0.014-60	0.9992	7	3	8
CYPER	0.3	0.9	6.1	20.3	1.9	6.4	83.4	278.0	1.4	4.7	0.030-60	0.9994	6	3	7
DELTA	0.5	1.7	19.3	64.4	13.5	44.9	380.1	1267.0	23.1	76.9	0.1-100	0.9997	12	2	6

[a] Split/splitless injection mode. n.d.: not detectable

***Electronic supporting information:** Extraction recoveries from various spiked edible seaweed at two addition levels (low level, 50 $pg\ g^{-1}$ and high level, 25 $ng\ g^{-1}$), obtained under optimized conditions of PLE-PTV-LVI-GC-MS/MS method (n=6)*

Analyte	Wakame				Nori				Sea Spaghetti			
	Recovery%		RSD%		Recovery%		RSD%		Recovery%		RSD%	
	Low level	High level	Low level	High level	Low level	High level	Low level	High level	Low level	High level	Low level	High level
CLP-M	102	107	7	7	103	103	7	7	106	96	8	8
CLP-E	93	95	2	7	91	95	5	5	85	98	9	9
CARB	89	88	9	5	93	92	5	5	102	92	7	9
PER	91	94	7	3	90	100	5	4	100	93	10	6
CYPER	105	82	6	3	105	87	5	6	103	83	9	10
DELTA	104	108	12	2	104	108	8	4	104	102	12	7

***Electronic supporting information:** Contiunued.*

Analyte	Irish Moss				Commercial edible seaweed			
	Recovery%		RSD%		Recovery%		RSD%	
	Low level	High level	Low level	High level	Low level	High level	Low level	High level
CLP-M	100	93	5	9	95	97	9	5
CLP-E	90	93	5	7	95	86	7	3
CARB	96	86	8	3	97	85	10	7
PER	94	89	5	6	89	92	6	5
CYPER	100	86	8	3	111	86	6	8
DELTA	90	99	8	9	103	108	9	9

3.5. Real samples analysis

Four samples of six wild seaweeds and five commercial edible seaweeds were analyzed by the proposed method. Based on the physico-chemical properties of the target pesticides, they may be accumulated in biota or provoke long-term toxic effects. The presence of CLP-M, CLP-E, PER and CYPER was confirmed with the PLE-PTV-LVI-GC-MS/MS (*Table 5*). Neither CARB nor DELTA were detected, perhaps because these pesticides are no systemic, very easily degradable in the natural environment, depending on their potential absorption, lipophilicity and the concentration of the active ingredients in the pesticide formulation. OP pesticides were detected in different samples and CLP-E was detected in almost all samples analyzed. The concentrations were below the limit of quantification for these two pesticides, except for Wakame sample with concentrations close to the LOQ level. This observation suggests that the OP pesticides were retained in seaweed rather than decomposed. PER and CYPER were detected at high concentrations in various wild seaweeds with the maximum input of CYPER in commercial edible sea spaghetti. No Maximum Residue Levels (MRLs) have been found for the target analytes and therefore the default level of 0.01 mg kg^{-1} is recommended to apply [55]. All the pesticide concentrations found in seaweed samples were below this recommended level. Nevertheless, it is important to note that although these concentrations are very low, they could represent a risk for the environment and health safety.

Table 5: *Concentration of target pesticides in seaweed samples using PLE-PTV-LVI-GC-MS/MS as analytical method (n=4)*

Sample	**Scientific name**	**Concentration ± SD (pg g^{-1})**					
		CLP-M	**CLP-E**	**CARB**	**PER**	**CYPER**	**DELTA**
	Wild seaweed						
EGG WRACK	*Ascophyllum nodosum*	-	<LOQ	-	-	1211±99	-
SEA SPAGHETTI	*Himanthalia elongata*	<LOQ	<LOQ	-	-	-	-
SEA LETTUCE	*Ulva lactuca*	22±2	<LOQ	-	207±11	-	-
BLADDER WRACK	*Fucus vesiculosus*	-	<LOQ	-	-	-	-
SEA POTATO	*Leathesia difformis*	-	-	-	30±1	-	-
IRISH MOSS	*Chondrus crispus*	-	<LOQ	-	-	-	-
	Commercial edible seaweed						
SEAWEED MIXTURE	*	-	<LOQ	-	-	-	-
SEA SPAGHETTI	*Himanthalia elongata*	-	<LOQ	-	-	2228±93	-
IRISH MOSS	*Chondrus crispus*	<LOQ	-	-	-	-	-
NORI	*Porphyra umbilicalis*	-	-	-	-	-	-
WAKAME	*Undaria pinnatifida*	15±1	11±1	-	-	-	-

** Undaria pinnatifida (30%),Ulva lactuca (19%),Laminaria ochroleuca (18%), Palmaria (18%) and Porphyra umbilicalis (15%)*

Figure 6 shows, as an example, the selected extracted ion GC-MS/MS chromatograms resulting from the analysis of two positive real samples: sea spaghetti and wakame, both commercial seaweeds. As can be observed, the spectra obtained by MS/MS confirm the identity of the analytes (CLP-M, CLP-E and CYPER) detected in the samples.

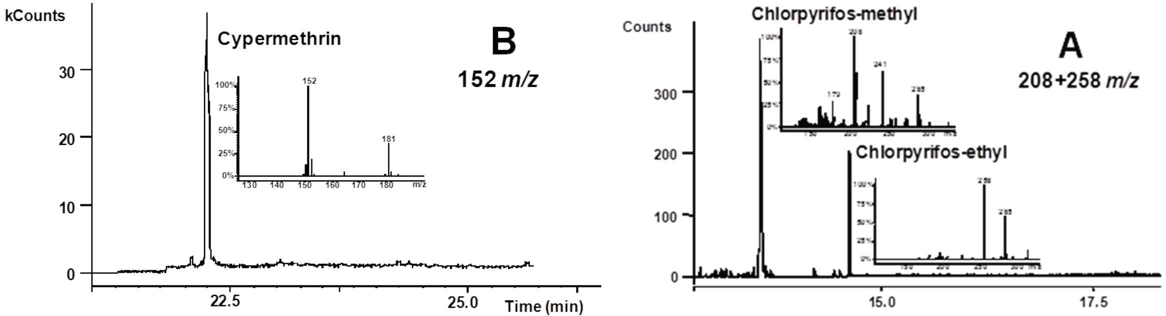

Fig. 6. *PLE–PTV-LVI–GC–MS/MS extracted chromatograms and spectra of A, commercial Sea Spaghetti; and B, commercial Wakame.*

4. Conclusions

The investigation of a new pressurized liquid extraction process for multi-class analysis of six pesticides showed that this new PLE was successful to recover all the selected compounds with a good precision and accuracy in seaweed. The procedure is easy to perform (clean-up integrated in cell) reducing costs, time and residue loss. The optimum conditions of pressurized liquid extraction and LVI-GC-MS/MS via PTV have been proven to be an efficient technology in the analysis of OP, PYR and CAR in seaweed at the parts per-trillion level, improving the selectivity and sensitivity. Various factors affecting this method have been studied in detail. To this end, experimental design has been a very helpful tool to provide the optimal conditions reducing the experimental effort. The overall method has been successfully validated by the study of different wild seaweed samples from Galicia (NW Spain) and edible seaweed samples form market with satisfactory results. The remarkable sensitivity and selectivity provided by PLE- PTV–LVI-GC–MS/MS in the analysis of OP, PYR and CAR pesticides in different seaweed matrices, suggest that the method could be established as a suitable routine procedure for screening ultra-trace levels in similar vegetable matrices. Furthermore, the reliability of the PLE-PTV-LVI-GC-MS/MS method should undoubtedly make it a valuable tool for monitoring of other OP, PYR and CAR pesticides. Assuming that CAR pesticides are thermally labile, more studies concerning their thermal instability are required.

Acknowledgment

This research was supported by the Project CTQ2006-03334/BQU MEC (Plan Nacional I+D, Spain). D. García-Rodríguez acknowledges Xunta de Galicia (Consellería de Innovación e Industria) for the financial support of his PhD.

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2.2. Extracción asistida por microondas y determinación de pesticidas multiresiduo en algas comestibles mediante inyección de grandes volúmenes con vaporización de temperatura programada-cromatografía de gases con espectrometría de masas en tándem.

El objetivo de este trabajo ha sido desarrollar un método que permita llevar a cabo la determinación de pesticidas en algas. El número de compuestos se amplía con respecto al estudio desarrollado para el análisis de pesticidas en algas empleando PLE que se ha presentado en este capítulo. De esta manera es posible integrar en un método multirresiduos el análisis de diversos pesticidas de frecuente uso para el control de plagas en la agricultura y la acuicultura. Los compuestos elegidos son diecisiete pesticidas neurotóxicos para el ser humano y constituyen un riesgo potencial para la salud si están presentes en alimentos.

El método presentado en el trabajo anterior se usó como base para desarrollar un procedimiento de extracción de amplio uso en los laboratorios analíticos, basado en la extracción asistida por microondas (MAE) y PTV-LVI-GC-MS/MS, para determinar los compuestos objeto de estudio. Se han obtenido límites de cuantificación inferiores a los niveles máximos de residuos (MRLs) establecidos por la Unión Europea para pesticidas en algas. Se ha utilizado un diseño central compuesto para evaluar los efectos de las principales variables que afectan a la extracción (temperatura, tiempo y volumen de disolvente). La purificación de los extractos se ha realizado mediante extracción en fase sólida (SPE). Las condiciones operacionales se han determinado mediante el estudio de las cantidades adecuadas de adsorbentes (Florisil y GCB) y el volumen de elución de hexano/acetato de etilo (80:20) con un diseño asimétrico de *screening* o cribado.

Aunque el proceso de extracción-purificación implica dos pasos (MAE-SPE), el tiempo total de análisis es semejante al de la PLE desarrollado en este capítulo. Las recuperaciones han sido próximas al 100% y la precisión, en términos de %RSD, inferior al 13%, valores comparables a los obtenidos mediante PLE.

El método desarrollado se aplicó al análisis de once algas distintas de las cuales cinco eran muestras comercializadas de algas y cinco muestras eran algas salvajes obtenidas de distintas zonas costeras de Galicia. Las once muestras de algas contenían residuos de al menos uno de los pesticidas estudiados. Nueve de los diecisiete residuos de pesticidas fueron detectados.

MICROWAVE-ASSISTED EXTRACTION AND LARGE VOLUME INJECTION GAS CHROMATOGRAPHY TANDEM MASS SPECTROMETRY DETERMINATION OF MULTIRESIDUE PESTICIDES IN EDIBLE SEAWEED SAMPLES

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Abstract

A microwave assisted extraction (MAE) method followed by clean-up with solid-phase extraction (SPE) combined with large volume injection gas chromatography–tandem mass spectrometry (LVI-GC-MS/MS) for the analysis of 17 pesticides in wild and aquaculture edible seaweeds has been developed. An experimental central composite design was employed to evaluate the effects of the main variables potentially affecting the extraction (temperature, time and solvent volume) and to optimize the process. The most effective microwave extraction conditions were achieved at 125 ºC and 12 min with 24 mL of hexane/ethyl acetate (80:20). SPE clean-up of the extracts with graphitized carbon and Florisil, optimized by means of the experimental design, proved to be efficient in the removal of matrix interferences The analytical recoveries were close to 100% for all the analytes, with relative standard deviations lower than 13%. The limits of detection ranged from 0.3 to 23.1 pg g^{-1} and the limits of quantification were between 2.3 and 76.9 pg g^{-1}, far below the Maximum Residue Levels (MRL) established by the European Union for pesticides in seaweed. The results obtained prove the suitability of the microwave-assisted extraction for the pesticides routine analysis of aquaculture and wild seaweed samples.

Keywords: Multiresidue pesticide; microwave-assisted extraction; GC-MS/MS; seaweed.

1. Introduction

Pesticides are present in different areas of the environment (i.e. water, soils or foodstuffs) as a consequence of the wide application as phytosanitary products in the modern agriculture and in the case of organophosphates (OP), carbamates (CAR) and pyrethroids (PYR) also as chemotherapeutants for controlling parasitic and microbial infestations in the aquaculture industry [1]. Due to their toxicity they represent a potential risk to consumers and, therefore, residues in food commodities are an issue of public concern [2]. The physicochemical characteristics facilitate bindings of the residue of pesticides to particulate material, such as marine sediments [3], seawater [4], fish [5] and seaweeds [6].

There are different types of interfering compounds in seaweeds affecting pesticides analysis, mainly chlorophyll and carotenoid pigments, polysaccharides (phycocolloids), polyunsaturated fatty acids, vitamins, mineral salts, oligo-elements and others, which are frequently co-extracted [7, 8]. The sample preparation procedure prior to chromatographic analysis is one of the most critical steps in the analytical processes. The main problem encountered during the analysis is the separation of the target analytes from matrix components, due to inefficient extraction, loss of sample, and low levels of analyte in the sample [9]. These difficulties restrict the determination of pesticides in seaweed; in fact, studies devoted to the analysis of pesticides in edible and wild seaweeds are scarce. In the analysis of organochlorine pesticides, PCBs, PAHs and petroleum hydrocarbons, the most commonly used sample pretreatment techniques were Soxhlet extraction [10], manual agitation [11] and ultrasonic bath [10, 12] with hexane, dichloromethane or hexane-based mixtures. Some of these procedures are time consuming and require large amounts of organic solvent. Following the extraction, a clean-up step based on adsorption chromatography (alumina, silica gel, Florisil), treatment with H_2SO_4 or gel permeation is usually required [10, 12, 13]. Also solid-phase microextraction (SPME) and solid-phase extraction (SPE) procedures coupled with microwave-assisted micellar extraction (MAME) for the determination of organochlorine pesticides [6], and supercritical fluid extraction (SFE) for PAHs [14] were applied to seaweed samples. Other instrumental based extraction techniques, performed at high pressure and/or temperature, such as pressurized liquid extraction (PLE) [15] and microwave-assisted extraction (MAE) might be appropriate and simplify the pretreatment stage. MAE has been widely applied in the extraction of PYR and OP from various matrices such as water [16], soils [17], and food [13, 18-21]. Several publications on the determination of pesticides in food matrices is available based on LC-MS [22-24], but the lack of published work based on gas chromatography on multiresidue pesticide studies in the aquaculture environment is evident [25-27].

The applicability of gas chromatography coupled with mass spectrometry (GC-MS) for the determination of pesticide residues at trace levels in complex food samples, such as vegetables or fatty foods, has been widely demonstrated [28-30]. In addition, international guidelines for pesticide residue

analyses in food [31] highlight the necessity of confirmatory methods giving structural information about target analytes. This is the case of GC–MS/MS, which performs the simultaneous identification, confirmation and quantification of trace analysis. Additionally, responding to continual decreases in maximum pesticide residue limits, it is necessary to develop methods with increased detection sensitivities taking benefit from approaches as large volume injection (LVI) techniques [30, 32-34]. A representative LVI method is a programmed-temperature vaporizer (PTV) method [35]. Combining a cool injection step with controlled vaporization eliminates or improves upon a number of major disadvantages associated with the use of conventional hot splitless injection. The quantitative performance of the PTV injection system appears to be comparable to that of on-column injection and much better than hot splitless injection [36]. Possible disadvantages of a PTV like careful method optimisation and contamination of the column inlet with no-volatile sample materials [36, 37] have been compensated with low limits of quantification obtained for all the analytes. Moreover, the proposed method ensures obtaining clean extracts, maintaining long-term instrument performance. To the best of our knowledge, no other previous detailed studies have been published on the MAE of pesticides in seaweeds followed by PTV-LVI-GC-MS/MS determination.

The aim of the present work was to develop a simple and sensitive method for the simultaneous determination of eleven pyrethroid insecticides (bioallethrin, tetramethrin, cyhalothrin, cyfluthrin, flucytrinate, fenvalerate, resmethrin, permethrin, deltamethrin, cypermethrin and empenthrin), two organophosphorous pesticides (chlorpyrifos-methyl and chlorpyrifos-ethyl), two carbamate pesticides (carbaryl and propoxur), a fungicide (2-phenylphenol) and a synergist (Piperonyl butoxide) in seaweeds, based on microwave-assisted extraction, cleanup and gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) operating in electron impact (EI) mode with large-volume injection having a programmed-temperature vaporizer (PTV-LVI). Several parameters affecting the MAE and sample clean-up were optimized in the present study by an experimental design approach.

2. Experimental

2.1 Chemicals and standard solutions

The seventeen compounds under consideration are summarized in Table 1.

Pestanal quality analytical standards of 2-phenylphenol, propoxur, carbaryl, chlorpyrifos-methyl, chlorpirifos-ethyl, piperonyl butoxide, empenthrin, bioallethrin, resmethrin, tetramethrin, λ-cyhalothrin, permethrin, cyfluthrin, α-cypermethrin, flucythrinate and fenvalerate were from Riedel-de-Haën (Seelze, Germany). Pestanal quality deltamethrin was from Chem Service Inc (West Chester, PA, USA). Internal

standards (diethyl-D_{10})-chlorpyrifos and (phenoxi-$^{13}C_6$)-*cis*-permethrin were from Cambridge Isotope Laboratories (Cambridge, UK).

Bulk sorbents Florisil (60-100 mesh) and graphitized carbon black (GCB) were from Aldrich (Madrid, Spain) and Supelco (Bellafonte, PA, USA), respectively. Analysis grade solvents, ethyl acetate, acetone and *n*-hexane were from VWR-Prolabo (Mollet del Vallés, Barcelona, Spain). Polypropylene solid-phase extraction syringes (15 mL capacity) and 20 µm polyethylene frits were from International Sorbent Technology (Mid Glamorgan, UK). Syringe filters (Millex GV, 13 mm and 0.22 µm) from Millipore (Billerica, MA, USA).

Ultra pure water was obtained using a Milli-Q□water purification system (Millipore). Individual standard stock solutions of 5000 µg mL^{-1} were prepared in acetone. A stock mixture solution of all the studied compounds at a concentration of 10 µg mL^{-1} was obtained by appropriate dilution of individual stock solutions in acetone.

Diluted solutions were prepared from the stock mixture solution in ethyl acetate. All solutions were stored in amber-colored vials at -20 ºC.

Table 1: *Structures, abbreviations and partition coefficients for each target analyte*

Compound	Abv	CAS	MW	Log K_{ow}	Structure
2-Phenylphenol	2-PP	90-43-7	170.2	2.94	
Bioallethrin	BIOAL	584-79-2	302.4	4.92	
Carbaryl	CARB	63-25-2	201.2	2.40	
Chlorpyrifos-ethyl	CLP-E	2921-88-2	350.6	4.77	
Chlorpyrifos-methyl	CLP-M	5598-13-0	322.5	3.71	
Cyfluthrin	CYFLU	68359-37-5	434.3	6.29	
Cyhalothrin	CYHAL	68085-85-8	449.9	6.20	

Table 1: *Continued*

Compound	Abv	CAS	MW	Log K_{ow}	Structure
Cypermethrin	CYPER	52315-07-8	416.3	6.27	
Deltamethrin	DELTA	52918-63-5	505.2	6.20	
Empenthrin	EMPEN	54406-48-3	274.4	6.35	
Fenvalerate	FENVA	51630-58-1	419.9	6.67	
Flucythrinate	FLUCY	70124-77-5	451.5	6.15	
Permethrin	PERM	52645-53-1	391.3	7.15	
Piperonyl butoxide	PBO	51-03-6	338.4	4.23	
Propoxur	PROP	114-26-1	209.2	1.60	
Resmethrin	RESME	10453-86-8	338.4	6.63	
Tetramethrin	TETRA	7696-12-0	331.4	4.78	

2.2 Seaweed samples

Dried edible seaweeds: Wakame (*Undaria pinnatifida*), Nori (*Porphyra umbilicalis*), Irish Moss (*Chondrus crispus*), Sea Spaghetti (*Himanthalia elongata*) and a commercial mixture of Wakame (30%), Sea Lettuce (*Ulva lactuca*, 19%), Kombu (*Laminaria ochroleuca*, 18%), Dulse (*Palmaria*, 18%) and Nori (15%),

coming from aquaculture production, were purchased at a local market. Samples were ground in an electric mill and stored in sealed vessels until the analysis.

Wild seaweed: *Ascophyllum nodosum*, *Himanthalia elongata*, *Ulva lactuca*, *Fucus vesiculosus*, *Leathesia difformis* and *Chondrus crispus* were collected in the vicinity of a seaport and beaches located on the coast of Galicia (NW Spain). The seaweed samples were stored at -18 ºC before processing. The frozen samples were dried in an oven at 50 ºC for 24 h, ground and homogenized in an electric mill and stored in sealed vessels until use.

Spiked Wakame seaweeds were used as the matrix to carry out the optimization study. Approximately 10 g of sample were placed in a beaker with a broad base and covered with 20 mL of acetone spiked with the pesticides to obtain a final concentration of each analyte in the seaweed of 60 ng g^{-1}. The sample was allowed to air-dry in the dark for one week and was stored at 4 ºC before extraction, in order to simulate the normal conditions of interaction between the seaweed and the pesticide compounds. Other edible seaweeds were similarly subjected to the spiking process, in order to demonstrate the suitability of the proposed method for the extraction of the target compounds from real samples.

2.3 Microwave-assisted extraction and cleanup

Dried seaweed samples were extracted using an Ethos Microwave Extraction System (Milestone, Leutkirch, Germany), equipped with 12 pressurized 100 mL volume Teflon vessels. Under optimized conditions, 0.2 g of seaweed were extracted with 24 mL of hexane/ethyl acetate (80:20) and 0.5 mL of ultrapure water at 125°C for 18 min. A ramp of 2 min and a microwave power of 800 W were employed to obtain these conditions. Then, extraction vessels were cooled down to room temperature, the slurry centrifuged for 2 min at 3000 rpm and the supernatant transferred to glass vials. This solution was then passed through a SPE cartridge loaded, from bottom to top, with 0.2 g of GCB and 1 g of Florisil. Analytes were recovered from the cartridge with 15 mL of hexane/ethyl acetate (80:20). This final extract was then evaporated to *ca.* 0.5 mL using a gentle stream of nitrogen in a Turvo Vap II concentrator (Zymark, Hopkington, MA, USA) and exactly adjusted to 1 mL with ethyl acetate. Extracts were filtered by means of a syringe filter with a pore size of 0.22 µm before injection of 50 µL into the PTV-LIV-GC-MS/MS system.

All experiments of the design used in MAE optimization, were carried out with 0.2 g of seaweed sample spiked at a level of 60 ng g^{-1}. The stirring of the vessels was set at the maximum power permitted by the instrument to ensure complete mixing of the solid (seaweed) and liquid (solvent) phases. Additionally, the presence in the extraction solution of water, a polar solvent with a higher dielectric constant than ethyl

acetate, improves the ability of the liquid phase to reach working temperatures in a short time, making the microwave extraction more efficient.

Seaweed is a complex matrix characterized by a high plant pigment content, which makes the extraction and further determination of target compounds difficult and requires extensive sample purification. Intense green extracts were obtained during the MAE process and an additional cleanup step was needed before GC injection in order to preserve the chromatographic system. The most commonly used method of purification is adsorption chromatography applying the SPE technique [13]. Florisil and GCB were selected as adsorbents because of the good results obtained in the extraction of several pollutants in fruit and vegetables by PLE [28], MSPD-PLE [38], MAE [39, 40] or QuEChERS method [41]. Furthermore, the selection of these adsorbents was based on our experience in the extraction of pesticides on seaweed by PLE with integrated clean-up [15]. For this reason, SPE cartridges initially containing GCB and Florisil were employed [39, 40]. The SPE eluate was then evaporated to a final volume of 1 mL and 1 µL was injected into the PTV-LVI-GC-MS/MS system. To improve recoveries obtained for pesticides and to achieve a better removal of matrix interferences, optimization of the SPE cleanup was required.

2.4 Chromatographic procedure

Determination of the studied compounds was achieved by GC-MS/MS using a Varian 450GC gas chromatograph (Walnut Creek, CA, USA) coupled to an ion-trap mass spectrometer Varian 240MS operating in external configuration and equipped with a Varian CP-8400 autosampler. Separation of the different compounds was achieved in a Varian Factor Four VF-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). Initial oven temperature was set at 70 ºC, held for 3.5 min; ramped at 25 ºC min^{-1} up to 180 ºC, held for 3 min; ramped at 10 ºC min^{-1} up to 300 ºC and held for 5 min. Helium (purity 99.999%, Carburos Metálicos, A Coruña, Spain) was employed as carrier gas with a constant flow of 1.0 mL min^{-1}. This instrument is equipped with a 1079 PTV injector and cryogenic CO_2 cooling. The PTV-LVI mode parameters were optimized in a previous work [15]. PTV injections were performed in four steps: injection, solvent evaporation, analyte transfer and cleaning. In the injection step the split valve was open at 20 mL min^{-1}, and 50 µL of the sample were introduced into a Siltek deactivated liner with frit (Restek, Bellafonte, PA, USA) at 70 ºC. During the evaporation step the temperature was raised to 85 ºC at a rate of 30 ºC min^{-1} for 30 s in order to eliminate the solvent, which was vented through the split valve at a flow of 36 mL min^{-1}. In the transfer step the split valve was closed and the temperature increased to 300 ºC at a rate of 75 ºC min^{-1} in splitless mode for 3 min. Finally, the injector was kept at 300 ºC with a purge flow of 50 mL min^{-1} until the end of the run for cleaning purposes. The mass spectrometer was operated in EI mode at 70 eV. The ion source, trap, manifold and transfer line temperatures were maintained at 150, 150, 40 and 290 ºC,

respectively. Analyses were performed with a filament-multiplier delay of 6.5 min. General parameters were as follows: multiplier offset, +200 V; AGC target value, 5000 counts; damping gas flow, 2.5 mL min^{-1} and emission current, 90 µA. Specific conditions for each analyte are listed in *Table 2*. A parent ion was chosen for each compound by taking the m/z and relative abundance of parent ions as high as possible in order to increase sensitivity. Good quality secondary spectra for every compound were obtained selecting a non-resonant waveform. The optimization of the excitation amplitude voltage for each pesticide was achieved using the automated method development (AMD) option included in the MS/MS software toolkit [42]. This value was considered optimum when the secondary spectra showed multiple and intense product ions while the parent ion intensity remained around 10%.

Quantification was accomplished by relative areas versus (phenoxi-$^{13}C_6$)-cis-permethrin used as internal standard (IS) for PYR while (diethyl-D_{10})-chlorpiryfos was used as IS for the remaining analytes. Thus, calibration curves were constructed by analyzing standard solutions in triplicate at seven concentration levels ranging from LOQ to 50 ng mL^{-1}. IS concentration was maintained at 100 pg mL^{-1}. Satisfactory linearity using least squares regression was assumed when the determination coefficient (R^2) was higher than 0.99, based on peak areas measurement, and the residuals lower than 30%.

2.5 Statistical treatments

A central composite design for three factors (temperature, time and solvent volume) was used to optimize the responses (% analyte recovery). This design consists of a 2^3 factorial design plus 6 axial points (α = 1.682), plus two points at the centre of the domain, and involves 16 experiments randomly performed to provide protection against the effects of lurking variables [43]. Thus, five levels of extraction temperature (70, 88, 110, 134 and 150 ºC), extraction time (4, 7, 12, 17 and 20 min) and solvent hexane/ethyl acetate (80:20) volume (10, 14, 20, 26 and 30 mL) were evaluated. The experimental design was generated and all analytical calculations were supported by Nemrod®W software [44]. For each compound a quadratic polynomial model was considered:

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} \sum_{j=1}^{n} b_{ij} x_i x_j$$

where x_i is the coded value of the factors studied (extraction temperature, time and solvent volume) and y is the response (peak area) obtained for each drug. The b values are the estimated polynomial coefficients: b_0 is the intercept term, b_i coefficients represent the main effect for each variable, b_{ij} coefficients in the quadratic terms are responsible for the curvature effects and $b_{ij(i \neq j)}$ coefficients describe the interaction effects.

Table 2: *Tandem mass spectroscopy acquisition parameters, for each compound.*

Compound	Parent Ion m/z	Exc. Amp.a V	Exc. Stg. Lvl.b m/z	Quant. Ion m/z
2-Phenylphenol	170	64	64.8	141
Bioallethrin	123	38	46.9	81
Carbaryl	144	51	54.9	115
Chlorpyrifos-ethyl	314	73	119.7	258
Chlorpyrifos-metil	286	99	109.0	208
Cyfluthrin	163	50	62.1	127
Cyhalothrin	181	73	69.0	152
Cypermethrin	181	71	69.0	152
Deltamethrin	181	70	69.0	152
Empentrhin	123	39	46.9	81
Fenvalerate	225	73	85.7	142
Flucythrinate	199	54	75.8	157
Permethrin	183	66	69.7	165
Piperonyl butoxide	176	57	67.1	117
Propoxur	110	43	41.9	81
Resmetrhin	143	49	54.5	128
Tetramethrin	164	54	62.5	107
Chlorpyrifos (IS)	324	76	123.5	260
Permethrin (IS)	189	64	72.0	171

[a]Excitation amplitude; [b]Excitation storage level

The estimates of the coefficients for the models were calculated by least-squares multi-linear regression and these models were validated by analysis of variance (ANOVA). In order to obtain the best operational conditions of clean-up of the MAE extracts, amounts of Florisil (1.0, 1.6 and 2 g), of GCB (0.2, 0.4 and 0.8 g) and elution volume (5, 10 and 15 mL) were the selected factors and levels studied by a three level symmetrical design for screening involving nine experiments [43]. Graphic chemometric tools have been used to help the interpretation of the factor effects in this design. Thus, delta weight plots show relative effects of level in each variable. These effects are shown as bars, the length of which is proportional to the relative magnitude of the effect, which is negative for bars going leftwards and positive for those going rightwards. The dotted lines represent the statistical significance levels as determined using the method of Lenth [43]. When the effect bars surpass the line, the effect of the level in question on the response is statistically significant at a 95% confidence interval. If an effect is negative, the response decreases when the factor moves from the low to the high level; the opposite occurs if the coefficient is positive. The sign and value of the effects make it possible the choice of the best level for each factor.

The paired *t*-test was applied to compare the values of means from the two sample preparation procedures MAE and PLE, using the Statgraphics Centurion XV statistical package (Manugistics, Rockville, MD). The test is based on the paired differences between these two measurement values. The usual null hypothesis (H_0) is that the difference in the mean values is zero [45].

3. Results and discussion

3.1 MAE optimization.

The experimental domain considered in the central composite design used for MAE optimization was defined taking into account preliminary experiments, the instrumental and operative limits and the stability of the compounds. Temperature values below 70 °C did not fulfill recovery requirements and temperatures above 150 °C could cause Teflon vessels to leak and the carbamate pesticides to degrade [46]. Furthermore, extraction times above 30 min would be excessive; and solvent volumes lower than 10 mL would not ensure complete immersion of the seaweed sample and the microwave temperature probe. Based on our previous experience regarding pesticide extraction from seaweeds [15], a mixture hexane/ethyl acetate (80:20) was selected as extraction solvent and subjected to further optimization.

Once the responses were obtained when de experimental design was applied, the analysis of variance (ANOVA) was carried out. The results showed that all three factors had a statistically significant effect for a significance level of 95%, on all the studied compounds, except for CAR pesticides. The application of Surface Response Methodology (SRM) revealed a similar tendency for OP and PYR pesticides, while CAR analytes behaved differently. The quadratic terms of the equation model and interactions between factors (temperature-time and temperature-solvent volume) were also statistically significant for several OP and PYR pesticides. The extraction of OP and PYR pesticides was favored by medium-high temperature values, while the extraction of CAR compounds was more efficient at low values of this factor. The MAE process was, in all cases, favored by high levels of the factors solvent volume and extraction time. *Figures 1A, 1B and 1C* show as an example the response surfaces obtained for CARB, CLP-E and PERM, respectively. In order to find the best simultaneous conditions, multicriteria decision-making strategies using desirability function optimization were applied without additional experimentation. The desirability functions were built as partial Derringer functions for each analyte response using the Nemrod® W 2000 software. The responses were transformed using a dimensionless desirability (d_i) scale, which ranged between d=0 for a completely undesirable response to d=1 for a fully desired response. Non-linear left unilateral desirability functions were required to maximize each response efficiency. In a second step, a global desirability function (D), which represents the global quality of the common optimum, was calculated by combining single desirability functions, usually as the geometric mean [43]. The maximum *D*

value obtained was 1.0 for a temperature of 125 ºC, an extraction time of 18 min and a solvent volume of 24 mL (*Figure 1D*). Under these conditions, predicted values of d_i were 1.0 for all analytes. Initially, 2-PP and PBO were not considered in the MAE optimization study. However, due to their interest and relationship with pyrethroids (PBO is a synergist of these compounds) or the use as food additives to prevent rancidification (2-PP is a commonly preservative used in vegetable and fruit to keep fresh) [47]. Finally, the optimal conditions of MAE were successfully applied for the extraction of these analytes.

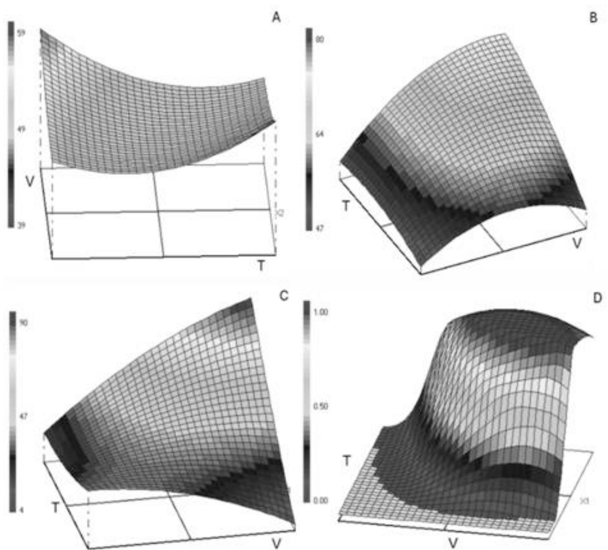

Fig. 1 *Response surfaces obtained for: a CARB; b CLPE; and c PERM versus temperature (°C) and solvent volume (mL). d overall desirability function for the target analytes*

3.2 SPE cleanup optimization.

Responses (% of recovery) from the experimental design were evaluated using ANOVA, and all the studied factors presented statistical significance at a 95% confidence interval, for most of the compounds under study (except PERM and CARB). Similar behavior was observed for all PYR, OP and CAR pesticide families. The results obtained were examined with the aid of graphic tools supplied by Nemrod®W 2000 software. The statistical significance on the response for CLP-E and CYPER is shown as an example in Figure 2.*Figures 2A and 2C* show the delta weight plots which are useful when comparing the relative effects of level changes in a variable. *Figures 2B and 2D* represent the total effects graphs, in which the length of the bars is proportional to the effect of each factor level on the analytical response. A low level of Florisil (1 g) and a high level of volume (15 mL) provided the best responses and were statistically significant for all compounds at a 95% confidence interval. A low level of GCB (0.2 g) was also statistically significant for CLP-M (Figure 2A) and attained near statistical significance for most PYRs. PERM presented slightly higher responses for the high value (0.8 g) than the low level (0.2 g), but good responses were also achieved at GCB 0.2 g; neither of the two values were statistically significant for PERM. The factors were not statistically

significant for CAR pesticides but they showed better responses with 1 g of Florisil, a medium level of volume (10 mL) and GCB (0.4 g). However, slightly lower responses with 15 mL and GCB 0.2 g provided equally good results for CAR pesticides.

The best common clean-up conditions were established on the basis of these results. Thus, 0.2 g of GCB and 1 g of Florisil were the selected sorbent amounts and 15 mL of *n*-hexane:ethyl acetate (80:20) was chosen as SPE elution volume.

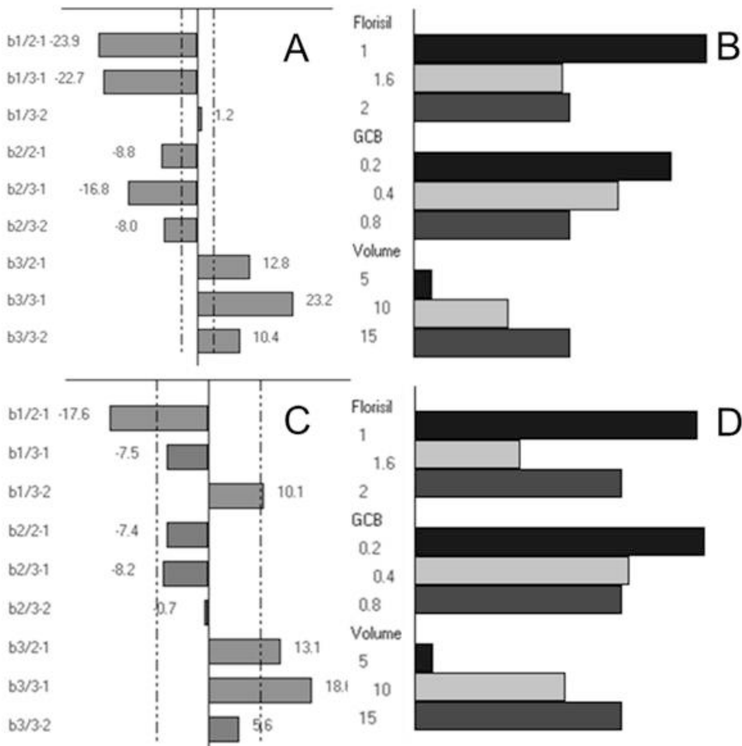

Fig. 2 *Delta weight plots for CLP-M (a) and CYPER (c); and total effect graphics for CLP-M (b) and CYPER (d). Bar units are recovery percentages.*

3.3 Method performance and validation

The analytical characteristics of the effective MAE coupled with LVI-GC-MS/MS method in terms of linearity, accuracy, precision and limits of detection (LOD) and quantification (LOQ) were investigated to evaluate the efficiency and the feasibility of applying the method developed to the analysis of seaweed samples. For quantification of the target analytes, linear calibration curves for all compounds over seven calibration levels from 0.01 to 50 ng mL^{-1} were prepared (except DELTA, from 0.025 to 50 ng mL^{-1}). The linearity was studied for each pesticide (each level in triplicate) considering the area of the peaks relative to internal standards, using isotopically labelled analogues (and in their absence, the most similar in terms of structure) as surrogate standards. Concentration of the IS along the calibration curve was maintained constant at 0.1 ng mL^{-1}. Analysis of variance (ANOVA) was performed to validate the regression calibration

data. Probability values for the lack-of-fit test ranged between 0.0563 (Permethrin) and 0.9710 (empenthrin), are greater than or equal to 0.05 (significant level). Linear regression is statistically significant for all calibration curves (probability values lower than 0.05). Good linearity of the response and suitable sensitivity values [48], were obtained for all pesticides at concentrations within the interval tested, with determination coefficients (R^2) ranging between 0.9985 for 2-PP and 0.9998 for CLP-E (see table 3). LODs and LOQs were defined as the concentration of the analyte that produced a signal-to-noise ratio of 3 times and 10 times, respectively [31]. *Table 3* shows these values in picograms per gram of seaweed. LODs ranged from 0.3 to 23.1 pg g^{-1} and LOQs ranged from 2.3 to 76.9 pg g^{-1}. These values are below the Maximum Residue Levels (MRL) established by European Union legislation [49, 50].

Table 3: *Determination coefficients, sensitivity, limits of detection, limits of quantification and MRLs for the studied compounds.*

Compound	R^2	Sensitivity[a]	LOD / pg g^{-1}	LOQ / pg g^{-1}	MRLs[b]/mg Kg^{-1}
2-PP	0.9985	8.2 10^{-3}	1.3	4.3	NS[c]
BIOAL	0.9997	3.4 10^{-3}	2.7	9.1	NS
CARB	0.9995	8.5 10^{-4}	3.0	10.0	NS
CLP-E	0.9998	7.4 10^{-3}	0.3	0.9	NS
CLP-M	0.9993	7.4 10^{-3}	0.7	2.3	NS
CYFLU	0.9997	1.5 10^{-3}	2.1	6.9	NS
CYHAL	0.9994	18.9 10^{-3}	2.0	6.8	0.02
CYPER	0.9997	1.6 10^{-4}	1.4	4.7	0.05
DELTA	0.9998	2.2 10^{-5}	23.1	76.9	NS
EMPEN	0.9994	3.4 10^{-4}	2.5	8.3	NS
FENVA	0.9997	4.8 10^{-4}	4.1	13.6	0.02
FLUCY	0.9989	3.7 10^{-3}	1.2	4.1	0.05
PERM	0.9996	2.1 10^{-4}	3.2	10.7	0.05
PBO	0.9989	18.5 10^{-3}	0.7	2.4	NS
PROP	0.9996	3.6 10^{-3}	3.0	10.0	NS
RESME	0.9994	8.9 10^{-3}	1.8	5.9	0.1
TETRA	0.9994	9.9 10^{-3}	2.5	8.3	NS

[a]The slope of the calibration curve [48]; [b]MRLs: Maximum Residue Levels established by European Union [49,50]; [c]NS: Not Specified

The accuracy and precision of the proposed method had to be tested to ensure the procurement of results suitable for the intended purpose. Therefore, the method was validated with five fortified representative market samples spiked at two different concentration levels (0.050 and 25 ng g^{-1}). Six replicates of spiked samples were extracted under optimal conditions given in the experimental section. As shown in Figure 3 recoveries obtained respect to theoretical spiked concentration from wakame seaweed ranged from 81.2 to 108.9%, with relative standard deviations (RSD) between 3.3 to 9.3% (at the high level 25 ng g^{-1}) and from 84.7 to 106.3%, with RSD between 2.7 to 11.0% (at the low level 0.050 ng g^{-1}). Remaining edible seaweed samples (Nori, Irish Moss, Sea Spaghetti and a commercial mixture of Wakame, Sea Letuce, Kombu, Dulse and Nori) were subjected to the same study. Results obtained with these fortified

seaweeds were similar to those obtained with Wakame and are included as electronic supporting information. Data reveal that the MAE procedure coupled with LVI-GC-MS/MS ensures good reproducibility with excellent linearity and sensitivity for the analysis of these seventeen multiresidue pesticides.

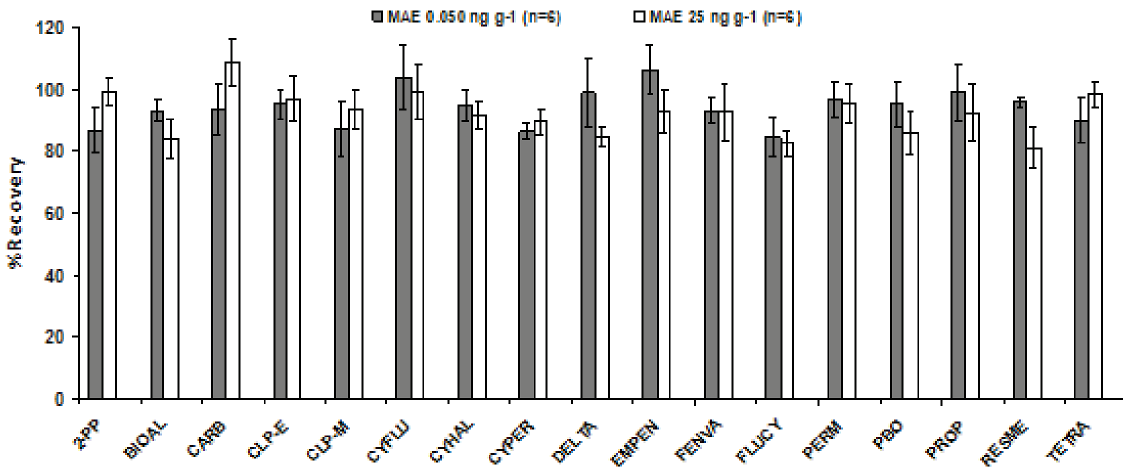

Fig. 3 *Recoveries of pesticides in Wakame samples (n=6) spiked at two levels and extracted with the proposed MAE method.*

The MAE method was also validated by comparison with a method based on pressurized liquid extraction (PLE) [15]. The recovery values of pesticides, extracted from 25 ng g^{-1} spiked samples, ranged from 82.4% to 113.9%, whereas RSD fluctuated between 3.9% and 11.6% for PLE, were acceptable for the analytical performace [31]. As can be seen, both methods provided good results for the determination of the selected pesticides in commercial and wild seaweed samples. The *t*-statistic, obtained for paired *t*-test, is 1.8476 for the 95% confidence interval and the *p*-value equals 0.0832. So H_0 is not rejected at α= 0.05 and the statistical analysis confirmed that MAE and PLE do not provide significantly different results for the extraction of target pesticides.

3.4 Real samples analysis

Based on these results, the optimized MAE-LVI-GC-MS/MS method is valid for the multiresidue pesticides analysis of seaweed samples. The proposed method was successfully applied to the analysis of six wild seaweed samples and five commercial edible seaweed samples. The results are summarized in *Table 4*. Nine of the 17 tested pesticides were detected in the studied samples. The highest detection rate was observed for the residues of CLP-E; 9 samples containing this pesticide, mostly below method LOQs. Moreover, TETRA, EMPEN and 2-PP were also found in the majority of samples analyzed. CAR pesticides were not detected in any sample, perhaps because these pesticides are easily degradable in the natural environment. None of the analyzed samples contained residues of the tested pesticides higher than the

MRLs established by the European legislation [49, 50]. The GC-MS/MS chromatogram and corresponding mass spectra of CLP-E, EMPEN and TETRA, found in a wild Irish Moss sample, are shown in *Figure 4*

Table (electronic supporting information): *Extraction recoveries from various spiked edible seaweed at two addition levels (50 pg g^{-1} and 25 ng g^{-1}), obtained under optimum conditions of MAE-PTV-LVI-GC-MS/MS method (n=6).*

Analyte	Wakame				Nori				Sea Spaghetti			
	Recovery%		RSD%		Recovery%		RSD%		Recovery%		RSD%	
	Low level	High level	Low level	High level	Low level	High level	Low level	High level	Low level	High level	Low level	High level
2-PP	86.8	99.3	7.6	4.4	85.8	102.6	4.0	4.6	89.1	97.0	8.6	4.4
PROP	98.9	92.4	9.1	9.0	106.6	96.5	14.5	5.7	95.9	95.4	6.6	3.5
CLP-M	87.1	93.6	8.7	6.3	99.2	94.1	8.1	6.8	86.2	90.0	3.8	5.5
CARB	93.4	108.9	8.2	7.7	92.8	89.4	9.1	11.7	98.0	84.0	4.9	4.2
CLP-E	95.1	96.8	4.7	7.5	99.6	108.0	3.6	7.6	97.3	107.5	4.7	4.7
EMPEN	106.3	92.9	8.1	7.1	100.3	100.0	12.9	7.8	106.2	85.4	2.5	6.5
BIOAL	92.9	84.2	3.6	6.2	97.7	83.3	9.1	8.5	92.6	82.7	8.7	5.6
RESME	95.8	81.2	1.7	6.4	98.0	100.1	4.4	2.6	98.4	88.6	3.4	8.5
PBO	95.3	85.8	7.1	6.7	97.1	97.8	11.4	3.7	94.7	95.2	8.1	5.0
TETRA	90.0	98.5	7.7	4.0	89.1	86.8	9.9	4.8	85.1	90.9	6.2	2.2
CYHAL	94.6	91.6	5.1	4.1	106.4	97.0	3.5	9.9	99.0	89.1	4.2	5.5
PERM	96.6	95.1	5.6	6.5	96.8	95.8	4.7	6.2	94.5	94.9	6.4	3.8
CYFLU	103.3	99.0	10.5	8.6	104.3	94.3	11.2	8.7	87.7	91.8	13.2	10.7
CYPER	86.4	89.3	2.7	4.0	89.3	90.9	5.8	7.0	88.7	95.0	5.9	11.4
FLUCY	84.7	82.6	6.6	4.0	96.9	98.4	6.7	8.5	94.9	97.0	10.4	12.3
FENVA	93.1	92.5	4.4	9.3	90.5	99.9	11.6	11.5	86.2	88.4	11.2	12.4
DELTA	99.1	84.7	11.0	3.3	105.5	109.2	11.0	6.0	96.5	108.3	7.1	9.8

Table (electronic supporting information): *Continued.*

Analyte	Irish Moss				Commercial seaweed mixture			
	Recovery%		RSD%		Recovery%		RSD%	
	Low level	High level	Low level	High level	Low level	High level	Low level	High level
2-PP	89.2	92.8	8.1	5.5	88.2	104.2	9.6	6.6
PROP	99.9	98.4	10.4	5.3	80.6	96.2	9.2	6.2
CLP-M	85.6	85.9	6.5	5.3	89.0	97.5	8.7	5.3
CARB	101.4	84.5	8.1	1.6	89.9	94.9	12.3	10.1
CLP-E	100.6	110.0	9.7	6.0	91.6	108.4	10.3	7.2
EMPEN	95.1	109.7	13.9	8.7	91.0	98.7	10.2	7.0
BIOAL	96.2	83.5	5.4	5.8	91.3	81.6	5.5	3.6
RESME	100.4	97.6	5.8	7.3	104.4	92.3	5.1	10.8
PBO	98.4	92.3	7.6	6.9	88.8	100.5	6.6	5.7
TETRA	87.5	89.3	5.9	4.3	93.8	88.4	11.6	4.6
CYHAL	99.2	84.0	6.0	9.1	103.7	93.7	3.7	8.3
PERM	95.0	91.6	4.9	4.4	86.7	96.9	8.2	6.1
CYFLU	100.5	95.0	5.1	10.0	100.1	95.0	10.4	10.6
CYPER	91.0	97.7	4.6	9.7	90.3	102.8	1.4	5.9
FLUCY	98.2	103.2	5.1	11.8	95.8	103.3	7.3	11.6
FENVA	89.6	92.3	7.9	10.9	91.5	96.3	11.6	9.3
DELTA	100.3	95.9	11.2	8.1	95.7	107.2	6.4	5.8

Table 4: *Pesticide concentrations found in several seaweed samples by MAE-LVI-GC-MS/MS.*
[a]Wild seaweed samples; [b] commercial edible seaweed samples

Sample	Concentration ($X±S$, $n=4$) / pg g^{-1}								
	2-PP	PROP	CLP-M	CARB	CLP-E	EMPEN	BIOAL	RESME	PBO
Egg Wrack[a]	-	-	-	-	<LOQ	1626±56	-	-	-
Sea Spaghetti[a]	2493±67	-	-	-	<LOQ	-	-	-	-
Sea Lettuce[a]	-	-	20±2	-	<LOQ	-	-	-	-
Bladder Wrack[a]	-	-	-	-	<LOQ	<LOQ	-	-	-
Sea Potato[a]	-	-	-	-	-	3679±205	-	-	194±12
Irish Moss[a]	-	-	-	-	<LOQ	3974±155	-	-	-
Seaweed Salad[b]	536±13	-	-	-	<LOQ	-	-	-	-
Sea Spaghetti[b]	-	-	-	-	<LOQ	-	-	-	-
Irish Moss[b]	524±66	-	<LOQ	-	<LOQ	-	-	-	-
Nori[b]	228±18	-	-	-	-	-	-	-	-
Wakame[b]	-	-	12±1	-	10±1	-	-	-	-

Table 4: *Continued.*

Sample	Concentration ($X±S$, $n=4$) / pg g^{-1}							
	TETRA	CYHAL	PERM	CYFLU	CYPER	FLUCY	FENVA	DELTA
Egg Wrack[a]	4229±155	-	-	-	1428±77	-	-	-
Sea Spaghetti[a]	-	-	-	-	-	-	-	-
Sea Lettuce[a]	2584±46	-	159±11	-	-	-	-	-
Bladder Wrack[a]	3051±166	-	-	-	-	-	-	-
Sea Potato[a]	-	-	47±4	-	-	-	-	-
Irish Moss[a]	2539±103	-	-	-	-	-	-	-
Seaweed Salad[b]	-	-	-	-	-	-	-	-
Sea Spaghetti[b]	285±19	-	-	-	2431±442	-	-	-
Irish Moss[b]	-	-	-	-	-	-	-	-
Nori[b]	596±64	-	-	-	-	1198±81	-	-
Wakame[b]	-	-	-	-	-	-	-	-

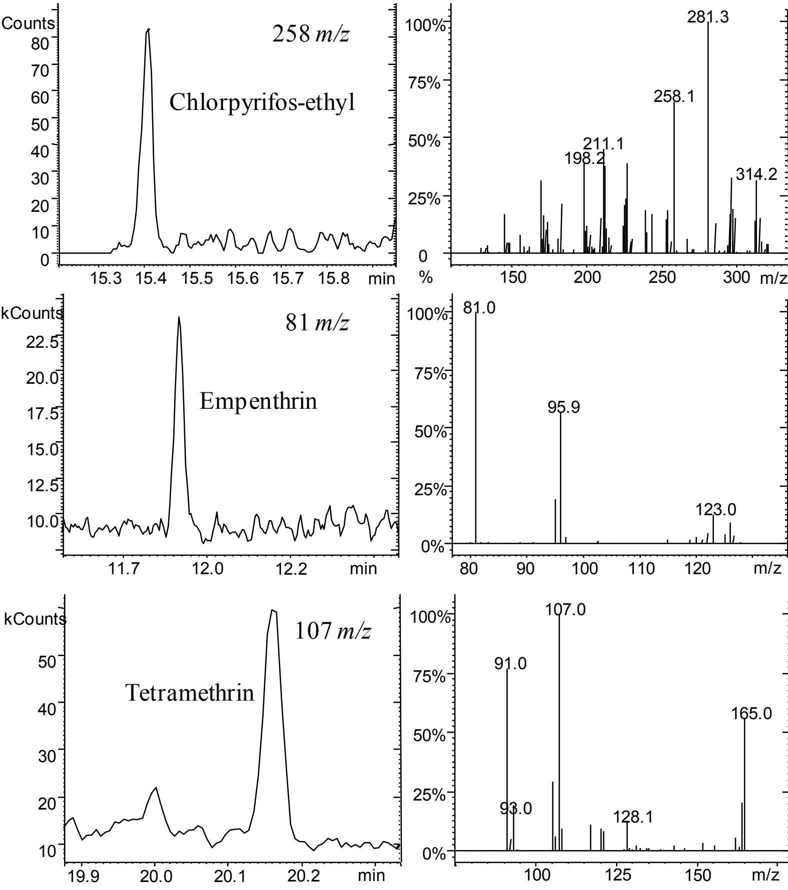

Fig. 4 *MAE-LVI-GC-MS/MS chromatograms and spectra of an extract of a wild Irish Moss sample. Positive peaks were obtained for chlorpyrifos-ethyl, empenthrin, and tetramethrin.*

4. Conclusions

A robust and sensitive multiresidue method based on microwave assisted extraction, clean-up and analysis of seventeen pesticides (i.e. OPs, CARs, PYRs and related compounds), with different physical–chemical properties, was developed for edible and wild seaweeds. Clean-up with GCB and Florisil provide good purification for extracts of samples, which improved the selectivity and accuracy of the procedure. Although the proposed MAE procedure requires this additional cleanup step by SPE, the overall time of analysis is comparable to that used in PLE.

The feasibility of the MAE method was successfully validated with laboratory-spiked seaweed and environmental and aquaculture samples. The range of linearity spans over 3 orders of magnitude for all the compounds. The results provide quantitative recoveries for the determination of selected analytes in seaweed samples with LODs and LOQs which are lower than MRL and other reported values. The optimized method has demonstrated to be effective separating and quantifying different pesticides in seaweed, below the Maximum Residue Levels (MRL) specified by the European Union. Overall, the MAE appears to be a suitable extraction technique for the determination of OPs, CARs and PYRs compounds in seaweed samples because it is quick and effective, permitting the processing of twelve samples simultaneously.

Therefore, the establishment of the MAE procedure completes the offer of other analytical techniques available in order that the analyst can choose the most suitable for monitoring these compounds in terms of the instrumentation available in your laboratory.

Acknowledgments

This research was supported by the Project CTQ2009-08377 MEC (Plan Nacional I+D, Spain). D. García-Rodríguez acknowledges Xunta de Galicia (Consellería de Innovación e Industria) for the financial support of his PhD.

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2.3. Análisis de residuos de pesticidas en algas mediante dispersión de la matriz en fase sólida y cromatografía de gases con detección de espectrometría de masas.

El objetivo de este trabajo ha sido desarrollar un método simple y de bajo coste que permita determinar simultáneamente diecisiete pesticidas, pertenecientes a distintas clases de compuestos de uso habitual en agricultura y acuicultura para el control de diversas plagas, en algas para consumo humano.

En los trabajos anteriores, la determinación de residuos de determinados pesticidas se había llevado a cabo empleando un método de PTV-LVI-GC-MS/MS para la cuantificación de los pesticidas objeto de estudio. Los límites de detección obtenidos eran muy bajos, del orden de partes por trillón, debido a la sofisticación de la técnica cromatográfica. Por ello se pensó en el desarrollo de otras estrategias analíticas que resultasen menos costosas y proporcionasen límites de detección lo suficientemente bajos, sin que la presencia de sustancias co-extraídas interfiriesen en la determinación de los pesticidas de interés.

Se ha propuesto la dispersión de la matriz en una fase sólida (MSPD) como técnica de extracción y limpieza simultánea y la separación y determinación de los analitos mediante cromatografía de gases con detección por espectrometría de masas (GC-MS). En el desarrollo del método se optimizaron varios parámetros como el tipo de dispersante (C_{18}, florisil y Na_2SO_4) y se ha seleccionado Na_2SO_4. La extracción se ensayó con distintos disolventes de polaridad relativa como el acetato de etilo, hexano y mezclas de hexano con acetato de etilo. Las mezclas de hexano:acetato de etilo tienen una polaridad intermedia, adecuada para la extracción de los pesticidas multiclase objeto de estudio. La proporción muestra:dispersante, la proporción de adsorbentes de purificación, florisil:GCB, el porcentaje de acetato de etilo en la mezcla con hexano (usado como disolvente de elución) y el volumen de disolvente de elución han sido optimizados mediante un diseño de superficie de respuesta Box-Behnken.

La técnica de extracción MSPD aporta numerosas ventajas: proporciona buenas recuperaciones, requiere baja cantidad de muestra y de disolvente, implica pocos pasos y la manipulación de muestra es simple. Por otro lado, no es necesario realizar tratamientos previos con lo que se consume poco tiempo. Se consiguen extractos con pocas interferencias usando los adsorbentes de limpieza adecuados, Florisil® y carbón grafitizad,o realizándose la extracción y limpieza simultáneamente. Los niveles de residuos de pesticidas se determinaron mediante GC-MS en el modo SIM. La cuantificación se realizó empleando patrones preparados enriqueciendo los extractos de algas, ya que se observó un efecto de la matriz en la determinación. La exactitud se evaluó en términos de % de recuperación, obteniendo valores en el intervalo entre 81.6 y 113.2 % con %RSD entre 1.6 y 13.2 %. Los límites de detección, entre 0.5 y 2.9 ng g^{-1}, fueron menores que los MRLs establecidos por la legislación europea, proporcionando una sensibilidad suficiente para el control de estos compuestos en el tipo de muestras seleccionado. Además este método

proporciona % de recuperación estadísticamente comparables con los proporcionados por el método de MAE-PTV-LVI-GC-MS/MS desarrollado en este capítulo.

ANALYSIS OF PESTICIDE RESIDUES IN SEAWEEDS USING MATRIX SOLID-PHASE DISPERSION AND GAS CHROMATOGRAPHY MASS SPECTROMETRY DETECTION

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Abstract

Products containing organophosphates, carbamates and pyrethroids pesticides, employed as chemotherapeutants in aquaculture, can remain as residues in the marine environment. Matrix solid-phase dispersion (MSPD) was developed to extract seventeen pesticides from seaweed samples using Florisil and graphitized carbon black as clean-up adsorbents prior to gas chromatography-mass spectrometry (GC-MS) determination. The extraction has been optimized by a Box–Behnken design. The optimal conditions were 1g of seaweed sample, 4 g of anhydrous sodium sulphate as dispersant, 3.6g of Florisil and 0.4g of GCB and an elution volume of 14 mL of a hexane:ethyl acetate mixture containing 40% ethyl acetate. The recoveries ranged from 81.6 to 113.2% with relative standard deviations (RSD) ranging from 1.6 to 13.2%. The limits of detection (LODs) ranged from 0.5 to 2.9 ng g^{-1}. Internal quality control was successfully carried out to verify the quality of the data obtained in the analysis of these pesticides in seaweed samples.

Keywords: Pyrethroid insecticides; carbamate pesticides; organophosphorous pesticides; seaweed; matrix-solid phase extraction; gas chromatography; mass spectrometry; experimental design.

1. Introduction

Historically, seaweeds have been used as food in Asian countries. World seaweed production is currently estimated to be 7.5 million metric tonnes produced by cultivation annually (Dhargalkar & Verlecar, 2009). From a nutritional point of view, edible seaweeds are a low calorie food, with a high concentration of minerals, vitamins and proteins and low lipid content. Seaweeds are an excellent source of vitamins A, B1, B12, C, D and E, riboflavin, niacin, pantothanic acid and folic acid as well as minerals such as Ca, P, Na, K. The quality of protein and lipid in seaweeds make them more acceptable for consumption compared to other vegetables, mainly due to their high content in essential amino acids and relatively high level of unsaturated fatty acids (Dhargalkar et al., 2009; Fleurence, 1999; Sánchez-Machado, López-Cervantes, López-Hernández & Paseiro-Losada, 2004; López-López, Cofrades, Ruiz-Capillas & Jiménez-Colmenero, 2009). The interest aroused by seaweeds as a healthy food in western countries has led to an increase in their presence in markets and also to the development of seaweed-based industries. Moreover, wild seaweeds act as soil conditioners because of their high fiber content and as fertilizers owing to their mineral content.The main way to solve pest and disease control problems (including salmon lice) in the aquaculture industry is the use of chemotherapeutants such as certain organophosphates (OP), carbamates (CAR) and pyrethroids (PYR) pesticides (Denholm et al., 2002). In addition, the marine environment also receives fluxes of pesticides mainly of agricultural origin, due to their widespread use in this field (Carafa et al., 2007). These compounds tend to associate with particulate matter like seaweeds due to their hydrophobicity and persistence (Vega-Moreno, Sosa-Ferrera & Santana-Rodríguez, 2007). There may be an effect of bioaccumulation through the food chain, affecting human health owing to their toxicity and their direct or indirect incorporation by ingestion through edible seaweeds. As a result, monitoring multiresidue pesticides is one of the most important aspects in minimizing potential hazards to human health from food contamination and from the standpoint of consumer safety, in terms of the MRL values specified by the European Union (Regulation EC No. 396/2005 of the European Parliament and Council, 2005). Furthermore, the occurrence of pesticide residues in food should be prevented with regard to OP, CAR and PYR pesticide groups which are known to be neurotoxic (Bjørling-Poulsen, Raun-Andersen & Grandjean, 2008). Although these pesticides have been approved for current use in Europe (Regulation EC No. 396/2005 of the European Parliament and Council, 2005; Plant Protection - Pesticide Residues. EU Annex 1 list), their neurotoxicity in humans has been reported in The Hazardous Substances Data Bank and/or in the NIOSH Pocket Guide to Chemical Hazards.

Some species such as Ulva, Gracilaria, Porphyra, Grateloupia, Undaria, Fucus and Cystoseira, which can be used as in several applications like human alimentation, animal feeding and cosmetics, have been employed as environmental biomonitors useful for displaying a mid-term contamination due to organic

micropollutants (polychlorinated biphenyls, PCBs; chlorinated pesticides; polycyclic aromatic hydrocarbons, PAHs)(Pavoni et al., 2003). Chlorophyta division: Ulva rigida and Valonia utricularis; Rhodophyta division: Coralina elongata, Solieria filiformis and Gracilaria cornea were also used for organochlorine pesticides monitoring (Vega-Moreno et al., 2007). Studies to provide data on processes and behaviour of some herbicides in the coastal environment (including seaweed) have well been investigated (Jianyi et al., 2006; Carafa et al., 2007).

For this purpose, analytical methods for a rapid, sensitive and selective determination of a broad range of pollutants in complex matrices are required. The determination of multiresidue pesticides in foods has frequently been performed by capillary gas chromatography coupled to mass spectrometry (GC-MS) for qualitative and quantitative purposes (Tanaka, Hori, Asada, Oikawa & Kawata, 2007; Garrido-Frenich, Plaza-Bolaños & Martínez-Vidal, 2008; Gilbert-López, García-Reyes & Molina-Díaz, 2009; Khay et al., 2009; Martinez-Vidal, Plaza-Bolaños, Romero-Gonzalez, Garrido-Frenich, 2009; García-Rodríguez, Carro-Díaz, Lorenzo-Ferreira & Cela-Torrijos, 2010).

The sample preparation procedure prior to chromatographic analysis is one of the most critical steps in analytical processes. Furthermore, the development of a simultaneous multiresidue extraction method entails difficulties due to the different physicochemical properties of pesticides (polarity, solubility, volatility). In recent years, many innovations have been developed in the analytical processes applied to prepare food samples for the extraction and determination of pesticide residues (Martinez-Vidal et al., 2009, Tadeo et al., 2010). These include matrix solid-phase dispersion, MSPD, which involves the dispersion of the sample in a solid sorbent, followed by preliminary purification and the elution of the analytes with a relatively low solvent volume and small sample size (Gilbert-López et al., 2009; Beyer & Biziuk, 2008). The quality of the MSPD performance depends on multiple factors, particularly the sorbent type and extraction solvent. A careful selection of a combination of factors specific to the analyte and the sample matrix is critical (García-López et al., 2008). MSPD has been successfully applied to residue pesticides of fruits and vegetables (Chu, Hu & Yao, 2005; Albero, Sánchez-Brunete & Tadeo, 2003; Wang, Xu, Pan & Jiang, 2007; Abhilash, Singh & Singh, 2009; Radišić, Grujić, Vasiljević & Lauševic, 2009; Fang, Min, He, Zhang, Qian & Wang, 2009; Lagunas-Allué et al. 2010) .

The aim of the current study was to develop a simple and reliable method based on MSPD followed by GC-MS determination to simultaneously extract and clean-up seventeen pesticides and related compounds from seaweeds: eleven pyrethroid insecticides (bioallethrin, tetramethrin, cyhalothrin, cyfluthrin, flucytrinate, fenvalerate, resmethrin, permethrin, deltamethrin, cypermethrin, and empenthrin), two organophosphorous pesticides (chlorpyrifos-methyl and chlorpyrifosethyl), two carbamate pesticides

(carbaryl and propoxur), a synergist (piperonyl butoxide) and a preservative commonly used in vegetables and fruit to keep them fresh (2-phenylphenol). Samples of various seaweeds were chosen to illustrate the applicability of the method.

2. Experimental

2.1. Standards and materials

Pestanal quality analytical standards of 2-phenylphenol, propoxur, carbaryl. chlorpyrifos-methyl, chlorpyrifos-ethyl, piperonyl butoxide, empenthrin, bioallethrin, resmethrin, tetramethrin, λ-cyhalotrhin, permethrin, cyfluthrin, α-cypermethrin, flucythrinate and fenvalerate were purchased from Riedel-de-Haën (Seelze, Germany). Pestanal quality deltamethrin was obtained from Chem Service Inc. (West Chester, PA, USA). Internal standards (diethyl-D_{10})-chlorpyrifos and (phenoxi-$^{13}C_6$)-*cis*-permethrin were purchased from Cambridge Isotope Laboratories (Cambridge, UK). Individual standard stock solutions of 5000 µg mL^{-1} were prepared in acetone. A stock mixture solution of all target analytes at a concentration of 10 µg mL^{-1} was obtained by appropriate dilution of individual standard solutions in acetone. All working solutions were prepared by convenient dilution of the stock mixture solution in ethyl acetate. All solutions were stored in amber-colored vials at -20 °C.

Ethyl acetate, acetone, and *n*-hexane, trace analysis grade solvents, were from VWR-Prolabo (Mollet del Vallés, Barcelona, Spain).

Florisil (60-100 mesh) and C_{18} were obtained from Aldrich (Madrid, Spain). Graphitized carbon black (GCB) was purchased from Supelco (Bellafonte, PA, USA). Sodium sulfate anhydrous was obtained from Panreac (Barcelona, Spain). Polypropylene solid-phase extraction syringes (15 mL capacity) and 20 µm polyethylene frits were purchased from International Sorbent Technology (Mid Glamorgan, UK). Syringe filters (Millex GV, 13 mm and 0.22 µm) were from Millipore (Billerica, MA, USA).

2.2. Sample preparation and fortification

All dried edible seaweed samples were purchased in a local market. Wakame (*Undaria pinnatifida*), Nori (*Porphyra umbilicalis*), Irish Moss (*Chondrus crispus*), Sea Spaghetti (*Himanthalia elongata*) and a commercial mixture of Wakame (30%), Sea Lettuce (*Ulva lactuca*, 19%), Kombu (*Laminaria ochroleuca*, 18%), Dulse (*Palmaria*, 18%) and Nori (15%) were ground by means of an electric mill and stored in sealed vessels until analysis.

Wild seaweed samples: *Ascophyllum nodosum*, *Himanthalia elongata*, *Ulva lactuca*, *Fucus vesiculosus*, *Leathesia difformis* and *Chondrus crispus* were collected in the vicinity of a seaport and beaches located on the coast of Galicia (NW Spain). The seaweed samples were stored at -18 ºC before processing. The frozen samples were dried in an oven at 50 ºC for 24 h, ground and homogenized in an electric mill and stored in sealed vessels until use. Spiked Wakame seaweeds were used as the matrix to carry out the optimization study. Approximately 10 g of sample were placed in a beaker with a broad base and covered with 20 mL of acetone spiked with the pesticides to obtain a final concentration in the seaweed of 100 ng g^{-1} in each analyte. The sample was allowed to air-dry in the dark for two days and stored at 4 ºC before extraction, in order to simulate the normal interaction between the seaweed and the pesticide compounds. Other types of edible seaweed were similarly subjected to the spiking process, so as to demonstrate the suitability of the proposed method for the extraction of the target compounds from real samples.

2.3. Extraction procedure

Under final working conditions, 1 g of seaweed sample was mixed with 4 g of anhydrous sodium sulphate in a glass mortar with a pestle. A polypropylene syringe containing a polyethylene frit at the bottom was filled (from bottom to top) with 0.4 g of GCB and 3.6 g of Florisil (as clean-up adsorbents) and, the homogenized matrix, in sandwich mode. Another frit was placed on top before applying slight compression with a syringe plunger. Elution was accomplished by gravity flow with hexane/ethyl acetate, containing 40% of ethyl acetate. 14 mL of eluent was collected into a graduated conical tube and the extract was evaporated to *ca.* 0.5 mL using a gentle stream of nitrogen in a Turvo Vap II concentrator (Zymark, Hopkinton, MA, USA). Finally, the MSPD extract was adjusted to 1 mL with ethyl acetate and filtered by means of a syringe filter with a pore size of 0.22 µm before GC injection.

2.4. Gas-chromatographic determination

Pesticide levels in the processed samples were determined by GC-MS using an Agilent 7890A gas chromatograph (Avondele, PA, USA) equipped with a split/splitless capillary inlet, an Agilent 5975C GC/MSD with Triple-Axis Detector, and an Agilent 7693 automatic liquid sampler. Separation of the different compounds was achieved in an Agilent J&W Scientific HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). Initial oven temperature was set at 70 ºC, held for 3 min; ramped at 25 ºC min^{-1} up to 180 ºC, held for 3 min; ramped at 10 ºC min^{-1} up to 300 ºC and held for 5 min. Helium (purity 99.999%, Carburos Metálicos) was employed as carrier gas with a constant flow of 1.5 mL min^{-1}. Injector, transfer line, ion source and quadrupole temperatures were 280, 290, 230 and 150 °C, respectively. The sample extracts (1 µL) were injected in splitless mode with a solvent delay of 6 min. The effluent of the GC column was introduced directly into the source of the MS. Spectra were obtained in EI mode using an ionization energy

of 70 eV. The mass spectrometer was operated under the selective ion monitoring (SIM) mode in order to improve detection limits. Specific conditions for each analyte are listed in *Table 1*.

Table 1: *Specific MS conditions for each pesticide and IS.*

Compound	Abv.	MS-SIM parameters	
		Quant. Ion (m/z)	Qualif. Ions (m/z)
2-Phenylphenol	2-PP	170	169, 141
Propoxur	PROP	110	152
Empentrhin	EMPEN	123	91
Chlorpyrifos-metil	CLP-M	286	125
Carbaryl	CARB	144	115
Chlorpyrifos-ethyl	CLP-E	97	197, 199
Bioallethrin	BIOAL	123	79
Piperonyl butoxide	PBO	176	149
Resmetrhin	RESME	123	171
Tetramethrin	TETRA	164	123
Cyhalothrin	CYHAL	182	197
Permethrin	PERM	183	163
Cyfluthrin	CYFL	163	165, 206

Table 1: *Continued.*

Compound	Abv.	MS-SIM parameters	
		Quant. Ion (m/z)	Qualif. Ions (m/z)
Cypermethrin	CYPER	163	181
Flucythrinate	FLUCY	199	157
Fenvalerate	FENVA	125	167
Deltamethrin	DELTA	253	181
Chlorpyrifos (IS)	-	99	198, 200
Permethrin (IS)	-	189	163

Quantification was accomplished by relative areas versus (phenoxi-$^{13}C_6$)-cis-permethrin used as internal standard (IS) for PYR compounds while (diethyl-D_{10})-chlorpiryfos was used as IS for the remaining analytes.

2.5. Experimental design

The optimization of an analytical procedure is frequently done following a step-by-step approach which requires a high number of experiments and does not consider interactions between the different variables. In this sense, alternative chemometric approaches like experimental design can help to quickly achieve optimum conditions. The effect of the dispersant/sample *(b_1)* and Florisil/GCB *(b_2)* ratios, as well as the percentage of ethyl acetate *(b_3)* on the *n*-hexane:ethyl acetate elution mixture and elution volume *(b_4)* were evaluated using a response surface Box-Behnken design (Lewis, Mathieu & Phan-Tan-Luu, 1999). For each compound a quadratic polynomial model was considered:

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} \sum_{j=1}^{n} b_{ij} x_i x_j$$

where xi is the coded value of the factors studied and y is the response (peak area) obtained for each drug. The *b* values are the estimated polynomial coefficients: b_0 is the intercept term, the b_i coefficients represent the main effect for each variable, the b_{ij} coefficients in the quadratic terms are responsible for the curvature effects and the b_{ij} ($i \neq j$) coefficients describe the interaction effects. The estimates of the coefficients for the models were calculated by least squares multi-linear regression and these models were validated by analysis of variance (ANOVA).

In order to find the best conditions for the simultaneous extraction of target analytes, multicriteria decision-making strategies using desirability function optimization were applied without additional experimentation. These functions were built as partial Derringer functions for each compound response

using the Nemrod®W 2000 software package. The responses were transformed using a dimensionless desirability (d_i) scale, which ranged between 0 for a completely undesirable response to 1 for a fully desired response. Non-linear left unilateral desirability functions were required to maximize each response efficiency. In a second step, a global desirability function (*D*), which represents the global quality of the common optimum, was calculated by combining single desirability functions, usually as the geometric mean (Lewis et al., 1999).

3. Results and discussion

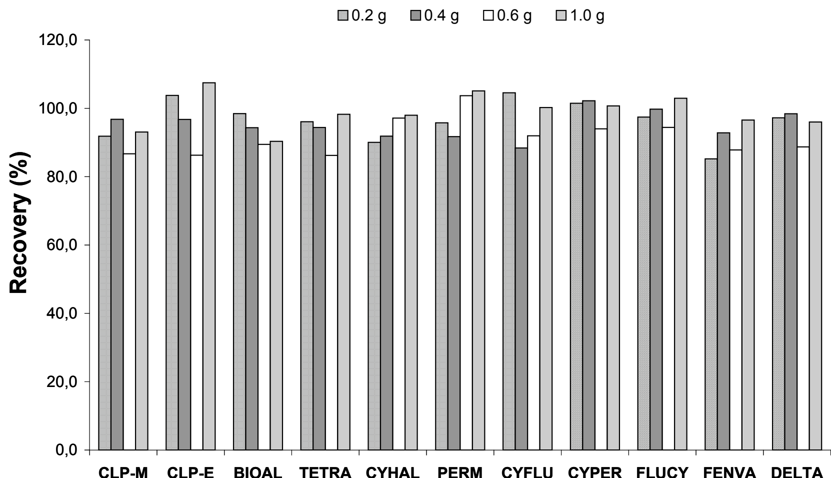
3.1. Preliminary assays

The extraction method proposed is based on the MSPD procedure. In this technique polarities of the sorbent and the elution solvent are known to be key factors, since they determine both the efficacy of the extraction and the purity of the final extracts (Poole, 2007). Preliminary assays to optimize the MSPD procedure for extraction of the selected pesticides from seaweed samples were performed using 0.5 g of a Wakame sample spiked with the studied compounds at 100 ng g^{-1}. Sodium sulfate anhydrous, C_{18} or Florisil (1 g each) were tested as dispersants. The use of a co-column was also desirable to enhance sample clean-up. Based on our experience and the literature on seaweed extracts clean-up, 1.5 g of Florisil and 0.5 g of graphitized carbon black (GCB) were selected as adsorbents (Banerjee et al., 2009; García-Rodríguez et al., 2010) for these preliminary assays. Elution of the cartridges was performed with 20 mL of a *n*-hexane:ethyl acetate (80:20) mixture. Recoveries obtained with Na_2SO_4 anhydrous and Florisil ranged from 59 to 119% while the use of C_{18} as dispersant in the MSPD yielded recoveries ranging from 60 to 145%. The higher peak areas obtained when using C_{18} can be explained by a matrix effect that enhances the chromatographic response to pesticides, as previously reported for the matrix effect in the determination of pesticides in different foodstuffs (Poole, 2007; Albero, Sanchez-Brunete & Tadeo, 2004; Lagunas-Allué et al. 2010). No significant differences on recoveries were observed when using Florisil or sodium sulphate anhydrous as matrix dispersants. Therefore, Na_2SO_4 anhydrous was finally selected as matrix dispersant due to its low cost and its ability to eliminate traces of water from the samples that could interfere with organic solvents in the extraction.

Elution solvent was also studied in order to provide a wide range of polarities compatible with both sorbents and pesticides. Ethyl acetate, *n*-hexane, *n*-hexane:ethyl acetate (50:50) and *n*-hexane:ethyl acetate (80:20) were tested. The aim was to find a solvent capable of extracting a high number of pesticides without additional clean-up steps. The use of *n*-hexane yielded low recoveries (0-20%) while elution with ethyl acetate resulted in high recoveries ranging between 70 and 170%. Both mixtures of *n*-hexane and ethyl acetate yielded recoveries lower than 120%. In view of these results, it seems that the elution solvent

would present a medium–high polarity. Therefore, the mixture *n*-hexane:ethyl acetate was selected as extraction solvent and subjected to detailed optimization.

Four different amounts of sample (0.2, 0.4, 0.6 and 1.0 g) were tested in order to minimize limits of detection and to check the roughness of the method using the GC-µECD system (García-Rodríguez et al., 2008). In all cases, the seaweed samples were spiked with 100 ng g^{-1}, mixed with sodium sulfate anhydrous, keeping the ratio dispersant/sample at 4/1. 1.5 g Florisil and 0.5 g GCB were used as clean-up sorbents and 20 mL of a mixture *n*-hexane:ethyl acetate (80:20) as elution solvent. Quantitative recoveries were obtained, ranging from 85 to 107%, for any sample amount (results included as *Electronic Supplementary Material 1*). Some of the studied compounds could not be detected in this chromatographic system, as is the case of PROP, CARB, EMPEN, PBO and 2-PP, represented by CLP-M in this study because all of them exhibit similar behavior in MSPD extraction. Similarly, BIOAL and RESME exhibit an analogous behavior with TETRA extraction.



Electronic Supplementary Material 1: *Recoveries obtained for different sample amounts (n=2).*

3.2. MSPD optimization

The experimental design involves 27 experiments (including 3 center points) randomly performed to provide protection against lurking variables. Nemrod®W software package (Mathieu, Nony & Phan-Than-Luu, 2000) was used for the generation of the matrix of experiments and to evaluate the effect of each factor on the efficiency of the extraction. All experiments were carried out with blank controlled samples spiked at 100 ng mL^{-1}. Values assigned to the high and low levels for each factor were: dispersant/sample ratio (1.0 – 0.2; using 0.2 g of seaweed sample), Florisil/GCB ratio (2.0 – 9.0; using 0.4 g of GCB), ethyl acetate percentage in the *n*-hexane elution mixture (20 – 50%) and elution volume (5 – 15 mL).

The analysis of variance (ANOVA) showed statistical signification for at least one factor to most of the responses (% recovery of each pesticide tested). However, the relative importance of model coefficients was not the same for the different families of pesticides. Thus, only the effect of elution volume (b_4) showed a positive effect for all the target analytes. The other principal factors presented both positive and negative effects. For OP the most significant factor was the elution volume (b_4). The ratio Florisil/GCB (b_2), the percentage of ethyl acetate (b_3) and the elution volume (b_4) were statistically significant factors on the MSPD of CAR pesticides. For most PYR insecticides the statistically significant factors proved to be the percentage of ethyl acetate (b_3) in the elution mixture as well as the elution volume (b_4). Also, interactions between these two factors and the sample/dispersant ratio (b_1) were significant for most PYR compounds and 2-PP. Looking at the estimates of the coefficients and their statistical significance (results included as Electronic Supplementary Material 2), the coefficients b_4 and b_3 of the model for all the responses, are the most significant together with the coefficients b_{33}, b_{12} and b_{24}. For PBO only the quadratic factor b_{11} was statistically significant. This means that second order interactions between factors are important and should be taken into consideration. The application of Surface Response Methodology (SRM) revealed trends that were similar within families of compounds but different between the families. As an example, *Figures 1A* and *1B* show surface responses for the percentage of ethyl acetate versus elution volume, obtained for CLP-M and CARB respectively.

Electronic Supplementary Material 2: *Estimates and statistical significance (* 95%, ** 99% and *** 99.9%) of the model coefficients for the responses.*

	2-PP		PROP		CLP-M		CARB		CLP-E	
	Coef.	**Signif. %**	**Coef.**	**Signif. %**	**Coef.**	**Signif. %**	**Coef.**	**Signif. %**	**Coef.**	**Signif. %**
b_0	694.3	0.103**	446.3	0.0356***	405.0	3.53*	54.7	3.40*	925.0	0.0883***
b_1	-99.8	1.23*	-12.5	78.8	-20.9	81.1	-2.7	65.8	32.5	76.3
b_2	-39.3	7.2	195.4	0.102**	10.8	90.1	18.3	7.2	22.5	83.5
b_3	156.8	0.502**	229.8	0.0279***	20.4	81.5	152.7	0.115**	46.8	66.5
b_4	328.9	0.115**	200.3	0.0845***	222.3	2.31*	124.3	0.173 **	240.3	4.16*
b_{11}	183.8	0.819**	-2.4	97.2	126.7	34.2	-74.5	1.07 *	-77.7	63.2
b_{22}	151.2	1.20*	16.7	81.0	114.0	39.1	49.3	2.39 *	38.3	81.3
b_{33}	172.3	0.930**	105.7	14.6	206.2	13.3	137.0	0.320**	271.0	11.2
b_{44}	-8.0	67.8	73.3	30.3	61.5	64.0	93.8	0.677**	36.8	82.0
b_{12}	349.5	0.304**	81.0	32.3	261.0	10.3	11.8	32.0	299.3	12.7
b_{13}	70.0	6.9	-139.3	10.2	29.5	84.5	6.3	55.8	-80.3	66.8
b_{23}	74.3	6.2	-22.8	77.7	-56.3	71.0	-8.0	46.6	70.3	70.7
b_{14}	-19.5	41.9	-81.8	31.9	-46.8	75.7	-1.5	88.2	-19.5	91.7
b_{24}	105.5	3.20*	-58.0	47.5	14.8	92.2	59.5	2.19*	-16.0	93.2
b_{34}	-12.8	57.7	-103.3	21.4	-163.5	29.1	265.8	0.114**	-252.5	19.2

Electronic Supplementary Material 2: *Continued*

	EMPEN		BIOAL		RESME		PBO		TETRA	
	Coef.	**Signif. %**	**Coef.**	**Signif. %**	**Coef.**	**Signif. %**	**Coef.**	**Signif. %**	**Coef.**	**Signif. %**
b_0	59.0	1.10*	1566.7	0.0622***	822.0	1.03*	59.0	1.10*	1566.7	0.0622***
b_1	-10.1	8.4	64.1	71.4	109.5	43.4	-10.1	8.4	64.1	71.4
b_2	58.4	0.284**	53.2	76.1	34.7	80.2	58.4	0.284**	53.2	76.1
b_3	77.1	0.164**	6.8	96.9	22.4	87.1	77.1	0.164**	6.8	96.9
b_4	219.3	0.0203***	371.7	5.0	142.9	31.2	219.3	0.0203***	371.7	5.0
b_{11}	-83.1	0.316**	151.8	56.4	134.2	52.1	-83.1	0.316**	151.8	56.4
b_{22}	21.1	4.58*	17.7	94.6	77.2	71.0	21.1	4.58*	17.7	94.6
b_{33}	211.6	0.0489***	174.6	50.8	210.6	32.0	211.6	0.0489***	174.6	50.8
b_{44}	160.9	0.0847***	8.5	97.4	34.1	86.9	160.9	0.0847***	8.5	97.4
b_{12}	40.0	1.78 *	472.8	13.6	168.3	48.7	40.0	1.78*	472.8	13.6
b_{13}	-2.3	71.8	-141.0	64.2	-391.8	12.0	-2.3	71.8	-141.0	64.2
b_{23}	123.5	0.191 **	78.5	79.5	124.5	60.5	123.5	0.191 **	78.5	79.5
b_{14}	8.0	27.7	53.0	86.1	194.5	42.3	8.0	27.7	53.0	86.1
b_{24}	64.8	0.690**	188.8	53.5	-34.8	88.5	64.8	0.690**	188.8	53.5
b_{34}	90.5	0.355**	-424.8	17.6	-208.5	39.1	90.5	0.355**	-424.8	17.6

Electronic Supplementary Material 2: *Continued.*

	CYHAL		PERM		CYFLU		CYPER		FLUCY	
	Coef.	**Signif. %**	**Coef.**	**Signif. %**	**Coef.**	**Signif. %**	**Coef.**	**Signif. %**	**Coef.**	**Signif. %**
b_0	24082.0	0.0117***	4814.7	0.0628***	7506.0	0.165**	22242.0	< 0.01***	5003.3	< 0.01***
b_1	1831.5	41.1	355.3	51.2	1002.5	30.2	1448.4	40.9	394.4	30.1
b_2	-1630.4	46.3	-291.9	58.9	-184.3	84.6	-891.8	60.8	-189.0	61.4
b_3	-4940.0	4.05*	-940.3	9.9	-2455.8	2.16*	-3802.3	4.43*	-892.2	3.10*
b_4	6765.6	0.847**	1433.4	1.83*	3677.5	0.191**	5628.7	0.606**	1025.8	1.58*
b_{11}	3344.0	32.1	1059.6	20.3	-486.6	73.3	1644.4	53.0	154.1	78.3
b_{22}	4552.9	18.4	1330.9	11.7	1640.9	26.2	3009.6	25.9	411.7	46.7
b_{33}	7546.5	3.75*	1998.6	2.61*	2700.9	7.7	5761.5	4.26*	1237.7	4.33*
b_{44}	2918.6	38.4	815.9	32.1	1585.1	27.8	751.5	77.2	265.9	63.6
b_{12}	8059.8	5.2	1943.5	5.4	1190.0	47.4	7169.8	3.09*	1380.3	4.97*
b_{13}	195.3	95.9	314.0	73.6	145.5	93.0	237.8	93.7	267.3	68.0
b_{23}	5945.3	13.7	1345.8	16.5	2324.3	17.5	4925.5	11.9	1243.8	7.3
b_{14}	4170.0	28.5	688.3	46.4	1485.5	37.5	1940.8	52.1	557.3	39.6
b_{24}	4217.3	28.0	1086.5	25.5	3600.8	4.52*	3448.5	26.2	1064.0	11.8
b_{34}	-7974.5	5.4	-1206.5	20.9	-1672.3	32.0	-4919.3	11.9	-466.0	47.5

Electronic Supplementary Material 2: *Continued.*

	FENVA		DELTA	
	Coef.	**Signif. %**	**Coef.**	**Signif. %**
b_0	260.7	0.0390***	8173.7	< 0.01***
b_1	10.1	71.3	461.3	42.2
b_2	-6.9	80.1	-48.8	93.1
b_3	-40.4	15.7	-1106.5	7.0
b_4	76.1	1.49*	2110.9	0.252**
b_{11}	56.8	18.3	288.4	73.5
b_{22}	-17.7	66.7	702.5	41.5
b_{33}	69.3	11.0	1766.3	5.5
b_{44}	28.8	48.8	-55.8	94.8
b_{12}	71.5	14.9	2296.3	3.43*
b_{13}	83.0	9.9	222.3	82.1
b_{23}	83.3	9.8	912.5	36.1
b_{14}	43.8	36.5	732.3	46.1
b_{24}	25.0	60.0	787.3	42.9
b_{34}	-81.5	10.5	-1691.3	10.4

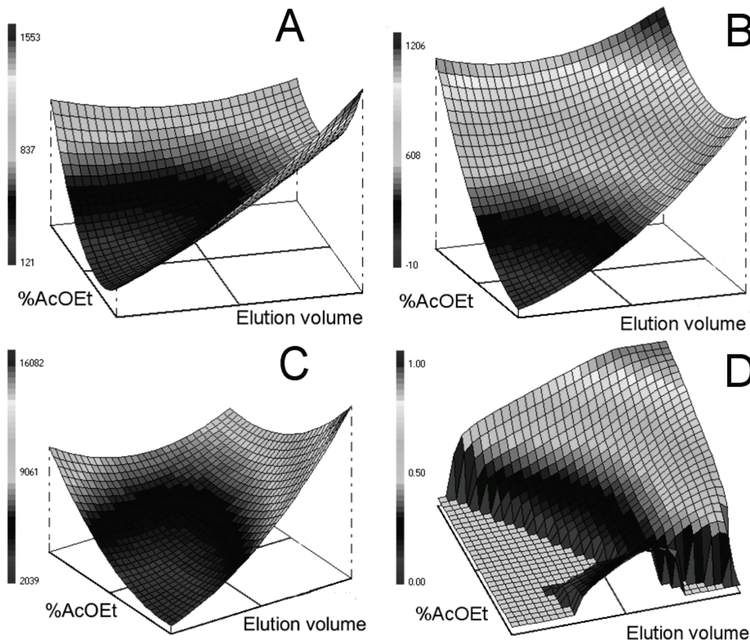


Fig. 1: *Response surfaces obtained for: percentage of ethyl acetate versus elution volume (A, CLP-M and B, CARB) and Florisil/GCB ratio versus Sample/dispersant ratio (C,TETRA and D, PBO).*

High values of elution volume provide good responses in both cases, while % of ethyl acetate in the mixture has the opposite effect in the extraction of CLP-M (better response with a low level of the factor) and CARB (better response with a high level). *Figures 1C* and *1D* show surface responses for Florisil/GCB ratio versus dispersant/sample ratio, obtained for TETRA and PBO respectively. Extraction of TETRA, and by extension that of the PYR insecticides, was favored by high values of dispersant/sample and Florisil/GCB ratios, as well as high values of elution volume and low percentages of ethyl acetate in the elution mixture. However, for PBO, higher responses were obtained when these factors were at their lower level. CAR pesticides extraction was favored by medium levels of the ratios dispersant/sample and Florisil/GCB and high levels of elution volume and % of ethyl acetate in the mixture. Low levels of dispersant/sample ratio and % of ethyl acetate and high levels of Florisil/GCB ratio and elution volume increased the response of the extraction of OP pesticides.

To find the optimal simultaneous conditions of the four variables, a global desirability function was used. Three-dimensional plots of the global desirability obtained are shown in *Figure 2*. The regions in red correspond to optimum values for *D*, where desirability was close to 1, while the regions in grey correspond to null values for *D* when level factors are not suitable to be chosen. The optimal compromise conditions were a dispersant/sample ratio of 4/1, a Florisil/GCB ratio of 9/1, using 0.4 g of GCB and an elution volume of 14 mL of a hexane:ethyl acetate mixture containing a 40% of ethyl acetate.

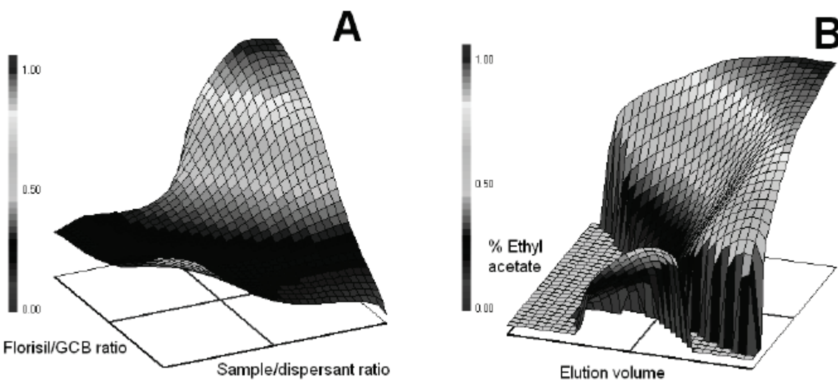


Fig. 2: *Response surfaces of overall desirability as a function of: Florisil/GCB ratio versus Sample/dispersant ratio (A) and percentage of ethyl acetate versus elution volume (B).*

3.3. Method validation

Once the ratio dispersant/sample was optimized, a sample amount of 1.0 g was selected in order to improve limits of detection. In all cases, Florisil/GCB ratio of 9/1 was maintained using 0.4 g of GCB.

Analytical parameters related to linearity, accuracy, precision, limits of detection (LOD) and limits of quantification (LOQ) where investigated to evaluate the feasibility of the proposed method.

Quantification, based on peak areas, was performed by the internal standard (IS) method using deuterated analogues of the compounds (and in their absence, the most similar ones in terms of structure) as surrogate standards. The linearity of the method was evaluated, considering peak areas relative to internal standards by constructing seven-point calibration curves (each level in triplicate) with a wide concentration range shown in *Table 2*. Concentration of IS along the calibration curves was maintained at 50 ng mL^{-1}. As is shown in *Table 2,* good linearity was observed for all compounds at the concentrations within the tested interval, with determination coefficients (R^2) ranging from 0.9981 for BIOAL to 0.9996 for CYPER. LODs and LOQs were defined as the concentration of the compound that produced a signal-to-noise ratio of 3 times and 10 times, respectively, determined experimentally from fortified samples (Directorate of General Health and Consumer Protection. Document No. SANCO/2007/3131, 2007). The range of LODs, summarized in *Table 2* was from 0.3 ng g^{-1} for TETRA to 2.9 ng g^{-1} for DELTA, whereas LOQs ranged from 0.9 to 9.6 ng g^{-1} for the same analytes. The LOQs established allow for the identification and quantification of target analytes below the Maximum Residue Levels (MRLs) established by the EU legislation (Commission Regulation No. 149/2008, 2008; Regulation No. 396/2005 of the European Parliament and Council, 2005).

Table 2: *Figures of merit of MSPD-GC-MS.*

Compound	GC-MS					Precision, RSD% (n=6)		
	Linearity (ng mL^{-1})	R^2	LOD (ng g^{-1})	LOQ (ng g^{-1})	MRLs[a] (mg Kg^{-1})	MSPD-GC-MS		MAE-LVI-GC-MS/MS
						10 ng g^{-1}	25 ng g^{-1}	25 ng g^{-1}
2-PP	1-1000	0.9991	0.5	1.7	NS[b]	5.3	7.8	4.4
PROP	1-1000	0.9991	0.6	2.0	NS	4.4	2.4	9.0
EMPEN	5-1000	0.9990	2.6	8.6	NS	3.7	9.0	7.1
CLP-M	1-1000	0.9991	1.0	3.5	NS	5.3	4.7	6.3
CARB	5-1000	0.9989	2.3	7.5	NS	6.2	7.0	7.7
CLP-E	1-1000	0.9993	0.5	1.6	NS	6.7	6.1	7.5
BIOAL	1-1000	0.9981	1.3	4.4	0.02	4.6	5.6	6.2
PBO	1-1000	0.9988	1.2	4.1	0.05	6.1	5.4	6.7
RESME	1-1000	0.9990	0.8	2.7	0.1	3.7	3.5	6.4
TETRA	1-1000	0.9994	0.3	0.9	NS	6.1	5.7	4.0
CYHAL	1-1000	0.9991	0.7	2.3	NS	10.5	11.5	4.1
PERM	1-1000	0.9991	0.6	2.1	0.05	4.5	4.4	6.5
CYFL	5-1000	0.9992	1.8	6.0	NS	12.0	11.9	8.6
CYPER	5-1000	0.9996	1.5	5.1	NS	12.6	13.0	4.0
FLUCY	1-1000	0.9993	2.4	7.9	0.05	12.4	12.9	4.0
FENVA	1-1000	0.9988	1.9	6.2	0.02	7.3	7.1	9.3
DELTA	5-1000	0.9987	2.9	9.6	NS	8.6	8.9	3.3

[a] MRLs: maximum residue levels established by European Union (Commission Regulation No. 149/2008, 2008; Regulation No. 396/2005 of the European Parliament and Council, 2005); [b] NS not specified

Method precision estimated in terms of inter-day variation is expressed as a relative standard deviations (RSD%) of the analysis of six replicates of spiked Wakame samples at two concentration levels (*see Table 2*). The RSDs ranged from 3.7% (EMPEN) to 12.6% (CYPER) at 10 ng g^{-1} and from 2.4% (PROP) to 13% (CYPER) at 25 ng g^{-1}. All RSDs are lower than 15%, indicating good precision of this method (Commission Regulation No. 149/2008, 2008; Regulation No. 396/2005 of the European Parliament and Council, 2005).

3.4. Application of the method to the analysis of seaweed samples

Internal quality control is an essential aspect of ensuring that data released from a laboratory are fit for the purpose (IUPAC, Harmonized guidelines for internal quality control in analytical chemistry laboratories, 1995). For this control, blank determinations were carried out to detect contamination of the analytical system originating from any source or during the analytical process. The simplest form of blank is the reagent blank, where the analytical procedure is executed in all respects apart from the addition of the test portion. In other instances, better execution of blank determinations was achieved using a simulated test material to assess the analytical method efficiency. In this case, a matrix blank, which is a seaweed sample with zero concentration of analytes, was used. Reliability was evaluated in terms of recovery by spiking five edible seaweeds samples (Wakame, Nori, Irish Moss, Sea Spaghetti, and a commercial mixture of Wakame, Sea Letuce, Kombu, Dulse, and Nori) at two concentration levels (10 ng g^{-1} and 25 ng g^{-1}). In *Figure 3* the results for Irish Moss and a commercial mixture (Seaweed Salad) are shown. From this it can be

seen that mean recoveries (n=6) ranged from 82.5% to 108.9% (Irish Moss at 25 ng g^{-1}), from 83.5% to 110% (Irish Moss at 10 ng g^{-1}), from 81.9% to 106% (Seaweed Salad at 25 ng g^{-1}) and from 81.6% to 108.4% (Seaweed Salad at 10 ng g^{-1}). RSD fluctuated between 2.9% and 9.9%, and between 1.6% and 10% for Irish Moss at 25 ng $g^{-1}$and 10 ng g^{-1}, respectively. Values of RSD ranged from 4.1% and 11.3%, and from 3.6% and 10.8% for Seaweed Salad at 25 ng g^{-1} and 10 ng g^{-1}, respectively. RSD were acceptable for the analytical performance (Commission Regulation No. 149/2008, 2008; Regulation No. 396/2005 of the European Parliament and Council, 2005). Similar recoveries and RSD were obtained from the other fortified seaweed samples and are included as Electronic Supplementary Material 3.

The proposed method was applied to analyze the different target analytes in five commercial edible seaweed samples and six wild seaweed samples (*Table 3*). The analysis showed that the concentrations of the pesticides were below the LOD in Wakame (edible commercial seaweed). Traces of analytes were detected in ten of the analyzed samples, included four edible commercial seaweed. Three pesticides (TETRA, EMPEN and CYPER) were detected simultaneously in Egg Wrack (wild seaweed). Two analytes were detected simultaneously in Nori (edible commercial seaweed, which showed traces of 2-PP and TETRA. EMPEN and TETRA were detected in Irish Moss (wild seaweed). It should be noted that TETRA was detected in five real samples, four of which had concentrations above the LOQ. Similarly, 2-PP was detected in four real samples but concentration above the LOQ was only detected in sea spaghetti (wild seaweed). EMPEN was detected in three real samples, whereas CYPER was detected in two real samples. The compounds detected in all analyzed samples are below the Maximum Residue Levels (MRLs) established by European Union.

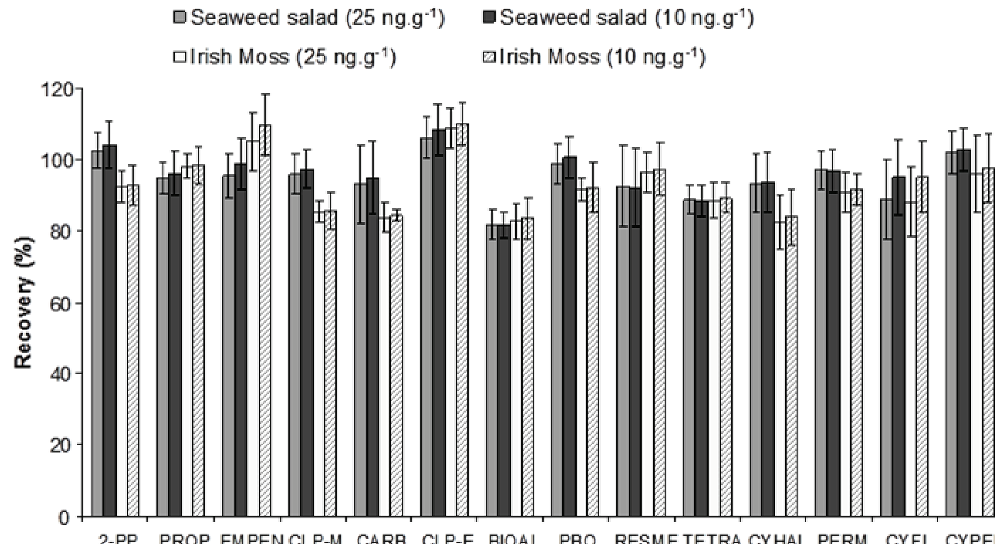


Fig. 3: *Recoveries in Irish Moss and a commercial mixture (seaweed salad) spiked at two concentration levels 10 and 25 ng g-1, applying MSPD-GC-MS method .Error bars represent the standard deviation (n=6).*

***Table 3**: Comparative results of the analysis of real seaweed samples with the MSPD-GC-MS proposed method and MAE-PTV-LVI-GC-MS/MS (n=4).*

Sample*	Concentration ±SD (ng g^{-1})							
	2-PP		EMPEN		TETRA		CYPER	
	MSPD	MAE	MSPD	MAE	MSPD	MAE	MSPD	MAE
EGG WRACK (A1)	-	-	<LOQ	1.6±0.05	3.6±0,3	4.2±0.15	<LOQ	1.4±0.08
SEA SPAGHETTI (A2)	2,5±0,1	2.5±0.07	-	-	-	-	-	-
SEA LETTUCE (A3)	-	-	-	-	1,9±0,1	2.5±0.05	-	-
BLADDER WRACK (A4)	-	-	-	-	2,9±0,2	3.0±0.2	-	-
SEA POTATO (A5)	-	-	<LOQ	3.6±0.2	-	-	-	-
IRISH MOSS (A6)	-	-	<LOQ	-	1,6±0,1	-	-	-
SEAWEED SALAD (B1)	<LOQ	0.5±0.01	-	-	-	-	-	-
SEA SPAGHETTI (B2)	-	-	-	-	-	-	<LOQ	2.4±0.4
IRISH MOSS (B3)	<LOQ	0.5±0.07	-	-	-	-	-	-
NORI (B4)	<LOQ	0.2±0.02	-	-	<LOQ	0.6±0.06	-	-
WAKAME (B5)	-	-	-	-	-	-	-	-

** A1 to A6: wild seaweed samples; B1 to B5: commercial edible seaweed samples*

***Electronic Supplementary Material 3:** Recoveries obtained by MSPD-GC-MS from fortified commercial seaweed samples. Two addition levels were studied: high, 25 ng g^{-1}; and low, 10 ng g^{-1}(n=6).*

Analyte	*Wakame*				*Nori*				*Sea Spaghetti*			
	%R		*RSD*		*%R*		*RSD*		*%R*		*RSD*	
	High	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*
2-PP	99.6	99.4	7.8	5.3	101.1	102.6	3.5	4.6	94.8	97.0	4.4	4.4
PROP	99.6	99.6	2.4	4.4	95.0	96.5	7.2	5.7	93.2	95.4	3.5	3.5
EMPEN	92.7	96.0	9.0	9.0	95.0	100.0	7.8	7.8	81.2	85.4	7.4	6.5
CLP-M	92.7	92.8	4.7	5.3	92.5	94.1	6.6	6.8	88.0	90.0	7.7	5.5
CARB	88.6	88.9	7.0	6.2	87.6	89.4	12.2	11.7	81.6	84.0	2.9	4.2
CLP-E	112.6	113.2	6.1	6.7	105.6	108.0	6.7	7.6	104.3	107.5	3.6	4.7
BIOAL	81.9	81.7	5.6	4.6	81.9	83.3	5.9	8.5	81.7	82.7	6.2	5.6
PBO	99.6	99.8	5.4	6.1	96.2	97.8	6.5	3.7	93.0	95.2	7.9	5.0
RESME	97.3	97.2	3.5	3.7	98.5	100.1	3.9	2.6	87.3	88.6	7.7	8.5
TETRA	88.5	88.3	5.7	6.1	85.5	86.8	3.6	4.8	89.8	90.9	2.3	2.2
CYHAL	95.3	95.7	11.5	10.5	95.1	97.0	11.7	9.9	87.3	89.1	5.5	5.5
PERM	92.6	92.4	4.4	4.5	94.4	95.8	5.7	6.2	93.8	94.9	4.6	3.8
CYFL	91.5	97.1	11.9	12.0	86.4	94.3	9.6	8.7	84.3	91.8	11.5	10.7
CYPER	88.0	88.7	13.1	12.6	88.6	90.9	7.0	7.0	92.8	95.0	11.9	11.4
FLUCY	101.5	103.6	12.9	12.4	94.7	98.4	10.3	8.5	93.2	97.0	13.1	12.3
FENVA	105.6	107.3	7.1	7.3	96.4	99.9	9.8	11.5	85.5	88.4	12.4	12.4
DELTA	108.8	115.8	8.9	8.6	99.6	109.2	7.3	6.0	99.3	108.3	10.9	9.8

Electronic Supplementary Material 3: *Continued.*

Analyte	Irish Mosh				Seaweed Salad			
	%R		RSD		%R		RSD	
	High	Low	High	Low	High	Low	High	Low
2-PP	92.5	92.8	4.3	5.5	102.7	104.2	4.9	6.6
PROP	98.0	98.4	3.4	5.3	94.7	96.2	4.6	6.2
EMPEN	105.2	109.7	8.1	8.7	95.5	98.7	6.2	7.0
CLP-M	85.4	85.9	2.9	5.3	96.0	97.5	5.7	5.3
CARB	83.7	84.5	4.3	1.6	93.1	94.9	11.1	10.1
CLP-E	108.9	110.0	5.7	6.0	106.0	108.4	5.8	7.2
BIOAL	82.7	83.5	5.1	5.8	81.9	81.6	4.3	3.6
PBO	91.6	92.3	3.3	6.9	98.8	100.5	5.6	5.7
RESME	96.5	97.3	5.6	7.3	92.6	92.3	11.3	10.8
TETRA	88.6	89.3	5.0	4.3	88.8	88.4	4.1	4.6
CYHAL	82.5	84.0	7.3	7.7	93.3	93.7	8.0	8.3
PERM	90.8	91.6	5.7	4.4	97.2	96.9	5.3	6.1
CYFL	88.2	95.0	9.9	10.0	88.8	95.0	11.1	10.6
CYPER	96.1	97.7	11.0	9.7	102.3	102.8	5.9	5.9
FLUCY	99.9	103.2	10.3	11.8	101.4	103.3	13.2	11.6
FENVA	89.4	92.3	9.8	11.8	94.6	96.3	8.7	9.3
DELTA	87.2	95.9	7.6	7.8	99.8	107.2	7.2	5.8

3.5. Comparison of MSPD-GC-MS with similar analytical methods

The performance data and results from the proposed method were compared with those from previous methods developed by the same authors, using microwave-assisted extraction (MAE) method followed by clean-up with solid-phase extraction (SPE) combined with large-volume injection gas chromatography–tandem mass spectrometry (LVI-GC-MS/MS) (Garcia-Rodriguez et al., 2010), by analyzing the same set of samples by both methods. MAE-LVI-GC-MS/MS improved sensitivity, with LOQs of 0.9 – 76.9 pgg^{-1} compared with 0.9 – 9.6 ngg^{-1} by MSPD-GC-MS. PTV-LVI coupled to GC–MS/MS appears to be a good alternative to GC-MS due the high sensitivity achieved but the limits obtained with the proposed methodology of MSPD-GC-MS are sufficiently low and also lower than the MRL.

The methods were compared with regard to real samples analysis (parallel analysis of six real Wakame, Nori, Irish Moss, Sea Spaghetti, and Seaweed Salad) fortified with all the analytes at a concentration level of 25 ng g^{-1}) and the analytical performance characteristics of both. Two-way ANOVA was used to evaluate differences between recovery values of following factors: A, two analytical methods (MAE-LVI-GC-MS/MS and MSPD-GC-MS) and B, five seaweed samples analysed. The significant differences of recovery means were compared by least-significant-difference methods at a confidence level of 95%. No significant differences were found ($p>0.05$) between the two methods ($p = 0.2824$). No difference was observed between the recoveries obtained when one or another methodology was applied to the seaweed samples studied, interaction AB ($p = 0.2127$). However, significant differences were found ($p<0.05$) between the five seaweed samples recoveries ($F=2.57$ and $p=0.0403$). The multiple range tests confirmed

these results and showed that the recovery means obtained with (Sea Spaguetti, Irish Moss and Wakame) form a homogeneous group, significantly different from (Irish Moss, Wakame, Nori and Seaweed Salad) recovery means group. According to the results of the ANOVA, the multiple range tests showed that the recovery means obtained with both analytical methods form a homogeneous group of means, demonstrated that both methods lead to similar accuracy results.

Method precision was also compared. Values for inter-day reproducibility were than 13.0% and 9.3%, for MSPD-GC-MS and MAE-LVI-GC-MS/MS, respectively (*Table 2*). The sensitivity obtained by use of the two methods was compared by looking at LODs and LOQs (*Table 2*) (Garcia-Rodriguez et al., 2010). The comparison, as expected, showed lower values for MAE-LVI-GC-MS/MS due LVI using PTV can improve GC system detection limits by one to two orders of magnitude over conventional split/splitless inlet. (Hu et al., 2008).

Finally, eleven seaweed samples were analyzed by both methods (n=4). The comparative results are shown in *Table 3*. The comparison showed good correlation between MSPD and MAE approaches, with results following the trend of higher responses via MAE-LVI-GC-MS/MS. Thus is due to the LOQs obtained with each method.

Overall comparison of the two methods revealed similar performance based on results for accuracy (%recovery) and precision but better results of MAE-LVI-GC-MS/MS for LODs and LOQs. However, the main advantages of MSPD-GC-MS method are lower cost, easy method, being more feasible for any analytical laboratory, enabling high-throughput analysis and providing a sufficient sensitivity to detect target analytes at concentrations below the levels set by legislation. Because of all these characteristics, the proposed method is applicable and could be deemed necessary within the field of food control and safety.

4. Conclusions.

Seaweeds must be considered not only as a resource capable of providing commercially important components for industrial development but also for their importance within the realm of nutrition.

The multi-residue MSPD-GC-MS method developed for the determination of pesticides in seaweed samples is fast because the on-column clean-up step is performed immediately after extraction. The method uses sodium sulfate anhydrous as dispersant with Florisil and Graphitized carbon black as clean-up adsorbents and a mixture of hexane/ethyl acetate (3:2) as elution solvent. It has been validated for different seaweed matrices and provides good selectivity, accuracy, precision, and sensitivity. The identification and quantification at low levels of concentration of seventeen pesticides have been possible and the LOQs

obtained were lower than the maximum residue levels established by European legislation. This method has been successfully applied to the analysis of pesticides in edible and wild seaweeds from the Galician coast (NW Spain). The method was then applied to the analysis of eleven seaweed samples, of which ten showed the presence of at least one pesticide at concentrations between <LOQ and 3.6 ngg^{-1}. 2-Phenylphenol and three pyrethroid pesticides (Tetramethrin, Cypermethrin and Empentrhin) were detected and the results showed that there were very low concentration levels. Comparison of the performance of this method with that of a previously developed method using MAE-LVI-GC-MS/MS showed similar recovery ranges for the analytes and good correlation with regard to results from eleven seaweed samples treated and analyzed by the two methods.

The results of this study should be considered for the future development of integrated poly-aquaculture systems, in which the seaweeds grown in fish farm effluents or shellfish serve as a dietary supplement for the animals themselves. Further research is directed at investigating the spatial and temporal distribution of pesticides in biota and their possible bioaccumulation in fish obtained from aquaculture areas.

Acknowledgments

This research was supported by the Project CTQ2009-08377 MEC (Plan Nacional I+D, Spain). D. García-Rodríguez acknowledges the Xunta de Galicia (Consellería de Innovación e Industria) for the financial support of his PhD.

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3. Desarrollo de métodos de análisis para la determinación de pesticidas en pescados y moluscos de cultivo

Relación de trabajos publicados en este capítulo:

Diego García-Rodríguez, Antonia M Carro-Diaz, Rosa A Lorenzo-Ferreira, *"Determination of pesticides in fish and shellfish by matrix solid phase dispersion and liquid chromatography-tandem mass spectrometry"* **J. Sep. Sci. (2012) Article first published online: 4 OCT 2012, DOI: 10.1002/jssc.201200440**

Póster: ***"Optimization of matrix solid-phase dispersion and LC-MS-MS confirmatory determination of pesticides residues in fish and shellfish"*** HPLC2011, 19–23 Junio 2011, Budapest (Hungría)

3.1. Determinación de pesticidas en pescados y moluscos mediante dispersión de la matriz en fase sólida y cromatrografía líquida con espectrometría de masas en tándem.

La acuicultura se encuentra en expansión, siendo la comunidad gallega líder en este sector, tanto en el mercado español como en mercados internacionales. Galicia presenta unas características climáticas y geográficas excelentes para el desarrollo de la acuicultura. Se han alcanzado valores mayores de 221000 toneladas en alimentos provenientes de la acuicultura en el año 2007. El producto más demandado en la acuicultura gallega es el mejillón, que representa un 96% de la cantidad total producida y un 98% del mejillón producido en España. La producción de otros bivalvos y de pescados también es importante, con más de 215000 toneladas cerca de 6000 tonelas respectivamente.

En este trabajo se ha llevado a cabo el desarrollo de un método que permita determinar ocho pesticidas pertenecientes a distintas familias de compuestos como son organofosforados, carbamatos, benzoilfenilureas y avermectinas en pescados de acuicultura y moluscos cultivados en las costas gallegas, mediante dispersión de la matriz en fase sólida seguida de una separación y cuantificación mediante cromatografía de líquidos con espectroscopia de masas en tándem (LC-MS/MS).

Algunos de los pesticidas seleccionados se emplean para controlar distintas enfermedades y plagas de la acuicultura. Son ingredientes activos de diversos productos comerciales como azametifos (Salmosan®), diflubenzuron (Lepsidon®), teflubenzuron (Calide®, Ektobann®) e ivermectin (Ivomec®).

En primer lugar, se ha realizado la optimización de los adsorbentes más adecuados que se utilizan en MSPD para retener los lípidos característicos de este tipo de muestras, que pueden interferir en la posterior determinación cromatográfica. También se han optimizado el disolvente más idóneo para la elución y las condiciones de extracción de los compuestos mediante un diseño central compuesto diseño de experimentos, consiguiendo un ahorro económico, de esfuerzo y de tiempo en el laboratorio.

En el método cromatográfico se han optimizado las energías de potencial del capilar y las energías de colisión para poder obtener los fragmentos característicos de cada uno de los compuestos. La detección por espectrometría de masas se lleva a cabo en modo positivo de ionización por electrospray para todos los compuestos, excepto para la familia de benzoilfenilureas, para la que se utilizó modo negativo. Los límites de detección están en el intervalo de 1.5-11.5 µg kg^{-1}. Las recuperaciones medias en muestras de salmón se encuentran entre 87.4–103.6 % y la precisión (%RSD) fue menor de 10.5 % en todos los casos.

Las muestras analizadas pescados de acuicultura (rodaballo, panga y salmón) y moluscos (vieira, almeja, mejillón y berberecho) presentan efecto matriz por lo que la cuantificación de las mismas tiene que realizarse a través del método de adición estándar. Carbaril, teflubenzurón y doramectina han sido

detectados en distintas cantidades en alguna de las muestras de pescado, mientras que algunas de las avermectinas han sido detectadas en vieira y almeja.

Determination of chemotherapeutic agents in fish and shellfish by matrix solid phase dispersion and liquid chromatography-tandem mass spectrometry

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Abstract

Chemicals are widely used in aquaculture and one of the main recipients of these analytes is the aquatic environment. The aim of this work was to develop and validate a simple and sensitive method for the determination of multiclass chemotherapeutic agents in farmed fish and shellfish using matrix solid-phase dispersion and liquid chromatography-tandem mass spectrometry. Residues of azamethiphos, three avermectins, two carbamates and two benzoylureas were extracted from samples using silica gel as clean-up adsorbent and 0.5% acetic acid in acetonitrile as elution solvent. The extraction conditions were investigated and optimized using an experimental design. Mass spectrometry detection was carried out in positive electrospray ionization mode with multiple-reaction monitoring scan (except for benzoylurea family). Matrix-matched standards were used for the drugs quantification. Good linearity ($R^2 \geq 0.996$) was observed in the range of 5-500 µg kg^{-1}. Limits of detection were in the range of 1.5-3.7 µg kg^{-1}. Recoveries from salmon samples spiked with veterinary drugs were in the range 84.9-118 %. Precision was satisfactory since relative standard deviations were lower than 10.6 %. The method can be successfully applied for the analysis of fish and shellfish from aquaculture.

Keywords: Matrix solid-phase dispersion; LC-MS/MS; aquaculture; chemotherapeutants; experimental design.

1 Introduction

During the past decade, a significant increase in the quantity of fish consumed from aquaculture occurred, and this trend is expected to increase further to meet future demand [1] Nevertheless, aquatic environments are under the pressure of direct and indirect pesticide discharges from urban, industrial and agricultural activities. Furthermore, several classes of chemotherapeutic agents are widely used to prevent diseases and pests, such as ectoparasitosis, of farmed fishes. For example, azamethiphos is an organophosphorus pesticide used to treat salmonids for sea lice infestations (Salmosan®), diflubenzuron (Lepsidon®) and teflubenzuron (Calide®, Ektobann®) are benzoylphenyl urea insecticides, ivermectin (Ivomec®), doramectin and abamectin are avermectin derivatives with potent anthelmintic and insecticidal properties, and carbaryl (Sevin® applied to control burrowing thalassinid shrimp and in oyster culture) and propoxur are two carbamate insecticides [2, 3]. Residues of these compounds, accumulated in the water environment and fish lipids, can be harmful to human health as well as to other marine organisms and farmed species [4]. The side effects include reproductive and developmental toxicity, neurotoxicity and bioaccumulation [5, 6] Therefore, in order to ensure consumer safety, monitoring of chemical residue levels in farmed fishes is essential, because this kind of food is weekly consumed in the European Union countries [7, 8]. The European Commission sets the regulation to protect consumers from exposure to unacceptable levels of pesticides residues in food and feed [9, 10]. A default maximum residue limit (MRL) of 0.01 mg kg^{-1} is established in this regulation for edible products without specific values [9].

The need to determine the pesticide residues in aquaculture products at trace levels has prompted the development of sensitive screening methods. Liquid chromatography-mass spectrometry (LC-MS) [11] and LC-MS/MS methods [12] have been applied to the analysis of pesticides in seafood because of its high sensitivity and ability to provide compound confirmation [13]. Separation of pesticides and chemotherapeutants from products of animal origin is difficult owing to the complexity of biological matrices, and particularly to the presence of fat [13, 14]. Traditional methods, mainly Soxhlet [15, 16] or solid-liquid extraction [17] for the extraction of pesticides from fish and shellfish can be replaced with instrumental techniques [6], such as supercritical fluid (SFE) [18], microwave-assisted (MAE) [19] and pressurized liquid extraction (PLE) [20]. These procedures provide low solvent consumption and relative short time extraction, but the purchase cost of the equipments is high [20]. On the other hand, it is difficult to identify a general method suitable for a wide range of analytes because the polarity of the extraction solvent mixture may not be adequate for all of them [21].

Several approaches have been attempted to eliminate lipids and co-extracted interference from fatty food extracts, including solid phase extraction (SPE), gel permeation chromatography (GPC) and solid-

phase micro-extraction (SPME) [14, 22-24]. The pesticide extracts may be cleaned up using SPE with single or combined layers of silica, alumina, carbon cartridges, Florisil or diatomaceous earth [22, 25-27]. Complex samples, such as fish or other fatty matrices, very often require a two-step clean-up which combines different chromatographic techniques in series [14]. Matrix solid-phase dispersion (MSPD) may be used as an alternative technique for the simultaneous extraction and purification (fat removal) of lipophilic chemical substances from biota samples [22, 26]. MSPD combines extraction and cleanup within a single process [28]. The main advantages of MSPD are the use of mild extraction conditions with a suitable combination of dispersant sorbent, moderate consumption of elution solvent, and low cost per extraction without expensive instrumentation [29].

The purpose of the current study was to develop a simple and fast method for the determination and monitoring of occurrence of eight pesticides in fish and shellfish by MSPD with anhydrous sodium sulphate and C18 as dispersants, silica as adsorbent and LC-MS/MS analysis. To the best of our knowledge this multi-residue methodology has not been tested yet in aquaculture species. The parameters that determine the extraction efficiency and the subsequent quality of analytical method were optimized and discussed in detail. The proposed method was validated and applied successfully to the analysis of these compounds from different aquaculture samples from a local food market.

2 Experimental sections

2.1 Reagent and materials

Pestanal quality analytical standards of azamethiphos (S-[(6-chloro-2-oxo[1,3]oxazolo[4,5-b]pyridin-3(2H)-yl)methyl]; propoxur (phenol, 2-(1-methylethoxy)-1-(N-methylcarbamate); carbaryl (1-naphthalenol, 1-(N-methylcarbamate); teflubenzuron (N-[[(3,5-dichloro-2,4-difluorophenyl)amino]carbonyl]-2,6-difluoro benzamide); diflubenzuron, (benzamide, N-[[(4-chlorophenyl)amino]carbonyl]-2,6-difluoro) were purchased from Riedel-de-Haën (Seelze, Germany). Pestanal quality abamectin; doramectin and ivermectin were purchased from Sigma-Aldrich (Madrid, Spain).

Individual standard stock solutions of 5000 μg mL^{-1} were prepared in methanol (MeOH). A stock mixture solution of all the studied compounds at a concentration of 100 μg mL^{-1} was obtained by appropriate dilution of individual stock solutions in MeOH. Diluted solutions were prepared from the stock mixture solution in acetonitrile (ACN). All solutions were stored in amber-colored vials at -20 °C.

ACN and methanol (gradient HPLC grade) and silica gel 60 Å (0.040–0.063 mm) were from Merck (Darmstadt, Germany). Florisil (60–100 mesh), diatomaceus earth (DE) in powder form, aluminium oxide

activated neutral (150 mesh) and C18 were from Sigma-Aldrich (Madrid, Spain). Sodium sulfate anhydrous was from Panreac (Barcelona, Spain). Neutral silica was activated overnight at 200 °C and then cooled to room temperature in a desiccating chamber. Ultra pure water was obtained using a Milli-Q® water purification system (Millipore). Syringe filters (Millex GV, 13mm, and 0.22µm) were from Millipore (Billerica, MA, USA).

2.2 Sample preparation

Culture fish (turbot, panga, and salmon) and shellfish (scallop, clam, mussel and cockle) were purchased in a local market. Prior to analysis, fillet muscle was freeze-dried for 48 h, homogenized in an electric mill to obtain a fine powder and stored in sealed vessels until analysis. Under final working conditions, sample portions (0.2 g) were then weighed, placed in a glass mortar and gently blended with 0.5 g of sodium sulphate anhydrous and 2 g of C18 for 3 min, using a glass pestle to obtain a homogeneous mixture. This mixture was transferred to a 6 mL SPE tube with a polypropylene frit at the bottom, filled (from bottom to top) with 2 g of silica (as clean-up adsorbent). Another frit was placed on top of the mixture before compression to form a column packing with the help of a modified syringe plunger. 0.5% acetic acid in ACN was used to elute the column by gravity flow. The eluent was collected in a graduated conical tube (18 mL) and evaporated to *ca.* 0.5 mL under a gentle stream of nitrogen in a Turvo Vap II concentrator (Zymark, Hopkinton, MA, USA). Finally, the extract was adjusted to 1 mL with acetonitrile and filtered by means of a syringe filter with a pore size of 0.22 µm before LC injection.

2.3 LC-MS/MS analysis

A Varian (Walnut Creek, CA, USA) liquid chromatograph equipped with two isocratic, high-pressure mixing pumps (Varian 410 Prostar), an autosampler, and a thermostated column compartment was used. The MS/MS system consisted of a U-shaped triple quadrupole (Varian MS 1200 L) equipped with an electrospray ionization interface (ESI). The LC-MS/MS instrument was entirely controlled by the Varian MS Workstation Version 6.9 software. Separations were carried out using a Hypersil ODS (100 mm × 3.2 mm i.d., 3 µm particle size) analytical column with a C18 security guard cartridge Phenomenex (4.0 x 2.0 mm). Eluent flow rate was set at 0.4 mL min^{-1} and the column was kept at 30 º C. The mobile phase was as follows: 5 mM ammonium acetate in ACN (A) and 5 mM ammonium acetate in water (B). The gradient conditions were: 0-14 min, from 50 % to 100 % A; 14-20 min, constant 100 % A; 20-22 min, back to 50 % A; 22-25 min constant 50 % A. The injection volume was 10 µL. The ESI interface was operated simultaneously was operated in both positive (PI) and negative (NI) ion modes according to the preferential ionization of each analyte and the voltage of the ESI needle fixed at 5.000 V. The optimized ESI conditions were established to furnish average maximum intensity of the precursor ions. The temperature of the ESI

housing was set at 50 °C. Argon (99.999%) was employed as collision gas (2.2 mTorr) in the mass spectrometer. The nitrogen nebulizer pressure was 50 psi and the nitrogen drying gas temperature (200 ºC,19 psi) in the ESI source, was provided by a high purity generator (Domnick Hunter, Durham, UK). The capillary potential was 5000 V/−4500 V (PI/NI). For MS/MS, high-purity nitrogen (99.999%) was used as collision gas. To optimize the multiple-reaction monitoring (MRM) transitions, each individual pesticide at a concentration of 10 μg mL^{-1} in ACN was injected directly. Two transitions were monitored per compound and a dwell time of 0.2 s per transition. The optimum conditions are summarized in Table 1. Compounds were confirmed by their retention times and the most abundant transition ion was used as quantifier and other transition used as qualifier.

***Table 1**. Conditions for mass spectrometry in MRM mode.*

Compound	Structure	Ioniz. mode	Potential (V)	Precursor ion	Product ions	Capillary voltaje (V)	Collision energy (eV)
Azamethiphos		ESI+	40.0	325	183* 139	40	11.0 18.5
Propoxur		ESI+	30.0	210	111* 153	30	10.5 5.0
Carbaryl		ESI+	30.0	202	145* 127	30	6.0; 24.5
Diflubenzuron		ESI-	36.0	309	289* 156	-36	8.0 10.0
Teflubenzuron		ESI-	30.0	379	339* 359	-30	10.0 6.0
Abamectin		ESI+	32.0	891	305* 567	32	19.0 12.0
Doramectin		ESI+	36.0	916.5	331* 593	36	21.0 10.0
Ivermectin		ESI+	36.0	893	307* 569	36	21.0 12.5

**Quantifier transition*

3 Results and discussion

3.1 Optimization of LC-MS/MS

The optimization of MS parameters (cone voltage and collision energy) was performed by direct infusion of 2 mL of standard solution (5 µg mL^{-1}) of each compound with 100 µL of 2% formic acid added. A flow rate of 0.05 mL min^{-1} of a mobile phase, MeOH/water (50:50) was used. Thus, the adsorption of standards to the glass walls of the vial and the possible formation of stable adducts with sodium ions, which are difficult to fragment, are avoided.

The ESI in positive mode (compounds ionized by adding a proton) was selected as ionization technique due to its sensitivity, ruggedness and easy handling and maintenance for all analytes, except for benzoylurea family, whose separation and determination were studied in negative mode (losing a proton), obtaining the best specificity and sensitivity. [30]. The ionization was optimized by a serial of preliminary experiments, testing different modifiers, such as acetic acid and ammonium acetate at various concentrations, in a binary gradient mobile phase comprising ACN and water. Finally, the addition of 5 mM ammonium acetate gave the best sensitivity [31]. Capillary voltages and collision energies were optimized in order to maximize the intensity for the precursor ion for each compound, and to identify a minimum of two transitions in the MS/MS spectra. The most intense transition was used for quantification and the second for confirmation for Mass Spectrometric detection. Full-scan spectra were acquired in order to select the most abundant m/z value, optimizing the cone voltage. Besides, the sensitivity of the mass spectrometer was further improved using the scheduled MRM mode (Table 1).

3.2 Optimization of the MSPD conditions

For efficient isolation of the eight analytes from fish or shellfish matrixes and evaluation of the analytical potential of different adsorbents, a serie of experiments were designed to optimize several relevant conditions affecting the extraction efficiency, including the type and amount of clean-up adsorbents, the percentage acetic acid in the elution solvent and the volume of eluent

The choice of solvents was based on solubility of the drugs in order to increase recoveries. Based on the literature data, ACN, several acetic acid:ACN mixtures [11, 32, 33] and a hexane:ethyl acetate mixture (60:40) [34] were tested as extraction solvents to extract the target compounds from the spiked (0.5 µg g^{-1}) salmon samples (0.2 g). To reduce the presence of fish co-extractives, 0.5 g of sodium sulfate anhydrous as dispersant and 2 g of florisil as adsorbent were used in the MSPD procedure. Large amounts of lipids were extracted when hexane:ethyl acetate mixture was used and hence the extracts were not injected in the

chromatographic system. The polarity of ACN is higher and thus it is a poor solvent of lipids, allowing good recoveries of the compounds studied. As shown Figure 1, the avermectins family was better extracted when acetic acid was added to ACN [11, 32, 33]. The acidification of the extraction medium could prevent the pH dependant degradation and improve the stability of problematic pesticides [35, 36]. Good normalized recovery results, between 75.6 % and 97.9 %, were obtained for all analytes with 1% acetic acid in ACN. In the case of ivermectin, the recovery was lower. Therefore, ACN with 1% acetic acid was the solvent selected for the simultaneous extraction of the target chemical agents from aquaculture samples.

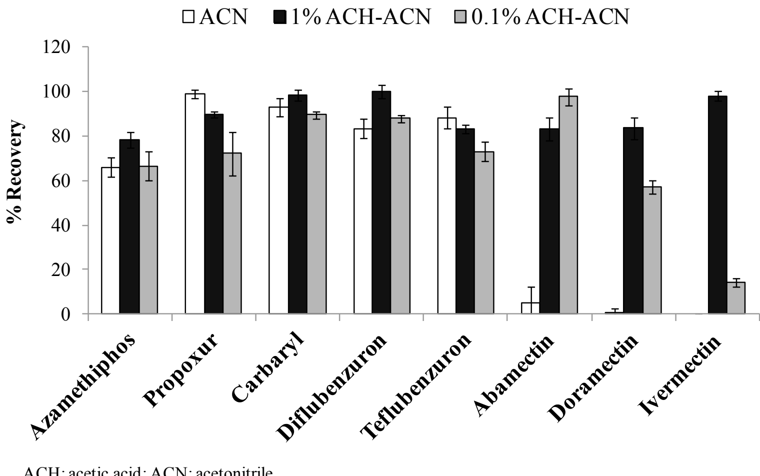
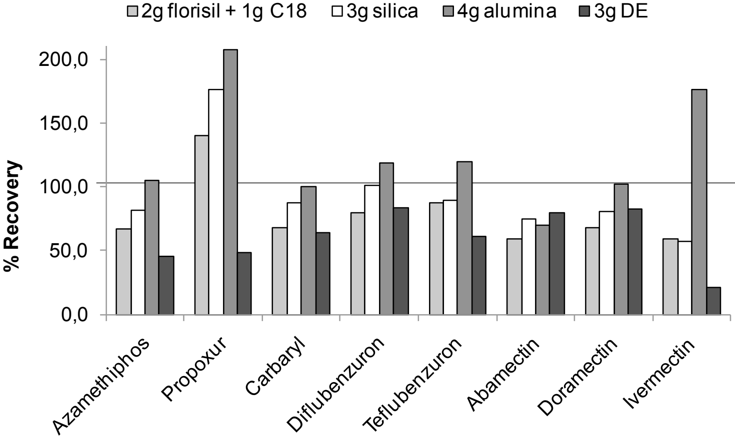


Figure 1. *MSPD recovery of target pesticides under different solvent elution conditions.*

To determine the suitability of the adsorbents, recovery studies were conducted by spiking blank samples with the standard mixture and then blending with 3 or 4 g of each adsorbent. Sodium sulfate anhydrous (0.5 g) was used in the extraction procedure to avoid the moisture presence in the samples [37]. C18 (2 g) was used as dispersant [13, 38]. MSPD experiments were performed to investigate different adsorbents commonly used as fat retainer: silica gel, neutral alumina, Florisil + C18 and DE [13, 27, 39]. A certain matrix effect was observed during LC-MS/MS analysis of such extracts due to the high lipid content (results included as Electronic Supporting Information File 1). 3 g of silica gave better recovery (93.5 %) than those obtained with 2 g of Florisil combined with 1 g of C18 (78.7 %) and with 3 g of DE (60.8 %). Excessively high values were achieved when 4 g of alumina were used (125 %). In this case, a low effectiveness on removing matrix interferences, especially lipids, could be the reason of this behavior [27]. It should be noticed that due to the good lipid removal efficiency of the MSPD using silica as adsorbent, no additional clean-up step was required.



Electroninc supporting information: File 1: *Study of different adsorbents used in MSPD.*

The effect of the solvent elution volume (7, 10, 14, 18 and 21 mL), the percentage of acetic acid in ACN (0, 0.2, 0.5, 0.8 and 1 %) and the amount of silica used as adsorbent (1, 2, 3, 4 and 5 g) in the MSPD procedure were evaluated using a response surface central composite design [40]. The experimental design involves 16 experiments (including 2 center points) randomly performed. Nemrod®W software package [41] was used for the generation of the matrix of experiments and to evaluate the effect of each factor on the efficiency of the extraction. All experiments were carried out with blank controlled salmon samples spiked at 0.1 µg g^{-1}. The estimates of the coefficients for the second-order models of the % recovery of each analyte (response function) were calculated by the least squares linear regression and these models were analysed and validated by ANOVA. The application of response surface methodology revealed that the relative importance of factor effects was not the same for the different drugs. Moreover, the principal factors presented both positive and negative effects. The extraction was favored by medium-high values of amount of silica, except for doramectin. For azamethiphos, higher responses were obtained when the percentage of acetic acid in ACN and the solvent elution volume were at their lower level. For doramectin, higher responses were obtained when these factors were at their higher level. However, for carbamates and teflubenzuron high levels of percentage of acetic acid in ACN and low levels of solvent elution volume provide good MSPD recoveries. Ivermectin, abamectin and diflubenzuron extraction was favored by medium-high levels of percentage of acetic acid in ACN and high levels of solvent elution volume. Searching for the best conditions for the simultaneous extraction of target analytes, multicriteria decision-making strategies using desirability function optimization were applied without additional experimentation by means of the Nemrod®W 2000 software [40]. The responses were transformed using a dimensionless desirability (d_i) scale, which ranged between d = 0 for a completely undesirable response to d = 1 for a fully desired response. The global desirability (D) that combines the individual desirability (d) of all the response variables into a single measure was maximized. Figure 2 shows the isoresponse curves with maximum desirability founded at high values for the volume of extraction solvent (Figure 2a) and at medium values

for percentage of acetic acid in ACN and amount of silica (Figure 2b). In the optimal zone, *D* was 0.957. Regions in grey correspond to null values of desirability when the levels of the factors are not suitable. Optimal numerical conditions resulted in 18 mL of ACN, 0.5 % of acetic acid in ACN and 2 g of silica.

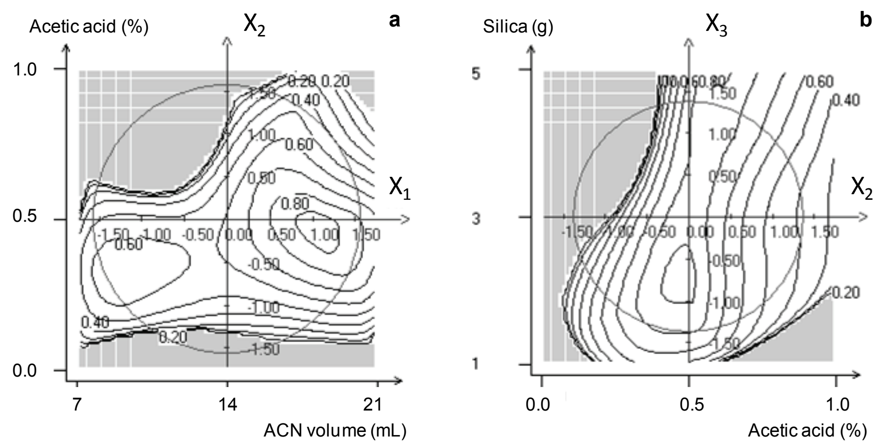


Figure 2. *Global desirability response surface plot using a central composite design. The arrow shows the region corresponding to optimal conditions.*

3.3 Method validation

Matrix effect was also evaluated during the validation of the method, since signal suppression or enhancement as a result of matrix effect can severely compromise quantitative analysis at trace levels, as well as it can greatly affect the method reproducibility and accuracy [42, 43]. The matrix effect was studied by comparison of the slopes of the calibration curves in solvent and in the obtained extract after MSPD procedure. If the first slope is less than the second one gives a positive matrix effect, and the signal enhancement occurred. Otherwise, negative values are indicative of signal suppression. Depending on the value of the percentage of the difference, different matrix effects could be observed. Both the solvent and matrix calibration curves showed good linearity with determination coefficients higher than 0.999 for solvent calibration curves and 0.996 for matrix-matched ones. Matrix effects were considered as relevant when the deviation of the matrix calibration slope was higher than 10 % from the solvent calibration slope. Several compounds presented relevant matrix effect in salmon samples (Table 2). Azamethiphos and carbamate drugs showed strong matrix effect and signal enhancement, while avermectins had medium matrix effect and signal suppression. The only drug that did not show matrix effect was diflubenzuron. Among the eight drugs evaluated, strong matrix effects, higher than 20%, were obtained for four of them. The highest matrix effect was observed in ESI+ for propoxur, which showed a signal enhancement above 90%.

Linearity was evaluated using matrix-matched standards in the wide range of 5 to 500 µg kg^{-1}. Good linearity was found for all drugs, with coefficients of determination higher than 0.996 (Table 2). The limits

of detection (LOD) and quantification (LOQ) of the method were calculated as the minimum amount of target analyte that produced a chromatogram peak with a signal-to-noise ratio of 3 and 10, respectively, and are shown in Table 2. The LOQs ranged from 4.7 μg kg^{-1} to 6.5 μg kg^{-1} and the LODs were in the range of 1.5- 2.5 μg kg^{-1}. The LODs obtained are lower than the MRLs.

The repeatability of the method was evaluated by measuring six replicate samples in the same day by spiking 0.2 g blank salmon with appropriate volumes of the composite working standard solution to furnish a concentration in the final extract of 50 μg kg^{-1}. Relative standard deviations (% RSD) of 2.2 to 6.3 were obtained for peak areas (Table 2).

Table 2. *Validation data for the MSPD-LC-MS/MS method.*

Compound	R^2	Matrix effect	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Intra-day precision (% RSD)[a] (n=6)	MRL (μg kg-[1])	Reference
Azamethiphos	0.9988	66.7	1.5	5.0	4.4	100	EC 508/1999
Propoxur	0.9991	97.0	1.5	5.0	4.8	10	EC 396/2005
Carbaryl	0.9992	57.6	2.3	4.8	2.2	250	US e-CFR/2012
Diflubenzuron	0.9988	5.8	1.5	4.7	4.9	1000	EC 2593/1999
Teflubenzuron	0.9991	18.1	1.5	4.7	5.8	500	EC 804/1999
Abamectin	0.9987	-11.3	1.7	5.6	3.8	20	EC 37/2010
Doramectin	0.9993	-15.5	2	5.9	3.7	10	EC 396/2005
Ivermectin	0.998	-24.7	2.5	6.5	6.3	10	EC 396/2005

[a]*Concentration added 50 μg kg^{-1}*

To evaluate the accuracy of the method, recovery was studied by spiking 0.2 g blank cockle, mussel, turbot, panga, clam, scallop and two salmon samples (matrices with different lipid content) with appropriate volumes of the composite working standard solution to furnish a concentration in the final extract of 25 μg kg^{-1}; except for carbaryl and ivermectin, for which the concentration was 50 μg kg^{-1}. Recovery and precision of the method were investigated by analysis of four replicate samples as described above. The results, listed in Table 3, show that the mean recovery of the eight analytes from blank samples are satisfactory. Average recovery for all compounds was 103.4 % (95.5-114.2 % interval) for scallop; 96.8 % (85.6-111.1 % interval) for mussel; 100.1 % (90.2 to 112.0 % interval) for turbot; 104.6 % (91.5 to 115.5 % interval) for panga; 100.6 % (84.9 to 116.2 % interval) for clam; 105.7 % (86.4 to 115.9 % interval) for cockle ; 91.9 % (83.8 to 118.0 % interval) for salmon 1; and 101.6 % (85.6 to 112.5% interval) for salmon 2. Reproducibility, expressed as % RSD, was used as an index of the precision of the method. Samples in quadruplicate (with the concentration indicated above) were analyzed and RSD was calculated for each compound (Table 3). The method showed to be precise, with RSD values ranging from 0.1 to 10.6 % for all the compounds studied.

Table 3. *Evaluation of recovery and inter-day precision at spiked level of 25 µg kg^{-1}, in several aquaculture samples for each analyte (n = 4)*

Compound	% Recovery *							
	Scallop	Mussel	Turbot	Panga	Clam	Cockle	Salmon 1	Salmon 2
Azamethiphos	102.4 (5.9)	90.3 (0.4)	97.7 (1.1)	91.5 (3.2)	92.4 (3.6)	86.4 (2.0)	83.8 (1.0)	85.6 (3.5)
Propoxur	103.3 (3.8)	85.6 (1.6)	104.0 (3.0)	95.8 (4.1)	110.8 (1.4)	115.9 (0.1)	88.5 (3.2)	96.3 (3.7)
Carbaryl	104.2 (0.5)	109.2 (3.2)	92.2 (1.6)	115.5 (3.3)	108.3 (4.0)	100.9 (2.1)	100.9 (2.1)	110.9 (4.4)
Diflubenzuron	102.2 (2.3)	104.4 (3.89	109.4 (3.6)	114.2 (3.4)	116.2 (1.7)	109.7 (6.0)	94.2 (2.0)	97.2 (2.4)
Teflubenzuron	95.5 (3.7)	90.7 (5.2)	101.5 (4.0)	102.8 (10.6)	103.6 (4.0)	114.9 (3.6)	118.0 (1.8)	112.5 (0.1)
Abamectin	114.2 (3.3)	111.1 (5.3)	112.0 (2.7)	97.5 (6.6)	101.7 (7.0)	95. 6 (3.4)	86.0 (3.6)	92.9 (4.8)
Doramectin	105.6 (10.6)	91.9 (1.49	95.9 (4.0)	107.1 (6.2)	87.0 (4.7)	114.6 (3.7)	86.5 (5.7)	111.2 (7.7)
Ivermectin	102.5 (2.6)	94.2 (1.0)	90.2 (3.6)	110.5 (1.6)	84.9 (4.7)	98.7 (2.1)	95.5 (3.6)	101.1 (3.6)

*%RSD values are given in brackets

3.4 Application of the method to the analysis of aquaculture products

The applicability of the method for the analysis of the eight chemical agents in real samples was tested in eight aquaculture products (cockle, mussel, turbot, panga, clam, scallop and two salmon samples) purchased from a local food market. The results are listed in Table 4. No pesticide residues, at concentrations above the detection limit, were found in cockle and mussel samples. Five out eight compouds were detected at different concentrations in four fishes and two shellfishes. The other three compounds were not detected. According to Regulations established by the European Union and United States to stipulate maximum levels of pesticide residues in food products, several concentrations obtained in this work exceed the specified MRLs [9, 44-47]. Figure 3 shows LC-MS/MS selected ion chromatograms of carbaryl and teflubenzuron detected in salmon 2 (a) and turbot (b) samples; selected ion chromatogram (c) and total ion chromatogram (d) obtained for free–analytes salmon sample spiked with 50 µg kg^{-1} of target pesticides. In the reported studies from the literature consulted, samples of animal origin were mostly analyzed for six main groups of pesticides, namely organochlorine pesticides, organophosphorus pesticides, carbamates, pyrethroids, triazines and avermectins [13, 14]. The sample treatment and determination of multi-residue pesticides from high-fat vegetable samples were also discussed [27]. The main class of pesticides analyzed in fish and shellfish were organochlorine pesticides, which were detected using GC-MS [22, 48]. Four avermectin residues in different animal-food products, fish included were simultaneously separated and determined by ultra-performance liquid chromatography–electrospray ionization tandem

mass spectrometry (UPLC-ESI-MS/MS) [13]. Samples were homogenized, extracted and de-proteinized by acetonitrile, cleaned via two-step cleaning procedure using Bond Elut C18 SPE columns and then alumina-N cartridges.

Table 4. *Amounts (µg kg^{-1}) of the pesticides in real aquaculture samples (n = 4).*

Compound	Concentration (µg Kg^{-1} ± SD)					
	Scallop	Turbot	Panga	Clam	Salmon 1	Salmon 2
Carbaryl	nd	nd	nd	nd	nd	5.4 ± 0.7
Teflubenzuron	nd	6.9 ± 0.6	nd	nd	nd	nd
Abamectin	7.1 ± 0.8	nd	nd	nd	nd	nd
Doramectin	18.3 ± 1.8	nd	26.0 ± 1.3	nd	32.3 ± 2.2	nd
Ivermectin	nd	nd	nd	16.2 ± 0.7	nd	nd

nd: not detected

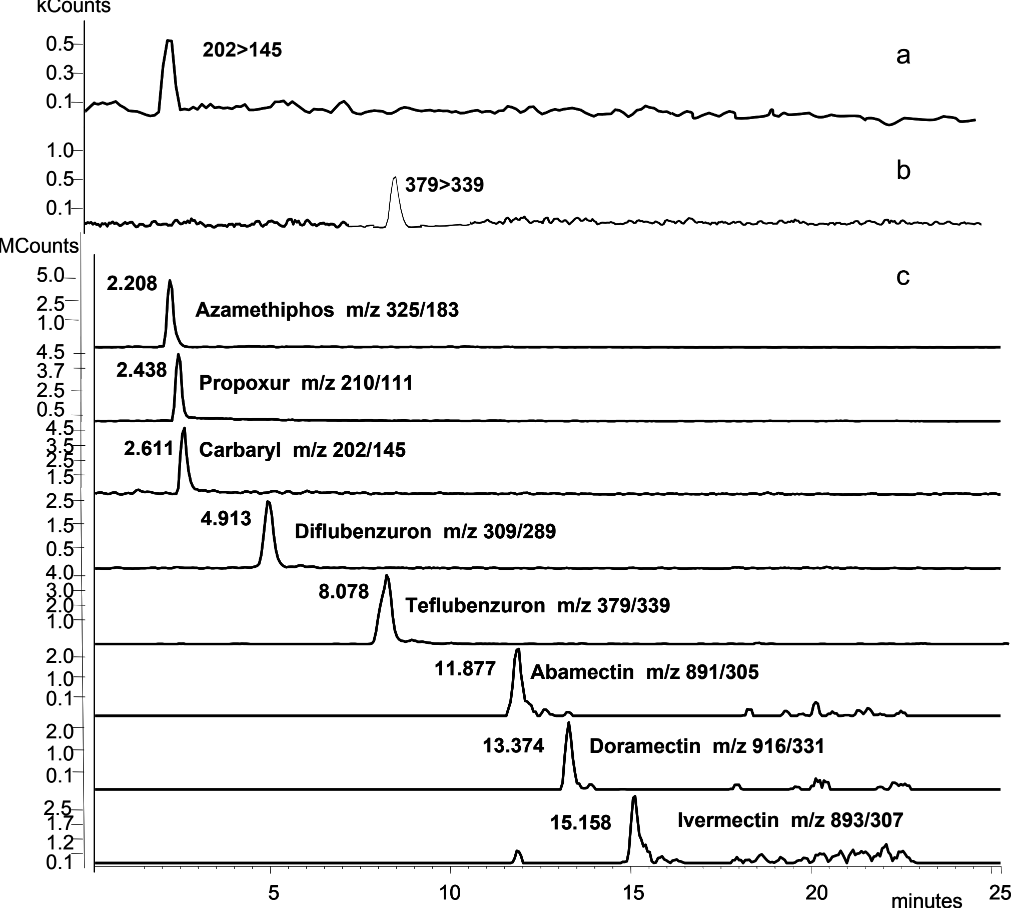


Figure 3. *LC–ESI-MS/MS selected ion chromatograms of a MSPD extract from salmon 2, containing carbaryl (202>145) in measured concentration of 5.4 µg kg^{-1} (a) from turbot, containing teflubenzuron (379>339) in measured concentration of 6.9 µg kg^{-1} (b) and a salmon sample spiked with 50 µg kg^{-1} of target analytes (c). For each detected analyte, the MRM transitions are shown.*

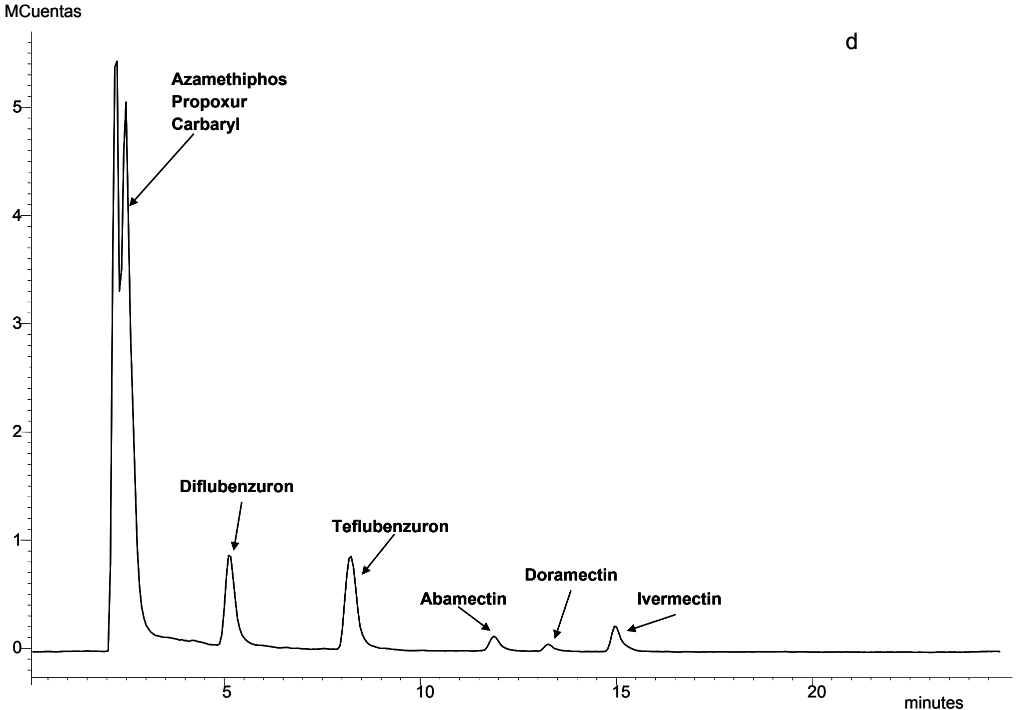


Figure 3 (continued). *Total ion chromatogram (TIC) of salmon sample fortified at 50 µg kg^{-1} (d).*

LODs were in the range 0.05-0.68 µgkg^{-1} and recoveries were ranged from 62.4 to 104.5%. Hollow fiber liquid-phase microextraction (HF-LPME) technique was used as a clean-up procedure for the determination of organophosphorus pesticides (OPPs) in fish tissue [49]. LODs were in the range 2.2-4.5 µgkg^{-1} and recoveries were ranged from 71.8% to 95.2%. A lack of data on the analysis of the proposed analytes in aquaculture samples is obvious and hence the importance of this work.

4 Conclusions

The developed method allows qualitative and quantitative analysis of eight chemical agents in farmed fishes and shellfishes by MSPD-LC-MS/MS, in a quick and simple manner. The use of 2 g of silica as adsorbent results in efficient extraction of the eight analytes tested. MSPD proved to be a technique suitable for routine extraction of low levels of pesticide multi-residues in real aquaculture samples. The accuracy, precision and selectivity of the proposed method are acceptable for multi-residue analyses of chemotherapeutic agents and that the LOQs achieved by the method are in good agreement with the MRL established by the European Union legislation. In addition, the method requires only small matrix size and offers considerable saving in terms of solvent consumption, cost of materials, matrix manipulation and analysis time. Furthermore, a SPE additional step, used for clean-up, is not required. Although many pesticides have been restricted from use in aquaculture, these compounds are still present in shellfish from regions to which the therapeutic substances had not been supplied. The environmental distribution of commercial chemicals, may potentially impact the development, the growth and the reproduction of wild

aquatic species. Our future work will focus on assessing the occurrence of other pesticides belonging to these families in water, sediments or seaweeds samples from aquaculture and sea.

Acknowledgments

This research was supported by the Projects 10TAL209005PR and 10PXIB208089PR Dirección Xeral de I+D (Xunta de Galicia, SPAIN) and FEDER.

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IV. CONCLUSIONES

IV. Conclusiones

Los resultados obtenidos de la metodología analítica desarrollada a lo largo de la Memoria han llevado a proponer procedimientos alternativos de preparación muestra para la determinación de agentes quimioterapeúticos y otros compuestos, utilizados como plaguicidas, en muestras relacionadas con la acuicultura y el medioambiente.

Dada la complejidad de las matrices analizadas (agua de mar, algas y pescados), se ha tratado de simplificar la etapa de preparación de muestra reduciendo el tamaño de la misma, el consumo de disolventes y su manipulación. Además, siempre que ha sido posible, se ha integrando la etapa de *clean-up* en el proceso de extracción.

El estudio de las variables relacionadas con los diferentes procesos de extracción se ha apoyado en la Quimiometría. Así, utilizando diseños de experimentos y optimización multicriterio (funciones de deseabilidad), se reduce el número de experiencias a realizar (menor coste y tiempo) y se consigue la máxima eficacia evaluando los factores influyentes y sus interacciones.

Dentro de las aportaciones científicas más relevantes e innovadoras derivadas de esta Tesis, cabe destacar la aplicación por primera vez de los procedimientos de extracción basados en PLE, MAE y MSPD al análisis de los compuestos estudiados en algas marinas.

A la vista de la investigación desarrollada a lo largo de la Memoria y los resultados alcanzados en la misma, se pueden establecer las siguientes conclusiones:

1. Determinación de pesticidas en aguas

Se han desarrollado y validado dos métodos de extracción: Microextracción en fase sólida (SPME) y microextracciónlíquido-líquido dispersiva (DLLME), utilizando la cromatografía de gases con detección de micro-captura electrónica (GC-µECD) y espectrometría de masas en tándem (GC-MS/MS), y con inyección de grandes volúmenes y temperatura programada (PTV-LVI-GC-MS/MS) para la identificación y cuantificación de los pesticidas en muestras de agua.

Determinación de niveles traza de agentes quimioterápicos empleados en acuicultura en auga de mar mediante SPME-GC-MS/MS.

- Se desarrollaron y validaron dos métodos multiresiduo para la determinación de dos pesticidas organofosforados y tres piretroides en muestras acuosas procedentes de la acuicultura. Ambos

métodos se basan en la extracción mediante SPME directa en 40 minutos a 75ºC, utilizando una fase de 100 µm de polidimetilsiloxano (PDMS) y 20 mL de muestra. La posterior separación de los compuestos extraídos se llevó a cabo mediante cromatografía de gases con detección de micro-captura electrónica o empleado espectrometría de masas en tándem.

- El método de SPME-GC-µECD presenta un buen rango dinámico lineal para todos los compuestos, con buenos coeficientes de regresión, obteniéndose una buena sensibilidad para todos los analitos. Los límites de cuantificación son muy bajos (2.8-38.8 pg mL^{-1}) para todos los pesticidas excepto para el diclorvos, cuyos límites de cuantificación son alrededor de tres órdenes de magnitud mayor. El método de SPME-GC-MS/MS presenta un rango dinámico lineal más amplio que el anterior, aunque la sensibilidad del sistema de detección es menor. Los límites de cuantificaciónson son bajos (5-308 pg mL^{-1}), aunque superiores a los alcanzados con el método de SPME-GC-µECD.
- También se estudió la precisión de ambos procedimientos analíticos, para lo cual se realizaron estudios de repetibilidad y reproducibilidad. Estas experiencias mostraron que ambos métodos, a pesar de la variabilidad intrínseca a la SPME, presentan una buena precisión (coeficientes de variación inferiores al 15%). También se estudió la exactitud en términos de % de recuperación (81-120% con RSDs inferiores al 12%), resultando igualmente satisfactoria.
- Se aplicó el método a varias muestras reales procedentes de la costa de Galicia (España), todas ellas tomadas en zonas cercanas a explotaciones acuícolas. Para realizar el análisis se utilizó el método de adiciones estándar y se comprobó que no había presencia de los compuestos estudiados en ninguna de las muestras, al menos por encima de los límites de detección del método aplicado.

Determinación multiresiduo de pesticidas en aguas mediante DLLME combinada con PTV-LVI-GC-MS/MS.

- Se propone la DLLME combinada con PTV-LVI-GC–MS/MS para la determinación de 17 pesticidas y compuestos relacionados en aguas. Este método de preparación de muestra, rápido y sencillo, fue optimizado mediante un diseño de superficie de respuesta Doehlert, siendo los volúmenes de disolvente extractante y dispersante las variables que más influyen en la eficacia de la extracción. Las condiciones optimas se fijaron en: 1.9 mL de acetonitrilo (disolvente dispersante), 178 µL de tricloroetano (disolvente extractante), 3 minutos de agitación manual y centrifiugación (5 minutos a 3600 rpm).
- En las condiciones óptimas y en el modo PTV-LVI se alcanzaron límites de cuantificación entre 0.3-150ng L^{-1}. La exactitud, evaluada como % recuperación, presentó valores satisfactorios entre 65 $\pm$ 3% y 130 $\pm$ 7 % en las diferentes matrices acuosas estudiadas.

- El método ha sido aplicado, con éxito, al análisis de 14 muestras de agua, algunas procedentes de zonas de acuicultura, en las que han sido detectados/cuantificados 11 pesticidasen muestras de agua de mar procedente de zonas de acuicultura y 9 en muestras de agua de rio y residual.

2. Determinación de pesticidas en algas

Las algas son matrices complejas de origen vegetal poco estudiadas que requieren de procedimientos de extracción y purificación. En esta Tesis se han desarrollado y validado tres métodos, que permiten la determinación multirresiduo de pesticidas de diferentes familias: Extracción con disolventes presurizados (PLE), extracción asistida por microondas (MAE) y dispersión de la matriz en fase sólida (MSPD). En dos de ellos (PLE y MSPD) se integra la extracción y purificación en una sola etapa.

Determinación multiresiduo de pesticidas en algas comestibles mediante PLE-PTV-LVI-GC-MS/MS.

- En primer lugar se abordó la optimización de la inyección de grandes volúmenes (LVI) de muestra utilizando un sistema de vaporización con temperatura programada (PTV–GC/MS). Las condiciones óptimas de flujo y de temperatura se inyectaron 50 µL de muestra mejorando la sensibilidad y selectividad de los 6 pesticidas determinados (2 organofosoforados, 3 piretroides y 1 carbamato).
- A continuación, mediante un diseño de screening, se estudiaron cinco parámetros que afectan a la PLE. Posteriormente, se evaluaron los adsorbentes (Florisil® y carbón grafitizado) añadidos a la celda para la purificación. En las condiciones optimas, utilizando 0.2 g de muestra, 1.6 g de Florisil® y 0.4 g of GCB, se extrajeron cuantitativamente los analitos a 100ºC con una mezcla de *n*-hexano/acetato de etilo (80:20) en tan solo 2 minutos.
- En la validación del método se estudiaron los parámetros de linealidad, sensibilidad, límites de detección y cuantificación, precisión y exactitud. Los bajos limites de cuantificación obtenidos (0.9-76.9 pg g^{-1}) están por debajo de los MRLs, establecidos por la legislación europea para algas. Se estudió la precisión del procedimiento analítico, los coeficientes de variación obtenidos en la reproducibilidad son, en todos los casos, inferiores al 12%. También se estudió la exactitud en términos de recuperación, los resultados obtenidos fueron igualmente satisfactorios, con recuperaciones cuantitativas (82-108%).
- La metodología desarrollada ha sido aplicada al análisis de 11 muestras de algas (5 comestibles y 6 tomadas en el litoral de Galicia). En algunas de estas muestras se detectaron trazas de clorpirifos-etil, clorpirifos-metil, permetrina, cipermetrina y deltametrina, pero no superaron los correspondientes MRLs.

Determinación multiresiduo de pesticidas en algas comestibles mediante MAE-PTV-LVI-GC-MS/MS.

- El método desarrollado, robusto y sensible, basado en la extracción asistida por microondas (MAE-LVI-GC-MS/MS) se presenta como una alternativa para la extracción de pesticidas en algas. Aunque requiere una etapa posterior de purificación del extracto mediante SPE, con carbón grafitizado y Florisil®, proporciona límites de cuantificación (2.3-76.9 pg g^{-1}) equiparables a los alcanzados con otras técnicas.
- Se ha observado que las variables que más influyen en la eficacia de la extracción son temperatura, tiempo y volumen de disolvente, y por tanto, las condiciones experimentales óptimas se fijaron en 125 °C, 12 min con 24 mL de hexano/acetato de etilo (80:20).
- Se demostró la aplicabilidad de la metodología propuesta al análisis de 17 pesticidas de diferentes familias en muestras de algas. Nueve muestras contenían trazas de clorpirifos-etil, pero la mayoría por debajo del LOQ. Además, pesticidas del grupo de los piretroides (tetrametrina, empentrina, cipermetrina, permetrina) y 2-fenilfenol fueron detectados en la mayoría de las muestras, aunque su concentración fue inferior al MRL establecido por la legislación europea. Ningún pesticida del grupo de los carbamatos fue detectado en las muestras analizadas, probablemente debido a que son fácilmente degradables.

Determinación de residuos de pesticidas en algas empleando MSPD-GC-MS(SIM).

- El método propuesto, basado en MSPD–GC-MS, es la primera aplicación de este procedimiento de preparación de muestra para la determinación de pesticidas en algas. Las principales ventajas que presenta frente a otros sistemas de extracción, son la simplicidad, bajo coste y la posibilidad de realizar de la extracción y purificación integradas en una sola etapa.
- Se han evaluado mediante diseño experimental la relación dispersante/cantidad de muestra, proporciones de adsorbentes (Florisil/GCB) y volumen de disolventes de elución. El volumen de la mezcla de disolventes de elución (14 mL hexano: acetato de etilo, 60: 40) ha sido la variable más influyente en la extracción de todos los pesticidas por MSPD. Con sulfato sódico anhidro como dispersante y una co-columna formada por 3.6 g of Florisil y 0.4 g de GCB, se obtuvieron recuperaciones entre 82 y 113 % con RSDs del 2 al 13%.
- Este método fue comparado con el desarrollado anteriormente, basado en MAE-LVI-GC-MS/MS, mostrando rangos similares de exactitud y precisión. Además, se encontraron buenas correlaciones para los resultados de las muestras reales analizadas por ambos métodos.

3. Determinación de pesticidas en pescados y moluscos.

En el último capítulo de esta Memoria se han seleccionado como matrices pescado y moluscos, procedentes de la acuicultura. El objetivo ha sido evaluar la presencia de agentes quimioterapeúticos utilizados en el tratamiento de patógenos, responsables de las enfermedades que se manifiestan en los cultivos intensivos de estas especies.

Determinación de agentes quimioterápicos en pescado y moluscos mediante MSPD-LC-MS/MS.

- Se ha optado por optimizar un procedimiento de preparación de muestra sencillo y de bajo coste como es la dispersión de la matriz en fase sólida. Los agentes quimioterápicos seleccionados, pertenecientes al grupo de los pesticidas organofosforados, carbamatos, derivados de la benzoilfenil urea y avermectinas, fueron determinados mediante LC-MS/MS.
- Para la reducción del efecto matriz derivado de la complejidad de las muestras y de su variado contenido lipídico, se utilizaron 2 g de sílice como adsorbente en la MSPD, dando lugar a una extracción eficaz de los ocho analitos estudiados.
- El método basado en MSPD-LC-MS/MS proporcionó límites de cuantificación del orden 5 µg kg^{-1}, inferiores a los MRL permitidos en la legislación europea. Así mismo, se demostró la aplicabilidad de la metodología propuesta mediante el análisis de 8 especies (berberecho, mejillón, rodaballo, panga, almeja, vieira y salmón) procedentes de acuicultura. En ninguna de ellas se encontraron los compuestos en concentraciones superiores a los MRL correspondientes.

ANEXO I. Publicaciones científicas derivadas de la Tesis Doctoral

- Diego García-Rodríguez, Antonia M. Carro, Rosa A. Lorenzo, Fátima Fernández, Rafael Cela *"Determination of trace levels of aquaculture chemotherapeutants in seawater samples by SPME-GC-MS/MS"* **J. Sep. Sci., 31 (2008) 2882-2890**
- Diego García-Rodríguez, Antonia M. Carro, Rosa A. Lorenzo, Rafael Cela *"Dermination Of Pesticides In Seaweeds By Pressurized Liquid Extraction And Programmed Temperature Vaporization-Based Large Volume Injection Gas-Chromatography-Tandem Mass Spectrometry"* **J. Chromatogr. A, 1217 (2010) 2940-2949**
- D. García-Rodríguez, A.M. Carro, R. Cela, R.A. Lorenzo *"Simultaneous Microwave-Assisted Extraction And Large Volume Injection Gas Chromatography Tandem Mass Spectrometry Determination Of Multiresidue Pesticides In Edible Seaweed Samples"* **Anal.Bioanal.Chem., 398 (2010) 1005-1016**
- D. García-Rodríguez, R.A. Lorenzo, R. Cela, A.M. Carro *"Analysis of Pesticide Residues In Seaweeds Using Matrix Solid-Phase Dispersion And Gas Chromatography Mass Spectrometry Detection"* **Food Chem., 135 (2012) 259-267**
- A.M. Carro, S. Fernández, I. Racamonde, D. García-Rodríguez, P. González, R.A. Lorenzo*"Dispersive Liquid-Liquid Microextraction Coupled With Programmed Temperature Vaporization-Large Volume Injection-Gas Chromatography-Tandem Mass Spectrometry For Multiclass Pesticides In Water"* **J. Chromatogr. A, 1253 (2012) 134-143**
- D. García-Rodríguez, P. Gonzalez-Siso, A.M. Carro, R.A. Lorenzo *"Determination Of Veterinary Drugs In Fish And Shellfish By Matrix Solid Phase Dispersion And Liquid Chromatography-Tandem Mass Spectrometry"* **Jounal of Separtion Science (2012)Article first published online : 4 OCT 2012, DOI: 10.1002/jssc.201200440**

ANEXO II. Otras publicaciones

J. Sep. Sci. 2008, *31*, 1333–1345 1333

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Review

Supercritical fluid extraction of polyhalogenated pollutants from aquaculture and marine environmental samples: A review

This article focuses on the state-of-the-art in sample preparation using supercritical fluid extraction (SFE), to monitor the content of polyhalogenated pollutants in aquaculture and marine environmental samples. Marine sediments and biological applications, including several types of samples matrices (fish, shellfish, seaweed and fish feed) and analyte groups (polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), polybrominated diphenylethers (PBDEs), polychlorinated dibenzo-*p*-dioxin (PCDD)/Fs and organochlorinated pesticide (OCPs)) are discussed with respect to SFE use and optimisation of conditions. We also discuss the great analytical potential of SFE, the integration of the extraction and clean-up steps for rapid sample processing justifying its use for routine work. The most recent SFE applications to the determination of these pollutants in marine environmental (biota and sediment) samples, published in the last 15 years, are reviewed.

Keywords: Aquaculture and marine environmental samples / Clean-up / Polyhalogenated pollutants / Supercritical fluid extraction

Received: December 5, 2007; revised: March 4, 2008; accepted: March 4, 2008

DOI 10.1002/jssc.200700637

1 Introduction

Both conventional and emerging persistent organic pollutants (POPs) cover a wide range of heterogeneous analytes (Table 1), which include, among others, the polyhalogenated pollutants considered in this review such as chlorinated pesticides, polychlorinated biphenyls (PCBs), polybrominated diphenylethers (PBDEs), polybrominated biphenyls (PBBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Status of active substances under EU review (doc. 3010), http://europa.eu.int/comm/food/plant/protection/evaluation/stat active subs 3010 en.xls.). They are characterised by a high chemical and biological stability, a high degree of lipophilicity and low water solubility. These characteristics make them persistent in the environment and bioaccumulatable throughout the food chain over a wide range of trophic levels [1]. The toxicity of POPs has been observed to adversely effect human and animal health, given that they are carcinogenic compounds which can damage the nervous system, cause immune system disorders, reproductive dysfunction as well as endocrine disruption. For all of the above reasons, these compounds are considered a priority target in monitoring environmental contamination at the trace level.

The sources of contamination are mainly of anthropogenic origin due to agricultural and industrial activities, disease control in aquaculture, the manufacture of animal feeds, incineration, insulation systems and flame retardants used formerly or currently in everyday household products. All of these potential pollution sources are linked to the marine environment and the aquaculture industry. Hence, the fact that POPs have been found in fish, sea birds and marine mammals would indicate that they have reached the highest level of the ecosystem since they have also been detected in adipose tissue and human body fluids [2–4], particularly in populations whose diets consist mainly of products from the sea [5].

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Abbreviations: BHC, hexachlorocyclohexane; **CRM,** certified reference material; **DDD,** dichlorodiphenyldichloroethane; **DDE,** dichlorodiphenyldichloroethylene; **DDT,** dichlorodiphenyltrichloroethane; **EPA,** U.S. Environmental Protection Agency; **GC-ECD,** GC-electron capture detector; **OCPs,** organochlorinated pesticides; **ODS,** octadecylsilane; **PAHs,** polycyclic aromatic hydrocarbons; **PBBs,** polybrominated biphenyls; **PBDEs,** polybrominated diphenylethers; **PCBs,** polychlorinated biphenyls; **PCDDs,** polychlorinated dibenzo-*p*-dioxins; **PCDFs,** polychlorinated dibenzofurans; **PFE,** pressurised fluid extraction; **POPs,** persistent organic pollutants; **SFE,** supercritical fluid extraction; **SPME,** solid-phase microextraction

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Table 1. Chemical structures, classification and uses of different POPs

POP	Chemical structure	Use
PCBs		Electrical insulators, pesticides, *etc.*
PBBs		Flame retardants
PBDEs		Flame retardants
PCDDs		Toxic by-products from different industrial processes
PCDFs		Toxic by-products from different industrial processes
*p,p*k-DDT		Organochlorine pesticide (insecticide)
Heptachlor		Organochlorine pesticide (insecticide)
Dieldrin		Organochlorine pesticide Aldrin breakdown product
β-BHC		Organochlorine pesticide (insecticide)

In recent decades, the consumption of fish reared in aquaculture has tripled. Therefore, it is of utmost importance to determine what these pollutants are in fish and to identify the source of the pollution in order to assess exposure to these substances through the diet for the purpose of protecting public health [6]. Analyses have also been carried out on the tissue of fish fed with commercial feed containing residues of these pollutants. The resulting data show that the amount of contamination in the tissues is similar to that found in the feed, which would demonstrate its biomagnification [7]. Products from aquaculture are linked to food safety since the risk of contamination from products and chemical agents is greater in the waters of fish farms and coastal ecosystems dedicated to marine culture than in areas of the open ocean [8]. Although the production, use and emission of most of these compounds is forbidden or subject to strict regulations (Introduction to EC pesticides residues legislation, http://europa.eu.int/comm/food/plant/protection/resources/introen.pdf; Accessed 2007 November 10; http://www.epa.gov/; Stockholm Convention on Persistent Organic Pollutants (POPs) May 17, 2004 http://www.pops.int/, [9, 10, 11]), it is still necessary to monitor and control the risk of contamination from these chemical substances by means of quality programs [2–4, 9].

Sample preparation is an area that has often been overlooked, receiving less attention than the stages of determination and detection in the analytical method [12]. However, the required information, sample type, analytes, available tools and measuring method are factors that directly affect the design of the sample preparation, playing a crucial role in the multiresidue analysis of environmental samples. These operations that must be carried out prior to sample determination are complex, involving a large amount of tedious manual work. Automation is difficult, which makes the process slow and time-consuming, accounting for between 70 and 90% of total analysis time (Soxhlet extraction method 3540C adopted by the U.S. Environmental Protection Agency, EPA) (http://www.epa.gov/, [13, 14]). In view of the above, sample preparation is a major source of errors – both random and systematic – which have a decisive impact on the quality of the analytical method (accuracy, precision). Furthermore, this step is a potential source of both personal and environmental risks, which are of great concern to "Green Chemistry". The solution is aimed at the use of new sample preparation methods that use automation and miniaturisation strategies to minimise or eliminate the use of reagents and solvents that may be toxic or harmful to the environment [14]. In general, the ideal properties of a sample preparation technique for the determination of POP residues would include the following: the largest possible number of POPs in a single procedure (multiresidual trial); recoveries of nearly 100%; the elimination of potential interference in the sample to increase selectivity and avoid undesirable matrix effects; allow for the preconcentration of analytes to increase analysis sensitivity; good accuracy; good ruggedness; low cost; quick and easy handling (low number of solvents, fewer harmful solvents) [15].

Supercritical fluid extraction (SFE) is a sample preparation technique used as an alternative to conventional extraction methods. Some of its advantages include the use of fewer organic solvents, making it possible to develop extractions with nontoxic, nonpollutant supercritical fluids, such as carbon dioxide (CO_2), which is the one most commonly used [16]. CO_2 is gaseous at ambient pressure and temperature, which makes for the easy recovery and separation of the halogenated compounds from the supercritical fluid. Moreover, by operating at low temperatures in a nonoxidating medium, it is possi-

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ble to extract thermolabile or easily oxidated compounds, lipophilic solutes and even moderately polar solutes like the POPs from complex biological matrices [1, 13, 17, 18]. The main limitation of this technique is that it is unable to extract polar compounds, although it is possible to change the polarity of the supercritical fluid, thereby increasing its solvating power towards the target analytes, through the use of polar modifiers. These modifiers are generally polar organic solvents, which when added in a low percentage, give rise to a supercritical fluid with higher polarity. Adding a modifier to an SFE system can reduce analytical – matrix interactions, improving the quantitative extraction [17, 19 – 23].

As regards solid matrices of a biological nature (of marine origin and aquaculture-related), owing to their complexity and structural variability as well as to the wide array of compounds included in the POP family (different molecular weights, polarity, how they bind to the matrix structure, *etc*.), SFE-based extraction processes have yet to be studied or applied in full [24]. This review does not include a discussion of the determination methods, as they have been widely studied and reviewed elsewhere [25, 26]. The main objective of this paper is to review the great analytical potential of the extraction method with supercritical fluids and to present a critical overview of the role of this technique in the separation of different groups of halogenated pollutants from aquaculture and marine environment-related samples.

2 Extraction with supercritical fluids

2.1 Characteristics of SFE

SFE is an instrumental extraction technique that uses a solvent under supercritical conditions. Supercritical fluids have densities very much like liquids but with lower viscosities, similar to gases, and high diffusion coefficients. This combination of properties makes the fluid more penetrating, affording it a high solvating power with the ability to extract solutes more quickly and efficiently that liquid solvents [12, 13]. Although numerous supercritical fluids have been tested, CO_2 is the one most commonly used in practice for several reasons. It reaches supercritical conditions under low pressure and temperature; it is nontoxic and nonflammable; it is not corrosive and it is almost chemically inert. While not polar, the polarity of the supercritical fluid can be increased by means of modifiers such as methanol, acetone, *etc*. Density is also increased with regard to pure supercritical CO_2 under the same conditions of pressure and temperature. Moreover, the modifiers act on the sample matrix, causing its deactivation and inflation, so as to improve mass transfer and the mobility of the analytes and their extraction from the matrix [24 – 27].

The advantages of SFE include the small sample quantities required (0.5 – 1.5 g) compared to 20 – 100 g of sample typically used in conventional liquid – solid extraction [28, 29]. Furthermore, solvents that are not environmentally friendly are rarely used, or only used in a low-impact way, as compared to the amounts needed (as much as several hundred mL) in liquid – solid extractions [27, 30].

2.2 Analytical considerations

SFE includes several aspects related to the sample matrix and the recovery of the analyte that warrant special consideration in order to obtain a quantitative extraction of the target analytes in complex samples of a biological or environmental nature. With regard to the nature of the sample matrix, the strategy is different depending on whether solid or liquid matrices are used. Solid matrices require a previous step that involves drying, lyophylisation, grinding and mixing with an inert agent like celite, diatomaceous earth or sea sand [31, 32]. Two different approaches have been used with liquid samples: the adsorption of the sample on an inert and porous substrate or the coinjection of the sample with supercritical fluid in the extraction container or in the packed column [33 – 35]. In terms of analytical recovery, the nature of sample matrix is one of the main factors involved in the reproducibility of SFE as a sample preparation method. The great many variables affecting SFE would justify the optimisation of its values in order to adapt the development of the technique to the proposed objectives: the procurement of an extraction that is effective, selective, quick and accurate.

The selection of the operating conditions will depend on the specific analyte or family of compounds that are to be extracted, taking into account their binding to the structure of the material, molecular weight, polarity and concentration, in each specific case.

A rise in temperature will reduce the density of the supercritical CO_2, for a given pressure, thus reducing its solvating power, increasing the volatility of the compound to be extracted and the mass transfer velocity (extraction kinetics). The temperature in SFE for organohalogenated compounds is generally set at between 35 and 150°C, although it should be as low as possible to avoid the degradation of the thermolabile compounds [24, 27, 28].

An important parameter in SFE processes is the extraction pressure that can be used to fine-tune the selectivity of the supercritical fluid. The higher the pressure, the greater the solvating power and the lower the extraction selectivity. The solvating power of the supercritical CO_2 is often described in terms of density, ranging from 0.15 to 1.0 g/cm^3, with a nonlinear relation between the two variables – temperature and pressure [28, 36].

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The flow of CO_2 is a critical parameter, which along with particle size and extraction time, has an effect on the thermodynamics (solubility) and kinetics (mass transfer) of the SFE process. An appropriate selection of these variables will lead to the complete extraction of the target compounds in a matter of minutes [24, 36]. Aspects such as particle size, sample quantity, pore size and matrix thickness are crucial to obtain satisfactory extraction yields within a reasonable time frame. Moreover sample homogeneity is decisive to the reproducibility of the process. Typically, particle sizes from 0.25 to 2.0 mm will reduce the extension of the diffusion of the supercritical solvent. However, excessively small particle sizes may lead to problems related to volatile compound loss and canalisation in the material. In this case, part of the solvent flows through the channels preventing contact with the material, resulting in efficiency and yield losses during the extraction process. The selection of the optimum sizes will depend on the type of material, its moisture content and liquid compound content, *etc*. [24, 27].

2.3 Optimisation of SFE parameters through the design of experiments

By optimising the analytical procedures through the development of methodologies using experimental design and combining them with formal optimisation strategies it has been possible to obtain optimum operating conditions with a minimal amount of labour, time and cost [37].

As discussed earlier, there are a number of experimental variables that may potentially affect the efficiency of SFE, namely the characteristics of the supercritical fluid (polarity, pressure, temperature, volume, modifier), the characteristics of the target analytes (volatility, polarity, concentration), the characteristics of the solid matrix (sample and particle size, nature, presence of interferences, fat content, moisture, pH), previous sample treatments (derivatisation, addition of solvents) and dynamic factors related to the extraction (extraction time, extracted fluid flow, design and geometry of the extraction cell) [38]. Given the large number and wide diversity of the factors, the optimisation of an extraction process with supercritical fluids is no easy task. The simplex programme has been employed to minimise the time used in developing extraction methods and to increase its effectiveness [39]. A statistical analysis has also been used to identify the key parameters in the extraction process and to reduce the number of experiments needed in the optimisation process [40, 41]. The design of experiments is commonly used to describe the following stages: identification of the factors that may affect the outcome of an experiment (screening), designing the experiment so as to minimise the effects of uncontrolled factors and the use of the statistical analysis to separate and evaluate the effects of the different factors involved (optimisation) [42].

The variables involved in the SFE process may be manipulated to achieve an end that is two-fold – to improve the efficiency and/or selectivity of the extraction. The extraction speed may be stepped up by shortening the extraction time and/or the extraction yield, the latter being a more interesting aspect. In some cases, it is not possible to recover the analytes above a certain level, even when the extraction time is extended.

Generally speaking, pressure and temperature are the most frequently optimised parameters through experimental design [43 – 45]. The CO_2 flow and particle size have also been investigated on different levels, using a central composite design, setting the extraction time at 4 h [43]. Also considered in other cases were CO_2 flow and extraction time in a Doehlert design [44] or the percentage of modifier in a factorial design [45 – 47].

In the case of POPs and other environmental pollutants, the variables involved in SFE may be divided into two groups – those affecting the extraction stage and those related to analyte collection. Both groups of variables are optimised independently through factorial designs that include the study of interactions between factors [48, 49]. However, in most of the literature, only the operating parameters directly affecting SFE are optimised [50 – 52].

The development of experimental designs entails a compromise between a number of different objectives such as the relative importance of the different parameters, the total number of experiments and instrumental limitations. The impact of pressure on recovery appears to be very important, with recoveries increasing as the pressure rises in a wide range of working pressures (16 – 43 MPa) depending on the behaviour of different groups of pollutants [41, 48, 53 – 56]. In these cases, temperature has a slight negative effect, which is not significant, so it would be advisable to set this parameter at a low level [48, 53, 55, 57]. High temperatures (between 35 and 85°C) and fluid densities were shown to benefit the extraction efficiency probably due to a simpler process of desorption of the semivolatile analytes from the active sites on the matrix and better solubilisation in the supercritical fluid [27, 58].

On the other hand, modifier content was demonstrated to be a crucial factor that affects the recovery of polar compounds. From the consideration of the chemical properties of POPs bearing a varied polar character, the use of a modifier (10% methanol) appeared to be essential to the improvement of SFE extraction efficiency. This suggestion is corroborated by the findings of several authors [53, 54, 58 – 61], in the sense that the addition of a cosolvent can compensate for CO_2 insufficiencies with the quantitative extraction of polar pollutants or pesticide metabolites. The modifier might increase

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the polarity of the supercritical fluid [27, 61] and/or interact with the sample matrix altering its physical properties or the sorption bonds that retain the analytes [48, 58]. Methanol behaves as a universal modifier for pesticide analysis, having very good properties, as confirmed by several studies [50, 54, 59–63].

It was observed that the CO_2 flow-rate and extraction time have no significant influence on the SFE optimisation for multiresidue pesticides. Moreover, the static extraction time exhibits a weak negative effect, hence its value should be set at a low level (5–10 min) [48, 49, 53, 55, 56]. In keeping with this, a short dynamic extraction time (10–30 min) may also be selected, largely benefiting the throughput, while also avoiding analyte degradation [53–56, 62]. The CO_2 flow-rate appeared to have small positive or negative effects depending on the analytes studied. A compromise value of 1–2 mL/min provides good recoveries in most cases [48, 54–56, 62].

When considering a variety of analytes, as is the case here, it is quite difficult to find a unique set of experimental settings that will provide overall optimal conditions for all the species under consideration. A good way to establish practical compromise settings for simultaneous multiresidue extraction conditions is to implement a multicriteria decision-making procedure using desirability function optimisation [44, 55, 56].

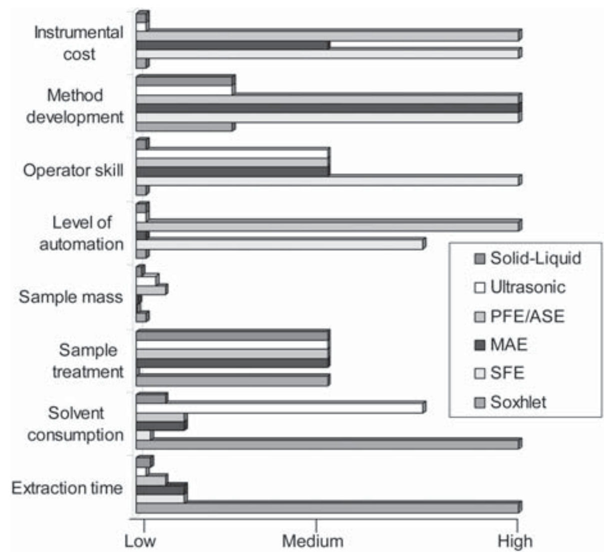

Figure 1. Comparison of different extraction techniques (data acquired from ref. [61, 64]).

3 Applicability of SFE in the determination of POPs in complex solid matrices

When considering any extraction technique, it is important to take into account the number of factors that may potentially influence extraction efficiency. A comparison of some of the traditional and instrumental extraction techniques used on polycyclic aromatic hydrocarbons (PAHs), PCBs and pesticides (Fig. 1) shows that selecting the optimum extraction technique is no simple matter [61]. Those most commonly used have been the conventional Soxhlet method and solid–liquid shake-flask extraction, which require huge amounts of chlorinated solvents. Soxhlet extraction is applied to a wide range of pollutants in solid matrices and, while the technique itself is slow (6–48 h), it is possible to extract several samples using various Soxhlet units. The cost of the equipment is low, however a large amount of solvent is consumed (150–500 mL), making the technique costly. Although solid–liquid extraction requires the use of few consumables and lower volumes of organic solvents (around 30 mL) and the technique is relatively fast (15 min), it does not produce quantitative extracts. Ultrasound extraction is tedious, but quick and requires a certain degree of operator skill to be able to obtain a good reproducibility, as can be seen in Fig. 1. For this reason, alternative instrumental strategies have been suggested for the extraction of these compounds in complex matrices.

The first technique of this kind to appear was SFE which was initially plagued with a number of different instrumental problems related to pressure regulation and investment costs [61, 64, 65]. Labs that work with certified quality systems only use methods that have been approved by the EPA or which have been suitably validated. In this case, solid–liquid extraction would have to be ruled out. SFE has been included in some of the EPA's reference methods for the extraction of pollutants (method 3562 for PCBs extraction, 3561 for PAHs extraction and method 3560 for total petroleum hydrocarbons extraction) [13, 61, 64, 65]. However, it is also necessary to validate SFE methods with reference materials and comparing them with official EPA-approved methods and with other extraction techniques. Despite the many advantages of SFE which include simultaneous clean-up and extraction (see Section 4) in only 30–60 min, the interest in this technique has waned over the course of the past decade in various analytical fields [61, 64, 65].

Microwave-assisted extraction (MAE) allows for the extraction of several samples simultaneously in less than 30 min with a moderate consumption of solvent (25–45 mL). However it does require an additional clean-up stage to eliminate the analytes coextracted with those being targeted and time for cooling the sample container (30 min). Moreover, there may be a limited number of solvents to choose from, as the process requires a type of solvent that can absorb the microwaves. With the application of forms of energy such as ultrasound or microwaves and laser – although they are part of automatic instrumental systems – it is possible to attack the sample

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more quickly and efficiently than with conventional systems like Soxhlet [61].

Pressurised fluid extraction (PFE) or accelerated solvent extraction (ASE) offer the sequential extraction of multiple samples in an automated system, but the drawbacks are a substantial initial capital investment, specific sample treatment for soils with high sulphur content, more solvent than SFE and external clean-up steps [61, 66].

SFE offers advantages in online fractioning or collection. It allows the compounds extracted to be separated into groups, adjusting the operating conditions such as the type and proportion of the modifier, the chelating agent added, variation in temperature/pressure, *etc*. [64, 67]. The three major types of collection, in a solvent, on a solid-phase trap and online coupling to a chromatograph, all require quite extensive optimisation procedures. However, solvent collection is probably the simplest system to use, and in many cases also the easiest to optimise [67].

The degree of automation provided by SFE makes it a suitable technique for quick, routine analysis. The effectiveness of online collection reduces contamination levels in transferring the extracts to the measuring instrument. Moreover, the online coupling of a supercritical fluid extractor to an analytical detector provides several advantages. Large amounts of extract can be passed through the instrument; very little sample manipulation is required, thus avoiding analyte losses; increased throughput is attained; where required, samples can be protected from light and air; substantially reduced amounts of solvent are used; and the coupling allows the development of sample screening methods, thereby avoiding the need to chromatograph every single extract in routine analyses [64].

Attempts to simplify sample preparation operations in the case of solid matrices generally focus on avoiding dissolution of the sample. SFE allows for the quick and efficient extraction of the analytes from a solid which is placed directly into the extractor with the clean-up step included [38].

SFE has not been used on a routine basis in the extraction of POPs in laboratory analysis, owing to the high cost of the instruments and the need to optimise a large number of operating parameters for each matrix. However, the greatest advantage of SFE in the analysis of complex biological and environmental samples is the possibility of obtaining highly selective extractions and relatively pure and preconcentrated extracts [15].

The areas in which SFE has been more widely developed in terms of analytical applications include environmental analysis (soils and sediments) and the analysis of food products (vegetables, fruits, cheese, fat animal, feed, fish) [16, 17, 24, 44, 54, 67, 68]. Moreover, there are many references in the literature that demonstrate the applicability of the SFE technique to determine the existence of POPs in several complex matrices: PCBs, PCDDs and OCPs (organochlorinated pesticides) in soils, sediments and fly ashes [19, 62, 67, 69], pesticide residues (OCPs, pyrethroids and organophosphorus pesticides, OPPs) and PCBs in food [15, 65, 70], human blood and tissues [65], pyrethroid pesticides in plants and seeds [24] or PCBs, PBBs, PBDEs, PCDDs, PCDFs and OCPs in biota and animal tissues [4, 13, 19, 67, 69].

3.1 SFE in aquaculture and marine environmental samples

As stated above, SFE has been used for the determination of halogenated pollutants, especially PCBs, in different types of samples. Determination of these compounds in marine environment samples is usually a difficult task due to the complexity of the samples. Conventional methodologies have been widely used, but they often employ hazardous organic solvents and numerous steps where contamination or loss of target analytes is likely. In this sense, SFE is a powerful technique that allows the simultaneous extraction, clean-up and concentration of the analytes from the solid matrix. A bibliographic review of applications of this technique to aquaculture-related samples is shown in Table 2.

3.1.1 Biological matrices

Johansen *et al.* [71] employed SFE coupled to HPLC for online extraction and clean-up of mono-*ortho*- and non-*ortho*-substituted PCBs from different biological samples including crab hepatopancreas. After supercritical extraction, analytes were focused inside the empty steel tubing of an impactor interface, assisted by the cooling effect obtained from the expansion of the supercritical fluid. Thus, PCBs were fractionated online with HPLC and quantified by GC-electron capture detector (GC-ECD). The method developed in this paper was validated against solvent extraction. Both methods performed similarly, with the SFE-HPLC method being faster for samples having high levels of lipids. Extraction, online clean-up, concentration and analysis by GC-ECD (GC-MS) took only 90 min. Another methodology for the determination of PCBs [72] was developed, where a partially automated SFE method for the determination of PCBs in fish tissues without the need for intermediate lipid removal steps, was used. In this work, the authors reported data on the effectiveness of different trapping conditions, comparing their method with Soxhlet extraction and with the values obtained by different laboratories on a candidate Standard Reference Material. In this paper, two types of trapping materials were examined: silanised glass beads and C_{18}-modified silica. Also, different trap temperatures and elution regimes were tested. A trapping temperature of −30°C followed by elution with iso-

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Table 2. Summary of SFE application to aquaculture and marine environmental samples

Analytes	Sample matrix	Pre-SFE-treatment	SFE conditions	Analyte collection	Analytical technique	Data recovery	Ref
Planar PCBs	Crab hepatopancreas	Sample mixed and homogenised with anhydrous sodium sulphate (1:4 w/w) Activated basic alumina placed in the outlet end of the extraction cell and in a separate cell after the extraction chamber (1:1 w/w)	CO_2, 60°C, 14.5 MPa, 40 min dynamic	Hexane employed as mobile phase for online HPLC coupling for extraction and clean-up	GC-ECD and GC-MS	71 – 101%	[71]
PCBs	Fish tissues	1 g of lyophilised fish tissue and 6 g of activated neutral alumina placed in the outlet of the extraction cell	CO_2, 150°C, 350 atm, 10 min static, 30 min dynamic	Glass beads and C_{18} as solid-phase trapping materials at 20, −10 and −30°C. Benzene and isooctane as elution solvents at 40 and 80°C	GC-ELCD	35.4 – 102.0%	[72]
PCDDs, PCDFs, PCBs and DDE	Edible marine species	Samples were homogenised and lyophilised prior to analysis. 0.2 – 10 g of sample was loaded in the extraction chamber	CO_2/CO_2 – MeOH, 20 min dynamic, 5 min static	Analyte collection on a C_{18} trap at 75°C. Elution with 3 mL of hexane. Concentration under N_2 stream and purified with alumina	HRGC-LRMS and HRGC-HRMS	0.07 pg/g to 32.4 ng/g	[21]
OCPs	Fish muscle	Lyophilised samples ground in a mechanical grinding device	CO_2, 328 K, 18 MPa, 60 min static	Concentration of extracts, clean-up and separation by means of a Florisil column	GC-ECD	70 – 130%	[57]
Organohalogented pesticides, PCBs, PBBs and PBDEs	Fish feed, farmed fish and shellfish	1 g of freeze-dried sample at the bottom of the thimble followed by 1.5 g of basic aluminium oxide and 1.5 g of acidic silica	CO_2, 60°C, 165 bar, 5 min static, 27 min dynamic	Analyte collection in ODS packed trap at 25°C. Elution with 2 mL hexane and concentration to dryness under N_2 steam. SPME extraction on the dry SFE extract	GC-MS/MS	65 – 101%	[55]
PCBs	Seaweed	0.5 g of lyophilised samples mixed with 3 g of alumina at the bottom of the thimble. 1 g of alumina on top with 200 μL of methanol as modifier	CO_2 + MeOH, 100°C, density 0.55 g/mL, 10 min static	Analyte collection on a stainless steel trap at 40°C. Elution with 5 × 1.5 mL of hexane. Concentration to 1 mL under a steam of air. Clean-up by means of activated silica column	GC-ECD	42 – 99%	[22]
PCBs and OCPs	Fish feed and shellfish	1 g of ground and homogenised sample placed on the bottom, followed by 1.5 g of aluminium oxide activated basic and 1.5 g of acidic silica gel on top	CO_2, 60°C, 165 bar, 5 min static, 27 min dynamic	Analyte collection in ODS packed trap at 25°C. Elution with 2 mL hexane and concentration to dryness under N_2 steam	GC-MS/MS	76 – 128%	[56]
PCBs	Marine sediments	Freeze-dried marine sediments. Activated Cu was added at the outlet end of the extraction cell to optimise the extraction efficiency	CO_2/CO_2 – MeOH, 150 – 400 bar, 80 – 110°C, 1.5 min static	Analyte collection in 7 mL of hexane. Concentration to 1 mL under N_2 stream at 40°C and sulphuric acid used for removal of traces of water	GC-ECD	84 – 93%	[20]

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Table 2. Continued

Analytes	Sample matrix	Pre-SFE-treatment	SFE conditions	Analyte collection	Analytical technique	Data recovery	Ref
PCBs	Marine sediment CRM	1 g of sample mixed with 7 g of anhydrous sodium sulphate and 2 g of Cu powder	CO_2, 80°C, 305 bar, 10 min static, 40 min dynamic	Analyte collection on a Florisil trap and elution with 1.5 mL of *n*-heptane	HRGC-ECD	79 – 141%	[73]
PCBs	Marine sediment CRM	5 g of sample mixed with 2 g of Cu powder. Dead volume filled with Na_2SO_4	CO_2, 150°C, 400 bar	Analytes collected in 10 mL of acetone	GC-ECD	44 – 108%	[74]
PCBs	Marine sediments	Mixture of 0.5 g of sample mixed with 0.5 – 1.0 g of Cu powder on top of a 1 cm layer of Na_2SO_4. Dead volume filled with Na_2SO_4 mixed with a small amount of Cu powder	CO_2, 80°C, 305 bar, 10 min static, 40 min dynamic	Analyte collection on a Florisil trap. Elution with 2 × 1.5 mL of hexane and 1.5 mL of DCM. Concentration to 20 μL and addition of 10 μL of nonane	GC-ECD	5 – 398 ng/g	[76]
Bioavailable PCBs	Marine sediments	0.5 g of sample mixed with 0.5 g of Cu powder. Dead volume filled with Na_2SO_4	CO_2, 40°C, 120 bar, 60 min static	Analyte collection in 10 mL of acetone at − 10°C. Concentration to 1 mL of isooctane under N_2 steam	GC-ECD	3 – 1060 ng/g	[75]
PCDDs and PCDFs	Marine sediments	3 g of homogenised, desiccated and sieved (<2 mm) sediment placed in the extraction chamber	CO_2 + H_2O, 130°C, 30 MPa, 50 min dynamic	Analyte collection on activated alumina trap at 150°C. Elution with 20 mL of hexane. Concentration of the extracts by a rotary evaporator and nitrogen flow	HRGC/HRMS	0.67 – 2000 pg/g	[23]
PCBs	Marine sediment CRMs	Sample was weighed in a stainless steel cell and on top of it sodium sulphate and activated copper power was added	CO_2, 140°C, 30 MPa, 15 min static, 30 min dynamic	Compound collection onto ODS packed column and eluted with 10 mL of acetone. Concentration under N_2 steam	GC/HRMS	0.15 – 58.20 μg/kg	[66]

octane from C_{18}-modified silica provided the best results. A comparison of the evaluated recoveries obtained with this method and the results from Soxhlet extraction and inter-laboratory studies showed that no significant differences were found. SFE proved to be less laborious and requiered less organic solvent than conventional methods, like Soxhlet extraction, but produced similar results.

The presence of halogenated contaminants like PCDDs, PCDFs, PCBs and OCPs was evaluated in edible marine species from the Adriatic Sea [21]. Only PCBs and *p*,*p*′-dichlorodiphenyldichloroethylene (DDE; a dichloro diphenyl trichloroethane (DDT) metabolite) were extracted by SFE, while PCDDs and PCDFs where extracted using ASE. This work found a trend towards high contamination levels associated with areas subject to increasing anthropogenic impact. As stated in the paper, this conclusion agrees with the fact that organochlorine pollutants accumulate preferentially in the lipid fractions of the food chain and that fish products with high fat levels are the ones most contaminated by OCPs. Antunes *et al.* [57] also developed an SFE method for the extraction of organochlorine pollutants from fish tissues. Optimisation of pressure, temperature and sample preparation for the determination of PCBs, *p*,*p*′-DDE, *p*,*p*′-dichlorodiphenyldichloroethane (DDD), *p*,*p*′-DDT and dieldrin is described in this paper. Optimum experimental SFE conditions (14 MPa, although temperature was not significant) were calculated based on a response surface methodology using a central composite design. Also, three different sample preparations of fish tissue were evaluated: fresh fillet, fresh fillet with anhydrous sodium sulphate and lyophilised fillet. The method developed showed a good performance in the determination of organochlorine compounds from freeze-dried fillets, but it did not efficiently extract these compounds from fresh fish. These results would suggest that the presence of water in the sample decreases CO_2 diffusion in the cell

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and therefore inhibits contact between the supercritical fluid and the analytes. The SFE methodology was also compared with Soxhlet extraction, producing comparable results. The efficiency of SFE was also compared with Soxhlet extraction for the analysis of PCBs from seaweed samples [22]. Data on analytical parameters such as detection limits, linearity, precision and recoveries from spiked samples were also reported in this paper. This study demonstrates that SFE yields similar recoveries and cleaner extracts than Soxhlet, which makes it possible to determine PCBs at lower concentrations. Moreover, the authors proved that sufficient accuracy and precision can be obtained with SFE, making this technique a good alternative to conventional methods for the determination of PCBs in seaweed samples. A similar comparison was studied by Miyawaki *et al.* [23]. This paper describes an SFE method for the determination of dioxins and dioxin-like PCBs in marine sediments. In this work the authors demonstrate that the quantitative determination of dioxins by SFE showed no significant differences as compared to the conventional Soxhlet extraction method in all the samples under study.

Recently, Rodil *et al.* [56] developed another SFE-based multiresidue analytical methodology for the determination of OCPs, PCBs, PBBs and PBDEs from marine biological samples. In this work, various fat retainers were tested and SFE parameters were optimised by means of the Doehlert design after the significant factors were identified by a screening study. The final experimental conditions were found by using desirability function optimisation (60°C, 165 bar and 27 min of dynamic extraction time). Fat interferences were eliminated by means of a combination of 1.5 g of acidic silica and the same amount of aluminium oxide basic in the extraction chamber. Under these conditions, the fat remaining in the extracts was below 0.1%. Data on the performance and validation of the method are also reported in this paper. Quantitative recoveries were obtained from two different certified reference materials (CRMs; RM IAEA 406 and WMF-01) with low RSD% values (less than 14% in all cases). The same authors [55] improved the analytical procedure described above by including a solid-phase microextraction (SPME) step after the supercritical extraction for the determination of the same organohalogenated pollutants from fish feed and cultured marine species. This methodology offers the efficiency, rapidness and selectivity of SFE combined with the increased sensitivity provided by the SPME. The optimisation of the SFE/SPME sample treatment was undertaken by an experimental design approach. In order to do this, the particular variables affecting each technique were screened using the factorial and Doehlert designs. Compromise experimental conditions were obtained by applying multicriteria decision-making strategies using desirability function optimisation. Experimental SFE conditions of 165 bar and 60°C with a flow of 2 mL/min of CO_2 for 5 min static extraction and 27 min dynamic extraction were used. This method was then validated against CRMs, from which good recoveries and repeatability were obtained. Both methods were then applied to real aquaculture samples including trout and turbot feed, turbot, cockle, clam and mussel. Various PCBs, PBBs and PBDEs were detected in the samples tested as well as α- and γ-hexachlorocyclohexane (BHC), and 4,4′-DDT. Detection limits were improved when SPME was used, reaching the pg/g level for all studied compounds.

3.1.2 Marine sediments

Berg *et al.* [20] studied PCBs along with PAHs and hydrocarbons in marine sediments using automated SFE for routine analysis. Analytes were collected in *n*-hexane, then diluted and divided into three aliquots to determine each family of compounds. Hydrocarbons were determined without any clean-up and only sulphuric acid was employed as a drying agent. This acid was also used before the PCB analysis to remove traces of water. The determination of PAHs was carried out after rinsing with dichloromethane to remove traces of interfering hydrocarbons. Methanol was added as a modifier directly into the extraction chamber in order to obtain comparable recoveries with ultrasound-assisted extraction (used as reference technique). In these conditions, recoveries obtained with the SFE method were similar to those obtained with the standard extraction technique. Furthermore, SFE showed better repeatabilities than ultrasound-assisted extraction. RSDs less than 9% were reported for the SFE method, while the RSD% for the standard technique varied between 13 and 25%.

Björklund *et al.* [73] also compared the extraction efficiency of SFE in marine sediment samples. This was done using the SFE-based EPA method 3562 as a reference technique for the validation of a PFE analytical procedure to determine PCBs. Comparison between both methods was performed by extracting sediment CRM (NIST SRM 1944). Recoveries obtained with the SFE method were close to the certified values (97%) without the need for offline clean-up steps. Higher recoveries were obtained with PFE (127% in some cases), probably due to the presence of interfering compounds, despite the clean-up step undertaken after the pressurised fluid extraction. In another paper [74], the same authors investigated the relative "tightness" of PCB binding to the real sediment sample. To do this the sequential extraction of rapidly and slowly desorbing PCBs was studied in historically contaminated samples. Kinetic profiles for marine sediment samples were obtained by using sequentially stronger SFE conditions, varying pressure from 120 to 400 bar and temperatures ranging from 40 to 150°C. Strong SFE conditions were applied to sediment CRMs yielding between 75 and 120% of the certified values for individual PCB conge-

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ners. Hence, through the application of different extraction conditions, they were able to identify the readily desorbing PCBs present in various sediment and soil samples by employing a mild SFE program. Different factors affecting the binding to the sediment matrix, such as PCB molecular weight, sample characteristics, particle size and organic content were also studied. Moreover, the PCB fraction that is bioavailable to benthic organisms can be evaluated by using the mild SFE conditions described above. Thus, selective removal of bioavailable PCBs fraction from contaminated marine sediment samples can be achieved [75].

Two different automated SFE methods for the determination of PCBs in marine sediments were compared in another paper [76]. One of the systems included a syringe pump and a liquid trap, while the other used a reciprocating pump and a solid-phase trapping device (EPA standard method 3562). The major difference between the two methods, in terms of the extraction process, is the extraction temperature (80°C in the EPA method and 150°C in the other method). In the marine sediment studied, no significant differences were found in the recoveries obtained with both methods. Still, the EPA standard method is a preferable choice for this particular sample because it yields lower and more homogeneous RSD values (between 1.8 and 4.2%). The lower RSD values obtained with the EPA method are most likely due to the lower extraction temperatures (cleaner extracts) and the compound trapping on a solid sorbent, which provides additional selectivity to the sample preparation.

Another comparison between SFE and other techniques for the extraction of organohalogenated pollutants from marine sediment samples was described by Numata *et al.* [66]. In this paper, different extraction techniques (PFE, MAE, saponification, Soxhlet extraction, SFE and ultrasonic extraction) were employed for the determination of PCBs and OCPs in two marine sediments CRMs (NMIJ CRM 7304-a and 7305-a). Compared with the other used techniques, SFE proved to be a highly specific method. PCB congeners were recovered effectively, no interferences with the detection system were detected and the clean-up step was not required. Optimal SFE conditions used were 140°C and 30 MPa with a combination of static and dynamic extraction modes. The addition of modifiers was also tested, but this did not seem to have a significant effect on the analytical results.

4 Clean-up

The selectivity of the SFE technique warrants special attention when developing extraction procedures for traces of halogenated organic pollutants with complex matrices like those related to aquaculture. Extract clean-up is a tedious stage, often associated with low recoveries which are generally done offline [77, 78] or it may be included in the extraction stage [79, 80]. Table 2 provides a summary of the clean-up procedures used in the different applications of SFE.

Lipophilic compounds tend to accumulate in fatty matrices, which lead to complex extracts with a high lipid content requiring additional clean-up stages to avoid interferences when making determinations with chromatography techniques. In GC, the presence of lipids may cause problems in the injector and at the inlet of the column [81, 82]. When the detection system is MS, contamination occurs in the ion source which affects the reliability and accuracy of the analysis [80].

The fat content of marine matrices is determined gravimetrically to express the results referring to fat content. The extract obtained after performing SFE is transferred to a previously weighed vial. The elution solvent is evaporated by drying under a nitrogen flow and the fat residue is determined by weighing. These types of matrices may be subjected to different purification and concentration procedures which are described below.

4.1 SPE offline

Commercial cartridges or LC columns prepared in the laboratory with different extractive phases (Florisil®, alumina, silica gel, C_{18}, *etc.*) are commonly used for the purification step by SPE. For the clean-up of PCDDs, PCDFs, PCBs and DDE in edible marine species, after the SFE was performed, the compounds were eluted from the trap with 3 mL of *n*-hexane and further purified on preactivated alumina [21]. The efficiency of SFE for the determination of 12 PCBs from algae samples was compared to Soxhlet extraction [22]. The PCBs were eluted with five fractions of 1.5 mL *n*-hexane, concentrated to 1 mL under a flow of air, purified on an activated silica SPE column and eluted with 10 mL of *n*-hexane.

4.2 Simultaneous extraction and clean-up

The simultaneous clean-up of extracts from SFE with the selective extraction of the PCBs from samples of Goldfish (*Carassius aurum*) and carp homogenates (*Cyprinus carpio*) was proposed by Hale and Gaylor [72]. Aliquots of lyophilised fish tissue (1 g dry weight) were loaded into 10 mL stainless steel extraction vessels. Next, the exit end of the extraction vessel was then filled with approximately 6 g of 150 mesh neutral alumina activated at 150°C, to retain lipids. SFE was shown to be effective in isolating native PCBs from lipid-rich fish tissues without the need for an intermediate offline lipid removal step. Two common fat retainers, basic alumina and Florisil, were investigated by Järemo *et al.* [79] to be used in simultaneous SFE and clean-up. In this study, the authors used basic alumina and Florisil activated and deactivated with 10%

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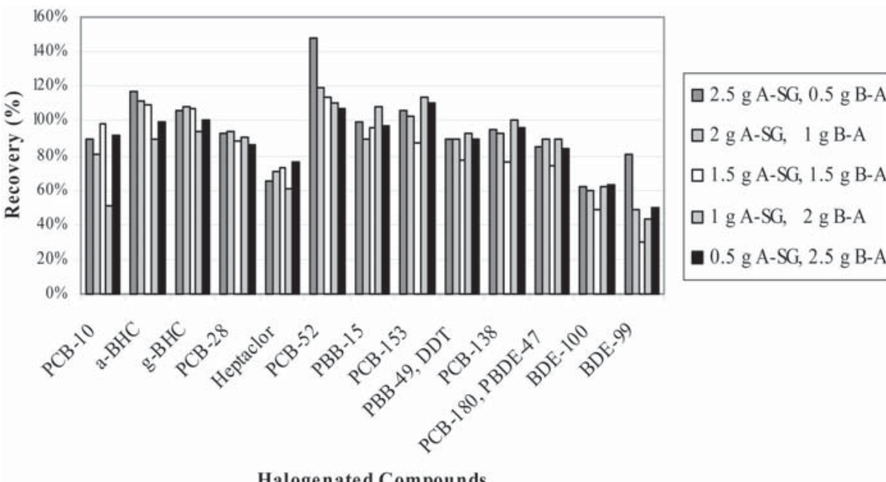

Figure 2. Recoveries obtained by online simultaneous clean-up and extraction using SFE. Different amounts and combinations of fat retainers were tested (A-SG, acidic silica gel; B-A, basic alumina) [85].

water w/w, observing that the fat retaining capacity drops sharply when the adsorbents are deactivated. Finally, they conclude that they have been able to obtain clean extracts and quantitative recoveries of PCBs with the addition of 2 – 4 g of basic alumina added directly to the extraction thimble as a fat retainer, maintaining a relation between the sample and the retainer material of at least around 1:3 w/w. The determination of planar PCBs in biological samples (crab hepatopancreas) by online coupling of SFE-HPLC as extraction and clean-up procedures, prior to analysis by GC-ECD or GC-MS, is carried out using basic alumina [71]. The alumina was activated at 190° and placed at the outlet end of the extraction cell and then in a separate cell after extraction. The good recovery of PCBs and the peak profile on the GC-ECD system would indicate that very low levels of fat were extracted together with the PCBs. A recent study has reported on the use of SFE for extracting different POPs from animal tissues [83]. PCBs, chlordane (including the chlordane MC6), toxaphenes and PBDEs were determinated in adult male walruses from the Arctic. Each blubber sample was homogenised in a mortar with sodium sulphate (1:5) and on the top of the sample around 4.5 g basic aluminium oxide (AlO*x*) was added as a fat retainer in the SFE procedure.

Recently, Rodil *et al.* [56] proposed a new procedure for the determination of halogenated pollutants, including OCPs, PCBs, PBDEs and PBBs in aquaculture samples (fish feed, cockle, clam and mussel), using supercritical fluid multiresidue extraction with supercritical CO_2 and online clean-up followed by GC with electron capture detection or MS. The study of simultaneous SFE and clean-up involved different adsorbents: basic alumina, Florisil, acidic silica gel and octadecylsilane (ODS). First, a study was done to determine the efficiency of each adsorbent in retaining lipids mixed at a ratio of 1:2 (sample/adsorbent w/w), placed on the sample in the cell. It was clearly observed that cleaner extracts were obtained when the adsorbent and the sample were not mixed. Florisil, basic alumina and acidic silicagel are more efficient for fat removal. This led to the investigation of the amount of adsorbent (0 – 3 g) and the combination of two fat retainers, acidic silica and basic alumina to obtain cleaner eluates and chromatograms without interferences. Figure 2 shows the recoveries obtained by using a combination of acidic silica and basic alumina, with the best results being found with 1.5 g of each adsorbent. The maximum amount used was 3 g owing to the limitation of the extraction chamber volume.

Another approach, SPME, has been used as an additional procedure for the clean-up and concentration of organohalogenated compounds from aquaculture feed and biological solid samples, after previous extraction using SFE [55]. The combination of the clean-up processes in the SFE cell with the subsequent SPME has resulted in a high degree of sensitivity and selectivity in the identification of pesticides, polyhalogenated and PBBs and polybrominated diphenyl ethers, reaching quantification limits on the order of pg/g.

4.3 Destruction of lipids by treatment with sulphuric acid

The procedure involving the direct treatment of the extracts with sulphuric acid is suitable for the destruction of lipids when the organohalogenated compounds are stable in a highly acidic medium like the PBDEs [25].

Three toxaphene congeners have been determined in salmon from the Swedish coastal environment using both supercritical fluid (SFE) and the traditional liquid – liquid extractions [84]. After each extraction, the extract was treated with sulphuric acid to remove lipid material, prior to group separation on a silica gel column where the bulk of the PCBs were removed with *n*-hexane in the first fraction. The toxaphene congeners, together with chlordane compounds and some other pesticides (*e.g.*

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DDT, BHCs), were eluted with a mixture of *n*-hexane/diethyl ether (3:1) in the second fraction. In a different study, supercritical carbon dioxide was used to extract PCBs, *p*,*p*′-DDE, *p*,*p*′-DDD, *p*,*p*′-DDT and dieldrin from fish muscle [12]. The SFE extracts were concentrated and passed through a Florisil column to separate the compounds. The first fraction eluted with hexane contained PCBs and *p*,*p*′-DDE and the second fraction eluted with dichloromethane/hexane (30:70) contained dieldrin, *p*,*p*′-DDD and *p*,*p*′-DDT. Further clean-up with sulphuric acid was also carried out. The recovery of the Florisil column was evaluated with a standard solution and more than 85% of each compound was obtained.

4.4 Elimination of sulphur from the sediments

Analytical problems associated with sediment samples are well documented in the literature. During the SFE step, many interfering components, such as large amounts of elemental sulphur, are coextracted from sediment samples together with the target analytes; so a clean-up procedure is generally required before the final chromatographic determination [25]. The simplest treatment that can be used on the sample to eliminate sulphur consists of a mixture of copper powder directly activated in the extraction cell [20, 66, 76]. Among the different extraction methods used for the certification of 14 PCB congeners and 4 OCPs in the new reference materials certified (NMIJ CRM 7304-a and NMIJ CRM 7305-a) [66], SFE stands out from the other procedures owing to its simplicity and high specificity. Another method involving SFE with a solid phase trap containing activated alumina was investigated for the rapid analysis of polychlorinated dibenzo-*p*-dioxins (PCDDs), PCDFs, and dioxin like PCBs (DL-PCBs) in soils and sediments [23]. The solid phase trap prepared with activated alumina was effective in the purification of extracts, since activated alumina can adsorb polar substances such as organic acids and sulphur compounds in soils and sediments.

5 Conclusions

This review shows clearly that the use of SFE makes possible the development of very attractive and powerful alternative sample preparation for the determination of polyhalogenated pollutants in aquaculture and marine environmental samples. Experimental design has been evaluated as a statistical tool for determining the best levels of operational SFE conditions. Furthermore, the suitability of SFE for fast extraction and clean-up of POPs from complex environmental and biota samples has been shown. SFE reduces extraction time and leads to final extracts with less interfering substances. The combination with an online clean-up using fat retainers seems to be an interesting and advantageous approach for this purpose in case of complex matrices.

The authors wish to thank Galician Government, Xunta de Galicia for financial support, project PGIDIT06PXIB237039PR. D. García-Rodríguez acknowledges Xunta de Galicia (Consellería de Innovación e Industria) for the financial support of his Ph.D.

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Anal Bioanal Chem (2012) 404:173–181
DOI 10.1007/s00216-012-6106-4

ORIGINAL PAPER

Pesticides in seaweed: optimization of pressurized liquid extraction and in-cell clean-up and analysis by liquid chromatography–mass spectrometry

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Received: 24 February 2012 / Revised: 27 April 2012 / Accepted: 7 May 2012 / Published online: 26 May 2012
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Abstract Chemical residues, such as insecticides and anthelmintics, are frequently redistributed from the aquatic environment to marine species. This work reports on a fast validated protocol for the analysis of azamethiphos, three avermectins, two carbamates and two benzoylurea pesticides and chemotherapeutic agents in seaweeds based on pressurized liquid extraction and separation of analytes by liquid chromatography coupled with tandem mass spectrometry. The variables affecting the efficiency of pressurized liquid extraction, including temperature, number of extraction cycles, static extraction time and percent acetonitrile flush volume, were studied using a Doehlert design. The optimum parameters were 100 °C and one cycle of 3 min with 70 % acetonitrile. Adequate in-cell clean-up of the seaweeds was achieved using 0.8 g of Florisil over 0.1 g of graphitized carbon black on the bottom of the cell. The optimized method was validated using an analyte-free seaweed sample fortified at different concentrations. The limits of quantification ranged from 3.6 μg kg^{-1} (azamethiphos) to 31.5 μg kg^{-1} (abamectin). The recovery was from 87 to 120 % in most cases at different spiking levels. Finally, the reproducibility of the method expressed as the relative standard deviation and evaluated at concentrations of 10 and 50 μg kg^{-1} was in the range 9–14.3 % and 6.1–12.3 %, respectively. The applicability of the method was evaluated with five commercial and 12 wild edible seaweeds, and four target compounds were detected in two wild seaweeds at a concentration below the quantification limit.

Keywords Seaweed · Pesticides · Pressurized liquid extraction · In-cell clean-up · Liquid chromatography coupled with tandem mass spectrometry · Experimental design

Electronic supplementary material The online version of this article (doi:10.1007/s00216-012-6106-4) contains supplementary material, which is available to authorized users.

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Introduction

Edible seaweeds are an abundant source of good-quality proteins, vitamins, essential unsaturated fatty acids, particularly long-chain *n*-3 polyunsaturated fatty acids, minerals and bioactive compounds with known antioxidant properties. Furthermore, they are an excellent source of dietary fibre. Therefore, the dietary value of edible seaweeds has prompted their consumption worldwide in recent years [1, 2]. The control of residues and contaminants is an important issue to ensure the quality of foods. Contamination with pesticides and chemotherapeutic agents can come from either agriculture or aquaculture activities, from where the contaminant compounds reach the aquatic environment. Most attention is focused on benzoylphenylurea, carbamates, avermectins and organophosphorus compounds, which are widely employed in aquaculture for prevention and control of several diseases [3, 4]. These toxic compounds tend to associate to particulate matter and seaweeds owing to their hydrophobicity [5]. As a consequence, they can enter the food chain and may cause a bioaccumulative effect with untoward consequences for human health [6]. The EU has established maximum residue levels (MRLs) in food products in order to prevent potential hazards to human

health [11]. Nevertheless, scarce information is available on the levels of these organic contaminants in seaweed.

The development of optimized multiresidue methods for monitoring pesticide residues is highly demanded, and various analytical methods have been reported for the determination of pesticide residues in fruits and vegetables [7–10]. The determination of pesticides in foods is a difficult task owing to the complexity of the matrices, their different physicochemical properties and the low concentrations in which these compounds are usually present. Simultaneous extraction and clean-up steps are particularly labour intensive and time consuming. This together with the relevant matrix-effect problems (ion enhancement or ion suppression) makes the quantitative analysis of some compounds extremely difficult [12]. Pressurized liquid extraction (PLE) combined with in situ (in-cell) clean-up of the extracts can avoid the application of exhaustive post-clean-up procedures, such as column and/or gel-permeation chromatography, and may be automated [13–16].

Several methods utilizing gas chromatography (GC) coupled with mass spectrometry (MS) for the analysis of organophosphorus and carbamate pesticides in fruits and vegetables have been published [17–19]. Liquid chromatography (LC) coupled with MS or tandem MS (MS/MS) has been applied for the determination of avermectins in biological tissues [20], milk [21, 22], and water, sediments and soils [23]. Benzoylurea insecticides have been analyzed by means of both GC-MS [24] and LC-MS [25].

The main objectives of the present study were (1) to develop an efficient extraction–purification method for the analysis of multiclass pesticides and chemotherapeutic agents—azamethiphos, three avermectins, two carbamates and two benzoylurea derivatives—in seaweeds based on PLE, (2) to validate the extraction method developed followed by analysis by LC–electrospray ionization (ESI) MS/MS, in both positive-ion and negative-ion modes, and (3) to test the performance of the analytical method by applying it to the analysis of real samples. To the best of our knowledge, no studies have been done to extract these chemical residues from edible seaweeds using this analytical method.

Experimental

Reagents and chemicals

Pestanal-quality analytical standards of azamethiphos (*S*-[(6-chloro-2-oxo[1,3]oxazolo[4,5-*b*]pyridin-3(2*H*)-yl)methyl]), propoxur (phenol 2-(1-methylethoxy)-1-(*N*-methylcarbamate)), carbaryl (1-naphthalenol 1-(*N*-methylcarbamate)), teflubenzuron (*N*-[[(3,5-dichloro-2,4-difluorophenyl)amino] carbonyl]-2,6-difluorobenzamide) and diflubenzuron (*N*-[[(4-chlorophenyl)amino]carbonyl]-2,6-difluorobenzamide) were purchased from Riedel-de Haën (Seelze, Germany). Pestanal-quality abamectin, doramectin and ivermectin were purchased from Sigma-Aldrich (Madrid, Spain).

Individual standard stock solutions of 5,000 μg mL^{-1} were prepared in methanol (MeOH). A stock mixture solution of all the compounds studied at a concentration of 100 μg mL^{-1} was obtained by appropriate dilution of individual stock solutions in MeOH. Diluted solutions were prepared from the stock mixture solution in acetonitrile (ACN). All solutions were stored in amber-coloured vials at −20 °C.

ACN and MeOH (gradient high-performance LC grade) and silica gel (60 Å, 0.040–0.063 mm) were purchased from Merck (Darmstadt, Germany). Florisil (60–100 mesh) and sea sand (50–70 mesh) were purchased from Sigma-Aldrich (Madrid, Spain). Anhydrous sodium sulphate was purchased from Panreac (Barcelona, Spain). Neutral silica was activated overnight at 200 °C and then cooled to room temperature in a desiccating chamber. Graphitized carbon black (GCB; bulk packing 120–400 μm) was from Supelco (Bellefonte, PA, USA). Ultrapure water was obtained using a Milli-Q® water purification system (Millipore, Billerica, MA, USA). Syringe filters (Millex GV, 13 mm and 0.22 μm) were obtained from Millipore. Cellulose filters (20-mm diameter) for the PLE cell were from Restek (Bellefonte, PA, USA).

Samples and sample preparation

To test the performance of the approach developed, 12 wild seaweed samples were collected in different areas located on the coast of Galicia (northwest Spain): sea spaghetti (*Himanthalia elongata*), spiral wrack (*Fucus spiralis*), bladder wrack (*Fucus vesiculosus*), *Ulva rigida (*common name of *glasán* in Irish), sea lettuce (*Ulva lactuca*), *Cystoseira* sp., oyster thief (*Codium tomentosum*), egg wrack (*Ascophyllum nodosum*), sea potato (*Leathesia difformis*) and Irish moss (*Chondrus crispus)*. The seaweed samples were stored at −18 °C before processing. The frozen samples were dried in an oven at 50 °C for 24 h, ground and homogenized in an electric mill and stored in sealed vessels until use. Dried edible seaweed samples were purchased in a local market. Wakame (*Undaria pinnatifida*), laver (*Porphyra umbilicalis*), Irish moss, sea spaghetti (*Himanthalia elongata*) and a commercial mixture of wakame (30 %), sea lettuce (19 %), kombu (*Laminaria ochroleuca*, 18 %), dulse (*Palmaria*, 18 %) and laver (15 %) were ground by means of an electric mill and stored in sealed vessels until analysis. Spiked laver seaweeds were used as the matrix to perform the optimization study.

Approximately 0.2 g of seaweed sample was spiked with 1 mL of a standard mixture at 100 ng mL^{-1} and left for 30 min before extraction. The spiked material was

homogenized in a mortar with 1 g of anhydrous sodium sulphate, which was used as a drying agent. PLE was conducted using a fully automated Dionex (Sunnyville, CA, USA) ASE 200 system. The PLE conditions were optimized for the extraction of the target residues from seaweed samples as discussed in “Results and discussion”. Loading of PLE cells was done in the following sequence: an 11-mL extraction cell was loaded by inserting two cellulose microfilters into each cell outlet, followed by addition of 0.1 g of GCB and 0.8 g of Florisil for in-cell clean-up; the spiked material was then added; the empty space above the mixture was filled with 0.5 g of sea sand. The cell contents were extracted during one cycle of 3 min, at 100 °C and 1,500 psi, with an ACN flush volume of 7.7 mL (70 % of PLE cell capacity) and purged with nitrogen (90 s). The extracts were evaporated down to 0.5 mL under a gentle stream of nitrogen gas in a TurboVap station. The extracts were filtered through a 0.22-μm syringe filter and transferred by adding ACN to reach a final volume of 1 mL in a 1.5-mL vial, and were then injected into an LC-MS/MS system.

LC-MS/MS conditions

A Varian (Walnut Creek, CA, USA) liquid chromatograph equipped with two isocratic, high-pressure mixing pumps (Varian 410 Prostar), an autosampler and a thermostated column compartment was used. The MS/MS system consisted of a U-shaped triple quadrupole (Varian MS 1200 L) equipped with an ESI interface. The LC-MS/MS instrument was entirely controlled by Varian MS Workstation version 6.9. Separations were done using a Hypersil ODS (100 mm×3.2-mm inner diameter, 3-μm particle size) analytical column with a Phenomenex C_{18} SecurityGuard cartridge (4.0×2.0 mm). The eluent flow rate was set at 0.4 mL min^{-1} and the column was kept at 30 °C. The mobile phase was 5 mM ammonium acetate in ACN (solvent A) and 5 mM ammonium acetate in water (solvent B). The gradient conditions were as follows: 0–14 min, from 50 to 100 % solvent A; 14–20 min, constant 100 % solvent A; 20–22 min, back to 50 % solvent A; 22–25 min, constant 50 % solvent A. The injection volume was 10 μL. The ESI interface was operated simultaneously in both positive-ion and negative-ion modes according to the preferential ionization of each analyte, and the voltage of the ESI needle was fixed at 5,000 V. The optimized ESI conditions were established to provide the average maximum intensity of the precursor ions. The temperature of the ESI housing was set at 50 °C. Argon (99.999 %) was employed as the collision gas (2.2 mTorr) in the mass spectrometer. The nitrogen nebulizer pressure was 50 psi, and the nitrogen drying gas (200 °C,19 psi) in the ESI source was provided by a high-purity generator (Domnick Hunter, Durham, UK). The capillary potential was 5,000 V (positive-ion mode) or −4,500 V (negative-ion mode). For MS/MS, high-purity nitrogen (99.999 %) was used as the collision gas. To optimize the multiple reaction monitoring transitions, each individual pesticide at a concentration of 10 μg mL^{-1} in ACN was injected directly. Two transitions were monitored per compound with a dwell time of 0.2 s per transition. The optimum conditions are summarized in Table 1. Compounds were confirmed by their retention times, and the most abundant transition ion was used as a quantifier and the next most abundant transition ion was used as a qualifier.

Results and discussion

LC-MS/MS optimization

The optimization of MS parameters (cone voltage and collision energy) was performed by direct infusion of 2 mL of standard solution (5 μg mL^{-1}) of each compound with 100 μL of 2 % formic acid added. A flow rate of 0.05 mL min^{-1} of a mobile phase of MeOH/water (50:50) was used. Thus, the adsorption of standards to the glass walls of the vial and the possible formation of stable adducts with sodium ions, which are difficult to fragment, were avoided.

ESI in positive-ion mode (compounds ionized by adding a proton) was selected as the ionization technique owing to its sensitivity, ruggedness and easy handling and maintenance for all analytes, except for the benzoylurea family, whose separation and determination were studied in negative-ion mode (losing a proton), which has better specificity and sensitivity [21, 29]. The optimization of the ionization was performed by a series of preliminary experiments, testing different modifiers in a binary gradient mobile phase comprising ACN and water, such as acetic acid and ammonium acetate at various concentrations. Finally, the addition of 5 mM ammonium acetate led to the best sensitivity [21, 30]. Capillary voltages and collision energies were optimized in order to maximize the intensity for the precursor ion for each compound, and to identify a minimum of two transitions in the MS/MS spectra. The most intense transition was used for quantification and the second most intense transition was used for confirmation following the criteria of European Commission Decision 2002/657/EC [31] for mass-spectrometric detection. Full-scan spectra were acquired in order to select the most abundant *m*/*z* value, with optimization of the cone voltage. Besides, the sensitivity of the mass spectrometer was further improved using the multiple reaction monitoring mode (Table 1).

Solvent optimization for PLE

Physicochemical properties such as polarity and specific density, which influence the penetration into the matrix,

Table 1 Conditions for mass spectrometry in multiple reaction monitoring mode

Compound	Structure	Retention time (min)	Ionization mode	Fragmentor potential (V)	Precursor ion	Product ions	CV	Collision energy/V
Azamethiphos		2.208	ESI+	40.0	325	183[a], 139	40	11.0, 18.5
Propoxur		2.438	ESI+	30.0	210	111[a], 153	30	10.5, 5.0
Carbaryl		2.611	ESI+	30.0	202	145[a], 127	30	6.0, 24.5
Diflubenzuron		4.913	ESI-	36.0	309	289[a], 156	-36	8.0, 10.0
Teflubenzuron		8.078	ESI-	30.0	379	339[a], 359	-30	10.0, 6.0
Abamectin		11.877	ESI+	32.0	891	305[a], 567	32	19.0, 12.0
Doramectin		13.374	ESI+	36.0	916,5	331[a], 593	36	21.0, 10.0
Ivermectin		15.158	ESI+	36.0	893	307[a], 569	36	21.0, 12.5

ESI electrospray ionization

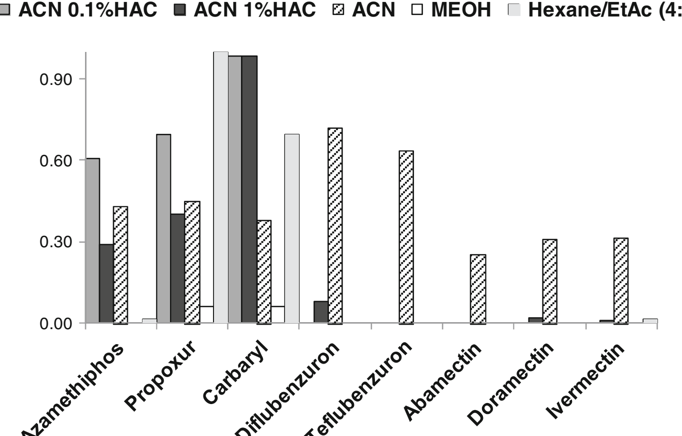
[a] Quantifier transition

are relevant for the selection of the extraction solvent, which must be able to solubilize the analytes of interest, minimizing the coextraction of other matrix components [26]. An important aim in the present study was the selection of a solvent system that enables the simultaneous extraction of the target pesticides and minimizes the extraction of matrix interferences. Previous studies suggest that ACN with a low percentage of acetic acid [21, 27, 28] and MeOH [32] are generally the preferred solvents for avermectins. On the other hand, a mixture of hexane and ethyl acetate (4:1) has been shown to be useful for carbamates and organophosphorus compounds [14, 29]. For benzoylureas, extraction was achieved with aqueous mixtures with MeOH [33]. In the present study, ACN with or without acetic acid, MeOH, hexane/ethyl acetate (4:1) and MeOH/H_2O (1:1) were assessed regarding the extraction efficiency using the following general conditions: two cycles of 5 min at 100 °C, 1,500 psi, solvent flush of 60 % and 90-s purge time [34]. The MeOH/H_2O (1:1) extracts were very dark, indicating coelution of matrix interferences, and were not analyzed further. The recovery results showed that ACN with 0.1 % acetic acid was the preferred solvent for azamethiphos and carbamates. ACN gave better extraction efficiency (Fig. 1a) for benzoylureas and avermectins than the other solvents or mixtures. Thus, ACN was chosen as the extraction solvent for further optimization tests of simultaneous extraction of chemical agents.

Selection of in-cell clean-up sorbents

Most vegetable tissues, such as seaweed, contain pigments that should be removed before the chromatographic analysis. GCB served as an adsorbent enabling in-cell clean-up during extraction of pesticides by PLE, and the resultant extracts were clean enough to be analysed using GC-MS [14, 17]. Since the extraction cell should have sufficient adsorbent capability [17], four different adsorbents were investigated. The use of silica and C_{18} combined with Florisil led to intensely coloured extracts. Florisil combined with GCB gave very clean extracts when extracting pyrethroid and organophosphorus pesticides from seaweed using ACN with 1 % acetic acid under the general conditions tested [34]. To obtain pigment-free extracts, a suitable ratio between Florisil and GCB was required; this is consistent with the ratio of 0.25 previously reported for the determination of some pesticides by GC-MS/MS [14]. Thus, in this experiment GCB was tested with Florisil. For 1.6 g of Florisil, ratios of 0.25, 0.125 and 0.0625 required 0.4, 0.2

Fig. 1 Effects of the type of extractant solvent on percent normalized recovery obtained for the target compounds using pressurized liquid extraction (**a**). Effects of the adsorbents used for online clean-up on percent normalized recovery by combining 1.6 g of Florisil with different amounts of graphitized carbon black (*GCB*) (**b**) and by combining 0.1 g of GCB and different amounts of Florisil (**c**). *ACN* acetonitrile, *HAC* acetic acid, *MEOH* methanol



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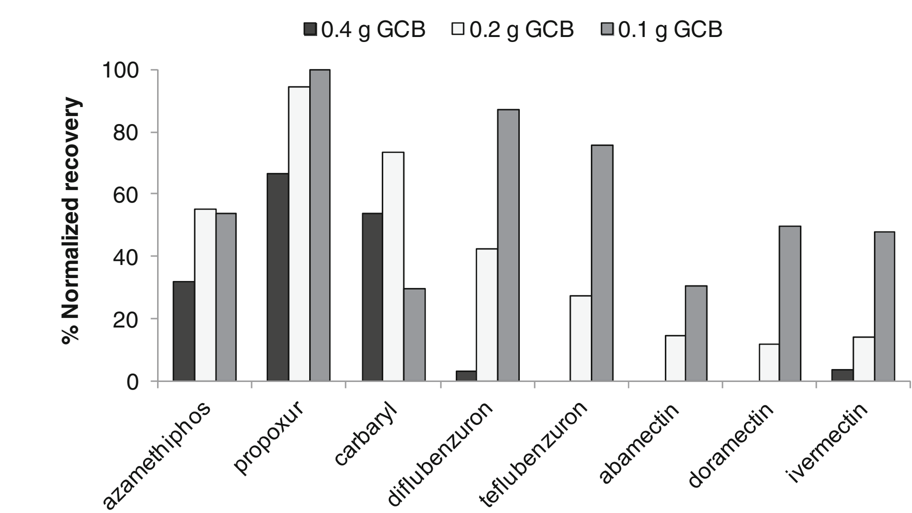

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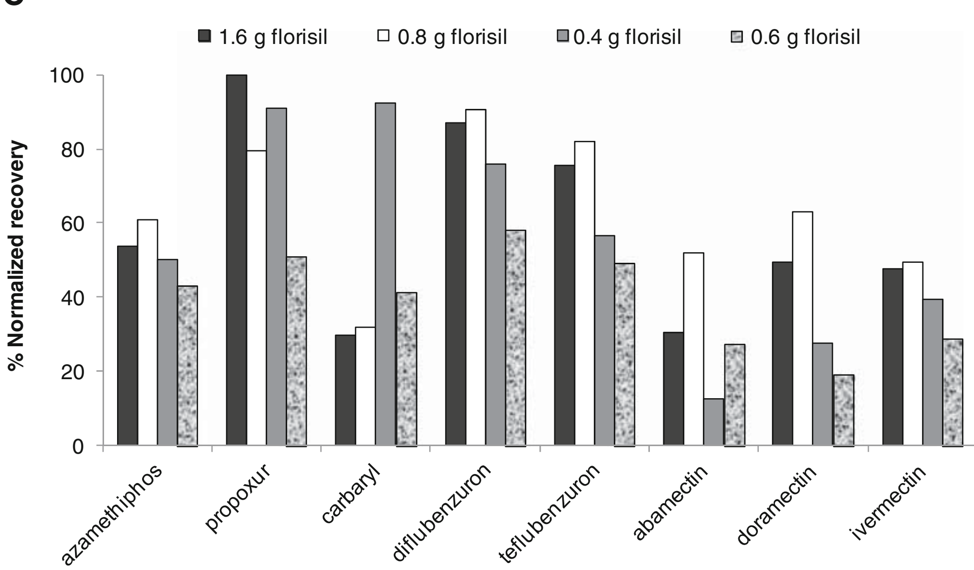


and 0.1 g of GCB (Fig. 1b). Higher retention of the analytes was obtained when the amount of GCB was increased. Colourless extracts and the best recoveries were obtained with 0.1 g of GCB, especially for the avermectin and benzoylurea families. Finally, different amounts of Florisil (1.6, 0.8, 0.4 and 0.2 g) were tested with a fixed amount of GCB (0.1 g) for ratios of 0.0625, 0.125, 0.167 and 0.25, respectively. Better PLE efficiency was achieved with high amounts of Florisil, as shown in Fig. 1c. No difference was found for 1.6 or 0.8 g of Florisil for most analytes. Although the best results for carbaryl were obtained with 0.4 g of Florisil, 0.8 g of Florisil (combined with 0.1 g of GCB) was chosen as a compromise solution because it significantly reduced the consumption of adsorbent and enhanced the recoveries of abamectin and doramectin, which had the least intense chromatographic signals.

Selection of number of cycles

The number of cycles was tested to ensure rapid extraction as well as high recovery. One and two extraction cycles were tested. In general, an increase in the number of extraction cycles allows the exposure of the matrix to fresh solvent and favours the solvent/sample equilibrium, improving partition into the liquid phase. The results showed that the recovery did not increase with the number of extraction cycles (results included in Fig. S1). When two cycles were used, the recovery was even a bit lower. Thus, considering the time taken and solvent consumption, the use of one cycle was considered optimal.

Experimental design for PLE optimization

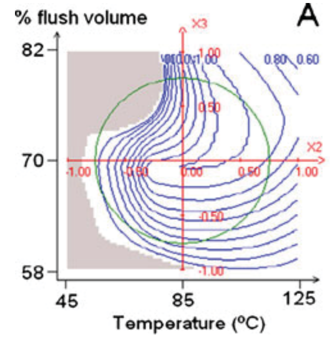
The effects of temperature, static extraction time and percent solvent flush were also evaluated. Pressure is a parameter without a significant effect on the extraction, in agreement with previous reports [13, 14]. Taking into account these aspects and considering a safety issue with the equipment, a pressure of 1,500 psi was selected and used throughout the study [14, 35]. Temperature (50, 62, 73, 85, 97,108 and 120 °C), static extraction time (2, 4, 6, 8 and 10 min) and percent ACN (60, 70 and 80 %) flush volume were the factors selected and levels studied in a response surface Doehlert design [36].

The responses were expressed as peak areas and were fitted by a multiple regression equation. The results obtained were evaluated using analysis of variance by Nemrod®W 2000 [37]. The analysis of variance demonstrated a high significance for diflubenzuron, teflubenzuron and ivermectin. Temperature was a statistically significant factor ($p<0.01$) for all of the target analytes, but the static extraction time was only statistically significant for teflubenzuron, whereas the percent ACN flush was not statistically significant for any of the compounds studied. The static extraction time–percent ACN flush interaction was statistically significant ($p<0.01$) for azamethiphos, carbamates and diflubenzuron. The static extraction time–temperature interaction was statistically significant ($p<0.01$) for the avermectin family. Several quadratic terms of the model were statistically significant ($p<0.001$) for azamethiphos, carbamates and diflubenzuron (percent ACN flush) and teflubenzuron (static extraction time). High levels of temperature provided good responses for all the pesticides, except for azamethiphos, whereas low levels of static extraction time led to high extraction efficiency for all the compounds. High levels of percent ACN flush showed a positive effect on the extraction of most of the target analytes.

To find the best-compromise conditions, desirability functions were applied without additional experimentation [36]. Two-dimensional plots of the isodesirability are shown in Fig. 2. The regions in grey correspond to null values for desirability when the factor levels are not suitable to be chosen. Optimal numerical conditions resulted in 100 °C, 3 min and 70 % ACN flush.

PLE-LC-MS/MS validation

The matrix effect was also evaluated during the validation of the method since signal suppression or enhancement can severely compromise quantitative analysis of the compounds at trace levels, and it can also greatly affect the method reproducibility and accuracy [38, 39]. The matrix effect was studied by comparison of the slopes of the calibration curves in solvent and in the extract obtained after the



Temperature (ºC)
B
Time (min)

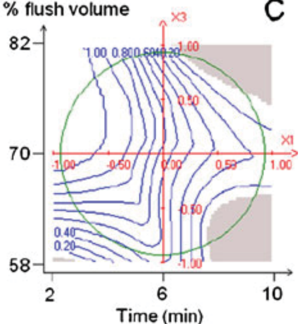


Fig. 2 Global desirability response surface plot using a Doehlert design as a function of percent flush volume versus temperature (**a**), temperature versus time (**b**) and percent flush volume versus time (**c**). The *arrow* shows the region corresponding to optimal conditions

PLE procedure. If the first slope minus the second one gives a positive value, signal enhancement occurred. Otherwise, negative values are indicative of signal suppression. Depending on the value of the percentage of the difference, different matrix effects could be observed. Both the solvent and the matrix calibration curves had good linearity, with determination coefficients higher than 0.999 for solvent calibration curves and 0.994 for matrix-matched ones. Matrix effects were considered as relevant when the deviation of the matrix calibration slope from the solvent calibration slope was higher than 10 %. Several compounds exhibited a matrix effect in seaweed samples (Table 2). Azamethiphos and carbamates evidenced a strong matrix effect and signal enhancement, whereas avermectin pesticides showed a medium matrix effect and signal suppression. Abamectin and doramectin did not show a matrix effect. Among the eight pesticides and chemotherapeutic agents evaluated, strong matrix effects, higher than 20 %, were obtained for five compounds. The highest was observed in ESI in positive-ion mode for azamethiphos and propoxur, and in ESI in negative-ion mode for teflubenzuron, which showed a signal enhancement of greater than 50 %. In the quantification of samples, problems arising from suppression or enhancement effects were avoided by using matrix-matched calibration standards [40]. Linearity was evaluated in the wide range from 25 to 1,250 μg kg^{-1} (except for the avermectin family, for which the range was between 50 and 1,250 μg kg^{-1}). Good linearity was found for all compounds, with coefficients of determination higher than 0.994 (Table 2).

The limits of detection (LOD) and limits of quantification (LOQ) of the method were calculated as the minimum amount of target analyte that led to a chromatogram peak with a signal-to-noise ratio of 3 and 10, respectively [41]. The LOQs ranged from 3.6 to 31.5 μg kg^{-1} and the LODs ranged from 1.1 to 12.5 μg kg^{-1}, with abamectin showing the highest values owing to the high background noise obtained at its retention time (Table 2). The LODs obtained are lower than the MRLs for diflubenzuron and teflubenzuron (50 μg kg^{-1}, valid for seaweeds) established by the European Commission [31]. The LOD is of the order of the MRL for abamectin (10 μg kg^{-1}) [11]. For similar vegetables such as lettuce, spinach and chard, the MRLs for carbaryl and propoxur are 50 μg kg^{-1}.

The precision of the method—expressed as the relative standard deviation (RSD)—was evaluated by measuring four replicate samples in 1 day (intraday precision) and four replicate samples on three consecutive days (interday precision) by spiking 0.2 g of blank laver seaweed with appropriate volumes of the composite working standard solution to provide a concentration in the final extract of 10 and 50 μg kg^{-1}. Replicate samples for each concentration were analyzed and the RSD was calculated for each pesticide

Table 2 Validation data for the pressurized liquid extraction–liquid chromatography–tandem mass spectrometry method

Compound	R^2	Matrix effect	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Intraday precision (% RSD) (n=4)		Interday precision (% RSD) (n=4)		Recovery (%) ($X \pm S$) (n=4)	
					10 μg kg^{-1}	50 μg kg^{-1}	10 μg kg^{-1}	50 μg kg^{-1}	10 μg kg^{-1}	50 μg kg^{-1}
Azamethiphos	0.997	56.8	1.1	3.6	10.1	11.3	10.3	10	107±8	107±11
Propoxur	0.998	50.5	1.8	5.9	8.7	8.8	10.4	9.2	105±6	104±6
Carbaryl	0.995	37.2	9.6	21.9	6.8	5.8	13.8	11.5	125±6	95±6
Diflubenzuron	0.994	42.8	1.3	4.3	13.8	2.8	12.1	12.3	108±6	87±7
Teflubenzuron	0.995	50.7	1.8	6	6.1	1.5	9	10.5	91±3	88±11
Abamectin	0.996	7.5	12.5	31.5	6.9	7.7	10.2	10.4	101±5	96±7
Doramectin	0.995	5.0	11.4	28.1	14.5	3	14.3	6.1	120±14	94±9
Ivermectin	0.996	−17.3	6.5	21.6	13.5	11.1	12.7	10.8	92±11	102±9

LOD limit of detection, *LOQ* limit of quantification, *RSD* relative standard deviation

(Table 2). The method was shown to be precise, with RSDs ranging from 1.5 to 14.5 % for all the compounds studied at all spiking levels, again meeting the requirements stipulated for such methods (RSD<20 %) [41]. The accuracy was investigated by analysis of four replicate samples as described above. The results, listed in Table 2, show that the mean recovery of the eight analytes from blank samples is satisfactory, ranging from 91 to 120 % for 10 μg kg^{-1}, except for carbaryl (125 %), and from 87 to 107 % for 50 μg kg^{-1}, which indicates the method meets the requirements stipulated for methods used for such analyses [41].

Application to real samples

The applicability of the method for real samples was tested in 12 wild seaweed samples and five edible seaweeds. According to Commission Regulation No. 149/2008, in which maximum levels of several of these residues in seaweed are stipulated [42], the analysis of the real samples led to concentrations of the target analytes not exceeding the specified values. Azametiphos, diflubenzuron, teflubenzuron and propoxur were detected in the wild species *Ulva rigida* collected at two different places on the Galician coast, but at concentrations lower than the LOQ. The LC-MS/MS selected ion chromatograms of propoxur detected in *Ulva rigida* and laver (*Porphyra umbilicalis*) spiked with 50 μg of target analytes per kilogram are shown in Fig. 3.

Conclusions

A multianalyte method was developed for eight veterinary compounds from three families in seaweeds that can be applied for routine analysis. The new robust and sensitive analytical method is based on a simultaneous extraction and in-cell clean-up by PLE and analysis by LC-MS/MS. The adsorbent GCB combined with Florisil provides good retention of coloured pigments but not of the target analytes. The validation showed high recovery rates. The LOQs were established at microgram per kilogram levels for all the compounds. However, for some of them, a high percentage of matrix effect was present, and therefore the standard addition method was used to compensate for these undesirable effects and to perform a correct quantification. The applicability of the method was proved by analysis of 17 seaweed samples. The results of the analysis of wild and edible seaweeds indicate that four compounds, including two benzoylurea derivatives, were present in a wild *Ulva rigida* sample. Further studies regarding the presence of chemotherapeutic agents in seaweeds are required because

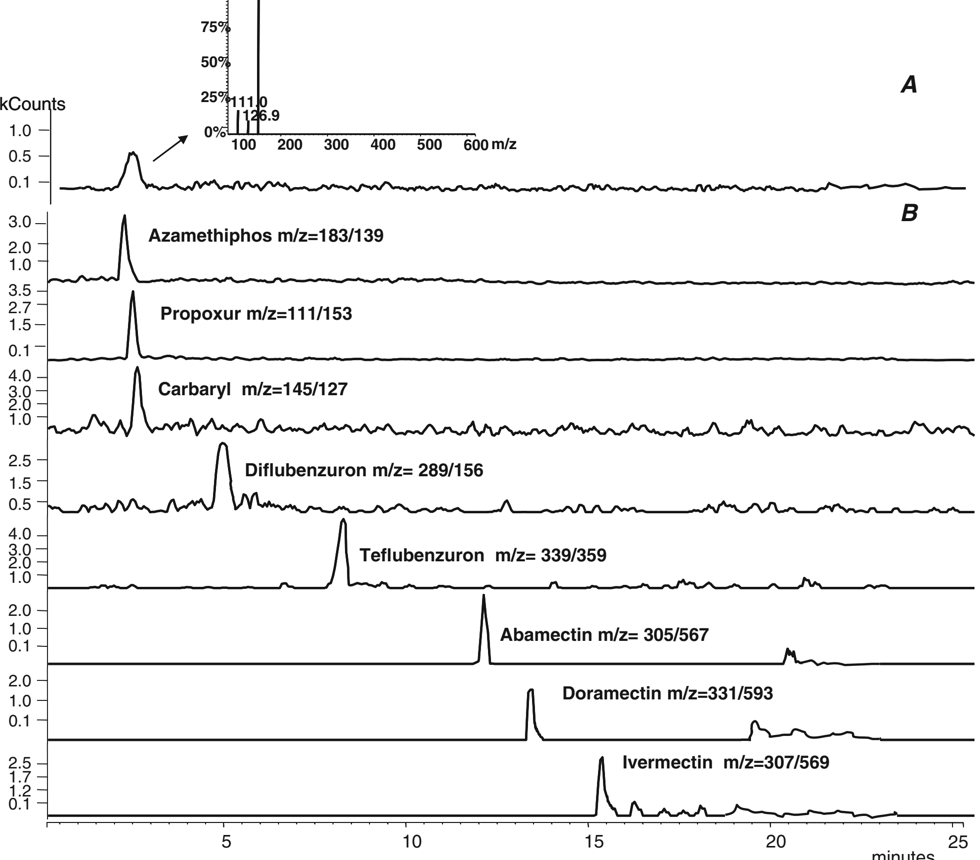


Fig. 3 Mass chromatograms obtained from a wild *Ulva rigida* sample (*A*), containing a detected peak of propoxur, and laver (*Porphyra umbilicalis*) spiked with 50 μg of target analytes per kilogram (*B*)

of the lack of data about some currently used compounds and to elucidate the transformation and biodegradation processes, because wild seaweeds can be a direct source of contaminants in the environment through their application as ecological fertilizer for agricultural soil and also an indirect source of human exposure through food as edible seaweeds.

Acknowledgments This research was supported by the project 10TAL209005PR Dirección Xeral de I+D (Xunta de Galicia, Spain). I.R. acknowledges an FPI grant from the Spanish Ministry of Education and Science.

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ANEXO III. Acrónimos

ACRÓNIMOS

ACN	Acetonitrile	**LLE**	Liquid-LiquidExtraction
ADI	Admissible Daily Intake	**LOD**	Limit Of Detection
ANOVA	Analysis Of Variance	**LOQ**	Limit Of Quantification
APCI	Atmospheric Pressure Chemical Ionization	**MECK**	Micellar Electrokinetic Chromatography
ASE	Accelerated Solven tExtraction	**MIPs**	Molecularly Imprinted Polymers
CAR	Carboxen	**MRL**	Maximum Residue Level
CAS	Chemical Abstracts Service	**MS**	MassSpectrometry
CI	Chemical Ionization	**MSPD**	Matrix Solid-Phase Dispersion
CW	Carbowax	**MWCNT**	Multiwalled Carbon Nanotubes
DAD	Diode Array Detector	**NPD**	Nitrogen Phosphorous Detector
DDD	Dichlorodiphenyldichloroethane	**OCPs**	Organochlorinated pesticides
DDE	1,1-Dichloro-2,2-di(chlorophenyl)ethylene	**OPs**	Organophosphorus compounds
DDT	Dichlorodiphenyltrichloroethane	**OPPs**	Organophosphorous pesticides
DLLME	Dispersive Liquid-Liquid Microextraction	**PA**	Polyacrylate
dSPE	dispersive Solid-PhaseExtraction	**PBB**	Polybrominated Biphenyl
DSPME	Direct Solid Phase Microextraction	**PBDE**	Polybrominated Diphenyl Ether
DVB	Divinylbenzene	**PBO**	Piperonyl Butoxide
ECD	Electron Capture Detector	**PCB**	Polychlorinated Biphenyl
EI	Electronic Impact	**PDMS**	Polydimethylsiloxane
EPA	Environmental Protection Agency	**PFE**	Pressurized Fluid Extraction
ESI	ElectroSprayIonization	**PLE**	Pressurized Liquid Extraction
EU	European Union	**POP**	Persistent Organic Pollutant
FAO	Food and Agricultural Organization	**PSA**	Primary and Secondary Amine
FID	Flame Ionization Detector	**PSE**	Pressurized Solvent Extraction
FPD	Flame Photometric Detector	**PTFE**	Politetrafluoroethylene
FTD	Flame Thermoionic Detector	**PVDF**	PolyvinylideneDifluoride
GABA	Gamma Amino-Butiric Acid	**PYR**	Pyretroid
GAC	Green Analytical Chemistry	**OMS**	OrganizacionMundial de la Salud
GC	Gas Chromatography	**QuEChERS**	Quick, Easy, Cheap, Effective, Rugged and Safe
GCB	Graphitized Carbon Black	**RSD**	Relative Standard Deviation
GLC	Gas-Liquid Chromatography	**SDME**	Single Drop Microextraction
GPC	Gel Permeation Chromatography	**SFE**	Supercritical Fluid Extraction
HCB	Hexachlorobenzene	**SPE**	Solid PhaseExtraction
HPLC	High Performance Liquid Chromatography	**SPME**	Solid Phase Microextraction
HSSPME	Headspace-Solid Phase Microextraction	**SRM**	Surface Response Methodology
ICP	Induced Coupled Plasma	**TOF**	Time Of Flight
ITD	Ion Trap Detector	**UV**	Ultra Violet
IUPAC	International Union of Pure and Applied Chemistry	**VSSC**	Voltage Sensitive Sodium Channels
JMPR	Joint FAO/WHO Meeting on Pesticide Residue	**WHO**	World Health Organization
K_{ow}	Partition Constant Octanol-Water	**WTP**	Waste Treatment Plant
LC	Liquid Chromatography		
LC_{50}	Lethal Concentration (50%)		

El objetivo de este trabajo es el desarrollo y validación de distintas metodologías analíticas para la determinación de pesticidas en muestras relacionadas con el medio marino: agua de mar, algas cultivadas y salvajes utilizadas con fines alimentarios, pescados y moluscos. Los compuestos que se determinan son pesticidas organofosforados, piretroides, carbamatos, derivados de la benzoilfenilurea y avermectinas. Todos ellos son usados, en mayor o menor medida, como agentes quimioterápicos en el tratamiento de infecciones parasitarias presentes en explotaciones acuícolas. Los métodos empleados para la determinación de residuos de pesticidas en agua de mar se basan en el empleo de técnicas de microextracción (SPME y DLLME) y en la determinación cromatográfica empleando detectores selectivos de captura de electrones y espectrometría de masas en tándem con inyección de grandes volúmenes. Para la determinación de los residuos de distintos grupos de pesticidas en algas, pescados y moluscos se han utilizando diferentes técnicas de extracción (PLE, MAE y MSPD) y de determinación por cromatografía de gases y líquidos con acoplamiento a espectrometría de masas en tándem.