Universitat Jaume I
Departament de Química Física y Analítica
Química Analítica

Institut Universitari de Plaguicides i Aigües

USO DE TÉCNICAS AVANZADAS CROMATOGRAFÍA DE GASES/ESPECTROMETRÍA DE MASAS CON ANALIZADORES DE TRIPLE CUADRUPOLO Y TIEMPO DE VUELO EN ANÁLISIS MEDIOAMBIENTAL Y BIOLÓGICO

Tesis Doctoral
TANIA PORTOLES NICOLAU
2010

Dr. Félix Hernández Hernáncez, Catedrático de Química Analítica, y Dr. Francisco J. López Benet, Catedrático de Química Analítica de la Universitat Jaume I de Castellón,

Certifican que: la Tesis Doctoral "Uso de técnicas avanzadas cromatografía de gases/espectrometría de masas con analizadores de triple cuadrupolo y tiempo de vuelo en análisis medioambiental y biológico" ha sido desarrollada bajo su dirección, en el área de Química Analítica del Departamento de Química Física y Analítica de la Universitat Jaume I de Castellón, por Tania Portolés Nicolau. Tras la creación del Instituto Universitario de Plaguicidas y Aguas (IUPA) por Decreto 260/2004, de 19 de Noviembre, la parte experimental de la Tesis Doctoral continuó y finalizó en las instalaciones del mencionado instituto.

Lo que certificamos para los efectos oportunos en Castellón de la Plana, a 27 de Septiembre de 2010.

Fdo. Dr. Félix Hernández Hernández Fdo. Dr. Francisco J. López Benet

Este trabajo responde al compromiso adquirido con el Ministerio de Ciencia y Tecnología por la concesión de una beca predoctoral para la Formación de Profesorado Universitario (FPU) desde el 01 de Abril de 2006.

Tania Portolés Nicolau ha realizado una estancia en el Department of Food Chemistry and Analysis del Institute of Chemical Technology, Praga, llevada a cabo desde el 1 de Marzo al 1 de Junio de 2006. El trabajo de investigación llevó por título: "*Development of a rapid screening of pesticides in tea samples by SPME-GCxGC-TOF MS*" bajo la dirección de Prof. Ing. Jana Hajšlová, Ing. Tomaš Čajka and Ing. Jakub Schůrek y permitió ampliar los conocimientos de la doctoranda en el uso de las técnicas de cromatografía de gases monodimensional (GC) y multidimensional (GC×GC) acopladas a espectrometría de masas con analizadores de tiempo de vuelo (TOF MS) de elevada resolución y elevada velocidad de adquisición, respectivamente. Los resultados obtenidos en este trabajo dieron lugar a la publicación de un artículo científico de título "*Application of head-space solid-phase microextraction coupled to comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry for the determination of multiple pesticide residues in tea samples*" en la revista Analytica Chimica Acta, 611, 163-172 (2008). Por otra parte, se realizaron 2 estancias, de dos semanas cada una, en Waters Corporation, Manchester, que permitió ampliar conocimientos en el uso de software de tratamientos de datos utilizados en la tesis doctoral.

Los trabajos realizados en la presente Memoria han sido en parte financiados por los proyectos del Ministerio de Ciencia y Tecnología "REN2002-01818", "CTQ2006-07594", "CTQ2009-12347", Bancaixa, "P1-1B2005-08", "PROMETEO/2009/054" y "Reciplasa"

Esta tesis ha sido realizada y será defendida de acuerdo con los requisitos exigidos para el título de Doctorado Europeo.

Previamente a la defensa de la Tesis Doctoral, este trabajo ha sido evaluado por tres censores independientes directamente relacionados con el área de investigación, Dra. Antonia Garrido Frenich (Catedrática del Departamento de Hidrogeología y Química Analítica, Universidad de Almería), Dr. Wilfried M.A (Extraordinary Professor of Faculty of Sciences, Free University of Amsterdam) y Tomáš Čajka (Associate Professor of the Department of Food Chemistry and Analysis, Institute of Chemical Technology, Prague)

Em pareix un bon moment, per expressar paraules d'agraïment a molta gent que ha format part de la meua vida durant aquests anys d'investigació.

En primer lloc als meus directors de Tesi. A Félix, a qui admire per la naturalitat amb que combina la seua tasca investigadora amb el bon tracte personal. Gracias por dirigirme y acompañarme en esta Tesis, por tu tiempo y esfuerzo, por darme esta oportunidad y por confiar en mí. A Paco, per ajudar-me a organitzar les idees, en això eres únic, i recolzar-me en moments de frustració; per mostrar-me confiança i donar-me l'espenteta que necessitava en alguns moments per continuar endavant. Quins "sustos" me pegues!

A la meua Elena Pi, per la incalculable ajuda, paciència i moments de rises; per creure en el que feia i dedicar-me el teu temps; per la teua capacitat de síntesi! I perquè mai oblidaré Praga. "Que bien explica Elena!".

Als meus companys del grup d'investigació, ara ja amics, als quals vullc. Açò no haguera sigut el mateix sense les veteranes María, Mercedes, Arantxa i Susana, quantes coses he aprés de les meues "germanes" majors, si les tinguera serien com vosaltres; gràcies per els bons consells. A Jose per ser "el meu Jose", saps que el despatx no és el mateix sense tu. A Edu i Elena per les llargues xerrades amb donuts i cafè i per ser els més simpàtics. A Ángel per permitir-me fer-te bromes (es que ho fiques fàcil!). Nadie me organiza las vacaciones como Robert, gracias (próximo destino New York). A la parella Cristina i Inés i als seus colors (cadascuna en un sentit). També a Jaime, qui em deu una actuació de Peces Gordos, a Ramón (ho vam passar bé a Manchester, que te parece?) i a Emma (agradable "ocupa" de nuestro despacho durante una temporada). A la futura mare Sandra, amb qui he compartit aquestos últims tràmits. Gràcies a Vima per ser la nº1 en organització d'esdeveniments. També a les noves incorporacions (Clara, Laura, Ana María, Neus i Mónica) en qui veig reflectida la il·lusió amb què jo vaig començar la investigació.

Juanvi, sempre és un plaer escoltar-te, ningú explica com tu la "photo finished". Ximo, m'agrada que em digues "Antonia", sé que ho fas amb bona intenció, gràcies per la teua ajuda en GC. El primer contacte amb la docència no haguera sigut el mateix sense l'ajuda de Toni, gràcies. També a Roque i Carmen, de qui sempre he rebut bona disponibilitat per ajudar-me quan calia.

Gràcies a tots aquells que han format part del grup d'investigació en alguna etapa d'aquesta Tesi: Sonia E., Sonia F., Alicia, Cru, Tatiana, Mari Carmen, Miguel Angel, Mireia, Óscar (gracias por tu desinteresada ayuda en mis inicios con el TOF), Carlos (eres únic), i sobretot Cecilia, companya de fatigues; juntes vam comprovar que les lleis de Murphy eren certes, totes!

A tots vosaltres, gràcies per contagiar-me d'alegria tots els dies, per l'ambient immillorable de treball que entre tots creem (així es més fàcil), per els trossos de vida compartits, per les bromes (algunes telefòniques), i perquè, sense saber-ho, heu sigut i sou per a mi de gran ajuda.

Thanks to Dr. Wilfried Niessen, for its valuable comments and help. I feel lucky to have had the opportunity of having intense discussions with him about some topics of this Thesis.

Thanks to Jana Hajšlova for allowing me to be a part of her research group and to the rest of the people of the lab, especially Jakub Schůrek and Tomas Čajka for dedicating part of their time in my TOF MS training.

Thanks to the people from Waters in Manchester who nicely welcomed me in their group. I enjoyed the atmosphere, their kindness, and their support.

Gràcies al Dr. Carlos Vázquez de l'Institut Valencià d'Oncologia per proporcionar-me les mostres biològiques humanes i a totes les persones anònimes que han donat el seu consentiment perquè els resultats d'aquestes formen part d'aquesta tesi.

Gràcies als meus amics, i a Naiara (guapíssima!!) per continuar sent la colla que érem encara que passe el temps. No heu entés mai ben bé a què hem dedicava, però sempre m'heu transmet que "devia de ser important". Gràcies per estar ahí sempre.

No vullc oblidar-me de les meues companyes d'estudi, amigues: Jéssica, Míriam, Mónica, Pamela i Teresa. Per les hores de biblioteca, que fàcil va ser amb vosaltres. Gràcies "equipo", m'alegra que continuem juntes en la distància.

I per últim a la meua família. A mon pare per ficar-m'ho tan fàcil, per la teua alegria, i pel teu valor; gràcies per "malcriar-me" i ser un exemple per a mi de sacrifici. A ma mare, bonica, per mirar-me amb eixos ulls plens de dolçor i somriure'm. I a tu, Fer, per mitja vida, per estar al meu costat "si o si", per escoltar-me i saber què dir-me per fer-me sentir bé. Per donar-me la mà; tantes vegades com fera falta...

Gràcies a tots,

Tania

.

Resumen

En la presente Tesis se pretende avanzar en el conocimiento del potencial que representa el acoplamiento cromatografía de gases-espectrometría de masas con analizadores de triple cuadrupolo y tiempo de vuelo como herramienta avanzada en el análisis medioambiental y biológico.

El trabajo se organiza en tres grandes bloques. En primer lugar, se investiga el uso de la técnica GC-MS/MS con analizador de triple cuadrupolo para el desarrollo de metodología analítica multirresidual que permita la cuantificación y confirmación de contaminantes orgánicos en aguas a los niveles de concentración requeridos por la legislación internacional (del orden de ng/L). Además de los requisitos relativos a la elevada sensibilidad y selectividad, se presta especial atención a los criterios exigidos para la correcta identificación y confirmación de los compuestos detectados con la finalidad de evitar falsos positivos. La metodología desarrollada se basa en una extracción en fase sólida, como técnica de extracción y preconcentración de la muestra, seguida del correspondiente análisis cromatográfico. El método se valida en términos de exactitud y precisión mediante ensayos de recuperación utilizando muestras fortificadas a distintos niveles de concentración y, posteriormente, se aplica al análisis de muestras reales. Siguiendo la misma metodología de trabajo se desarrolla un método basado en GC-MS/MS para la determinación de compuestos con carácter xenoestrógeno en muestras de tejido adiposo mamario y en tejidos tumorales, en colaboración con el Instituto Valenciano de Oncología (IVO) de Valencia, que suministró las muestras objeto de análisis. La determinación de estos contaminantes en este tipo de muestras resulta de especial interés por su elevada liposolubilidad y tendencia a la bioacumulación, así como por su carácter estrogénico, por lo que podrían ejercer alguna influencia en procesos cancerígenos que tienen un cierto componente hormonal. Los dos métodos anteriormente indicados hacen uso de una fuente de ionización electrónica. En el análisis de aguas, también se ha optimizado y validado el método para compuestos organoclorados haciendo uso de la fuente de ionización química en modo negativo con el fin de estudiar la ventajas y/o desventajas que este modo de ionización puede aportar con respecto a la más comúnmente utilizada fuente de ionización electrónica.

En segundo lugar, se explora el potencial del acoplamiento GC-TOF MS para el desarrollo de métodos avanzados y rápidos de *screening* para un amplio rango de

contaminantes. Con el objetivo de realizar la menor manipulación posible de muestra y tener bajo consumo de disolventes se aplica una etapa de extracción mediante SPE ó SPME a las muestras de agua. La capacidad del analizador TOF de adquirir el espectro completo de iones con elevada sensibilidad y exactitud de masa, hace que sea una técnica muy adecuada para este fin. La búsqueda de contaminantes se realiza por dos vías: (a) análisis *post-target*, donde los compuestos de interés se buscan *a posteriori*, una vez adquiridos los datos MS, bien inmediatamente o en cualquier otro momento posterior, sin necesidad de re-analizar la muestra (b) análisis *non-target,* donde no se lleva a cabo ninguna selección previa de los compuestos, siendo en este caso necesario alguna herramienta de software que sea capaz de "detectar" la presencia de componentes relevantes en la muestra, para posteriormente proceder a su identificación gracias a la información obtenida. El trabajo se centra principalmente en aspectos cualitativos y de elucidación estructural, campos en donde la gran cantidad de información aportada por GC-TOF MS es fundamental, al permitir la correcta identificación de las especies detectadas. Este estudio se aborda en dos campos de aplicación donde la tecnología TOF MS tiene un gran futuro, como son el análisis medioambiental, representado por el análisis de aguas, y el análisis biológico, en nuestro caso muestras de tejido adiposo humano. Adicionalmente, se contempla la necesidad de validar cualitativamente los métodos de *screening* desarrollados, con el fin de asegurar la calidad de los resultados obtenidos desde el punto de vista de la correcta identificación de los compuestos detectados. El método se valida en términos cualitativos (identificación absolutamente fiable) para distintos tipos de aguas: naturales (superficiales y subterráneas) y aguas residuales y para un elevado número de contaminantes orgánicos. Adicionalmente, se explora el potencial de GC-TOF MS para la elucidación de compuestos en aquellos casos en los que el espectro de EI obtenido experimentalmente no se encuentra en las librerías comerciales utilizadas. En este caso, el uso combinado de fuentes de ionización fuertes y suaves, como ionización electrónica e ionización química, respectivamente, aporta información relevante sobre la identidad del compuesto detectado, pudiendo en la mayoría de los casos proponer una estructura plausible para el mismo. Finalmente, se exploran las capacidades de un nuevo prototipo de fuente de ionización ampliamente utilizada en combinación con LC –la fuente de ionización química a presión atmosférica– diseñada recientemente para su acoplamiento a sistemas de GC. El trabajo consiste en estudiar las ventajas del acoplamiento GC-QTOF MS con la nueva fuente APCI con fines de *screening*.

En último lugar, se combinan las técnicas GC-MS/MS y LC-MS/MS con triple cuadrupolo para investigar con fines cuantitativos una amplia variedad de contaminantes orgánicos en aguas de lixiviado de residuos sólidos urbanos. Los métodos aplicados habían sido previamente optimizados y validados en nuestro laboratorio, en términos cuantitativos. A continuación, se investiga la presencia de otros contaminantes no incluidos en los métodos mencionados con el fin de ampliar el nivel de multirresidualidad del *screening*. Para ello, se analizan las muestras por GC–TOF MS y LC–QTOF MS, procesándose los dados siguiendo una metodología *non-target*. Asimismo, se explota el potencial que presenta el uso combinado de GC-TOF MS y UHPLC-QTOF MS para investigar las posibles causas de un episodio de mortandad de abejas en entornos apícolas de la Comunidad Valenciana. Ante el desconocimiento sobre el origen del problema, se realiza un *screening non-target* por GC-TOF MS y UHPLC-QTOF MS, el cual genera una gran cantidad de información de utilidad cualitativa, al disponer de espectros de iones completos medidos con elevada exactitud de masa. Esta metodología se ha aplicado en un segundo episodio de mortandad de abejas, en el que no sólo se analizaron muestras de abejas, sino también hojas y flores de nectarina cercanas al área de las abejas y sospechosas de ser responsables del envenenamiento de las mismas. Finalmente, se aprovecha el gran potencial de las técnicas empleadas para investigar metabolitos de los principales compuestos detectados en las abejas.

Summary

USE OF ADVANCED ANALYTICAL TECHNIQUES BASED ON GAS CHROMATOGRAPHY/MASS SPECTROMETRY WITH TRIPLE QUADRUPOLE AND TIME OF FLIGHT ANALYZERS IN ENVIRONMENT AND BIOLOGICAL ANALYSIS

In this Thesis the potential of coupling gas chromatography-mass spectrometry with triple quadrupole and time-of-flight analyzers is investigated as an advanced analytical technique in environmental and biological analysis.

The work is divided in three main parts. Firstly, the use of GC-MS/MS with triple quadrupole is investigated for the development of multiresidual analytical methodology that allows the quantification and confirmation of organic contaminants in water at the low concentrations established by the international legislation (ng/L levels). In addition to the requirements of elevated sensitivity and selectivity, special attention is given to the strict criteria needed for the correct identification and confirmation of detected compounds in order to avoid reporting false positives. The methodology developed is based on a solid phase extraction, as a sample extraction and preconcentration technique, followed by GC-MS/MS analysis. The method is validated in terms of accuracy and precision by recovery experiments in different water matrices spiked at several concentration levels. It has been applied to the analysis of real samples with the result of several contaminants being detected and confirmed, mainly herbicides. Following similar methodology, a method based on GC-MS/MS is developed for the determination of xenoestrogen compounds in adipose and tumoral tissue samples. This work has been carried out in collaboration with the Instituto Valenciano de Oncología (IVO), which provided the samples. The determination of these contaminants in these kind of samples has special interest due to their high liposolubility and tendency to bioaccumulation, and also due to its estrogenic character, which might have some influence in carcinogenic processes with a certain hormonal component. The methods previously described both used an electron ionization source. In addition, a method for organochlorine compounds using negative ion chemical ionization source has been optimized and validated for water analysis in order to test the advantages and drawbacks of this ionization mode in comparison to the most commonly used electron ionization source.

Secondly, the potential of GC-TOF MS for the development of advanced and rapid screening methods for a wide-range of organic contaminants is explored. Keeping in mind the minimization of samples manipulation and solvent consumption, an extraction step based on SPE has been applied to water samples. The capability of the TOF analyzer to obtain full spectrum data at high sensitivity and mass accuracy makes this technique highly appropriate for this purpose. Searching of contaminants is carried out in two different ways: (a) *post-target* analysis, where the compounds of interest are investigated *a posteriori*, once MS data have been acquired, without the need of reinjecting the sample (b) *non-target* analysis, where no previous selection of compounds is carried out, being necessary in this case a powerful software able to "detect" the presence of "relevant" compounds in the sample in order to proceed to their identification thanks to the useful information given by TOF MS. The work is mainly focused on qualitative aspects and on structural elucidation, where the huge amount of information given by GC-TOF MS is crucial, allowing the correct identification of the compounds detected. This study is carried out in two application fields where TOF MS technology has great interest, as environmental analysis, focussed on water samples, and biological analysis, specifically human breast adipose tissue samples. A qualitative validation of the screening methods has been performed, in order to assure the quality of the results from a correct identification point of view. The method is validated in qualitative terms (confident identification) for different types of water: natural (surface and ground) and waste water and for a large number of organic contaminants. Additionally, we have also investigated the potential of GC-TOF MS for the elucidation of those compounds for which the electron ionization spectrum is not available in our commercial libraries. In this case, the combined use of soft and hard ionization sources, like electron ionization and chemical ionization, respectively, gives relevant information for the identification of the compound. In many cases, it is feasible to propose a plausible structure for the detected compound thanks to the accurate masses given by TOF MS in both ionization modes. Finally, the capabilities of a new prototype of ionization source widely used in LC analysis –the atmospheric pressure chemical ionization source– designed for its coupling to GC systems are explored. The work consists on a preliminary study on the use of GC-QTOF MS with the new APCI source for *screening* purposes.

Finally, GC-MS/MS and LC-MS/MS with triple quadrupole are combined for quantification of a wide variety of organic contaminants in treated and raw untreated leachates from a municipal solid waste treatment plant. Later, the presence of other contaminants not included in the mentioned *target* methods was investigated. For this purpose, the samples were also analyzed by GC–TOF MS and LC–QTOF MS, processing the MS data following a *non-target* methodology. This has allowed the discovering of several contaminants in the samples, which can be included in future monitoring programs based on GC-MS/MS or LC-MS/MS with triple quadrupole. The potential of the combined used of GC-TOF MS and UHPLC-QTOF MS has been explored to investigate the possible causes of honeybee poisonings. As no information about the origin of death was available, a *non-target screening* by GC-TOF MS and UHPLC-QTOF MS was applied, which generates a huge amount of qualitative information, with the accurate mass full spectra being generated. Finally, both TOF MS techniques are applied to investigate the presence of metabolites of the compounds detected in samples, mainly the insecticide coumaphos.

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CAPITULO 5

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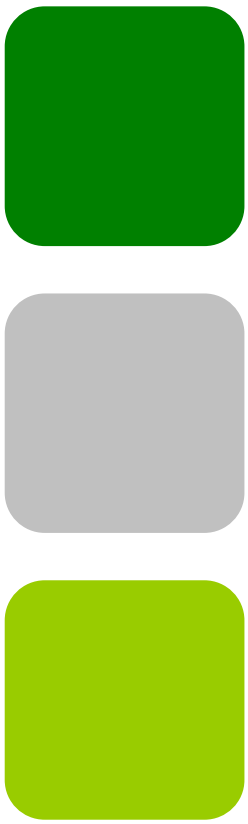
CHAPTER 4. Combined use of GC-MS and LC-MS with triple quadrupole and time-of-flight analyzers in environmental and toxicological studies

CHAPTER 5

Objetivos y plan de trabajo



OBJETIVOS

El **objetivo principal** de la presente Tesis Doctoral es explorar el potencial analítico del acoplamiento instrumental cromatografía de gases – espectrometría de masas, tanto en modo simple (GC-MS) como en tandem (GC-MS/MS), usando analizadores de triple quadrupolo (QqQ) y tiempo de vuelo (TOF), para la investigación de contaminantes orgánicos, principalmente en los campos medioambiental y biológico.

En primer lugar, se aplica la técnica GC-MS/MS con analizador de triple cuadrupolo para la determinación cuantitativa de contaminantes orgánicos en muestras de tejido adiposo humano y en aguas, prestando especial atención al poder de confirmación de dicha técnica. En segundo lugar, se estudia el potencial del acoplamiento GC-TOF MS, tanto como técnica de confirmación como para el desarrollo de métodos de *screening* de amplio rango, abordando la problemática desde una metodología *target* así como *non-target*. Finalmente, se comparan las dos técnicas estudiadas desde el punto de vista de su poder cuantitativo y cualitativo y se compara su potencial para el *screening* amplio de contaminantes orgánicos en distintos campos de aplicación.

Para alcanzar este objetivo principal, se establecen los siguientes **objetivos específicos**:

1. Desarrollar y validar metodología analítica basada en GC-(QqQ)MS/MS para la cuantificación y confirmación de contaminantes orgánicos prioritarios, a niveles de sub-ppb, en aguas utilizando la extracción en fase sólida (SPE) como técnica de extracción y preconcentración.

2. Evaluar el modo de ionización química negativa, en términos de sensibilidad y selectividad, como técnica alternativa a la ionización electrónica para ciertos contaminantes prioritarios.

3. Desarrollar y validar metodología analítica para la cuantificación de compuestos xenoestrógenos en muestras de tejido adiposo humano mediante GC-(QqQ)MS/MS, prestando especial énfasis a su correcta identificación y confirmación.

4. Explorar las capacidades identificativas de la técnica GC-TOF MS para el *screening* y confirmación de contaminantes orgánicos, tanto en modo *target* como *non-target*. Estudiar sus ventajas e inconvenientes.

5. Establecer una metodología de trabajo para el tratamiento de la gran cantidad de datos generados por GC-TOF MS. Investigar la capacidad de las herramientas de software disponibles para este fin.

6. Aplicar la metodología de trabajo desarrollada al *screening* y confirmación de contaminantes prioritarios en aguas mediante un tratamiento de muestra basado en microextracción en fase sólida (SPME) seguida de un análisis por GC-TOF MS.

7. Validar la metodología de trabajo desarrollada en términos cualitativos, usando para ello muestras de agua de distintos orígenes y características que se fortifican con un amplio grupo de contaminantes seleccionados, con objeto de comprobar que el método de *screening* es fiable con fines de detección e identificación de dichos compuestos.

8. Investigar la presencia de contaminantes de origen antropogénico en muestras de tejido adiposo humano mediante GC-TOF MS. Comparar los resultados con los obtenidos previamente mediante el análisis con GC-(QqQ)MS/MS.

9. Estudiar las posibilidades de la técnica GC-TOF MS, en cuanto a la elevada exactitud de masa que proporciona, para la elucidación de compuestos cuyo espectro de ionización electrónica no se encuentra en librerías comerciales. Explorar el uso combinado de las fuentes de ionización electrónica e ionización química para este fin.

10. Estudiar el potencial de la nueva fuente de ionización química a presión atmosférica en combinación con GC-TOF MS para el análisis de residuos de plaguicidas en muestras de alimentos.

11. Investigar el potencial que aporta el uso combinado de las técnicas GC-MS y LC-MS con analizadores de triple cuadrupolo y TOF para el *screening* de un elevado número de contaminantes, muy superior al considerado en las aproximaciones analíticas convencionales.

12. Utilizar de forma complementaria las técnicas GC-TOF MS y UHPLC-(Q)TOF MS para la investigación *non-target* de contaminantes y metabolitos en un caso real de envenenamiento de abejas.

PLAN DE TRABAJO

De manera general, el análisis cuantitativo desarrollado en la presente Tesis Doctoral se ha seguido abordando de acuerdo con el siguiente plan de trabajo:

1. Revisión bibliográfica sobre el estado actual de la determinación de contaminantes orgánicos en aguas y selección de los compuestos a estudiar en función de su persistencia, toxicidad y carácter emergente.

2. En el caso del tejido adiposo humano, estudio bibliográfico sobre los compuestos con carácter estrogénico con persistencia media o alta y con capacidad de acumularse en muestras grasas de origen biológico.

3. Estudio de las condiciones óptimas de MS en GC-(QqQ)MS/MS mediante la inyección de patrones. Estudio de los espectros de ionización electrónica y selección de posibles iones precursores para cada analito. Una vez seleccionado el ion precursor, aislamiento del mismo en el primer cuadrupolo y optimización de la energía de colisión para la obtención de iones productos característicos en el segundo cuadrupolo. Finalmente, selección de 2 transiciones SRM por compuesto teniendo en cuenta su sensibilidad y selectividad.

4. Optimización de la separación cromatográfica mediante inyección de patrones. Selección del gradiente de temperatura optimizando el número de compuestos a incluir en cada ventana cromatográfica de tiempo. Estudio del parámetro *dwell time* en función de la anchura de los picos, del número de transiciones por ventana y de la sensibilidad requerida.

5. Para las muestras de agua, optimización de la etapa de extracción en fase sólida (volumen de muestra, tipo de eluyente y volumen del mismo) con objeto de obtener un elevado grado de multirresidualidad y un tratamiento sencillo del extracto para su inyección en GC.

6. Estudio del efecto matriz en aguas mediante el análisis de muestras fortificadas y de patrones en solvente de la misma concentración.

7. Validación de la metodología optimizada estudiando parámetros de calidad como linealidad, especificidad, exactitud y precisión, mediante ensayos de recuperación a varios niveles de concentración, sobre la base de las guías SANCO de la Unión Europea. Inclusión del parámetro de confirmación Q/q *ratio* en la validación.

8. Aplicación de la metodología desarrollada al análisis de muestras reales, aplicando criterios de control de calidad, y discusión de resultados.

9. En el caso de muestras de grasa, estudio de la etapa de purificación por HPLC en fase normal previa al análisis por GC-MS, estableciendo el patrón de elución de los lípidos presentes en tejido adiposo mamario así como de los compuestos OCs y OBrs objeto de análisis.

10. Validación de la metodología desarrollada estudiando parámetros como linealidad, especificidad, exactitud y precisión, , mediante ensayos de recuperación en muestras fortificadas a varios niveles de concentración sobre la base de las guías SANCO de la Unión Europea. Inclusión del parámetro de confirmación Q/q *ratio* en la validación (cabe resaltar la dificultad de encontrar una muestra blanco para los ensayos de recuperación, debido a la ubicuicidad de los contaminantes clorados. Así pues, se utilizará una mezcla de varias muestras, previamente homogeneizada y analizada. Se tendrán en cuenta las señales de los picos encontrados en el blanco y se restarán de las señales obtenidas en muestras fortificadas en el proceso de validación).

11. Aplicación de la metodología desarrollada al análisis de muestras reales, aplicando criterios de control de calidad, y discusión de resultados.

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

1. Recopilación de información sobre contaminantes orgánicos más frecuentemente detectados en aguas naturales y residuales urbanas. Selección de un elevado número de contaminantes, analizados por GC, abarcando diferentes familias fisico-químicas.

2. Selección de la estrategia más adecuada en cuanto a procesamiento de datos TOF MS, en modo manual o automatizado, mediante el uso de softwares específicos para el desarrollo de métodos *target*.

3. Desarrollo de metodología *target* mediante GC-TOF MS, para compuestos de los que se dispone de patrón, mediante el uso del software de procesamiento de datos

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

4. Estudio de los parámetros críticos que afectan al método de procesamiento de datos *target*: selección de iones, ventana de extracción de masa, posibles interferencias procedentes del *lock mass*, problemas de saturación del detector, etc.

5. Desarrollo de metodología *target* mediante GC-TOF MS basada en el uso de espectros de librería para compuestos sin patrones disponibles. Valoración de ventajas e inconvenientes respecto a la metodología para compuestos con patrones disponibles.

6. Desarrollo de metodología *non-target* mediante GC-TOF MS basada en el uso del software de deconvulación de datos Chromalynx que proporciona, de manera automática, el espectro de iones en masa exacta, permitiendo su comparación con los espectros disponibles en librerías comerciales (para EI) así como el estudio del error de masa de los distintos fragmentos.

7. Aplicación de procedimientos de extracción para muestras de agua que sean adecuados para métodos rápidos de *screening*, con la menor manipulación de muestra posible, persiguiendo una elevada multirresidualidad y que sean adecuados para bajos niveles de concentración (normalmente sub-microg/L). Se considerarán las técnicas de SPE y SPME por inmersión directa, que se optimizarán en cuanto a los parámetros más importantes.

8. Establecimiento de criterios para la adecuada identificación y confirmación de los compuestos detectados en las muestras. Entre estos criterios se encontrarán: tiempo de retención (y desviación máxima permitida), errores de masa, número de iones necesarios para la identificación, calidad de la información aportada, y relevancia del patrón de distribución isotópica en cada uno de los compuestos ensayados.

9. Validación cualitativa del método de *screening* basado en GC-TOF MS para contaminantes orgánicos en aguas con objeto de establecer los niveles de concentración más bajos para los que se pueda detectar e identificar correctamente los analitos. La validación se lleva a cabo con distintos tipos de agua, incluyendo aguas

residuales urbanas (influente y efluente), industriales, aguas superficiales y aguas subterráneas).

10. Aplicación de la metodología analítica de amplio *screening* desarrollada a muestras de agua, usando las aproximaciones *target* y *non-target*.

11. Aplicación de la metodología desarrollada por GC-TOF MS al análisis *target* y *non-target* de extractos de tejido adiposo humano previamente analizados mediante la técnica GC-(QqQ)MS/MS. Comparación con los datos obtenidos y evaluación de ventajas e inconvenientes. Estudio de la complementariedad de ambas técnicas.

12. Aplicación de la fuente de ionización química para determinar el ion molecular de compuestos detectados en modo *non-target*, en aquellos casos en los que los espectros de masas de ionización electrónica no se encuentran disponibles en librerías comerciales.

13. Propuesta de la composición elemental a partir de la masa exacta del ion molecular obtenido en el espectro de ionización química y de las intensidades relativas observadas en su distribución isotópica (especialmente de los heteroátomos cloro, bromo y azufre).

14. Confirmación de la composición elemental propuesta utilizando los fragmentos generados en la fuente de ionización química, así como los fragmentos del espectro de ionización electrónica.

15. Búsqueda en bases de datos de la composición elemental propuesta con objeto de asignar una estructura molecular. La confirmación definitiva requerirá la adquisición del patrón correspondiente y su inyección el en sistema cromatográfico.

16. Estudio de la ionización/fragmentación de plaguicidas seleccionados en la nueva fuente de ionización química a presión atmosférica (APCI) en combinación con GC-TOF MS. Optimización de las condiciones de trabajo que favorezcan la formación de ion molecular como pico base del espectro.

17. Uso de las condiciones establecidas para el desarrollo de un método rápido de *screening* de plaguicidas en muestras de alimentos y vegetales mediante GC-QTOF MS con APCI.

18. Uso de GC-QTOF MS con la nueva fuente de APCI como técnica de confirmación de los plaguicidas detectados en el *screening*.

19. Uso complementario de métodos basados en GC-MS y LC-MS con analizadores de triple cuadrupolo y TOF para fines de *screening*, detección y cuantificación de un elevado número de contaminantes, muy superior a las aproximaciones analíticas más convencionales, en lixiviados (muestras brutas y depuradas mediante ósmosis inversa) procedentes de una planta de tratamiento de residuos sólidos urbanos.

20. Uso combinado de las técnicas GC-TOF MS y UHPLC-QTOF MS para la investigación de las posibles causas de envenenamiento en un episodio de mortandad masiva de abejas. Aplicación de ambas técnicas al estudio de metabolitos de los principales compuestos encontrados en abejas.

Objetives and working plan

OBJECTIVES

The **main objective** of this Thesis is to explore the analytical capabilities of gas chromatography coupled to mass spectrometry in single (GC-MS) and tandem (GC-MS/MS) modes using triple quadrupole (QqQ) and time of flight (TOF) analyzers, for the investigation of organic contaminants, mainly in environmental and biological fields.

Firstly, the potential of GC-MS/MS with triple quadrupole analyzer is studied for the quantitative determination of organic contaminants in human adipose tissue and water, giving special attention to the confirmation potential of this technique. Secondly, the potential of GC-TOF MS is explored in both applied fields, as a confirmation technique and for wide-scope screening purposes following *target* and *non-target* strategies. Both techniques are compared from the quantitative and qualitative point of view, and their potential for wide-scope screening of organic contaminants is investigated in the above mentioned fields.

Specific objectives of the Thesis are the following:

1. Development and validation of analytical methodology based on GC-(QqQ)MS/MS for quantification and confirmation of priority organic contaminants at sub-pbb concentration levels in water, using solid phase extraction (SPE) as extraction/preconcentration technique.

2. Evaluation of negative chemical ionization mode in terms of sensitivity and selectivity as an alternative technique to electron ionization for selected priority contaminants.

3. Development and validation of analytical methodology for quantification of xenoestrogen compounds in human adipose tissue samples by GC-(QqQ)MS/MS, giving special attention to the correct identification and confirmation of detected compounds.

4. Investigation of the capabilities of GC-TOF MS for the screening and confirmation of organic contaminants, using *target* and *non-target* approaches.

5. Development of a methodical approach to deal with the huge amount of MS data generated in GC-TOF MS. Evaluation of different software tools available for this purpose.

6. Application of the developed methodology to the screening and confirmation of organic contaminants in environmental and waste water with a sample treatment based on solid-phase microextraction (SPME) followed by GC-TOF MS analysis.

7. Qualitative validation of the GC-TOF MS screening method, using water samples from different origin and characteristics spiked with a wide number of organic contaminants.

8. Investigation of the presence of anthropogenic organic contaminants in human adipose tissue samples by GC-TOF MS. Comparison with previous data obtained by GC-(QqQ)MS/MS analysis.

9. Study of the GC-TOF MS potential for elucidation of compounds which electron ionization MS spectrum is not available in commercial libraries. Combined used of electron ionization and chemical ionization sources for this purpose.

10. Study of the potential of the new atmospheric pressure chemical ionization source in combination with GC-TOF MS for pesticide residue analysis in food samples.

11. Combined used of GC-MS and LC-MS with triple quadrupole and TOF analyzers for the screening of a large number of contaminants, much higher to that considered in the conventional analytical approaches.

12. Study of the complementary use of GC-TOF MS and UHPLC-QTOF MS for investigation of *non-target* contaminants and metabolites in a real case of honeybees poisoning.

WORKING PLAN

Quantitative analysis method development has been carried out accordingly to the following general working plan.

1. Bibliographic revision on the state-of-the-art of the determination of priority organic contaminants in water. Selection of the organic contaminants to be studied based on their persistence, toxicity and emerging character.

2. In the case of human adipose tissue, bibliographic revisions about those compounds with medium or high persistence that show estrogenic character and tend to bioaccumulate in biological fatty samples.

3. Optimization of GC-(QqQ)MS/MS conditions by injection of reference standards. Study of electron ionization spectra and selection of the possible precursor ions for each analyte. Once the precursor ion is isolated in the first quadrupole, the collision energy is optimized to obtain characteristics product ions. Finally, selection of 2 SRM transitions per compound taking into account their sensitivity and selectivity.

4. Optimization of the chromatographic separation by injection of reference standards. Temperature gradient selection keeping in mind the inclusion of the maximum number of compounds in each chromatographic time window. Study of the *dwell time* parameter based on the peak width, number of transitions per window and required sensitivity.

5. Development of analytical methodology based on GC-(QqQ)MS/MS for water samples. Optimization of the solid phase extraction step (sample volume, elution solvent and eluting solvent volume) to obtain a high degree of multiresiduality with a simple sample treatment previous to injection in GC.

6. Study of the matrix effect in different water matrices by analyzing spiked samples and reference standards in solvent at the same concentration level.

7. Method validation based on the SANCO European Union guidelines, by performing recovery experiments at different concentration levels. Study of quality parameters, such as linearity, specificity, accuracy, precision, LODs and LOQs. Inclusion of *Q/q ratio* confirmation parameter in the validation process.

8. Application of the methodology developed to real water sample analysis, applying quality control criteria, followed by discussion of the results.

9. Development of analytical methodology based on GC-(QqQ)MS/MS for biological fatty samples. Study of the purification step by HPLC in normal phase previous to GC-MS analysis. Establishment of the elution patterns for lipids present in the human adipose tissue and for the OCs and OBrs compounds to be studied.

10. Method validation based on the SANCO European Union guidelines, by performing recovery experiments at different concentration levels. Study of quality parameters, such as linearity, specificity, accuracy and precision. Inclusion of *Q/q ratio* confirmation parameter in the validation process (it is worth to mention the difficulty to find blank samples for recovery experiments, due to the ubiquity of chlorinated contaminants. Thus, a pool of different samples, previously homogeneized and analyzed, will be used. The signals from the analyte peaks detected in the "blank" will be subtracted from the signals of the spiked samples used in the validation process).

11. Application of the methodology developed to real sample analysis, by applying the quality control criteria, followed by discussion of results.

Qualitative screening method development has been carried out accordingly to the following general working plan.

1. Reporting information about organic contaminants more frequently detected in natural and waste water. Selection of a large number of potential contaminants belonging to rather different physico-chemical families.

2. Selection of the strategy for TOF MS data processing. Comparison between manual and automatic processing using specific software for the *target* methods development.

3. Development of a *target* screening methodology by GC-TOF MS for those compounds for which reference standards are available, using the processing software Targetlynx. Development of processing methods that manage relevant information about the compound to allow its safe identification. The most relevant information is

based on its retention time, the elemental composition and the exact mass of the main fragments observed in the electron ionization spectrum together with their relative intensity.

4. Study of the critical parameters that affect the *target* processing method development: ion selection, extracted-ion mass window width, possible interferences coming from the lock mass, detector saturation problems, etc.

5. Development of GC-TOF MS *target* screening methodology based on the use of library spectra for those compounds for which reference standard is not available.

6. GC-TOF MS *non-target* methodology development based on the use of the deconvolution software Chromalynx, which automatically displays the accurate mass full spectrum and compares it with those available in commercial libraries (for EI), followed by mass error for evaluation the different fragments observed.

7. Application of sample extraction methods for screening purposes in water, pursuing little sample manipulation, in order to get the maximum multiresiduality, and adequate sensitivity to reach low analyte concentration levels (normally sub-microg/L). SPE and direct immersion SPME extraction techniques will be considered, for which the most important parameters will be optimized.

8. Establishment of strict criteria for identification and confirmation of compounds detected in the samples. These criteria will be based on retention time (and maximum allowed deviation), mass errors, number of ions necessary for a safe identification, quality of the information managed, and evaluation of the isotopic pattern.

9. Qualitative validation of the screening GC-TOF MS method for identification of organic contaminants in water in order to establish the lowest concentration levels for which the analytes can be correctly detected and identified. The validation is carried out with different water matrices, including urban waste water (influent and effluent), industrial waste water, surface and ground water.

10. Application of the developed wide-scope screening methodology to investigate the presence of organic contaminants in water samples, using *target* and *non-target* approaches.

11. Application of the GC-TOF MS developed methodology for *target* and *non-target* screening of human adipose tissue. Comparison with data previously obtained by GC-(QqQ)MS/MS analysis. Complementariness of both techniques.

12. Application of the chemical ionization source for the determination of the molecular ion of compounds detected in *non-target* way, for which mass spectrum is not available in commercial libraries.

13. Elemental composition proposal for the *unknown* compound using the exact mass of the molecular ion, obtained in the accurate mass chemical ionization spectrum, and the relative intensities observed from the isotopic pattern distribution (especially those obtained from heteroatoms like chlorine, bromine and sulphur).

14. Confirmation of the proposed elemental composition using the *m/z* fragments from both the chemical ionization and the electron ionization spectra.

15. Databases search of the elemental composition proposed in order to assign a molecular structure to the unknown. The definitive confirmation will require the acquisition of the reference standard and the injection in the chromatographic system.

16. Study of the ionization/fragmentation of selected pesticides in the new atmospheric pressure chemical ionization source (APCI) in combination with GC-TOF MS. Working conditions optimization in order to improve the formation of the molecular ion as the base peak of the spectrum.

17. Use of the established conditions for the development of a rapid GC-(APCI)TOF MS screening method for pesticides in food and vegetable samples.

18. Use of GC-QTOF MS with the new APCI source as a powerful confirmation technique for pesticides detected in the screening.

19. Complementary use of GC-MS and LC-MS with triple quadrupole and TOF analyzers for the screening, identification and quantification of a large amount of contaminants (much higher than in conventional analytical approaches) in treated (submitted to a reversed osmosis process) and raw leachate water from a municipal solid waste treatment plant

20. Combined used of GC-TOF MS and UHPLC-QTOF MS for the investigation of several cases of honeybee poisoning. Application of both techniques to investigate the presence of metabolites of the main compounds found in honeybees.

CAPÍTULO 1

Introducción general

ACOPLAMIENTO GC-MS

La cromatografía de gases (GC) constituye la técnica de separación más adecuada para la determinación de residuos de compuestos orgánicos apolares o con polaridad moderada, volátiles y semi-volátiles y térmicamente estables (1). Asímismo, la espectrometría de masas (MS) se ha convertido en una técnica muy poderosa en el campo del análisis de compuestos orgánicos gracias a su capacidad de dar información sobre la composición y estructura de los compuestos detectados. En este sentido, la cromatografía de gases acoplada a espectrometría de masas combina el poder de separación de GC con las excelentes características de detección e identificación de MS. Por ello, GC-MS es una herramienta analítica ampliamente utilizada en análisis ambiental, alimentario y toxicológico, en otros campos de interés, ya que permite desarrollar métodos de gran sensibilidad y poder de confirmación. La compatibilidad de fases entre GC y MS ha facilitado su acoplamiento, siendo posible conectar directamente la salida del sistema cromatográfico a la fuente de ionización.

Con respecto a las fuentes de ionización más comúnmente utilizadas en GC-MS destacan las de ionización electrónica (EI) e ionización química (CI), ambas caracterizadas por su modo de trabajo en condiciones de elevado vacío. Recientemente, se han llevado a cabo estudios basados en GC-MS con nuevos prototipos de interfase de

ionización química a presión atmosférica (APCI), ampliamente utilizadas en LC-MS, cuyo uso en GC-MS puede resultar muy interesante en determinadas aplicaciones.

En la fuente de EI un haz de electrones acelerados, procedentes de la emisión termoiónica desde un filamento de volframio o renio, bombardean las moléculas en estado gaseoso provocando su ionización y convirtiéndolas en un catión radical $M^{+\cdot}$. Como la energía generada es mucho mayor a la necesaria para arrancar un electrón, el exceso de energía se invierte en fragmentar la molécula en iones característicos, dando lugar a espectros de masa generalmente de elevada fragmentación. Este modo de ionización es universal y muy reproducible, por lo que permite generar librerías espectrales. Como desventaja se podría subrayar que la fragmentación, en ocasiones, es excesiva para determinadas aplicaciones, como más adelante veremos en esta Tesis.

En la fuente de CI, el haz de electrones ioniza al gas introducido (normalmente metano, isobutano o amoniaco). Este gas se fragmenta dando lugar a otras especies, que a su vez transfieren un protón (u otra molécula cargada) al analito convirtiéndolo en un ion. Es un proceso menos energético y se produce una menor fragmentación, por lo que, en general, permite determinar el peso molecular, pero, por el contrario, ofrece menos información estructural. Este modo de ionización aporta mayor selectividad y sensibilidad en el análisis de determinados compuestos; sin embargo, ha sido poco utilizado en métodos multirresiduo, principalmente por no ser una técnica de ionización universal. Se trata de una ionización menos reproducible que la EI, lo que dificulta la creación de librerías espectrales.

En lo referente a analizadores de masa, entre los más utilizados en el acoplamiento GC-MS destacan el analizador de filtro de iones cuadrupolar (Q), la trampa de iones cuadrupolar (IT) y el analizador de tiempo de vuelo (TOF). En lo referente a analizadores híbridos, podríamos destacar el triple cuadrupolo (QqQ). No obstante, cabe mencionar el analizador híbrido cuadrupolo-tiempo de vuelo (QTOF) por sus excelentes resultados en combinación con LC y su incipiente uso en GC.

El analizador de filtro de iones cuadrupolar, o cuadrupolo, ha sido tradicionalmente uno de los más populares por su relativo bajo coste, fácil mantenimiento y elevada aplicabilidad. Está formado por cuatro barras alineadas paralelamente entre sí y equidistantes una distancia r_0 de un eje central imaginario. Sobre las barras del cuadrupolo se aplican (dos a dos) voltajes de corriente continua

(DC) y de radiofrecuencia (RF). A un valor específico de DC y RF, sólo los iones con una particular relación *m/z* siguen una trayectoria estable a través de las barras y alcanzan el detector. Variando los voltajes RF y DC de manera sistemática se puede realizar un barrido espectral. Debido a sus características intrínsecas permite trabajar en modo barrido de iones completo o *full scan*, ofreciendo espectros clásicos y reproducibles de resolución de masa unidad. Sin embargo, la necesidad de determinar niveles de concentración tan bajos como los actualmente requeridos, obliga a trabajar en modo *Selected Ion Monitoring* (SIM), donde solo se filtran los iones de unas relaciones *m/z* concretas, de manera que se aumenta la sensibilidad y selectividad del análisis, pero se pierde la información cualitativa obtenida a partir del espectro en modo *full scan*. Además, la respuesta lineal a la concentración es muy buena, por lo que son muy utilizados para llevar a cabo análisis cuantitativos.

El analizador de trampa de iones cuadrupolar está formado por tres electrodos hiperbólicos: uno con forma de anillo y dos electrodos colectores. Las moléculas del compuesto de interés son ionizadas (en el interior o exterior de la trampa) y fragmentadas en la trampa y los iones generados son retenidos o expulsados de la misma en función de su relación *m/z*, ya que, para unos voltajes de RF y DC dados, sólo determinadas relaciones *m/z* describirán trayectorias tales que permitan que el ion se encuentre confinado en la trampa. Una vez que los iones son expulsados de la trampa pasan al detector. Este analizador destaca por su elevada sensibilidad en modo *full scan*, si se compara con el analizador de cuadrupolo, así como por la posibilidad de obtener espectros de masas de iones productos (MS/MS y MS^n), lo que tiene especial interés en la elucidación estructural.

El analizador de tiempo de vuelo separa los iones en función del tiempo que tardan en atravesar un tubo de vuelo de longitud conocida, el cual depende de la relación *m/z,* ya que aquellos más ligeros llegarán al detector antes que los más pesados. La introducción de espejos iónicos o reflectrón y, más recientemente, de doble reflectrón ha incrementado notablemente la resolución alcanzada por este diseño, llegando a valores típicos de 5.000-20.000 FWHM, lo cual repercute en la posibilidad de obtener medidas con masa exacta de los iones detectados. Sin embargo, debido a la variabilidad que presenta este tipo de instrumentos con respecto a parámetros como, por ejemplo, la temperatura, es necesario, aparte de la calibración diaria del eje de masas, que el equipo se encuentre en constante calibración. Esto se consigue mediante

la adición de un compuesto, cuyas masas exactas de sus fragmentos en la fuente son conocidas, simultáneamente a la entrada de la muestra en la fuente de ionización. Su único modo de trabajo es *full scan*. Aunque investigaciones recientes están aportando mejoras a este respecto, su limitado rango de linealidad lo ha hecho menos adecuado para el análisis cuantitativo hasta el momento.

En lo referente a analizadores híbridos, el triple cuadrupolo consiste en "tres cuadrupolos" conectados en serie. Los cuadrupolos Q1 y Q3 funcionan como dos analizadores conectados en serie. El "cuadrupolo" Q2 (normalmente es un hexapolo u octapolo, para favorecer la transmisión de los iones), o celda de colisión, se sitúa en medio de Q1 y Q3. Se pueden utilizar distintos modos de trabajo en función del objetivo final del análisis. Así, en modo MS se puede trabajar tanto *full scan* como en SIM. Cuando se trabaja en modo tandem MS (MS/MS) se pueden realizar barridos de iones producto (*product ion scan*), de iones precursores (*precursor ion scan*), de pérdidas neutras (*neutral loss scan*), o bien monitorizar una transición concreta (*Selected Reaction Monitoring*, SRM), aumentando la sensibilidad y la selectividad en la detección. La fragmentación se produce en la celda de colisión, por colisión del ion seleccionado en el primer cuadrupolo (ion precursor) con moléculas de gas inerte (generalmente argón). Este proceso recibe el nombre de disociación inducida por colisión (CID) y produce la fragmentación del ion en función de la estructura del analito, dando lugar a iones producto. A continuación, se produce la transmisión de los iones desde la celda de colisión al tercer cuadrupolo donde se realiza un barrido de los iones o se selecciona uno de éstos con el fin de obtener una transición selectiva del analito, en función del objetivo del análisis. Las principales características de los triples cuadrupolos son su elevada sensibilidad en modo SRM y su amplio rango lineal, que los hacen ideales para el análisis cuantitativo de contaminantes orgánicos a niveles traza. Sin embargo, su aplicación en el campo cualitativo es limitada debido a su bajo poder de resolución (del orden de 1 Da), lo que se traduce en medidas de masa nominal y baja sensibilidad en modo *full scan*.

En el caso del QTOF, la adquisición en modo MS/MS se realiza con dos analizadores distintos, un cuadrupolo y un TOF, por lo que constituye un instrumento híbrido. De esta manera, una vez fragmentado el ion precursor en la celda de colisión, todos los iones producto son medidos mediante el TOF (*product ion scan*) obteniendo el espectro de iones producto con elevada sensibilidad y exactitud de masa. Estas

características lo hacen ideal para el análisis cualitativo, tanto para la búsqueda de moléculas desconocidas como para la elucidación estructural. Sin embargo, su aplicación en el campo cuantitativo, y especialmente en el análisis de trazas, es limitada debido a su bajo rango lineal (apenas dos órdenes de magnitud) y su menor sensibilidad con respecto a instrumentos de triple cuadrupolo en modo SRM.

En la presente Tesis Doctoral se han utilizado básicamente dos analizadores, el triple cuadrupolo y el analizador TOF. Adicionalmente, se han realizado experiencias preliminares con el analizador híbrido QTOF y un prototipo de interfase APCI.

ANALIZADOR DE TRIPLE CUADRUPOLO

A continuación, se discuten brevemente aplicaciones seleccionadas de las técnicas descritas anteriormente en diferentes campos de interés. El acoplamiento GC-MS con analizador de cuadrupolo en modo SIM ha sido en las últimas décadas ampliamente utilizado en la determinación de microcontaminantes orgánicos en muestras medioambientales, biológicas y alimentos, pero presenta limitaciones en el análisis de muestras complejas, sobretodo cuando los niveles de concentración son muy bajos (sub-ppb), donde la presencia de interferencias de matriz se hace más patente (2-11). A este respecto, la espectrometría de masas en tandem (MS/MS) aporta un valor añadido por su capacidad de obtener el espectro de iones producto formados a partir de un ion precursor seleccionado. Se consigue así una disminución de interferencias y una mayor especificidad en el análisis, aumentando la selectividad y sensibilidad, y proporcionando un mayor grado de fiabilidad en la identificación de un analito. Mediante la adecuada selección de los iones precursores y de los iones producto se pueden eliminar interferencias isobáricas y resolver coeluciones espectrales, por lo que disminuye la necesidad de disponer de una resolución cromatográfica perfecta entre analitos y componentes de la matriz, incluso a niveles traza en matrices complejas. Estas características permiten desarrollar métodos de elevada sensibilidad, selectividad y nivel de multirresidualidad, difícilmente alcanzables por la espectrometría de masas simple, y reducir considerablemente algunas etapas tediosas de purificación en determinadas matrices. Por otro lado, según los criterios establecidos por las guías europeas en vigor (12, 13), se requiere que los métodos analíticos aporten información estructural sobre el compuesto detectado para una identificación y confirmación

correcta de la identidad. En este sentido, los métodos basados en GC-MS/MS llevan a cabo simultáneamente la identificación, confirmación y cuantificación de los compuestos, convirtiéndola en una técnica muy adecuada para el análisis de un amplio número de compuestos *target* a niveles traza.

La espectrometría de masas en tandem (MS/MS), tal como se ha indicado, implica una serie de procesos que ocurren de manera secuencial: ionización de las moléculas en la fuente de ionización, selección del ion precursor, fragmentación del ion precursor seleccionado por colisión con moléculas de gas inerte (generalmente argón o helio) y, finalmente, análisis de los fragmentos producidos (iones producto). Estas etapas pueden llevarse a cabo en el tiempo (*tandem-in-time*) o en el espacio (*tandem-in-space*). En los instrumentos *tandem-in-time*, como por ejemplo la trampa de iones, estos procesos ocurren secuencialmente en el mismo espacio físico, y por tanto, separados en el tiempo. Por el contrario, en los instrumentos *tandem-in-space* se requiere la presencia de dos analizadores dispuestos en serie, como ocurre en un triple cuadrupolo, produciéndose las etapas secuencialmente en regiones separadas del instrumento, y por tanto, diferenciadas en el espacio. Ambos instrumentos presentan ventajas y desventajas desde el punto de vista de la determinación de microcontaminantes orgánicos en muestras medioambientales, biológicas y alimentos.

Los analizadores de trampa de iones han sido muy utilizados en los últimos años en el campo medioambiental, biológico y alimentario, y entre sus principales ventajas se podría destacar su elevada sensibilidad en modo *full scan*, su capacidad para trabajar en modo MS^n, así como su capacidad de proporcionar el espectro de iones producto (*product ion scan*), lo cual es especialmente conveniente para la confirmación de positivos en muestras complejas (5, 14-18). Sin embargo, presenta algunas limitaciones entre las que podríamos destacar su dificultad de detectar iones producto con un valor de *m/z* menor al 30 % del valor de *m/z* del ion precursor y su vulnerabilidad a efectos espacio-carga que puede degradar la calidad del espectro de masas, incluyendo exactitud de masa y resolución. Esto puede ser un problema en el análisis de componentes a niveles traza en matrices complejas, ya que la trampa se llena de muchos iones de la matriz. Además, el modo de trabajo *product ion scan* limita el número de compuestos que pueden ser determinados simultáneamente, lo cual obliga a prestar especial atención a la separación cromatográfica, dando lugar a

métodos cromatográficos largos, muchas veces inapropiados para el análisis multirresidual en rutina.

En cambio, el analizador de triple cuadrupolo presenta una mayor versatilidad, ya que puede operar en los cuatro modos de trabajo MS/MS mencionados anteriormente: *product ion scan, precursor ion scan, neutral loss* y SRM. Este último modo de trabajo es más rápido que el modo *product ion scan* (utilizado en las trampas de iones) y permite la medida simultánea de un mayor número de transiciones debido a su mayor velocidad de barrido y a que únicamente monitoriza unos pocos iones producto para cada compuesto. Además, el modo SRM es más sensible y permite seleccionar transiciones selectivas haciéndolo más adecuado para el análisis *target* y cuantitativo de contaminantes orgánicos a nivel traza en matrices complejas. Así pues, la opción MS/MS utilizando equipos de triple cuadrupolo parece la más selectiva y sensible para la cuantificación y confirmación, especialmente cuando el número de compuestos a estudiar es elevado y la complejidad de la matriz aumenta.

Hasta el momento han sido varios los campos de aplicación de la técnica GC-MS/MS con analizador de triple cuadrupolo. Cabe destacar su elevado uso en el campo de la seguridad alimentaria en el que se han llevado a cabo un importante número de trabajos relacionados con el análisis multirresidual de contaminantes orgánicos, entre los que destacan plaguicidas (organoclorados, organofosforados, triazinas, carbamatos, cloroacetanilidas, etc), hidrocarburos policíclicos aromáticos (PAHs) y bifenilos policlorados (PCBs) en diversas matrices como frutas, vegetales, alimentos infantiles, aceites, productos de origen cárnico, bebidas alcohólicas, cereales, pienso, tabaco, etc (19-25). Esta técnica ha supuesto un gran avance en este campo ya que ha permitido reducir considerablemente determinadas etapas de tratamiento de muestra y purificación junto con el tiempo de análisis cromatográfico en la mayoría de los casos, lo cual resulta crucial en los laboratorios de control donde el número de muestras diarias a analizar, así como los contaminantes a controlar, son muy elevados y la respuesta analítica ha de ser rápida.

Así pues, desde el punto de vista del análisis multirresidual en productos alimentarios, la técnica GC-MS/MS ha permitido desarrollar estrategias analíticas capaces de determinar desde pocas decenas hasta un total de 200-300 compuestos en un análisis. La mayoría de los métodos contemplan la adquisición simultánea de 2 (y a

veces 3) transiciones para cada compuesto, de manera que la detección, cuantificación y confirmación de la identidad del analito se realiza en una única inyección. Otra opción ha sido la de elaborar un primer método con fines de *screening*, con una única transición por compuesto con la finalidad de detectar posibles positivos por encima de un nivel de concentración establecido. En segundo lugar, las muestras con potenciales positivos se reinyectan con un método que adquiere 2 ó 3 transiciones por compuesto con fines confirmativos. Esta segunda estrategia permite reducir los tiempos de análisis, pero implica la inyección por duplicado de aquellas muestras que presenten al menos un posible positivo. En la elaboración de estos métodos multirresiduales en modo SRM, no es extraña la monitorización de algún compuesto en modo SIM, ya que algunos compuestos presentan respuestas más sensibles y/o selectivas en este modo. En cualquiera de las estrategias seguidas, los límites de detección alcanzados han sido en general satisfactorios de acuerdo a los exigidos en este campo y los rangos de linealidad adecuados para una cuantificación correcta de manera rápida y robusta en matrices de diferente complejidad.

De todas maneras, en matrices vegetales muy complejas, el efecto matriz todavía es un problema y ni tan solo el uso de MS/MS en modo SRM es capaz de eliminar las interferencias de matriz que dan lugar a un aumento o disminución en la respuesta cromatográfica, pues en algunos casos dicha variación en la señal es consecuencia del sistema cromatográfico (inyector y columna) y no del sistema de detección (26). Así pues, para llevar a cabo una correcta cuantificación de los compuestos detectados, es necesario, en ocasiones, recurrir a etapas de dilución, calibrados preparados en matriz, etapas previas de purificación, uso de superficies inertes en el sistema GC, utilización de otras técnicas de inyección, o aplicación de factores de corrección, entre otros (26, 27).

Aunque menos numerosos, también se han realizado trabajos en el campo ambiental mediante GC-MS/MS con triple cuadrupolo, sobretodo en lo relativo a la determinación de PAHs, PCBs y plaguicidas organoclorados en muestras de suelo y aire (28-30). Con respecto al análisis en muestras biológicas, el acoplamiento GC-MS/MS con triple cuadrupolo también ha mostrado resultados satisfactorios en aplicaciones como el análisis de canabinoides y algunos fármacos en sangre y orina (31, 32), así como drogas de abuso en muestras de pelo (33). En dichas aplicaciones, el número de analitos a determinar simultáneamente no suele ser tan elevado como en seguridad

alimentaria, pero en la mayoría de ellas también destacan las mejores características analíticas de los métodos GC-MS/MS con triple cuadrupolo respecto a los métodos previos basados en GC-MS en modo SIM y GC-MS/MS con trampas de iones. Generalmente se utilizan calibrados preparados en matriz para corregir los efectos matriz. Al igual que en análisis alimentario la ionización por EI es la más utilizada, aunque ha habido algunas aplicaciones concretas, en el análisis de fármacos y drogas, en donde, por su carácter electronegativo, se han obtenido buenos resultados usando ionización quimica en modo negativo (32, 34).

Finalmente, cabe comentar que, a pesar de las ventajas que ha demostrado la técnica GC-MS/MS con triple cuadrupolo en los diferentes trabajos publicados, su uso todavía no está muy extendido en los laboratorio analíticos. A excepción del análisis de alimentos, en otros campos como el biológico y el ambiental se han publicado escasas aplicaciones y muy específicas. Además de su mayor coste, en comparación con otras técnicas alternativas, algunos autores justifican estas limitaciones por la ausencia de una técnica de ionización suave para GC, tan universal como hasta ahora ha resultado ser la fuente de EI, que sea capaz de proporcionar eficientemente iones moleculares de mayor abundancia, factibles de ser utilizados como iones precursores a la hora de aplicar la espectrometría de masas en tandem (7).

ANALIZADOR DE TIEMPO DE VUELO

Los analizadores TOF MS se caracterizan por su elevada sensibilidad en modo de adquisición de espectro de iones completo y por su elevada resolución espectral dando lugar a medidas de masa con elevada exactitud. Estas características reducen considerablemente el número de posibles composiciones elementales que puedan dar la masa experimental medida, al tiempo que confieren excelente selectividad por la capacidad de extraer iones en un rango muy estrecho de masa. Estas características resultan muy ventajosas en el desarrollo de métodos de *screening* por GC-TOF MS resultando también muy atractivos por la elucidación de compuestos *non-target*.

El acoplamiento GC-TOF MS se revela como una poderosa técnica que ofrece nuevas perspectivas en la determinación de compuestos orgánicos a nivel de traza en matrices de elevada complejidad. Estos equipos presentan un elevado potencial en la identificación de contaminantes orgánicos volátiles y semi-volátiles y permiten afrontar

problemas analíticos hasta ahora restringidos a la utilización de los grandes equipos basados en sector magnético.

En lo referente a características, campos de aplicación y perspectivas de la técnica GC-TOF MS, se adjunta el **artículo científico 1**.

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Artículo científico 1

Trends in Analytical Chemistry (submitted)

GAS CHROMATOGRAPHY / HIGH-RESOLUTION TIME-OF-FLIGHT MASS SPECTROMETRY: AN ADVANCED ANALYTICAL TOOL TO INVESTIGATE THE PRESENCE OF ORGANIC COMPOUNDS AT TRACE LEVELS IN ENVIRONMENTAL, FOOD SAFETY AND TOXICOLOGY FIELDS

Félix Hernández*, Tania Portolés, Elena Pitarch, Francisco J. López

Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain,

ABSTRACT

Gas chromatography coupled to high-resolution time-of flight mass spectrometry (GC-HR TOFMS) is a powerful analytical technique with excellent capabilities due to its high sensitivity in full spectrum acquisition mode together with its resolving power and accurate mass measurements. These features make this technique very attractive in qualitative analysis, especially for wide-scope screening of a large number of organic contaminants and residues at trace levels.

The availability of full MS spectrum allows data processing, in principle, of an unlimited number of compounds in the samples, as no analyte-specific information is required before the injection. Additionally, as all data remain available, a retrospective analysis is always possible without the need to reinject the sample. This definitely represents an important advantage of full spectrum techniques. Despite these advantages, GC-HRTOF MS has scarcely been applied to date, so promising results are expected in different applied fields in coming years.

In this paper, a detailed discussion on the characteristics and potentiality of GC-HRTOF MS is made. Different analytical strategies are described from wide-scope target screening to the investigation of unknowns, in biological, environmental and food-safety fields. Recent instrumental developments, such as high-speed Analog-to-Digital Converter or soft ionization sources, and advances in software for processing the huge amount of data available, open new perspectives, making GC-TOF MS one of the

most promising techniques to investigate the presence of organic compounds in different applied fields.

KEYWORDS

Gas chromatography, high resolution time-of-flight, mass spectrometry, target and non-target screening, food safety, environment, biological samples, drugs of abuse

1. INTRODUCTION

Modern analytical methodologies to face with a large number of organic contaminants are currently required in many applied fields, such as environment, toxicology, and food safety, where an increasing number of potentially dangerous compounds may be present in samples. Most analytical methods applied until now are focused on quantification measurement of target analytes, and their scope rarely exceeds more than 100 compounds. However, target analysis does not normally provide a complete overview of the organic pollution pattern, and consequently the need arises for developing screening methods able of detecting, identifying, and even quantifying a large number of organic contaminants and residues. This would allow discriminating samples with no detectable residue from those with residues at a certain concentration level. Then the analytical efforts could be focused on quantitation and, if necessary, confirmation of presumptive positive samples [1].

Gas chromatography (GC) coupled to mass spectrometry (MS) is the technique of choice for many priority organic compounds, typically (semi)volatile and/or of low polarity. The large number of GC-MS applications reported [2-6] is the result of the efficient GC separation, together with the spectral information and satisfactory sensitivity provided by MS. Until recently, low-resolution mass spectrometric detectors working in selected ion monitoring (SIM) have been normally used [6-8] due to its relatively low cost, compactness and simplicity. The widely used single quadrupole analyzers show limitations in analysis of complex matrices. Ion trap (ITD) and, more recently, triple quadrupole analyzers (QqQ) allow operation in MS/MS mode which achieves unquestionable sensitivity and selectivity, as demonstrated in an increasing number of applications reported in different fields [9-19]. Using these MS/MS

configurations, identification and quantification of pre-defined contaminants (those for which MS data have been acquired) can be successfully carried out at very low concentrations. However, other relevant compounds that might be present in the samples would not be detected under the optimized MS conditions. In many applied fields, like environmental pollution, toxicology, or food-safety, full-spectrum acquisition and sensitive methods would be welcome as they would facilitate the detection and identification of a large number of compounds. Unfortunately, sensitivity of the above mentioned MS/MS analyzers in scan mode is not sufficient to this aim. In addition, their nominal mass resolving power is a severe limitation for identification and elucidation purposes.

Recent progress in instrumentation design (mainly optics), and the use of fast recording electronics together with improvements in signal-processing has led to renaissance of the time-of-flight mass analyzer (TOF MS) for investigation of organic compounds in complex matrices [20]. High-resolution (HR)TOF MS (~7000 FWHM) are instruments capable of achieving a mass accuracy as low as 5 ppm, which allows nominally isobaric ions to be mass resolved. They present moderate acquisition speed (maximum acquisition rate 10 s^{-1}) and linearity range of around three orders of magnitude. Unit-resolution is another type of TOF instruments, with high acquisition speed (maximum acquisition rate of 500 s^{-1}) and linearity of around four orders of magnitude. High speed (HS)TOF MS are suitable for detection of very narrow chromatographic peaks generated by fast and ultra-fast GC, or by GC×GC, which has been the main application of this analyzer. [20].

TOF MS provides high sensitivity in full spectrum acquisition mode when compared to conventional scanning instruments, principally due to its high mass analyzer efficiency (25% compared to ~0.1% in a quadrupole). In the case of HRTOF MS, the sensitive full spectrum acquisition is complemented with mass accuracy, which gives it extraordinary potential for qualitative purposes. GC-TOF MS is able to screen hundreds of compounds at high sensitivity within one run [21]. In addition, data can be acquired and reprocessed without needing prior knowledge of the presence of certain compounds, i.e. no analyte-specific information is required. Of equal importance, the presence of any other compounds of interest might be investigated at any time by simply reprocessing the data. Therefore, TOF MS characteristics fit perfectly with the requirements for wide-scope screening methods.

The high mass resolving power and mass accuracy provided by GC-HRTOF MS make it feasible to obtain Extracted Ion Chromatograms using narrow mass windows (nw-XICs), in this way excluding a large proportion of the chemical background and isobaric interferences, significantly improving signal-to-noise ratios. Under these conditions, the identification of the analyte is vastly improved in comparison to other conventional analyzers. Figure 1 shows the effect of reducing the mass window in a surface water sample where the fungicide thiabendazole was detected. In contrast to 1 Da or 0.5 Da mass window, reducing it to 0.02 Da led to only the thiabendazole peak being present with an improvement of the limit of detection. It is worth noting that there is a limit to the narrowness of the mass windows, especially at low analyte concentrations in complex matrices. This could result in underestimation of the peak area, or even to loss of the analyte peak due to mass accuracy deterioration at low ion intensity [21-23].

Despite the excellent features of GC-HRTOF MS, this technique has seldom been explored for the determination of organic contaminants up until now, as recently highlighted [21]. Almost all applications reported deal with the determination of persistent and other priority GC-amenable pollutants in environmental [23-25] and biological samples [7, 26, 27]. Accurate mass data generated by GC-HRTOF MS have been also used in a few applications of doping and drug control [28, 29] or in elucidation processes such us the identification of impurities generated in organic synthesis or in flavour research fields [30, 31].

The limited dynamic range of HRTOF MS instruments reduces their potential for quantitative analysis. For this reason, most applications related to quantitative GC-TOF MS are based on the use of high-speed TOF MS analyzers [32, 33]. Only a few quantitative applications have been reported in the screening of pesticides, polybrominated diphenyl ethers (PBDEs) and polycyclic binephyls (PCBs) contaminants in food and environmental samples [7, 24, 34-36]. Lastest advances in instrumentation design have included inbuilt Dynamic Range Enhancement (DRE), making quantification easier in HRTOF MS instruments [20, 36].

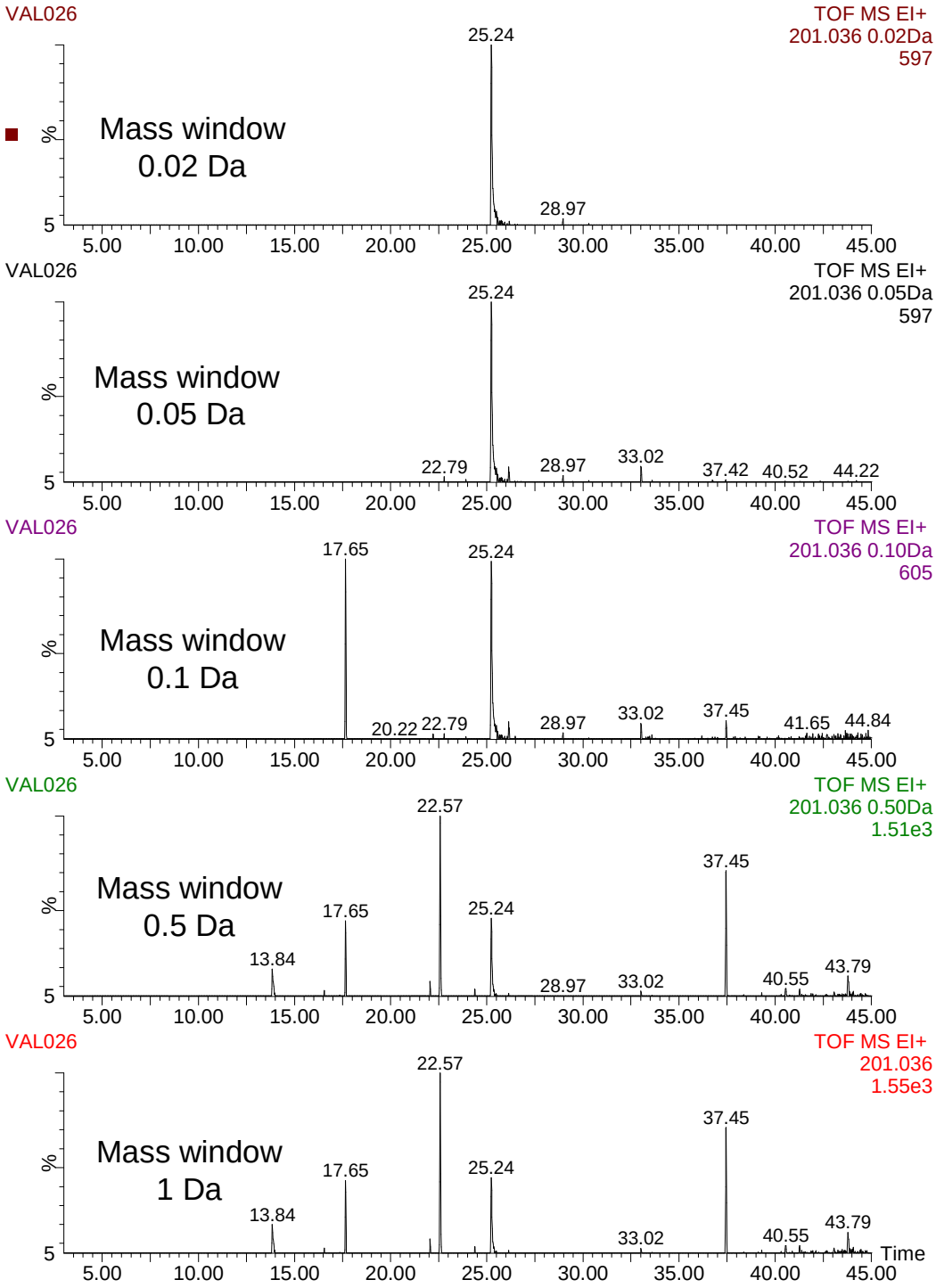


Figure 1. Effect of the mass window width on thiabendazole detection in a surface water sample when applying GC-TOF MS target screening.

The characteristics of GC-HRTOF MS also make this technique appropriate for elucidation of unknowns. The discovery of non-target analytes at trace level is a challenging task because the electron ionization (EI) spectrum does not yield a conclusive match with a database record in many cases. This occurs from being unable to assess the molecular mass due to the abundant fragmentation resulting from this hard ionization source. The use of soft ionization modes, such as chemical ionization (CI), negative ion chemical ionization (NICI), field ionization (FI) or atmospheric pressure ionization sources (APCI) seems attractive for unknowns' research [37].

The aim of this work is to illustrate the potential of GC-HRTOF MS in different applied fields, mainly environmental pollution, food-safety and toxicology and focused on the usefulness of this technique for screening purposes. Some additional applications on elucidation of unknowns are also presented and discussed.

2. GC-TOF MS SCREENING APPLICATIONS

GC-TOF MS is especially suited to screening purposes. Different strategies can be followed to manage the accurate mass information provided by TOF instruments, depending on the availability of information on the compounds to be searched, i.e. target and non-target screening [24, 26, 27] (see Figure 2).

Advanced processing software is necessary to manage the large amount of data available and to avoid otherwise highly time-consuming processes. The development of processing methods capable of simplifying the review data step is laborious and should be performed with special care for a successful analysis.

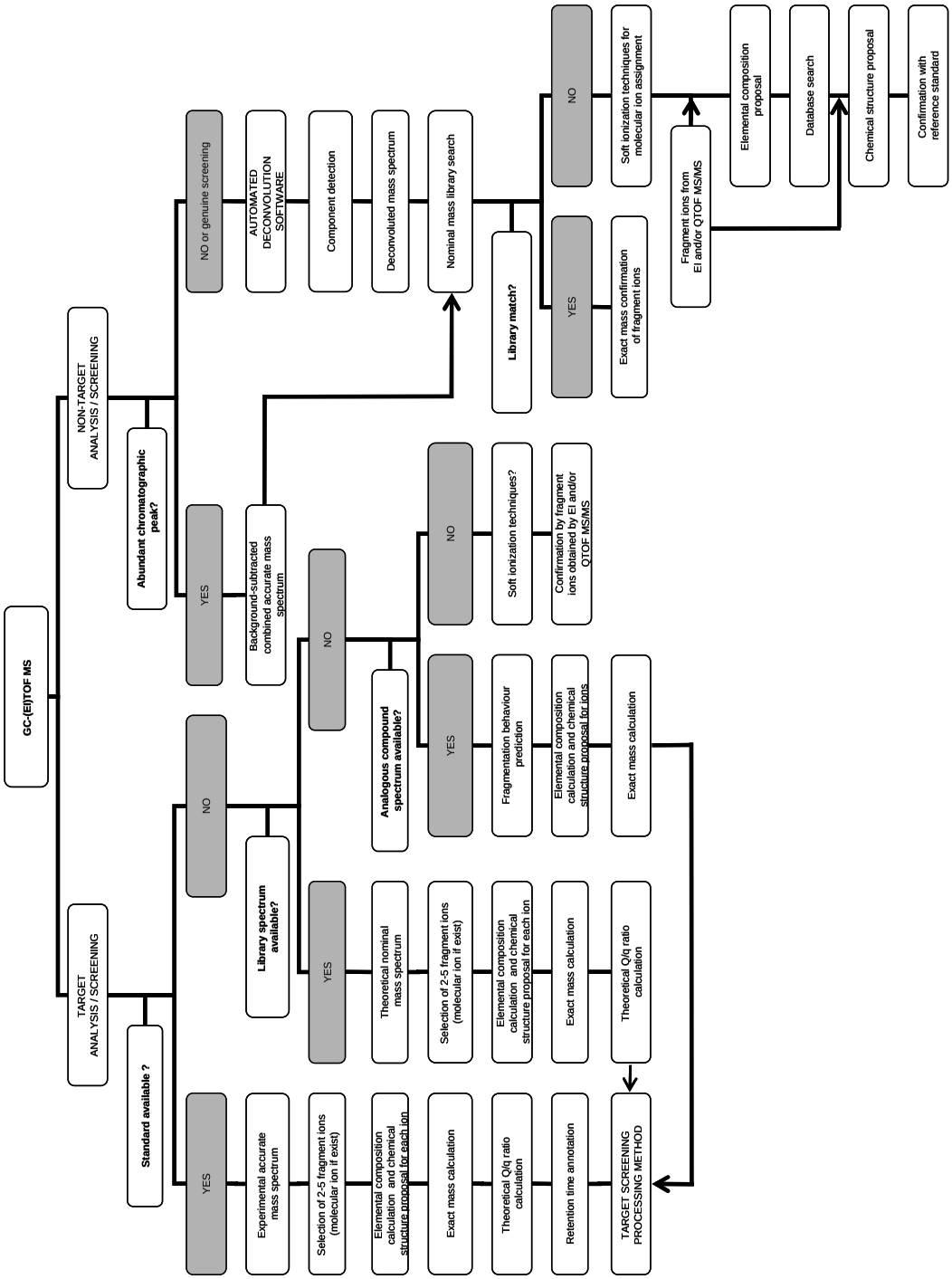


Figure 2. Different GC-TOF MS strategies for screening purposes

2.1. GC-TOF MS target screening

In a target method, the number of compounds investigated is always limited. In most methods reported, analyte-specific information is required before injecting a sample (pre-target analysis); so, other potentially harmful compounds that might be present would not be detected. However, accurate mass full spectrum data generated by GC-TOF MS remain available over time, even when dealing with target analysis. Thus, a retrospective analysis is always feasible and, in principle, any compound could be investigated a posteriori (post-target analysis), provided such residues have passed the sample preparation, chromatographic separations and ionization process with sufficient efficiency [38], which clearly represents an important advantage of full scan MS.

To obtain maximum information from full-spectrum data a processing method capable of managing all data in a user friendly manner becomes necessary. It should contain qualitative information on target analytes to facilitate their correct identification, i.e. accurate masses of the most abundant ions to perform the corresponding nw-XICs. The processing method is highly dependent on the raw data generated by the instrument, which is directly related to the ionisation mode. EI is the most popular ionization source used in GC-TOF MS. It achieves highly reproducible spectra, facilitating the use of EI mass spectral libraries for identification of unknowns. However, EI produces extensive fragmentation of the molecule, and the prediction of the most abundant fragment ions at accurate mass can be difficult when reference standards are not available. Oppositely, LC-TOF MS with electrospray ionization source (ESI) typically generates the (de)protonated molecule as the base peak of the spectrum. Monitoring this ion is usually the first step of the identification strategy (except for some compounds that exclusively show adducts formation). As a consequence, the strategy applied for target screening by GC-TOF MS is rather different to that used in LC-TOF MS. Development of the MS processing method is an important task in target analysis. This method should be easily updated with as many compounds as desired. The strategy applied highly depends on the availability of reference standards as discussed in the following paragraphs.

Availability of reference standards

The most efficient and simplest way to develop a target method when reference standards are available is to inject them under the selected experimental conditions. In this way, the most relevant information will be available: retention time, exact mass of main ions, and Q/q_i ratios, where Q is the most abundant ion and q_i the rest of the ions selected. Between 2 and 5 ions (molecular ion included, if available) are normally selected for each analyte from its EI accurate mass spectrum. Accurate masses are of great importance in this process, as they can be used for elemental composition calculation and chemical structure proposal. The presence of at least two ions and the attainment of their Q/q_i intensity ratio within specified tolerances are normally required for the reliable confirmation of target analytes [23, 39]. The use of nw-XICs (e.g. 0.02 Da) notably improves selectivity and sensitivity of the screening method.

This strategy has been applied for screening of organic pollutants in water [23, 24]. Combination of SPME for sample extraction and GC-TOF MS allowed the investigation of 60 target pollutants, including pesticides, octyl/nonyl phenols, pentachlorobenzene and PAHs in surface, ground and wastewater. Making a retrospective analysis, full-scan accurate MS data were examined to test the presence of PBDEs and some additional fungicides in the samples.

In a subsequent step, the number of target contaminants was widened and a SPE treatment (C_{18} cartridges) was used instead of SPME. Around 150 organic contaminants from different chemical families were investigated increasing the number of pesticides and including several metabolites. The screening was applied for qualitative purposes and it was validated [40] in surface, ground and wastewater samples spiked with all target analytes at different sub-ppb levels (between 0.02 and 1 µg/L). All samples were analyzed by sextuplicate. Most of the compounds were correctly identified at 1µg/L in all samples analyzed. Identification at 0.1 µg/L was more problematic for some compounds, especially in complex-matrix samples like influent wastewater. On the contrary, many contaminants could be identified properly at the lowest level tested, i.e. 0.02 µg/L, in cleaner matrices (ground and surface water, effluent wastewater). The screening procedure was applied to different types of water samples and allowed detecting several PAHs (naphthalene and pyrene), triazine

herbicides (simazine, terbumeton, terbuthylazine and terbutryn), organophosphorus insecticides (malathion, chlorpyriphos, diazinon), among others. All positive findings could be correctly identified following the strict established criteria based on the use of accurate mass measurements and Q/q ratios accomplishment.

GC-TOF MS has been also applied for target screening and confirmation of anthropogenic contaminants in human breast adipose tissues [27], for a list of 125 compounds, which included persistent halogen pollutants such as organochlorine (OC) pesticides, PCBs, and PBDEs, as well as polycyclic aromatic hydrocarbons (PAHs), alkylphenols, and a notable number of pesticides (fungicides, herbicides and insecticides). Target pollutants were investigated by evaluating the presence of up to five representative ions, all measured at accurate mass (0.02 Da mass window), and experimental Q/q_i ratios were compared with reference standards for confirmation. This strategy allowed the detection of several compounds (HCB, β-HCH, p,p'-DDE, trans-nonachlor, and some PCBs). Oppositely to GC-MS/MS with triple quadrupole, full spectrum acquisition in TOF MS allowed widening the screening to around 100 additional compounds not included in a previous list of 30 target analytes investigated by GC-MS/MS QqQ [14]. The result was the detection of selected contaminants, like some PAHs and other PCB congeners, that could not be detected by QqQ due to the specific-analyte information required in that case.

In other works an screening GC-TOF MS method was applied for anabolic steroids and other representative prohibited substances in human urine [28]. Specifications for matrix interferences, carryover and specificity were met, after the analysis of the derivatized samples. The low dynamic range was proved to be a limiting feature of this technique, since it influenced the chromatographic peak shape and height in the case of coelution of the peak of interest with another abundant peak. The presence of only one ion was used for identification and confirmation purposes of target analytes using narrow mass window. The combined use of LC-TOF MS and GC-TOF MS was proposed for the steroid designer drugs screening in a blind but accurate and generic way.

GC-TOF MS was demonstrated to be a challenging solution in analysis of multiple pesticide residues in baby food, typically at 0.01 mg/kg level, under quality control requirements of SANCO/207/3131. The presence of only one ion was required

for the identification and quantification of target analytes in a narrow mass window of 0.02 Da [34].

Quantification of approximately one hundred pesticides and transformation products at 0.01 and 0.1 mg/kg in fruit-based baby food and fruits and vegetables could be satisfactorily made by GC-TOF MS. The presence of three ions was used for identification and confirmation purposes of target analytes. The use of DRE improved mass accuracy across the tested concentration range, thus enhancing detection and quantification at higher analyte concentrations. The TOF instrument also led to an improvement in selectivity by narrowing the m/z window, giving better separation of the target pesticides from coeluting compounds, which is very important when analysing complex matrices [36].

A target screening method, using at least three accurate masses for each pesticide, was applied to the determination of 170 organohalogen and organophosphorus pesticides, isomers, and metabolites in dried ground ginseng root. It allowed detecting several pesticides in the samples in the range of 1-460 ng/g [7].

The necessity to run all standards is a limiting factor and impractical in some cases, especially when a wide-scope screening is pursued, as not all standards are always available in the laboratory when a large number of compounds is investigated. In those cases, it would be desirable to perform a (post-target) screening for detection of analytes without the need to inject standards. In a subsequent step (when sufficient, solid evidence exist on the presence of such compound in the samples) unequivocal confirmation of suspect positives could be performed by acquiring the reference standard. GC-TOF MS screening without reference standards can be performed efficiently thanks to the high-quality useful information provided, although some difficulties are obviously associated to this approach, as discussed in the following section.

Unavailability of reference standards

In this case, the information available from the list of compounds selected would normally be limited to their molecular formula and, consequently, their theoretical exact mass. With only this information, target screening would not be easily

performed by GC-TOF MS, as only those compounds presenting M+• in the EI spectrum, with relatively high abundance, would be detected, an event that rarely occurs. Consequently, it is necessary to know the main fragment ions in the EI spectrum to perform a reliable detection. In principle, this information might be collected from commercial spectra libraries that are accessible in most laboratories. The way to proceed would be similar than when the reference standard is available, although with some relevant differences. First, the retention time is unknown. Second, the information would be obtained from a theoretical spectrum, and accurate masses for each fragment ion would not be accessible because commercial libraries are offered in nominal mass. The third difference is that Q/q_i ratios resulting from the library spectra might differ from those obtained experimentally. The difficulties to assess the elemental composition and chemical structure for a given fragment ion, without the help of accurate mass measurement, must also be taken into account.

It is worth mentioning that not all theoretical spectra are available in commercial libraries (e.g. many metabolites and some emerging contaminants). In these cases, target analyte identification becomes still more difficult, even if accurate mass data have been acquired. This was the case of an investigation on honeybee poisoning carried out at our laboratory [26]. After a non-target screening (see next section for more details on this approach) performed on samples of dead honeybees, the insecticide coumaphos was identified at high concentrations. The presence of metabolites was then investigated in a post-target way, i.e. searching for specific compounds after MS data acquisition, based on information available in the scientific literature. Several coumaphos metabolites reported in human urine, soils and animals were searched, for which nominal EI mass spectra from NIST library were available. The metabolite 3-chloro-7-hydroxy-4-methyl-chromen-2-one (CMHC) was found in the samples, resulting chromatographic peaks for 5 of its pre-selected ions at the same retention time, with ion intensity ratios within specified tolerance and mass errors below 2 mDa. Investigation of other metabolites, like the dechlorination product potasan, was more difficult as, although being reported in the bibliography, its EI spectrum was unavailable in the library. Despite no spectral data were accessible, the nw-XIC at its theoretical exact *m/z* (328.0543) was performed with the result that a notable chromatographic peak appeared at 12.65 min. Accurate masses from its

combined spectrum fit well with fragments compatible with potasan structure, leading to the conclusion that the compound detected was the metabolite potasan.

It is interesting to comment another option when neither the reference standard nor the library spectrum is available. The example presented illustrates a particular case on pesticide investigation, as a consequence of a food-safety European alert in December 2006 due to the presence of isofenphos-methyl in peppers, a compound that might be considered an OP pesticide, although never been approved in any EU Member State. A pepper extract suspected to be positive to isofenphos methyl was sent to our laboratory by a Spanish Public Health laboratory. The sample had been previously analyzed by GC-NPD in that laboratory obtaining an abundant peak at a retention time different to target pesticides commonly investigated in the routine analytical method. The extract was analyzed by GC-TOF MS resulting in a total ion chromatogram with a large number of unknown peaks. In order to investigate the presence of isofenphos methyl in the sample, a nw-XIC at its exact mass (331.1007) was performed, but no chromatographic peak was obtained. However, this fact did not allow us to discard the presence of isofenphos methyl, as the occurrence of the molecular ion in the EI spectrum was not assured. As the theoretical EI spectrum was not available in the NIST library, we used the spectrum of an analogue compound, isofenphos ethyl, which enabled us to predict the EI fragmentation of the suspicious compound. The elemental composition proposed for the main fragments of isofenphos ethyl (*m/z* 255, 213 and 121) and analogue fragments of isofenphos methyl (*m/z* 241, 199 and 121) are shown in Figure 3a. Three nw-XICs at predicted *m/z* ions were performed (*m/z* 241.0630, 199.0160 and 121.0290) obtaining good chromatographic peaks in the three cases, all at the same retention time (Figure 3b). The EI accurate mass spectrum generated by TOF MS is also shown in Figure 3c. Up to six m/z fragment ions were in accordance with predicted chemical structures of isofenphos methyl fragment ions, with mass errors below 3.1 mDa. All this information allowed us to confirm the presence of isofenphos methyl in the pepper samples, even in the absence of reference standard.

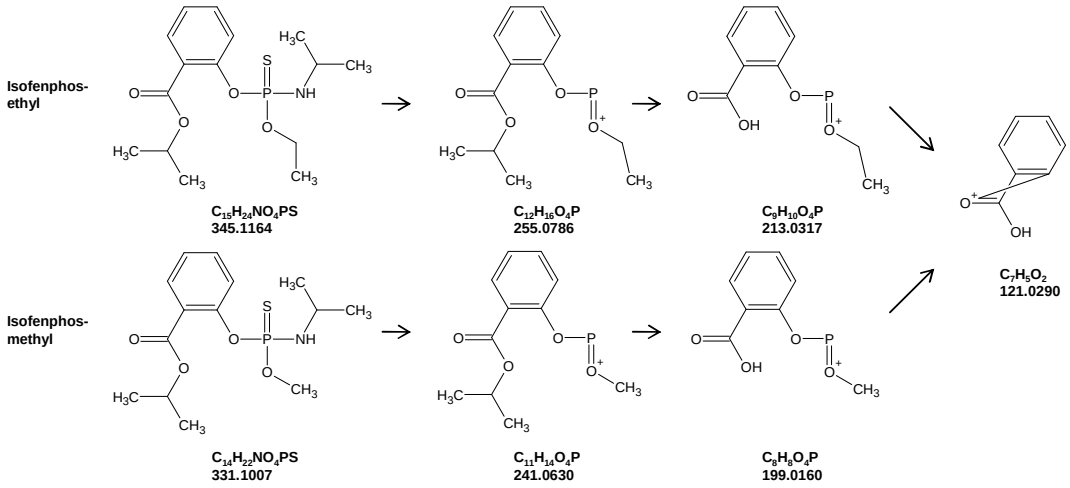
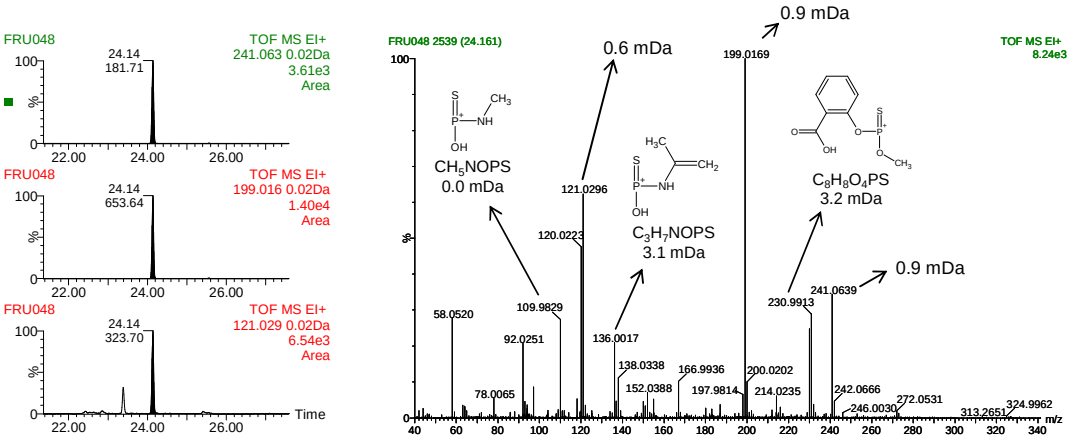


Figure 3. (a) Chemical structures proposed and exact masses for EI fragment ions for isofenphos ethyl and isofenphos methyl.



(b) (c)

Figure 3. (b) Extracted-ion chromatograms at different m/z (mass window 0.02 Da) for a positive finding of isofenphos methyl in pepper. (c) Experimental EI accurate mass spectrum and chemical structures proposed for the most abundant EI fragment ions and mass errors.

2.2. GC-TOF MS non-target screening

In non-target screening there is neither previous compound selection nor specific-analyte information used to facilitate the detection of the compounds. The analytical system must detect the presence of a component in the sample and proceed to its correct identification. To develop a non-target screening method a number of requirements should be taken into account. These include minimal, non-selective, sample preparation for a wide range of compound groups with different polarities; conventional GC separation to minimize matrix interference while maintaining a reasonable run time; and automated peak detection and mass spectrum deconvolution of components detected. In principle, the relevance of an unknown component would only be associated to its ion abundance as no other information is normally accessible to the analyst in a true non-target screening.

The investigation of trace level non-target compounds is a laborious and time-consuming task that rarely succeeds due to absence of information and the large amount of ions/peaks coming from the matrix, etc., that mask and sometimes coelute with the compounds that might be of interest. This fact makes it difficult, and sometimes unfeasible, to get a pure spectrum that can be searched in the library, which notably limits the possibilities of the unknown elucidation. In addition, unless an abundant peak is present in the total ion chromatogram (TIC), the manual investigation of peaks in the TIC seems unhelpful. Under these circumstances, the use of powerful software is necessary to detect the presence of multiple components (in many cases not visible in the TIC), and to show the deconvoluted MS spectra for each individual component detected. As an example, ChromaLynx Application Manager (Waters) has been used by our group to automatically process data in non-target GC-TOF MS analysis. It can plot reconstructed ion chromatograms of up to eight ions. When a peak is found to satisfy user-defined parameters (such as scan width, spectra rejection factor, peak width at 5% height) the software displays its deconvoluted mass spectrum, which is automatically submitted to library search routine. At this point, the identification of an unknown is highly dependent on the availability of the spectrum searched.

Components can be reduced to a list of possible candidates by using the fit factor from the mass library search (a library match >700 is normally required). Then, accurate mass confirmation is automatically performed. The formula from the library

hit is submitted to an elemental composition calculator and accurate mass measurements of up to 5 of the most abundant ions are evaluated for confirmation/rejection of the finding. It is important to carefully check the elemental compositions automatically assigned to the fragment ions in order to assess whether they are compatible with the molecular structure proposed. Although some tools can facilitate this task (e.g. ACD/MS Fragmenter software from Advanced Chemistry Developments, Inc), it is necessary to be familiar with the main mass fragmentation rules to succeed in the elucidation process.

This non-target strategy allowed the detection in environmental and wastewater of pollutants not included in the target list of contaminants investigated, like bisphenol A, the antioxidant 3,5-di-tert-butyl-4-hydroxy-toluene (BHT), its metabolite BHT-CHO, the polycyclic musk galaxolide, or the UV filter benzophenone [24]. Obviously, some limitations were observed, which prevented the investigation of non-target compounds at low levels indicating that, at present, screening of organic (micro)contaminants in the environment can not only be performed treating samples and analytes as unknown. Both approaches, target and non-target, are complementary when searching for organic pollutants in the environment in order to have a realistic overview.

Similarly, full-spectral acquisition and accurate mass data generated by GC-TOF MS allowed the application of a non-target approach in human breast tissues [27]. Several compounds (not included in the list of target analytes) were discovered, like BHT, BHT-CHO, dibenzylamine, N-butylbenzenesulfonamide (N-BBSA), 1,2-dimethylnaphthalene, 2-methylnapththalene and several PCB congeners. Data obtained by both, target and non-target approaches, illustrated the strong potential of GC-TOF MS for screening purposes in biological samples.

GC-HRTOF MS has also been used to investigate drugs of abuse. As illustrative example we will present the following real-world case. Three unknown plant seeds, suspected of being from *Datura stramonium*, were brought to our laboratory by the police in February 2008. *Datura stramonium* contains tropane alkaloids that might be used as hallucinogen. The active ingredients are atropine, hyoscyamine and scopolamine, which are classified as deliriants or anticholinergics. Several alkaloids reported in the literature on genus *Datura* were investigated as target analytes but

none of them were detected in the sample analyzed. At this point, a non-target approach was applied in order to identify relevant compounds that could give information on what kind of plant seeds were under study. Accurate mass confirmation/rejection of the library findings (match>700) allowed several compounds to be identified, ricinine being one that caught our attention. Ricinine is an alkaloid that shares a common plant source with ricin, a toxalbumin derived from the castor bean plant, *Ricinus communis* [41]. Its seed oil is used as a purgative in medicine and the toxicity of its lectin, ricin, is well known.

Figure 4 shows the positive finding of ricinine when using the non-target approach. Accurate mass scoring applied to three representative ions led to the confirmation of the identity of ricinine, with mass errors lower than 1 mDa for all ions. Also, chemical structures for the most abundant EI fragment ions were suggested based on their elemental compositions according to accurate mass data. Therefore, the plant seeds analyzed corresponded to *Ricinus communis* not to *Datura stramonium*.

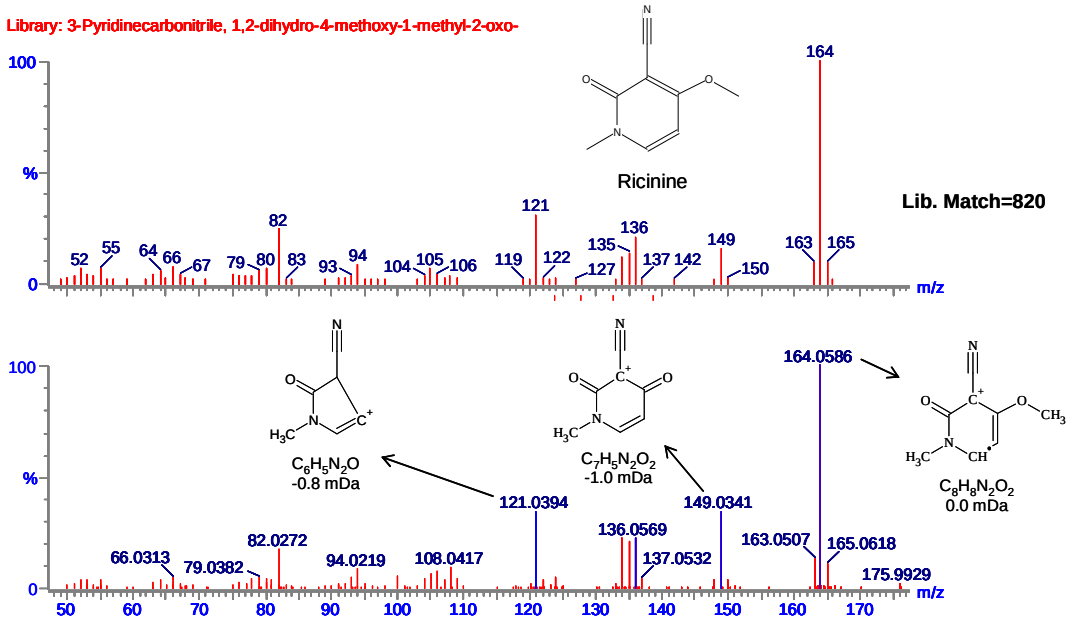


Figure 4. Experimental EI accurate mass spectrum of a positive finding of ricinine in a seed sample. Chemical structures proposed for the three most abundant EI fragment ions and mass errors (bottom). Library nominal mass spectrum for ricinine (top).

Our laboratory was also involved in a case of contamination related to drugs of abuse. A sample of hemp seed oil (*Cannabis sativa*) was analyzed because the oil had supposedly caused severe intoxication of a 2 years-old child. Δ^9-Tetrahydrocannabinol (Δ^9-THC) is the primary psychoactive constituent of cannabis, which predominantly acts on the central nervous and cardiovascular systems and may produce behavioural effects [42]. Hemp seed oil is obtained through first cold pressing in order to preserve its high and balanced content of polyunsaturated fatty acids (57% linolenic acid, 18.6% alpha-linolenic acid), and it would not be expected to contain the mentioned psychoactive constituent. After dilution of the seed oil with hexane and partitioning with acetonitrile, non-target GC-TOF MS analysis revealed three abundant chromatographic peaks in the TIC, so that deconvolution software was not necessary to detect the presence of these components (see Figure 5, where only the spectra of Δ^9-THC is shown for simplification). The three experimental accurate mass spectra were submitted to the nominal NIST library search and they were assigned to three compounds related with the cannabis: Δ^8-THC, Δ^9-THC, cannabinol, (library match > 800). Their identity was confirmed by evaluating the exact mass of the fragments. Therefore, the presence of these cannabis derivatives was confirmed in the seed oil analyzed.

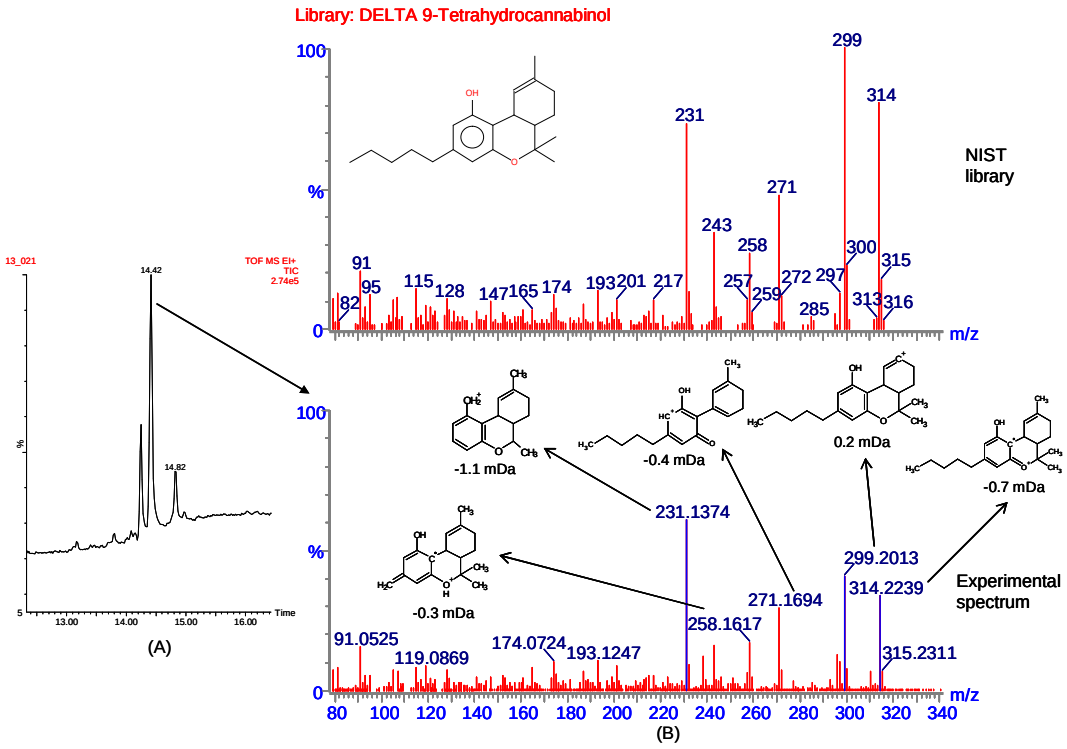


Figure 5. (a) TIC chromatogram of hemp seed oil extract. (b) Experimental accurate mass spectrum of Δ^9-THC and chemical structures proposed for the most abundant EI fragment ions and mass errors (bottom). Library mass spectrum for Δ^9-THC at nominal mass (top).

When no library match, or unacceptable match, is found for an experimental EI spectrum, the elucidation process gets more difficult. The absence of the molecular ion in many EI spectra creates more difficulties in the elucidation procedure. Oppositely, the use of soft-ionization techniques would facilitate identification of the molecular ion. The elucidation process normally requires the combined use of hard and soft ionization techniques and/or performing tandem MS experiments by QTOF MS. In the best cases, a unique elemental composition might be proposed for the unknown based on accurate mass measurements of the molecular and fragment ions. Isotopic patterns observed are helpful for the prediction of appropriate number of elements like Cl, Br and S. Similarly, a carbon prediction number filter can be applied to reduce the number of possible elemental compositions based on the relative abundance of the isotopic peak

corresponding to ^{13}C. The Nitrogen rule can also be used to fix the ion as an "even-electron ion" or "odd-electron ion". Finally, the candidate elemental compositions can be searched in available databases (Index Merck, Sigma Aldrich, ChemSpider, Pubchem, Reaxys, etc) and a chemical structure can hopefully be proposed. Fragment ions should be compatible with the chemical structure assigned. In order to get a definitive confirmation, the reference standard would be required in a final step to check the retention time and experimentally confirm the presence of fragment ions by GC-TOF MS analysis.

A few applications have been reported on the use of GC-TOF MS for elucidation of unknowns when library mass spectra are not available. One of the first papers was reported by Grange et. al. [25], who investigated extracts of well water. The authors concluded that GC-TOF MS was not as powerful for determining ion compositions as double-focusing mass spectrometers.

Although not related to the applied fields considered in this article, we will present a few applications on the use of GC-TOF MS in metabolomics as very little has been published on the subject treated here. GC-TOF MS using APCI source was evaluated as regards repeatability, reproducibility, linearity and detection limits, for 31 selected compounds (amino acid, organic acids, alcohols, xantines, etc) for which standard mixture was available in the automated analysis of metabolites in biological samples. The developed method was applied to human cerebrospinal fluid (CSF) for metabolic profiling. More than 300 compounds with different isotopic features were determined. The identity of some of them could be corroborated by the standards included in the mixture, but in the absence of reference standard only mass position and isotopic distribution could be used to achieve the identification of the compounds present in the CSF according to their molecular formula [43].

More recently, combination of hard and soft ionization techniques has been proposed for elucidation purposes [44]. Despite the lower ion yields obtained by FI, the superiority of this source when coupled with TOF MS in producing molecular ions and chromatograms with good S/N was impressive. When this is considered along with the ability to measure molecular mass accurately, and to narrow down the possible formula of an unknown, FI in combination with EI results very attractive. Such a configuration operating with a postcolumn split 10:1 FI:EI would generate spectra that would be of

generally similar quality and allow accurate molecular weight and fragmentation data to be collected simultaneously. This would be of potential benefit in fields like metabolomics where structures that cannot be confidently identified by library matching, or interpretation of EI spectra alone, are regularly encountered.

In metabolomic applications that use GC-(EI)MS, the low abundance of the molecular ion has impeded the calculation of elemental compositions in the elucidation process of unknowns. On changing the beam-steering voltage of the ion source, the relative abundances of molecular ions at 70 eV EI were increased up to ten-fold for alkanes, fatty acid methyl esters and trimethylsilylated metabolites, concomitant with 2-fold absolute increases in ion intensities. The abundance, mass accuracy and isotope ratio accuracy of molecular species in EI were compared with those in CI with methane as reagent gas under high-mass tuning. When constraining lists of calculated elemental compositions by chemical and heuristic rules using the Seven Golden Rules algorithm and PubChem queries, the correct formula was retrieved as top hit in 60% of the cases and within the top-3 hits in 80% of the cases [45].

3. CONCLUSIONS AND FUTURE PERSPECTIVES

The preceding sections show that GC-HRTOF MS opens up the possibility of identifying a large number of GC-amenable, target or non-target, compounds with improved reliability and sensitivity. This technique has offered promising results when applied for screening purposes in the environmental field. Full-spectrum accurate mass data available, widen the options of detecting more compounds in the samples, giving more realistic organic pollution overview in comparison to conventional target methods. Despite the great qualitative potential of GC-HRTOF MS, to date it has seldom been applied, and normally in environmental and biological samples. In other fields, like veterinary drug residue analysis and doping control it has scarcely been applied [21].

One of the main drawbacks of TOF MS has traditionally been its low dynamic range, which affects not only mass accuracy but mostly quantitative applications. Recent advances in instrumental design, especially the detector and its electronics, have allowed the dynamic range to be extended (e.g. DRE for TDC detector and the use of high-speed Analog-to-Digital Converter (ADC) technology). ADC-based systems present greater intrinsic dynamic range, and facilitate the detection of lower-

abundance compounds in the presence of higher-abundance ones. To our knowledge, high speed ADC has only been used in combination with LC. Its use in GC-HRTOF instruments would be welcome to improve the dynamic range and it would surely facilitate GC-HRTOF MS quantitative applications.

Several improvements are also being made in the acquisition rates of HRTOF instruments. When deconvolution software is used, especially in complex matrices where coelution of analytes with matrix interferences is highly probably, it might be necessary to acquire MS full-spectrum data at higher speed. HRTOF MS instruments used to acquire data at maximum of 10 spectra s^{-1}, although 20 or 25 spectra s^{-1} could be acquired in some cases [20]. However, working at the maximum speed capability leads to a notable decrease in sensitivity [36]. Recent advances have allowed HRTOF MS, coupled to LC, to acquire data at 40 spectra s^{-1} maintaining high-resolution, mass accuracy and sensitivity. Rapid full spectrum acquisition rates improve the coupling of TOF to fast chromatography, facilitating the detection and improving peak-shape of the narrow chromatographic peaks obtained. At the moment, high-acquisition rates have been only applied in LC-TOF MS configurations. It is expected this feature will be also applied in GC-HRTOF MS instruments soon.

One of the most promising advances in GC-TOF MS instrumentation is the development of new APCI sources, which offer interesting benefits over traditional EI and CI. Using this soft ionization source, the protonated molecule is, in most cases, the base peak of the spectrum, and very low fragmentation of the molecule is observed in comparison to EI source. The predictable presence of the protonated molecule when using APCI in GC-TOF MS would facilitate performing rapid, wide-scope and more sensitive screening, as detection would be more effective based on molecular ion searching. A reliable confirmation of the compounds detected could be carried out by using a QTOF instrument, which allows performing MS/MS experiments after isolation of the precursor ion. The high degree of fragmentation in EI is a problem when one tries to select the precursor ion in MS/MS experiments. Possibly for this reason, GC has normally been coupled to TOF MS instead of hybrid QTOF MS. The absence (or low abundance) of the molecular ion in EI spectra also has the consequence of higher LODs, and reduces selectivity and sensitivity. In the low fragmented APCI spectra, the precursor ion selection would no longer require a compromise between selectivity and sensitivity, allowing more useful MS/MS experiments to be carried out.

QTOF MS analyzer also allows fragmentation in the collision cell. To improve the identification reliability, sample analysis could be made using two acquisition functions in the same injection: the first one, with low collision energy, to detect (typically) the protonated molecule; and the second one, with high collision energy, as a confirmatory function, where fragmentation of the molecule would be promoted. This acquisition mode, known as MS^E, has shown excellent results in LC-QTOF MS applications, improving reliability of the identification process for a wide-variety of contaminants in water matrices [46, 47]. Although preliminary results have been obtained with GC-(APCI)QTOF-MS, further research is required to fully establish the achievements and pitfalls of this promising source [37].

Future instrumentation development able to generate accurate mass data on both molecular ion and fragment ions would be of great interest for wide-scope screening and identification of compounds not included in current MS libraries [44, 47-49]. Combination of GC-QTOF MS and LC-QTOF MS will be an excellent way in the near future to investigate the presence of target and non-target organic compounds of very different physico-chemical characteristics in many applied fields like environment, toxicology, food-safety, sport, doping control or metabolomics.

ACKNOWLEDGMENTS

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of GC-TOF MS (GCT). This work has received financial support from the Spanish Ministry of Science and Education (Project reference: CTQ2009-12347) and from Generalitat Valenciana, as research group of excellence PROMETEO/2009/054. The authors acknowledge the constructive comments and advices of our colleague Juan V. Sancho. T. Portolés is indebted to University Jaume I for her post-doctoral grant.

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CAPÍTULO 2

Desarrollo de métodos cuantitativos basados en cromatografía de gases acoplada a espectrometría de masas con analizador de triple cuadrupolo

2.1 INTRODUCCION

En el presente capítulo se explora la capacidad analítica de la técnica GC-MS/MS con analizador de triple cuadrupolo para el análisis *target* cuantitativo en dos campos de aplicación, el medioambiental y el biológico.

En un primer apartado se lleva a cabo el desarrollo y optimización de un método multirresidual para la determinación de alrededor de 50 contaminantes orgánicos considerados de carácter prioritario en el análisis de aguas, entre los que se incluyen insecticidas organoclorados, organofosforados, herbicidas, PCBs, PAHs, PBDEs y octil/nonil fenoles. Los resultados de este trabajo se recogen en el **artículo científico 2**. En el segundo apartado se desarrolla un método para la determinación de contaminantes xenoestrógenos en muestras de tejido adiposo mamario y en tejidos tumorales. Este trabajo ha sido enfocado a la determinación de alrededor de 30 compuestos organoclorados (PCBs y plaguicidas) y compuestos organobromados (PBDEs) cuya liposolubilidad y tendencia a la bioacumulación, así como su carácter estrogénico, han sido reportados en la literatura. Los resultados de este trabajo se detallan en el **artículo científico 3**.

En ambos trabajos se presta especial atención a la optimización de los parámetros instrumentales para la correcta identificación y confirmación de los compuestos detectados con la finalidad de evitar reportar falsos positivos. Debido a sus

características, los métodos optimizados cumplen rigurosamente con los requisitos exigidos para una cuantificación exacta y una confirmación fiable de la identidad de los compuestos detectados, de acuerdo con las Guías Europeas más recientes.

En cuanto al tratamiento de muestra, se ha intentado minimizando lo más posible. En el análisis de aguas, se ha basado en una extracción en fase sólida con cartuchos C_{18}, que ha permitido la extracción y preconcentración de los analitos. Las muestras de tejido adiposo se han sometido a una extracción con hexano seguida de una purificación por HPLC en fase normal para eliminar los interferentes procedentes de la matriz, principalmente lípidos.

Los métodos desarrollados han sido validados en términos de exactitud y precisión mediante ensayos de recuperación utilizando muestras fortificadas a distintos niveles de concentración y posteriormente se han aplicado al análisis de muestras reales. En cuanto a las aguas, se han analizado distintos tipos de aguas de diferente complejidad (superficiales, subterráneas y residuales). En el caso de muestras biológicas, la metodología desarrollada ha sido aplicada al análisis de muestras de tejido adiposo mamario (tejido adiposo y tumoral).

Los métodos anteriormente indicados hacen uso de una fuente de EI. En el análisis de aguas, también se ha optimizado y validado el método para el análisis de compuestos organoclorados mediante el uso de la fuente de CI en modo negativo con el fin de estudiar la ventajas y/o desventajas que este modo de ionización puede aportar con respecto a la más comúnmente utilizada fuente de EI.

En el caso de las muestras de tejido adiposo, se ha estudiado el potencial de la técnica GC-TOF MS como técnica de confirmación adicional de la identidad de los compuestos detectados mediante GC-MS/MS con triple cuadrupolo.

2.2 DETERMINACIÓN DE CONTAMINANTES PRIORITARIOS EN AGUA POR GC-MS/MS CON TRIPLE CUADRUPOLO

En la actualidad, un elevado número de contaminantes puede llegar a estar presente en las aguas, afectando de modo importante a su calidad y convirtiéndose en un problema medioambiental de relevancia. Debido a las consecuencias potencialmente peligrosas para el ser humano que pueden originar los contaminantes orgánicos en las aguas, resulta necesario desde el punto de vista medioambiental y de salud pública, disponer de datos relacionados con la concentración, características y comportamiento de estos contaminantes en las aguas.

Para ello, se requieren métodos analíticos avanzados capaces de afrontar este problema, aportando información realista sobre el grado de contaminación de las aguas. La detección, correcta identificación y cuantificación de cientos e incluso miles de potenciales contaminantes orgánicos es, sin duda, uno de los mayores retos de la química analítica moderna. Además, muchos de ellos son todavía desconocidos, pues pueden proceder de la transformación de otros contaminantes primarios en el medio acuático. El desarrollo de metodologías analíticas multirresiduales se está convirtiendo en una de las prioridades en este campo, ya que proporcionan amplia información acerca de la contaminación de las aguas y reducen el tiempo de análisis, el tratamiento de muestra y el coste. En los últimos años se han incrementado el número de trabajos publicados sobre análisis multirresidual, la mayoría de ellos focalizados hacia un análisis en modo *target*. En general, estos métodos se basan en el acoplamiento de GC o LC con MS. La elección entre ambas técnicas cromatográficas está basada principalmente en las propiedades físico-químicas de los analitos, aunque en los últimos años la gran versatilidad de LC la está convirtiendo en la técnica de análisis por excelencia. En el caso de compuestos apolares o semi-apolares, y volátiles o semi-volátiles, normalmente la técnica de elección sigue siendo GC. Así, la determinación de trazas de antibióticos (1, 2) o drogas de abuso (3, 4) se basa mayoritariamente en métodos LC-MS/MS, mientras que la determinación de compuestos clorados y bromados persistentes (5, 6) o hidrocarburos policíclicos aromáticos (7, 8) está basada en GC-MS, con distintos tipos de analizadores. En el caso particular del análisis de residuos de plaguicidas (ARP) se han impuesto las técnicas GC-MS/MS (9-12) y LC-MS/MS (14-16) siendo la tendencia actual el uso combinado y complementario de ambas técnicas, con el fin de abarcar el mayor número posible de analitos (13, 14).

Por lo general, el análisis de aguas en modo *target* se ha llevado a cabo mediante GC-MS con analizadores de cuadrupolo simple en modo SIM (15-19) o trampa de iones en modo tandem MS (20-24), siendo ambas técnicas todavía en la actualidad ampliamente utilizadas por los buenos resultados demostrados. Más novedosa resulta la técnica GC acoplada a triple cuadrupolo, cuya elevada sensibilidad en modo de adquisición SRM y amplio rango lineal la hace ideal para el análisis cuantitativo en modo *target*, especialmente a los bajos niveles de concentración normalmente requeridos en este campo. El modo de adquisición en SRM implica la necesidad de establecer *a priori* las transiciones que se van a monitorizar y optimizar las condiciones MS/MS para cada analito. Por ello, es necesario definir los contaminantes a determinar previamente al análisis, lo cual, en la mayoría de las ocasiones, se lleva a cabo en base a listas prioritarias establecidas por diferentes organismos según criterios de peligrosidad.

La optimización de las condiciones MS/MS para cada compuesto (ion precursor, energía de colisión, iones producto, *dwell time*,…) se lleva a cabo mediante la inyección de patrones en el sistema cromatográfico. Como metodología general, se seleccionan uno o más iones precursores para cada analito (el ion molecular entre ellos, si está disponible) y se fragmentan a distintas energías (normalmente valores entre 10 y 30 eV) en la celda de colisión, obteniéndose distintos iones producto. Una vez obtenida esta información, se seleccionan las transiciones, normalmente las más selectivas y sensibles. En principio es suficiente con seleccionar dos transiciones para cada compuesto, preferiblemente lo más específicas posible, aunque, obviamente, lo deseable desde el punto de vista de la identificación y confirmación inequívoca del compuesto detectado sería adquirir todas las transiciones disponibles para cada compuesto. Sin embargo, esto no es factible en el desarrollo de métodos multirresiduales, ya que el número de transiciones que contiene un método tiene una relación directa sobre la sensibilidad y la forma del pico cromatográfico y, por tanto, sobre la calidad de la determinación, provocando que el número de transiciones monitorizadas se limitado.

En general, la sensibilidad depende directamente de la velocidad de barrido, lo cual, en el equipo utilizado en la presente Tesis Doctoral, en modo de adquisición SRM, viene determinado por el valor de *dwell time* (s) (tiempo empleado en la monitorización de un ion). Es decir, cuanto más tiempo se esté midiendo un ion, más

sensible será la técnica. Así pues, si se persiguen límites de detección muy bajos o el compuesto a detectar presenta buena sensibilidad, se requieren valores de *dwell time* de entre 0.1 y 0.3 s, con lo cual no se pueden adquirir más de 5 ó 6 transiciones simultáneamente. Esto es debido a que los picos cromatográficos tienen normalmente una anchura de entre 5 y 8 s, ya que en análisis cuantitativo la forma de pico es muy importante en términos de reproducibilidad siendo necesario que los picos cromatográficos estén definidos al menos por diez puntos. Por el contrario, si no se requieren límites de detección tan bajos o el compuesto en cuestión es muy sensible, se pueden utilizar valores de *dwell time* de 0.01 – 0.05 s, con lo que se pueden llegar a adquirir del orden de entre 20 y 25 transiciones simultáneamente. Cabe resaltar que el equipo utilizado permite aplicar un valor de *dwell time* diferente para cada transición dentro de una misma ventana de adquisición, a diferencia de otros equipos de triple cuadrupolo, cuyo tiempo de barrido debe ser el mismo para todas las transiciones que se encuentren en la misma ventana de adquisición. Esto añade mayor versatilidad a la hora de programar distintos valores de *dwell time* para compuestos que se encuentran en la misma ventana de tiempo cromatográfico, pero que, sin embargo, presentan distintas sensibilidades y/o anchuras de pico.

En este trabajo, se determinó un total de 51 contaminantes orgánicos en aguas de distinta naturaleza, incluyendo compuestos de diferentes familias como insecticidas organoclorados y organofosforados, herbicidas, PCBs, PAHs, PBDEs y octil/nonil fenoles. El método aplicado presenta la ventaja de adquirir dos transiciones para cada analito. La primera de ellas se ha utilizado para la cuantificación (Q) y la otra con fines de confirmación (q_1). De este modo, la confirmación de la identidad del compuesto detectado es extremadamente fiable y segura. El método desarrollado en modo SRM ha permitido la cuantificación y confirmación simultánea de los compuestos seleccionados con una excelente sensibilidad y fiabilidad.

Los niveles de sensibilidad y los bajos LODs requeridos (nivel bajo de validación 25 ng/L), obligaban a utilizar valores de *dwell time* preferiblemente superiores a 0.1 s, por lo que el número de transiciones adquiridas simultáneamente no podían ser mayor de 5 ó 6. De todas formas, se estudió cada caso en particular, aplicándose el valor de *dwell time* óptimo en función de la anchura de pico y del número de transiciones a monitorizar. Por ello, fue necesario optimizar la separación cromatográfica con el fin

de tener grupos cromatográficos diferenciados, de modo que se limitó el número de compuestos en una ventana de tiempo determinado.

El tratamiento de muestra consistió en una extracción en fase sólida (SPE) con cartuchos C_{18} (500 mg). En el caso de las aguas brutas de lixiviado, previamente a la etapa de extracción, se diluyen 50 veces con agua HPLC para rebajar su alta carga orgánica.

Como patrones para el control de calidad del procedimiento (surrogates) se utilizaron compuestos marcados isotópicamente (HCB-$^{13}C_6$, lindano-D_6, terbutilazina-D_6, *p,p'*-DDE-D_8 y b(a)antraceno-D_{12}), los cuales se añadieron a cada una de las muestras analizadas en la etapa inicial del proceso, es decir, antes de la extracción por SPE. Con ello, se persigue corregir los posibles errores que pudieran ocurrir a lo largo de la etapa de tratamiento de muestra, así como posibles variaciones en las señales del instrumento utilizado.

Los métodos desarrollados se validaron en términos de análisis cuantitativo, mostrando LODs y LOQs satisfactorios. La exactitud y precisión de los análisis se evaluaron mediante ensayos de recuperación utilizando muestras fortificadas a distintos niveles, obteniendo resultados dentro de los límites establecidos en la gran mayoría de los casos. La excelente selectividad y sensibilidad de la técnica de GC-MS/MS con analizador de triple cuadrupolo en modo de SRM permitió la cuantificación y confirmación satisfactoria de los compuestos estudiados a niveles de concentración del orden de 25 ng/L en muestras de agua. El método desarrollado se aplicó a distintos tipos de aguas, de diferente complejidad en la matriz, como son las aguas superficiales, subterráneas y residuales.

Una desventaja inherente al uso de la ionización electrónica es, en ocasiones, la selección del ion precursor. Al partir de espectros generalmente muy fragmentados, existen casos en los que la selección del ion precursor es complicada. Cabe decir que, a la hora de seleccionar transiciones selectivas y específicas para cada compuesto objeto de estudio, lo más adecuado sería siempre seleccionar el ion molecular como ion precursor, y como iones fragmentos, aquellos que resulten de la pérdida de un fragmento específico de la molécula. Esto, en la práctica, especialmente cuando se utilizan fuentes de EI, resulta complicado y para muchos analitos esta opción no es posible.

Así pues, se utilizó la fuente de ionización química en modo negativo (NICI), también disponible en el equipo, con el fin de estudiar transiciones más selectivas, escogiendo como iones precursores los iones moleculares generados o, en su defecto, algun ion fragmento de mayor masa que en el caso de los espectros de EI. El estudio se centró en los compuestos organoclorados incluidos en la lista de compuestos estudiados, por su conocida elevada sensibilidad en la fuente NICI.

El uso de esta técnica mejoró considerablemente la sensibilidad en la detección de la mayoría de los compuestos organoclorados estudiados, haciendo posible, en algunos casos la detección y confirmación de positivos en la muestra que no habían sido detectados mediante EI. Sin embargo, no fue posible desarrollar un método en modo SRM, ya que sólo estaba disponible una transición para cada compuesto, y además era bastante inespecífica, por lo que con la fuente de NICI se optó por desarrollar un método en modo SIR con dos iones para cada compuesto.

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2.2.2 Artículo científico 2

Anal. Chim. Acta. 583 (2), 246-258, 2007

DETERMINATION OF PRIORITY ORGANIC MICRO-POLLUTANTS IN WATER BY GAS CHROMATOGRAPHY COUPLED TO TRIPLE QUADRUPOLE MASS SPECTROMETRY

Elena Pitarch, Cecilia Medina, Tania Portolés, Francisco J. López, Félix Hernández

Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain,

ABSTRACT

A multiclass method has been developed for screening, quantification and confirmation of organic micro-pollutants in water by gas chromatography coupled to mass spectrometry with a triple quadrupole analyzer. The work has been focused on the determination of more than 50 compounds belonging to different chemical families: 19 organochlorine and organophosphorus insecticides, 6 herbicides, 7 polychlorinated biphenyls, 16 polycyclic aromatics hydrocarbons, 2 brominated diphenyl ethers, and 3 octyl/nonyl phenols and pentachlorobenzene. Most of these analytes are included in the list of priority substances in the framework on European Water Policy.

Analyte extraction was performed by solid phase extraction using C_{18} cartridges, and five isotopically labeled standards were added before extraction as surrogates. Analyses were performed by gas chromatography with tandem mass spectrometry (MS/MS) in electron impact mode. Accuracy and precision were evaluated by means of recovery experiments using water samples fortified at two concentration levels (25 and 250 ng L^{-1}), with satisfactory results for most of analytes. The excellent selectivity and sensitivity reached in selected reaction monitoring mode allowed us satisfactory quantification and confirmation at levels as low as 25 ng L^{-1}. Two MS/MS transitions were acquired for each analyte, using the Q/q intensity ratio as a confirmatory parameter. The method developed was applied to the analysis of surface, ground and wastewater samples collected from the Valencia Region (Spain).

Analytical methodology using negative chemical ionization mode was also validated for the organochlorine compounds selected, showing a superior sensitivity and lower detection limits.

Keywords: Water analysis; Organic pollutants; Gas chromatography tandem mass spectrometry; Triple quadrupole; Analyte confirmation

INTRODUCTION

Many organic pollutants can be present in environmental water, normally at the μg L^{-1} level or below, as a result of different sources of pollution, from anthropogenic activities, as industrial chemical production or agricultural applications, or natural origin. The extensive use of organochlorine (OC) pesticides and polychlorinated biphenyls (PCBs) in agriculture and their industrial applications played an important role in the last century. This fact, together with the persistence of these compounds, can explain their wide presence in the soil–water environment (1). Most of OC pesticides are banned at present, but many other chemical families are nowadays applied in agriculture, and in domestic and public health treatments and their presence in surface and ground water have been widely investigated. Polybrominated diphenyl ethers (PBDEs) are also emerging as important environmental contaminants, as they have been widely used as flame retardants in most types of polymers employed in electrical appliances including TV sets, computers and other electronic household equipment, over the past two decades (2-5). Octyl and nonyl phenols are used as precursors in the manufacture of non-ionic surfactants (6). Polycyclic aromatic hydrocarbons (PAHs) represent an important class of hazardous organic chemicals derived from anthropogenic (e.g. emissions in the environment as a result of vehicle exhaust, asphalt pavements, unvented radiant and convective kerosene space heaters, heating appliances) and natural sources (all incomplete combustion at high temperature and pyrolytic processes involving fossil fuels, such as peat, coal and petroleum) (7).

Several of these compounds are relevant in the field of Water Policy of the European Union, and are included in Annex X of the Directive 2000/60/EC (8), so regulation and environmental monitoring programs have to be adopted to control the concentration levels of these compounds in water. Consequently, analytical methodologies should be able to accurately determine the very low concentration levels set up by the legislation and should also provide unambiguous evidence to confirm both the identity and the magnitude of any pollutant detected. Gas chromatography (GC) has been the technique of choice for the analysis of environmental samples containing semivolatile and volatile organic compounds due to its favorable combination of high selectivity and resolution, good accuracy and precision, wide dynamic concentration range and high sensitivity (9-11). Numerous applications in water analysis based on GC coupled to mass spectrometry (MS) have been reported such as the determination of PAHs (12,13), pesticides (14-16), PCBs (17), octyl/nonyl phenols (18) and some multiresidue procedures for the determination of priority and persistent organic pollutants (19-21). Moreover, Santos and Galceran (22) reviewed modern developments in GC–MS based methodology for environmental samples reporting the use of different instruments, from simple linear quadrupoles to multi sector analyzers, with electron impact (EI) and positive/negative chemical ionization (CI). Although GC–MS has proved to be an advantageous and powerful technique for the determination of organic compounds in environmental samples, in recent years the application of tandem mass spectrometry (MS/MS) has been considered as a valuable approach which allows high selectivity and low analyte detectability, minimizing or even removing many of the interferences.

The use of MS/MS, using ion trap detector (ITD) or triple quadrupole (QqQ) analyzers leads to the possibility of adequate precursor and product ion selection and allows reducing the chemical noise in the chromatograms. The utility of product ion spectra for absolute identification at trace levels in environmental samples together with the ease of use and low cost (compared with QqQ) has made ITD a widely used technique for the determination of organic compounds in water (23-27). However, the use of two stages of mass analysis in MS/MS systems based on QqQ offers the possibility of applying selected reaction monitoring (SRM), one of the most selective and sensitive approaches for quantification and confirmation, especially in trace analysis where normally there is high background chemical noise. Moreover, in regard to quantification,

QqQ in SRM mode seems to be more powerful when compared with ITD in MS/MS mode, which is very important in trace analysis of complex matrices (28).

Very little has been published until now on organic compound analysis by GC–MS/MS with triple quadrupole analyzers, in spite of its evident advantages. The first works on the use of triple quadrupole analyzers were published in the 80 and 90 s where QqQ was tested for the determination of organics, dioxins and dibenzofurans in environment (29-31). However, first application with new generation of triple quadrupole instruments date from 2003, where the potential of the technique was investigated for the dioxin/furan analysis in fish and flyash (32). In the last 2 years, this approach has been mainly applied to the determination of pesticides in fruits and vegetables (33-35), food (36-39), fats and oils (40) and PCBs in environmental samples (41). All these authors have emphasized the fact that GC–MS/MS (QqQ) provides excellent selectivity, sensitivity and gain on total analysis time, allowing simultaneous identification and quantification of target analytes. Our own research group also proved that GC–MS/MS (QqQ) is a powerful technique for the reliable determination of organohalogen xenoestrogen compounds in human breast tissues (28).

As far as we know, GC–MS/MS with QqQ analyzer has not been explored in water analysis, where the reliable quantification and confirmation of many organic pollutants is required at very low concentration levels, according to the present regulations. The excellent sensitivity of this technique in SRM mode together with its strong potential for identification/confirmation of the compounds detected in unknown samples, by adequate acquisition of specific MS/MS transitions, make this technique very attractive with strong potential in the determination of sub-ppb analyte levels in water with superior analytical characteristics compared to GC–MS with single quadrupole or GC–MS with ion trap analyzers.

In this paper, we have developed analytical methodology for the rapid and sensitive determination of organic pollutants in water using GC(EI)–MS/MS with triple quadrupole analyzer. The procedure uses solid phase extraction (SPE) as sample treatment and it has allowed the quantification and reliable confirmation of 54 semivolatile organic contaminants, belonging to quite different chemical families. Alternatively, GC(NCI)–MS has also been investigated for the most sensitive determination of organochlorine (OC) pesticides. In both cases, the confirmation

criteria have been detailed studied in the line of the European Commission guidelines (42).

EXPERIMENTAL

Reagents

Organic pollutants investigated in this work are shown in Table 1, where compounds regulated by the Directive 2000/60/EC are highlighted in italic. All pesticide standards (OC and organophosphorus (OP) insecticides and herbicides), octyl/nonyl phenols and pentachlorobenzene were purchased from Dr. Ehrenstorfer (Augsburg, Germany). PCB Mix 3 from Dr. Ehrenstorfer (100 μg mL^{-1} in cyclohexane) was used for single quantification of PCBs congeners IUPAC number 28, 52, 101, 118, 138, 153 and 180. Standards of BDEs (brominated diphenyl ethers), BDE-100 and BDE-99 (50 μg mL^{-1} in nonane) were obtained from Wellington Laboratories (Guelph, Ontario, Canada). US Environmental Protection Agency (EPA) 525 polycyclic aromatic hydrocarbons, PAH Mix 25, was purchased from Dr. Ehrenstrofer (100 μg L^{-1} in acetone). Acenaphthene and naphthalene (Fluka, Buchs, Switzerland) and fluoranthene (Riedel de Haen, Seelze, Germany) were also used.

Stock solutions (around 500 μg mL^{-1}) were prepared by dissolving reference standards in acetone and stored in a freezer at −20 °C. Working solutions were prepared by diluting stock solutions in acetone for sample fortification and in hexane for GC injection.

Acetone (pesticide residue analysis), ethyl acetate, dichloromethane (DCM) and hexane (ultra-trace quality) were purchased from Scharlab (Barcelona, Spain). About 500 mg Bond Elut cartridges C_{18} (Varian, Harbor City, CA, USA) were used for solid-phase extraction.

Five isotopically labeled surrogates were used: *p*,*p*′-DDE-D_8, lindane-D_6, benzo(*a*)anthracene-D_{12} and terbutylazine-D_5 (Dr. Ehrenstorfer) and hexachlorobenzene (HCB)-$^{13}C_6$ (Cambridge Isotope Labs Inc., Andover, MA, USA). A working mixed solution of labeled standards (ca. 100 ng mL^{-1}) were prepared by dilution of individual stock solutions (ca. 100 μg mL^{-1} of *p*,*p*′-DDE-D_8, terbutylazine-D_5 and (HCB)-$^{13}C_6$; 10 μg mL^{-1} of

lindane-D_6 and benzo(*a*)anthracene-D_{12}) with hexane for calibration preparation and with acetone for sample fortification and stored at 4 °C.

Table 1. Experimental conditions of the optimized EI (SRM) method (compounds regulated by Directive 2000/60/EC are shown in italic)

t_R (min)	Window (min)	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Q/q	Dwell time (s)	Collision energy (eV)	Q/q ratio[a]
6.06	3–9.2	Naphthalene	128	102	Q	0.1	30	1.26 (5)
11.88	9.2–12.5	Acenaphthylene	152	150	Q	0.1	30	1.16 (3)
12.69	12.5–13.2	Acenaphthene	154	152	Q	0.1	35	4.54 (5)
13.51	13.0–14.6	Pentachlorobenzene	248	142	Q	0.1	30	1.16 (4)
14.89	14.6–16.5	Fluorene	166	164	Q	0.05	35	2.19 (10)
			165	115	q		30	
15.42		4-t-Octylphenol	135	107	Q	0.05	10	3.48 (3)
			135	77	q		20	
17.25	16.5–17.8	Trifluraline	306	264	Q	0.3	10	1.95 (8)
			264	160	q		20	
17.69	17.5–18.0	HCB-$^{13}C_6$	292	257		0.05	20	
17.71		HCB	284	249	Q	0.05	20	1.22 (8)
			284	214	q		20	
18.38	18.0–18.7	Simazine	201	173	Q	0.05	10	1.86 (8)
			186	91	q		10	
18.62	18.4–19.0	Atrazine	200	122	Q	0.05	10	1.26 (3)
			200	132	q		10	
18.65	18.5–19.2	Lindane-D_6	224	150		0.05	10	
18.79		Lindane	219	183	Q	0.05	10	1.56 (4)
			217	181	q		10	
18.88		4-n-Octylphenol	107	77	Q	0.05	20	4.15 (4)
			206	107	q		20	
19.11	19.0–20.2	Phenanthrene	178	152	Q	0.05	20	2.15 (3)
			178	176	q		35	
19.14		Terbutylazine-D_6	234	178		0.05	10	
19.20		Terbutylazine	214	132	Q	0.05	10	1.08 (6)
19.31		Anthracene	178	152	Q	0.05	20	1.85 (3)
			178	176	q		35	
20.44	20.1–20.9	Endosulfan ether	239	204	Q	0.1	10	1.14 (6)
			272	237	q		10	
20.99	20.7–21.5	4-n-Nonylphenol	107	77	Q	0.1	20	2.31 (5)
			220	107	q		10	

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Table 1. Experimental conditions of the optimized EI (SRM) method (compounds regulated by Directive 2000/60/EC are shown in italic)

t_R (min)	Window (min)	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Q/q	Dwell time (s)	Collision energy (eV)	Q/q ratio[a]
21.09		PCB 28	256	186	Q	0.1	20	1.20 (4)
			258	186	q		20	
21.6	21.3-22.2	Heptachlor	272	237	Q	0.1	10	1.17 (4)
			274	239	q		10	
21.82		Alachlor	188	160	Q	0.1	10	1.97 (2)
			188	131	q		20	
22.46	22.2-23.0	PCB 52	290	220	Q	0.3	20	2.33 (4)
			290	255	q		10	
22.91	22.7-23.2	Aldrin	261	191	Q	0.3	30	1.70 (5)
			263	193	q		20	
23.19	23.0-24.0	Metolachlor	162	132	Q	0.1	20	1.08 (9)
			238	162	q		10	
23.41		Chlorpyrifos	199	171	Q	0.1	10	2.26 (9)
			316	260	q		10	
23.99	23.7-24.4	Isodrin	193	157	Q	0.3	20	1.63 (4)
			195	123	q		30	
24.46	24.2-25.0	Heptachlor epox B	355	265	Q	0.05	20	1.47 (9)
			351	261	q		10	
24.56		Fluoranthene	202	200	Q	0.05	30	8.40 (3)
			202	152	q		20	
24.64		Heptaclor epox A	183	119	Q	0.05	10	1.23 (8)
			185	157	q		20	
24.99	24.8-25.3	Chlorfenvinphos	267	159	Q	0.3	10	2.75 (4)
			323	267	q		20	
25.50	25.2-26.3	Pyrene	202	200	Q	0.1	35	5.33 (5)
			202	150	q		45	
25.75		PCB 101	326	256	Q	0.1	20	1.20 (4)
			324	254	q		20	
25.79		α-Endosulfan	239	204	Q	0.1	20	1.18 (9)
			272	237	q		10	
26.76	26.3-27.5	Dieldrin	263	193	Q	0.1	30	1.23 (9)
			261	191	q		20	
26.78		*p,p'*-DDE-D_8	324	254		0.05	20	

Table 1. Experimental conditions of the optimized EI (SRM) method (compounds regulated by Directive 2000/60/EC are shown in italic)

t_R (min)	Window (min)	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Q/q	Dwell time (s)	Collision energy (eV)	Q/q ratio[a]
26.84		*p,p'*-DDE	216	246	Q	0.1	20	1.36 (5)
			318	246	q		20	
27.89	27.3-28.3	ß-Endosulfan	241	170	Q	0.3	30	1.33 (6)
			193	123	q		20	
28.01		PCB 118	326	256	Q	0.3	20	1.60 (2)
			326	254	q		20	
28.36	28.0-29.4	*p,p'*-DDD	235	165	Q	0.1	20	1.50 (1)
			237	165	q		20	
28.85		PCB 153	360	190	Q	0.1	20	1.62 (2)
			358	288	q		20	
29.45	29.2-30.8	Endosulfan sulfate	272	237	Q	0.1	20	1.26 (5)
			274	239	q		10	
29.69		*p,p'*-DDT	235	165	Q	0.1	30	3.10 (5)
29.82			237	165	q		10	
		PCB 138	360	290	Q	0.1	20	1.57 (3)
			358	288	q		20	
31.03	30.8-31.8	B(a)anthracene-D_{12}	240	236		0.3	30	
31.17		B(a)anthracene[b]	228	226	Q	0.3	20	3.01 (3)
31.34		Chrysene[b]	228	224	q	0.3	55	2.75 (4)
31.81	31.5-32.9	Metoxychlor	227	169	Q	0.1	30	1.13 (4)
			227	141	q		20	
32.20		PCB 180	394	324	Q	0.1	20	1.84 (4)
			392	322	q		30	
32.96	32.6-34.4	Mirex	272	237	Q	0.1	10	1.59 (2)
			274	239	q		10	
35.31	34.4-36.5	BDE 100	404	297	Q	0.3	20	1.39 (19)
			564	404	q		20	
35.86		B(b)fluoranthene[b]	252	250	Q	0.1	35	3.23 (3)
35.96		B(k)fluoranthene[b]	250	248	q	0.1	35	3.62 (6)
36.14		BDE 99	404	297	Q	0.3	20	1.48 (22)
			564	404	q		20	
37.08	36.5-39.0	B(a)pyrene	252	250	Q	0.3	35	4.12 (3)
			250	248	q		30	

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Table 1. Experimental conditions of the optimized EI (SRM) method (compounds regulated by Directive 2000/60/EC are shown in italic)

t_R (min)	Window (min)	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Q/q	Dwell time (s)	Collision energy (eV)	Q/q ratio[a]
40.70	39.0–44.0	Indeno(1,2,3,cd)pyrene	276	274	Q	0.1	40	3.04 (3)
			276	272	q		60	
40.83		Dibenzo(a,h)anthracene	278	276	Q	0.1	30	2.55 (7)
			278	274	q		50	
41.36		B(g,h,i)perylene	276	274	Q	0.1	30	3.88 (7)
			274	272	q		30	

[a] Average value calculated from nine injectiions of standards solutions (three replicates, three concentration levels), and R.S.D. in parenthesis.

Sample material

Water samples of different types and origin were collected on September 2005 from different sites at the Valencia area (Spain), and they were analyzed in order to investigate the presence of selected organic contaminants. Three surface water samples were collected from Borriana (Clot), Vila-Real (Mijares River) and Alcora (M^a Cristina Dam). Two ground water samples were collected from wells located at Puerto de Sagunto and Almazora. Moreover, two samples – before and after treatment – were obtained from a water urban treatment plant sited at Vila-Real.

All samples with suspended solids or turbidity were filtered through Nylon filter (45 μm, under vacuum) before analysis. Raw urban water was centrifuged before filtering.

GC instrumentation

A GC system (Agilent 6890N, Palo Alto, USA) equipped with an autosampler (Agilent 7683) was coupled to a triple quadrupole (QqQ) mass spectrometer, Quattro Micro GC (Micromass, Boston, USA), operating in EI and CI modes. The GC separation was performed using a fused silica HP-5MS capillary column with a length of 30 m × 0.25 mm i.d. and a film thickness of 0.25 μm (J&W Scientific, Folson, CA, USA). The oven temperature was programmed as follows: 90 °C (1 min); 20 °C min^{-1} to 180 °C; 3 °C min^{-1} to 280 °C; 30 °C min^{-1} to 300 °C (2.5 min). Splitless injections of 1 μL sample were carried out. Helium 99.999% (Carburos Metálicos, Valencia, Spain) was used as carrier gas at a flow of 1 mL min^{-1}. The interface temperature was set to 250 °C and a solvent delay of 3 min was selected.

Working in EI, the source temperature was set at 250 °C and the system operated in MS/MS (SRM) mode using argon 99.995% (Carburos Metálicos) as collision gas at a pressure of 2.8 × 10^{-3} mbar in the collision cell. Dwell times/channel between 0.05 and 0.3 s was chosen.

Working in negative chemical ionization (NCI), the source temperature was selected at 110°C and the QqQ system operated in SIR (selected ion recording) mode. Methane 99.9995% (Carburos Metálicos) was used with an optimal flow of 40%.

The application manager Quanlynx was used to process the quantitative data obtained from calibration standards and from samples.

Analytical procedure

About 1 mL of surrogate solution mixture (five internal labeled standards) was added to 100 mL of water sample and passed through the SPE cartridge previously conditioned by passing 6 mL methanol, 6 mL ethyl acetate:DCM (50:50), 6 mL methanol and 6 mL deionized water avoiding dryness. After loading the sample, cartridges were washed with 3 mL of deionized water. The cartridge was dried by passing air, using vacuum for at least 15 min, and then the elution was performed by passing 5 mL of ethyl acetate:DCM (50:50). The extract collected was evaporated under a gentle nitrogen stream at 40 °C and redissolved in 1 mL of hexane.

The extract obtained was injected into the Quattro Micro GC system in (EI)MS/MS or in (NCI)MS under the experimental conditions shown in Table 1 and Table 2, respectively. Quantification of samples was carried out by using calibration curves prepared with standards in solvent containing also the surrogates, using relative responses to the corresponding internal labeled standard added as surrogate to the samples. The selection of each internal standard (IS) was made according to its retention/elution behavior in the SPE procedure and/or its gas chromatographic retention time.

Table 2. Experimental conditions of the optimized NCI (SIR) method

t_R (min)	Window (min)	Compounds	Ion (m/z)	Q/q	Dwell time (s)	Q/q ratio[a]
13.51	12.0–16.0	Pentachlorobenzene	250	*Q*	0.3	1.67 (1)
			248	*q*		
17.25	16.0–17.5	Trifluraline	335	*Q*	0.1	3.23 (4)
			305	*q*		
17.69	17.5–18.3	HCB-$^{13}C_6$	290		0.3	
17.71		HCB	284	*Q*	0.3	1.26 (1)
			286	*q*		
18.65	18.3–20.0	Lindane-D_6	261		0.3	
18.79		Lindane	255	*Q*	0.3	1.63 (2)
			257	*q*		
20.44	20.0–21.2	Endosulfan ether	308	*Q*	0.3	1.71 (7)
			342	*q*		
21.6	21.2–22.5	Heptachlor	300	*Q*	0.1	1.82 (5)
			266	*q*		
22.91	22.5–23.6	Aldrin	330	*Q*	0.3	5.72 (5)
			237	*q*		
23.99	23.6–24.3	Isodrin	330	*Q*	0.3	5.65 (9)
			364	*q*		
23.49	24.3–25.4	Heptachlor epox B	318	*Q*	0.1	1.64 (9)
			388	*q*		
24.64		Heptaclor epox A	354	*Q*	0.1	1.70 (8)
			388	*q*		
25.79	25.4–26.4	α-Endosulfan	406	*Q*	0.1	2.64 (8)
			372	*q*		
26.76	26.4–27.5	Dieldrin	346	*Q*	0.1	1.30 (3)
			380	*q*		
26.78		*p,p'*-DDE-D_8	326		0.1	
26.84		*p,p'*-DDE	318	*Q*	0.1	1.30 (5)
			316	*q*		
27.89	27.5–28.2	ß-Endosulfan	406	*Q*	0.3	2.50 (6)
			372	*q*		
28.36	28.2–29.0	*p,p'*-DDD	285	*Q*	0.1	7.16 (14)
			320	*q*		
29.45	29.0–30.0	Endosulfan sulfate	386	*Q*	0.1	8.50 (6)
			421	*q*		
29.69		*p,p'*-DDT	248	*Q*	0.1	2.92 (9)
			284	*q*		
31.81	30.0–32.0	Metoxychlor	166	*Q*	0.3	12.81 (5)
			167	*q*		
32.96	32.0–44.0	Mirex	368	*Q*	0.1	2.87 (3)
			404	*q*		

[a] Average value calculated from nine injections of standard solutions (three replicates, three concentration levels), and R.S.D. in parenthesis.

Validation study

Statistical validation of the method developed was performed evaluating the following parameters:

• Linearity. The calibration curves were obtained by analyzing reference standard solutions in duplicate. The range of concentration studied was 0.5–50 μg L^{-1} which corresponded to 5–500 ng L^{-1} in the water samples. Linearity was assumed when regression coefficient was >0.99 with residuals lower than 30%.

• Accuracy. The accuracy was estimated by means of recovery experiments, analyzing deionized water (n = 5) spiked at two concentrations levels (25 and 250 ng L^{-1}).

• Precision. The precision, expressed as repeatability of the method, was determined in terms of relative standard deviation (R.S.D., %) from the recovery experiments (n = 5) at each fortification level.

• Selectivity. The selectivity of the GC–(EI)MS/MS procedure was based on monitoring the appropriate MS/MS transitions for each analyte by selecting the adequate precursor and product ions. In the GC–(NCI)MS procedure, the selectivity was based on the appropriate m/z ions selection.

• Limit of quantification (LOQ). The LOQ was established as the lowest concentration level that was fully validated by applying the overall analytical procedure, with satisfactory recovery (70–120%) and precision (R.S.D. < 20%).

• Limit of detection (LOD). The LOD was estimated as the analyte concentration that produced a peak signal of three times the background noise from the chromatogram at the lowest fortification level tested.

• Confirmation criteria. The Q/q ratio, defined as the ratio between the intensity of the quantification transition (Q) and the confirmation transition (q), was used to confirm peak identity in samples. Firstly, the theoretical average Q/q for each compound was calculated as the mean value obtained from three standard solutions injected in triplicate each (n = 9) (see Table 1 and Table 2). The identity of a peak was confirmed by comparison of the experimental Q/q ratio in the sample with the theoretical Q/q ratio of the reference standard.

To confirm a finding as an actual positive, a maximum ratio tolerance ±20% was accepted when the intensity of the confirmative transition was higher than 50% of the quantitative one (Q/q ratio value <2). This was the case for the majority of the target analytes. For higher Q/q ratios, the tolerances were increased. So, deviations of ±25% (relative intensity 20–50%, Q/q ratio 2–5), ±30% (relative intensity 10–20%, Q/q ratio 5–10) and ±50% (relative intensity ≤10%, Q/q ratio >10) were accepted. These criteria are in the line of the European Commission Decision 2002/657/EC, which was originally defined for the determination of organic contaminants in food samples, although it is being increasingly used in environmental and biological samples (40,43).

Obviously, the agreement in the retention time in sample and standard was also required to confirm a positive.

RESULTS AND DISCUSSION

GC optimization

Electron impact (EI)MS/MS

Optimization of the MS/MS method was performed for all organic contaminants selected using hexanic standard solutions with the GC–MS operating in EI ionization mode. After obtaining the full scan spectra, the precursor ion for every analyte was selected as base peak of the spectra. Once the precursor ion was selected, different values of collision energy (between 10 and 60 eV) were tested to perform the subsequent fragmentation. The final purpose was to develop a selected reaction monitoring (SRM) method with at least two MS/MS transitions (with the exceptions of surrogates with only required one transition), normally the most sensitive ones, for each compound in order to have a reliable confirmation of the identity of the analyte. Table 1 shows the quantitative (Q) and confirmative (q) transitions acquired and the collision energy selected for every compound in EI ionization mode. Optimum values of collision energy were found to be normally lower than 20 eV, except for PAHs, which required higher collision energy (30–60 eV) due to their poor fragmentation in EI ionization (aromatic rings and high stability). Moreover, the loss of two or four hydrogen atoms were commonly chosen as quantitative and/or confirmative transitions for the determination of PAHs due to the improved selectivity and sensitivity attainable.

The theoretical values of the Q/q intensity ratios are also shown in Table 1. Average Q/q ratios, used for confirmation, were calculated for every analyte from standard solutions at three concentration levels (10, 25 and 50 μg L^{-1}) injected each in triplicate (n = 9), obtaining satisfactory R.S.D. (≤10%) except for BDEs (>15%), probably due to their low sensitivity. Average Q/q ratio values, similarly to retention times, suffered slight variations along the experiments performed in this work. So, they were corrected with the calibration standards included in every sample sequence.

The dwell time parameter was also optimized in order to obtain a good chromatographic peak (with at least 10 points/peak) maintaining satisfactory sensitivity for each compound. This parameter was modified between 0.01 and 0.5 s. The best results, which allowed a compromise between sensitivity and peak shape, are also shown in Table 1.

Linearity of relative response (analyte versus internal standard) was studied by injecting standard solutions, in duplicate, in the range 0.5–50 μg L^{-1} (PAHs with the exceptions of naphthalene and acenaphthylene, 5–50 μg L^{-1}), 2.5–50 μg L^{-1} (dieldrin and simazine), 5–50 μg L^{-1} (heptachlor epoxide A and B, α- and ß-endosulfan and BDE 99) and 1–50 μg L^{-1} (the rest of compounds). The values of regression coefficient were higher than 0.99 for all compounds over the whole range tested, with residuals lower than 20%.

Negative chemical ionization (NCI)MS

NCI was tested for OC pesticides as this ionization mode allowed improving the sensitivity in comparison to EI. The development of a MS/MS procedure was not feasible in NCI for these compounds as the only transition observed was the fragmentation of the precursor ion to give a chlorine atom. The low selectivity of this transition unadvised to use it, and therefore a SIR method was optimized, using at least two *m/z* ions, the most sensitive ones, selected from the full scan spectra of each compound.

To optimize NCI(SIR) method, different values of source temperature (100–150 °C), electron energy (30–100 eV), emission current (100–500 μA) and methane flow (20–60%) were tested in order to increase the sensitivity. The values selected were 110 °C, 100 eV, 300 μA and 40%, respectively.

As a summary, Table 2 shows dwell time values and both the quantitative (Q) and confirmative (q) *m/z* ions chosen for every compound. Average Q/q intensity ratios, used for confirmation, were calculated for every analyte from standard solutions at three concentration levels (10, 25 and 50 μg L^{-1}) injected each in triplicate (n = 9), obtaining R.S.D. < 15%.

Linearity of relative response of analytes versus internal standard was established by analyzing standard solutions in duplicate, with concentrations ranging from 1 to 50 μg L^{-1}, with the exceptions of *p,p'*-DDD and *p,p'*-DDT in the range 5-50 μg L^{-1}. The values of regression coefficient were higher than 0.99 for all compounds over the whole range tested, with residuals lower than 20%, except for pentachlorobenzene, trifluraline, HCB, isodrin and *p,p'*-DDE (30%) although no tendencies were observed.

SPE optimization

The objective of this paper was not to investigate in detail the extraction step, so SPE optimization was not extensively studied as it is a well known and established technique for the extraction of analytes selected. Based on the literature and on our own experience (44-47), C_{18} cartridges were chosen to perform the SPE procedure.

Different organic solvents (DCM, ethyl acetate and ethyl acetate:DCM, 50:50) and elution volumes (between 2 and 10 mL) were tested to optimize the elution step. Based on the results obtained, 5 mL of ethyl acetate:DCM (50:50) was selected for further SPE experiments. Moreover, different volumes of fortified water, until 100 mL, were tested and no significant losses by breakthrough were observed. So, 100 mL of water was selected as volume loaded into the cartridge.

Analytical parameters

GC-(EI)MS/MS procedure

Validation of the multiresidue procedure using EI mode was carried out in terms of accuracy, precision, LODs and LOQs. Five labeled internal standards were added at the initial stage of the procedure as quality control (surrogates) in order to correct for

possible losses along the overall procedure and/or instrumental deviations. Surrogates were used as follows: benzo(*a*)anthracene-D_{12} (for PAHs), terbutylazine-D_5 (for herbicides and OP insecticides), *p*,*p*′-DDE-D_8 (for octyl/nonyl phenols, BDEs, PCBs and OC pesticides such as trifluraline, aldrin, isodrin, *p*,*p*′-DDE, *p*,*p*′-DDD and *p*,*p*′-DDT), HCB-$^{13}C_6$ (for pentachlorobenzene and HCB) and lindane-D_6 for the rest of OC pesticides (lindane, endosulfan and derivatives, heptachlor, heptachlor epoxides, dieldrin, methoxychlor and mirex).

Precision and accuracy of the developed EI procedure were studied using deionized water fortified at two concentrations (25 and 250 ng L^{-1}). All experiments were performed in quintuplicate (Table 3). In general, recoveries were satisfactory with average values between 70 and 120%. Among OC insecticides, the exceptions were aldrin, isodrin and mirex whose recoveries were slightly lower (around 60%) probably due to the surrogate used, which did not adequately correct their low absolute recovery (around 50%). In the case of PCBs, the 28 and 52 congeners showed recoveries higher than 150% at the lowest level assayed, although precision was better than 9%. The two more volatile PAHs, naphthalene and acenaphthylene, were poorly recovered (<60%), which is in compliance with the literature (47,48).

Table 3. Average recovery (%) and R.S.D. (in parenthesis) after the application of the GC–(EI)MS/MS procedure to deionised water sample fortified (*n*=5) at two concentration levels

Compounds	Fortification levels (ng L^{-1})		LOD (ng L^{-1})	LOQ (ng L^{-1})
	25	250		
Pentachlorobenzene	94 (11)	97 (10)	5	25
Trifluraline	105 (10)	103 (5)	5	25
HCB	96 (12)	105 (4)	5	25
Lindane	101 (8)	104 (6)	5	25
Endosulfan ether	95 (11)	104 (5)	5	25
Heptachlor	82 (13)	69 (5)	5	25
Aldrin	59 (14)	62 (7)	25	25
Isodrin	60 (5)	65 (10)	10	25
Heptachlor epox B	-	101 (4)	30	250
Heptachlor epox A	-	98 (4)	30	250
α-Endosulfan	-	101 (8)	130	250
Dieldrin	94 (17)	95 (5)	25	25
p,*p*′-DDE	88 (2)	101 (5)	15	25
ß-Endosulfan	-	97 (4)	100	250
p,*p*′-DDD	96 (5)	110 (3)	5	25
Endosulfan sulfate	92 (5)	106 (3)	10	25
p,*p*′-DDT	96 (5)	107 (7)	10	25
Metoxychlor	111 (9)	97 (6)	5	25
Mirex	64 (23)	63 (7)	15	25
PCB 28	182 (3)	105 (8)	5	250
PCB 52	149 (6)	99 (9)	5	250
PCB 101	117 (10)	98 (5)	15	25
PCB 118	119 (4)	91 (6)	5	25
PCB 153	90 (11)	83 (10)	20	25
PCB 138	102 (15)	92 (10)	15	25
PCB 180	73 (19)	68 (6)	25	25
BDE 100	-	78 (10)	60	250
BDE 99	-	70 (6)	150	250
Naphthalene	-	57 (9)	100	[a]
Acenaphthylene	-	23 (14)	130	[a]
Acenaphthene	103 (11)	85 (12)	5	25

Table 3. Average recovery (%) and R.S.D. (in parenthesis) after the application of the GC–(EI)MS/MS procedure to deionised water sample fortified (n=5) at two concentration levels

Compounds	Fortification levels (ng L^{-1})		LOD (ng L^{-1})	LOQ (ng L^{-1})
	25	**250**		
Fluorene	113 (9)	89 (8)	5	25
Phenanthrene	104 (8)	90 (4)	5	25
Anthracene	107 (10)	84 (2)	5	25
Fluoranthene	106 (5)	93 (5)	5	25
Pyrene	117 (3)	92 (3)	5	25
B(*a*)anthracene	105 (3)	86 (2)	1	25
Chrysene	100 (3)	90 (6)	1	25
B(*b*)fluoranthene	94 (9)	86 (7)	5	25
B(*k*)fluoranthene	84 (18)	94 (5)	5	25
B(*a*)pyrene	83 (12)	82 (6)	5	25
Indeno(1,2,3,*cd*)pyrene	66 (23)	89 (6)	5	25
Dibenzo(*a*,*h*)anthracene	74 (16)	99 (10)	5	25
B(*g*,*h*,*l*)perylene	68 (20)	101 (5)	5	25
4-*t*-Octylphenol	74 (12)	101 (13)	5	25
Simazine	–	108 (4)	30	250
Atrazine	124 (9)	101 (6)	10	25
4-*n*-Octylphenol	90 (7)	88 (11)	20	25
Terbutylazine	109 (18)	110 (8)	10	25
4-*n*-Nonylphenol	80 (12)	82 (12)	10	25
Alachlor	106 (8)	118 (11)	10	25
Chlorpyrifos	75 (13)	100 (17)	10	25
Chlorfenvinphos	98 (10)	68 (12)	5	25

Underlined, not acceptable results. Detection (LOD) and quantification (LOQ) limits.

[a] LOQ not estimated as validation parameters at both fortification levels were not satisfactory.

R.S.D. were better than 20% for the majority of compounds studied, with the only exceptions of mirex and indeno(1,2,3,*cd*)pyrene at the lowest level assayed with a slightly higher value (23%). The limit of quantification (LOQ) objective, defined as the lowest concentration level that the method was fully validated for with satisfactory recoveries and precision, was 25 ng L^{-1} for most of compounds. In some cases the LOQ objective could not be reached due to the lower sensitivity attained (heptachlor epoxides, α- and ß-endosulfan, BDEs, naphthalene and simazine). For other compounds, such as PCB 28 and 52, the LOQ was established at 250 ng L^{-1} as the validation was not fully satisfactory at the lowest level assayed. Limits of detection (LOD), calculated as the analyte concentration giving a peak of three times the signal-to-noise ratio in the chromatograms obtained at the LOQ level, were in the range of 1–30 ng L^{-1} for most of compounds, with the exceptions of α- and ß-endosulfan, BDE 100 and BDE 99 (130, 100, 60 and 150 ng L^{-1}, respectively). These LOD values were obtained from the quantification transition (*Q*).

Regarding LOD, it is troublesome to make a realistic comparison with those reported in the literature using other GC techniques, because the criteria and methodology used for estimating the LODs are highly variable in the bibliography and the values are continuously decreasing with the new generation of mass spectrometers. However, it seems evident the excellent sensitivity of the SRM approach in tandem MS for quantification and confirmation as recently reported by several authors (28,36,40).

Moreover, the experimental intensity *Q*/*q* ratios obtained from deionized water sample extracts fortified at the lowest level validated (i.e. the worst case) were compared to those calculated from reference standards prepared in solvent (see Table 1) in order to test the robustness of the values for the confirmation process. Compounds selected were confirmed at such a low level, showing *Q*/*q* deviations in compliance with the confirmation criteria (see Section 2.5). Aldrin was an exception because the confirmation (*q*) transition gave no signal at 250 ng L^{-1} due to its poor sensitivity. As an example, Fig. 1 shows MS/MS chromatograms for several of the compounds studied prepared in solvent and in fortified water samples at 25 ng L^{-1}, including also the *Q*/*q* ratios.

Additionally, the procedure was extended to other type of water matrices, selecting five additional samples: a tap water from Castellón city, two ground water

samples from Piles (Valencia) and Benlloch (Castellón), and two surface water collected from sites (Vall d'Uixó and Vila-Real) of the Castellón province. In a first step, matrix effects were checked comparing responses of standards prepared in hexane (25 μg L^{-1}) with those of standards added into the final hexanic water extract obtained after applying the overall SPE procedure (250 ng L^{-1} in sample, as the concentration factor applied in sample treatment was 100). No severe matrix effects were observed that affected the response of the analytes in any of the samples selected, so calibration could be prepared with standards in solvent independently of the water samples analyzed.

Later, validation was also extended to these five water samples fortified at 250 ng L^{-1} (n = 10), analyzing them in duplicate. The average recoveries were between 70 and 120% with the reproducibility better than 22% for the majority of compounds studied, in the line of the results obtained previously for deionized water.

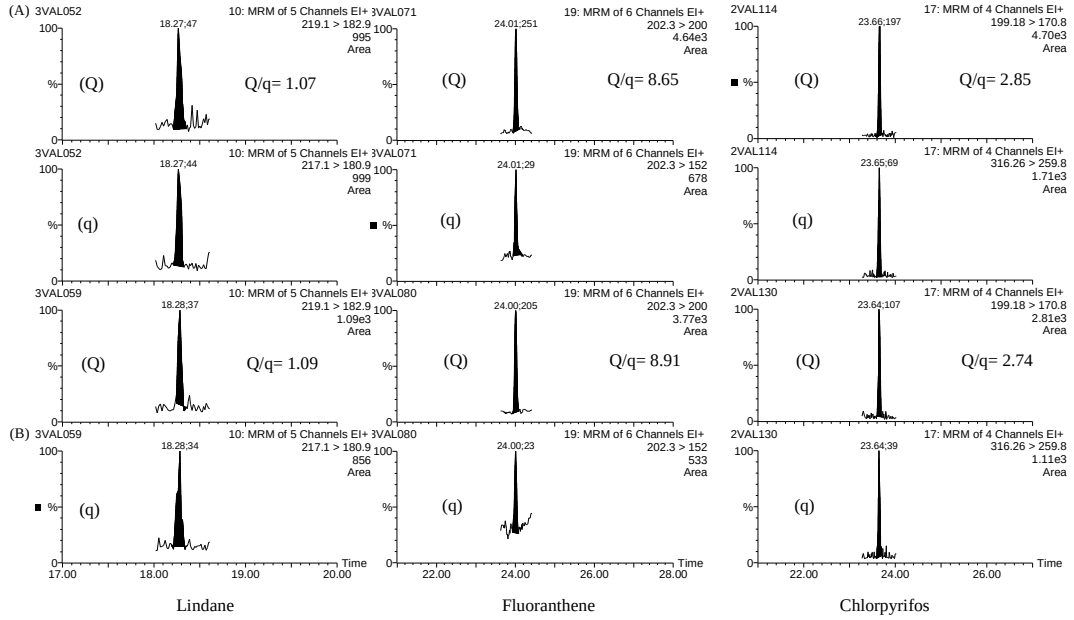


Fig. 1. (EI)SRM chromatograms for selected compounds. (A) Standard solution at 2.5 μg L^{-1}. (B) Water sample fortified at 25 ng L^{-1} (concentration of the extract to be injected into GC system is 2.5 μg L^{-1}). Quantification transition (Q). Confirmation transition (q). Q/q intensity ratios for standard solutions and for fortified samples are shown.

GC-(NCI)MS procedure

The GC–(NCI)MS procedure was only applied for the determination of OC pesticides with the aim to increase the sensitivity. Three labeled internal standards were added at the initial stage of the procedure as surrogates: HCB-$^{13}C_6$ (used for quantification of pentachlorobenzene and HCB), *p*,*p*′-DDE-D_8 (for trifluraline, aldrin, isodrin, *p*,*p*′-DDE, *p*,*p*′-DDD, *p*,*p*′-DDT and mirex) and lindane-D_6 for the rest of OC pesticides.

Precision and accuracy were calculated by analyzing five replicates of deionized water fortified at two levels, 25 and 250 ng L^{-1} (Table 4). Recoveries were satisfactory although heptachlor, at the highest level, and aldrin and isodrin, at the lowest level showed values around 60%. R.S.D. were also satisfactory with values better than 13% for the majority of compounds studied.

Table 4. Average recovery (%) and R.S.D. (in parenthesis) after the application of the GC–(NCI)MS procedure to deionised water sample fortified (n = 5) at two concentration levels

Compounds	Fortification levels (ng L^{-1})		LOD (ng L^{-1})	LOQ (ng L^{-1})
	25	250		
Pentachlorobenzene	91 (4)	113 (1)	1	25
Trifluraline	91 (6)	102 (7)	1	25
HCB	93 (5)	103 (3)	0.2	25
Lindane	102 (1)	101 (2)	2	25
Endosulfan ether	95 (5)	99 (1)	2	25
Heptachlor	72 (6)	59 (8)	9	25
Aldrin	65 (9)	65 (5)	25	25
Isodrin	61 (7)	73 (2)	25	25
Heptachlor epox B	94 (13)	93 (4)	3	25
Heptachlor epox A	91 (8)	87 (2)	4	25
α-Endosulfan	79 (2)	87 (1)	0.3	25
Dieldrin	93 (11)	86 (6)	9	25
p,*p*′-DDE	109 (4)	97 (4)	3	25
ß-Endosulfan	72 (6)	77 (3)	0.2	25
p,*p*′-DDD	-	114 (20)	30	250
Endosulfan sulfate	73 (3)	83 (3)	9	25
p,*p*′-DDT	-	83 (11)	190	250
Mirex	93 (13)	94 (6)	3	25

Detection (LOD) and quantification (LOQ) limits.

Methoxychlor could not be studied by NCI because, under the conditions selected, it was not possible to obtain satisfactory fragmentation (spectra).

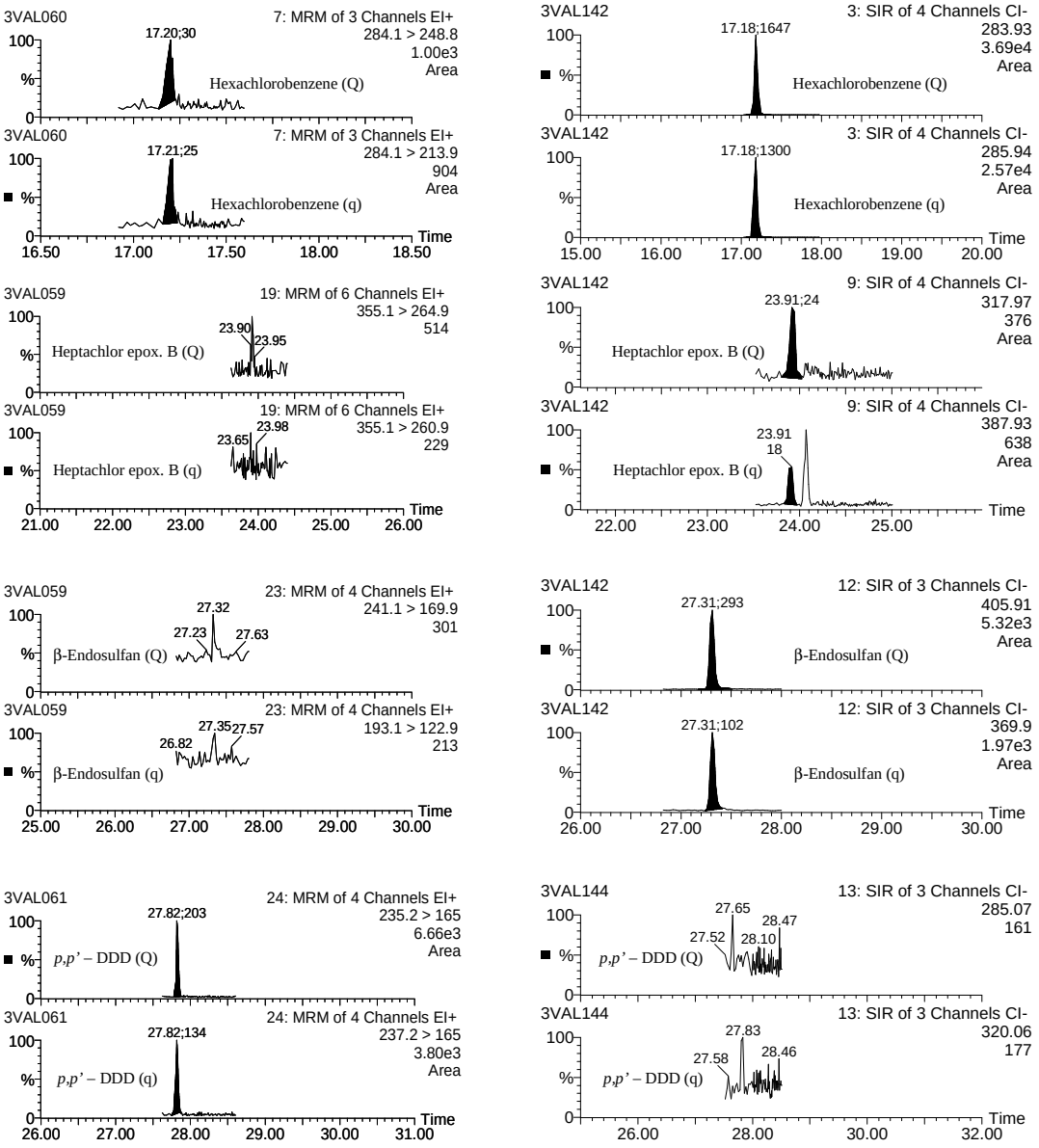
Table 4 also shows LOQs and LODs for OC insecticides by applying the NCI procedure. For most of analytes, LOQ value was set up at 25 ng L^{-1}, except for *p*,*p*′-DDD and *p*,*p*′-DDT due to their low sensitivity. LODs were in the range of 0.2–10 ng L^{-1}, except for *p*,*p*′-DDD (30 ng L^{-1}) and *p*,*p*′-DDT (190 ng L^{-1}).

The experimental intensity Q/q ratios obtained from sample extracts fortified at the lowest level validated were also compared to those calculated from standards prepared in solvent (see Table 2). Compounds selected could be confirmed at that level, showing Q/q deviations in compliance with the confirmation criteria employed (see

Section 2.5). Two exceptions were aldrin and isodrin where the *q* transition at 25 ng L^{-1} gave very low signal, insufficient to use the *Q*/*q* intensity ratio as a criterion for confirmation.

The NCI procedure allowed a considerable improvement of sensitivity for most of OC analytes studied, making feasible their quantification and confirmation, for example heptachlor epoxides and/or α- and ß-endosulfan included, at 25 ng L^{-1} or even at lower concentration. Two notable exceptions were *p*,*p*'-DDD and *p*,*p*'-DDT, which showed much lower sensitivity by using NCI procedure. This has been also reported by other authors (49). Fig. 2 shows MS/MS chromatograms for several selected OCs after applying EI and NCI procedure, and also emphasizes the notably higher sensitivity reached by NCI.

As regards the confirmation potential, obviously two transitions in the (EI)GC–MS/MS is more valuable for identification purposes that the use of two ions in the (NCI)GC–MS approach. However, the whole isotopic pattern might be considered taking into account the number of chlorine atoms present in the molecule, leading to a notable improvement in the confirmation capability of the NCI procedure.



(EI) (NCI)

Fig. 2. Chromatograms for several compounds in water fortified at 25 ng L^{-1} after application of the EI and the NCI procedures. Quantification transition (*Q*). Confirmation transition (*q*).

Application to real samples

The optimized (EI)MS/MS procedure was applied to the analysis of several water samples, collected at different sites of the Valencia Region (Spain). Samples consisted on two ground waters, three surface waters and two waters provided from an urban treatment plant (one treated and one raw obtained before treatment).

The majority of analytes detected were pesticides, as a consequence of the wide use of these compounds in our area, where citric crops are predominant. The herbicides simazine and terbutylazine were detected in all the seven samples, at concentration levels in the range 25–8100 ng L^{-1}. The other triazine herbicide monitored, atrazine, was only detected in surface and urban waste water (both raw and treated water), at 40–400 ng L^{-1}. The same situation as atrazine was observed for the insecticide chlorpyrifos, although it was found at concentrations lower than 100 ng L^{-1} (between 25 and 82 ng L^{-1}). 4-*t*-Octylphenol was detected in five samples, but it was quantified in only one surface water (30 ng L^{-1}). Among the rest of compounds investigated, lindane, some PAHs (acenaphthylene, phenanthrene and pyrene), alachlor and chlorfenvinphos were occasionally detected, but always at concentrations below 25 ng L^{-1}.

As regards confirmation of positive findings, all the compounds that could be quantified (concentration > LOQ) as well as those detected at levels below the LOQ (between LOD and LOQ) were confirmed by the use of the two transitions selected and the compliance of the theoretical Q/q ratios. The acquisition of two transitions allowed the simultaneous quantification and confirmation of analytes in only one injection as an alternative method to the propose elsewhere where one injection with only one transition is used as a screening method (39,41) and a second injection of only potentially positive samples is required for confirmation and quantification purposes (33, 36).

The water samples collected were also analyzed by the optimized (NCI)MS method for OC pesticides. NCI mode allowed to confirm positives found in EI mode (lindane), but also to detect other compounds, which were not observed by EI, demonstrating the higher sensitivity provided by NCI. Lindane was found in six samples, ß-endosulfan in four and endosulfan-sulfate in two samples. None of the analytes detected in NCI mode was quantified as the concentrations were always below 25 ng L^{-1},

but all of them were confirmed by the use of the two *m/z* ions selected and the compliance of the theoretical *Q/q* ratios.

The excellent sensitivity of both methods applied would surely allow a reliable quantification below 25 ng L^{-1}, as Fig. 3 shows for several of the compounds detected in one surface water sample. However, concentrations < LOQ have not been reported in this paper, as the method was not fully validated below this level.

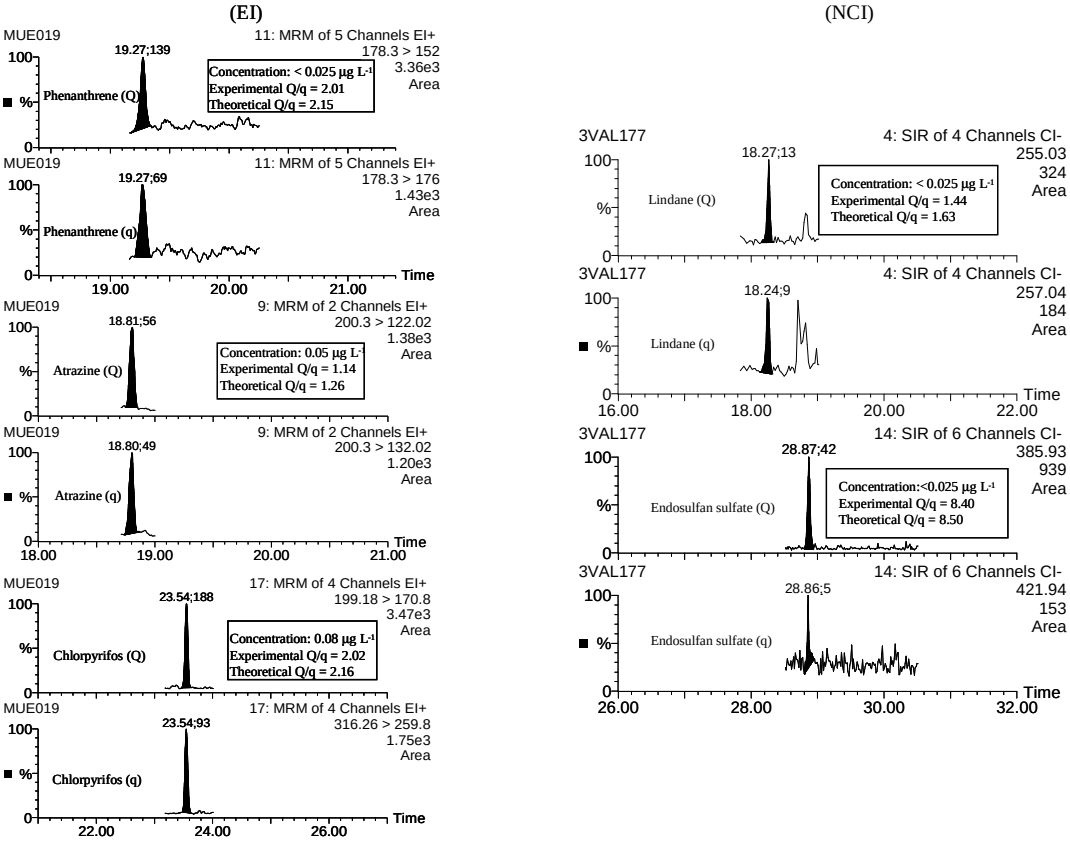


Fig. 3.(EI)SRM and (NCI)SIR chromatograms for several compounds detected in surface water. Quantification transition (*Q*). Confirmation transition (*q*). *Q/q* intensity ratios for the sample analyzed and for the reference standard are shown.

CONCLUSIONS

The potential of GC–MS/MS with triple quadrupole analyzer for multiresidue determination of semivolatile organic pollutants in water has been proved in this paper. A method based on GC–(EI)MS/MS has been developed for the simultaneous quantification and confirmation of 54 organic compounds, belonging to different chemical families, in one single determination step. The use of QqQ in selected reaction monitoring led to excellent selectivity and sensitivity, recording two transitions for each analyte and using the *Q*/*q* ratio as a confirmatory parameter. The procedure, which included an SPE extraction step with C_{18} cartridges, was validated obtaining satisfactory accuracy and precision for most of analytes, with the exceptions of naphthalene and acenaphtylene, probably due to their high volatility, and PCBs 28 and 52 that showed excessive recoveries at the lowest level assayed. The limit of quantification was 25 ng L^{-1} for the majority of the compounds, with the overall procedure satisfactorily validated at this level.

GC–(NCI)MS method was also developed for the quantification and confirmation of OC pesticides. The procedure, optimized in SIR mode with at least two *m*/*z* ions for every compound, allowed notable sensitivity improvement and was also validated obtaining satisfactory results and better LODs for most of analytes. The usefulness of the developed methods was tested by the analysis of several water samples, with the result of detecting several analytes, mainly triazines, that were all confirmed even at concentration levels below the LOQ.

ACKNOWLEDGMENTS

The Quattro Micro GC was funded by the European Union (Fondos Feder-Reino de España, Ministerio de Ciencia y Tecnología). This work forms a part of the project REN2002-01818 developed with the financial support of Ministerio de Ciencia y Tecnología. T. Portolés is very grateful to Ministerio de Educación y Ciencia for her pre-doctoral grant.

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2.3 POTENCIAL DE LA TÉCNICA GC-MS/MS CON TRIPLE CUADRUPOLO PARA LA CUANTIFICACIÓN Y CONFIRMACIÓN DE COMPUESTOS XENOESTROGENOS ORGANOHALOGENADOS EN TEJIDO ADIPOSO MAMARIO

Es conocida la amplia dispersión de los compuestos organoclorados (OCs), incluyendo tanto plaguicidas como bifenilos policlorados (PCBs), en el compartimiento biótico del medio ambiente. Su demostrada toxicidad, persistencia, y el elevado uso en el pasado en todo el planeta, así como las posibilidades de contaminación de distintos tipos de muestras (suelo, aguas, aire, productos alimenticios,...) son, sin duda, las causas de su carácter prioritario como contaminantes a controlar. Este tipo de compuestos se detectan habitualmente en una gran variedad de muestras biológicas, tales como peces, mamíferos marinos, pájaros y animales terrestres. La elevada liposolubilidad y resistencia a metabolizarse de los compuestos OCs provoca su bioacumulación a través de la cadena trófica, llegando a alcanzar niveles de concentración elevados en los últimos escalones de la misma. La población, en general, se encuentra expuesta a estos contaminantes OCs principalmente a través de la dieta (en especial alimentos grasos). En definitiva, pueden llegar a encontrarse elevados niveles de concentración de OCs en la grasa corporal humana, incluyendo tejidos adiposos, los lípidos de la sangre y la leche humana. Muchos de estos compuestos OCs pueden tener efectos adversos sobre la salud de un organismo o de su progenie como consecuencia de alteraciones en la función endocrina.

Por otra parte, los difenil éteres polibromados (PBDEs) son compuestos que, desde 1960, se han venido utilizando ampliamente como retardantes de llama, adicionándolos a termoplásticos utilizados en aplicaciones eléctricas, televisores, tarjetas electrónicas y a materiales de construcción. Estos compuestos son estructuralmente similares a los PCBs, diferenciándose por la presencia de un grupo éter, utilizándose la misma nomenclatura y presentando un número semejante de congéneres. Debido a su persistencia y bioacumulación, los PBDEs también han sido estudiados en matrices medioambientales y en seres humanos. Estos compuestos muestran una toxicidad relativamente baja, aunque datos recientes indican que pueden actuar como disruptores endocrinos.

Los disruptores endocrinos son un grupo de compuestos químicos exógenos que presentan actividad biológica hormonal; también se conocen como xenobióticos o xenoestrógenos. Muchos de estos compuestos pueden interferir en el desarrollo de los sistemas endocrinos y afectar a determinados órganos que responden a señales endocrinas. Parece que los efectos endocrinos y reproductivos de los xenobióticos son debidos a su capacidad de simular los efectos de las hormonas endógenas, producir efectos antagónicos a los de las hormonas endógenas normales, alterar el modo de síntesis y el metabolismo de las hormonas naturales y modificar los niveles de hormonas receptoras. Se trata de un grupo de compuestos muy amplio a los que los seres vivos se encuentran expuestos dado su carácter ubicuo como consecuencia de un uso generalizado, así como por su baja biodegradabilidad, el transporte de los mismos por aire y agua, y la bioacumulación en la cadena trófica. Estudios realizados durante la década de los 60 y 70 mostraron el carácter estrogénico de un elevado número de compuestos industriales y pesticidas OCs como DDT, kepone, methoxychlor y PCBs. Durante los últimos años, se han añadido a la lista de xenoestrógenos varios compuestos encontrados habitualmente en el medioambiente de modo que, en la actualidad, son muchos los contaminantes clasificados como xenoestrógenos, destacando los compuestos OCs. La preocupación por los efectos nocivos de este tipo de compuestos debería ser una constante de nuestra sociedad actual ya que todos nosotros presentamos en nuestro organismo concentraciones más o menos elevadas de contaminantes organoclorados persistentes como *p,p'*-DDE, HCB, β-HCH o algunos PCBs.

Debido a la bioacumulación de xenoestrógenos en muestras grasas, resulta de especial interés el análisis de muestras de tejido adiposo que constituyen una matriz difícil y compleja de analizar. Normalmente, debido a la baja selectividad del método de extracción utilizado para la determinación de contaminantes, gran parte de los materiales interferentes presentes en la matriz, principalmente lípidos, son co-extraídos junto con los analitos. Esto hace necesario un sistema de purificación eficaz que elimine las sustancias interferentes previamente a la determinación cromatográfica, de manera que también se eviten daños en la columna y la contaminación de los detectores. Además, la adecuada purificación de los extractos grasos permite mejorar los límites de detección del método analítico, lo cual es de gran importancia para una mayor fiabilidad de los resultados. A pesar de la aplicación de etapas de purificación,

se requieren técnicas analíticas con gran poder de identificación para confirmar la identidad de los compuestos.

Los métodos convencionales para la determinación de OCs y PBDEs en muestras ambientales y biológicas suelen implicar varios pasos, incluyendo una etapa de extracción, otra de purificación (mediante digestión con ácidos, cromatografía de adsorción en columna o extracción en fase sólida) y la determinación analítica, generalmente, por GC-ECD ó GC-MS. Aunque la técnica GC-MS en modo SIM se ha utilizado ampliamente para contaminantes orgánicos en muestras ambientales y biológicas, la detección mediante espectrometría de masas en tandem, tanto utilizando trampa de iones como triple cuadrupolo, aporta una mayor especificidad y sensibilidad, por lo que se trata de la mejor opción para el análisis de muestras complejas. La opción MS/MS utilizando equipos de triple cuadrupolo en modo SRM es la más selectiva y sensible para la cuantificación y confirmación, especialmente en el análisis de trazas donde puede aparecer un elevado ruido químico.

En el trabajo que se presenta a continuación se ha desarrollado un procedimiento analítico novedoso, eficaz y fiable para la determinación de OCs y PBDEs en muestras de tejido mamario, basado en el uso de la GC-MS/MS con analizador de triple cuadrupolo tras una etapa de tratamiento de muestra que se ha simplificado considerablemente al utilizar HPLC en fase normal con columnas de silicagel. Se ha prestado especial atención a la correcta identificación y confirmación de los compuestos detectados para evitar falsos positivos, habiéndose seleccionado dos transiciones SRM para cada compuesto y estudiando su relación de abundancia.

El procedimiento analítico se ha validado en cuanto a linealidad, exactitud, precisión y límite de detección, obteniéndose en general recuperaciones entre 80-120 % con coeficientes de variación inferiores al 15% y límites de detección entre 1-20 ng/g. Se han establecido asimismo los criterios de confirmación mediante el uso de transiciones MS/MS adicionales y evaluación de la Q/q ratios.

La metodología analítica desarrollada se ha aplicado a 51 muestras de tejido mamario de pacientes con cáncer de mama, 25 de ellas correspondientes a fragmento tumoral y las restantes a tejido graso intramamario no tumoral. En cuanto a los DDTs, el principal metabolito *p,p'*-DDE se ha detectado en un 92% de las muestras (con un valor máximo de 11.5 µg/g), mientras que *p,p'*-DDD se ha detectado, siempre a bajas

concentraciones, en un 50% de las muestras. *p,p'*-DDT sólo se ha detectado en 7 muestras (27-128 ng/g). HCB y β-HCH también han sido detectados frecuentemente (94% y 78% respectivamente), normalmente a concentraciones mayores a 10 ng/g. Respecto a PCBs, los congéneres 138, 153 y 180 han sido los más detectados a niveles normalmente inferiores a 200 ng/g.

Como confirmación última e inequívoca de la identidad de los compuestos detectados en muestras de tejido adiposo, algunas de estas muestras fueron re-analizadas mediante cromatografía de gases acoplada a espectrometría de masas con analizador de tiempo de vuelo (TOF). La confirmación de los analitos presentes a elevada concentración fue satisfactoria con errores de masa menores a 5 mDa. Sin embargo, la confirmación por TOF MS no fue posible a bajos niveles de concentración (del orden de pocos ng/g), como consecuencia de la menor sensibilidad de ésta última técnica comparada con la de triple cuadrupolo en modo SRM.

Posteriores trabajos de nuestro grupo de investigación han conseguido mejorar la sensibilidad y rapidez del método aumentando el factor de preconcentración de los extractos mediante el uso de cartuchos de SPE de silica, en lugar de la purificación por HPLC en fase normal. Además, el uso combinado de EI y NCI ha permitido aumentar la multirresidualidad del método, añadiendo a la lista de compuestos un mayor número de congéneres de PBDEs (1).

Cabe comentar que estas investigaciones se enmarcan en un proyecto de colaboración con el Instituto Valenciano de Oncología cuyo objetivo inicial era estudiar la relación existente entre los principales xenoestrógenos medidos en grasa y en tejido tumoral de pacientes operadas de cáncer de mama y su posible efecto cancerígeno. Así pues, el método desarrollado se aplicó a un total de 162 pacientes, la mayoría de ellas intervenidas por presentar un cáncer de mama en el periodo comprendido entre julio 2004 y enero de 2007. Los datos obtenidos se encuentran bajo estudio en el citado Instituto. Los resultados preliminares indican que existen mayores niveles de estos compuestos en la grasa de la mama que en el propio tumor. Esto, en principio, no permite aventurar ninguna conclusión a propósito de la influencia que estos productos puedan tener en la carcinogénesis, ya que de momento el número de muestras de tejido mamario analizadas no parece ser suficiente para realizar estudios

epidemiológicos. La importancia de la problemática abordada en este estudio está siendo objeto de investigación en los últimos años (2-7).

2.3.1. Bibliografía

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2.3.2 Artículo científico 3

Anal. Chem. 77 (23), 7662-7672, 2005

POTENTIAL OF GAS CHROMATOGRAPHY COUPLED TO TRIPLE QUADRUPOLE MASS SPECTROMETRY FOR QUANTIFICATION AND CONFIRMATION OF ORGANOHALOGEN XENOESTROGEN COMPOUNDS IN HUMAN BREAST TISSUES

Félix Hernández[1], Tania Portolés[1], Elena Pitarch[1], Francisco J. López[1], Joaquín Beltrán[1], Carlos Vázquez[2]

[1]Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain,

[2]Servicio de Cirugía, Fundación Instituto Valenciano de Oncología (FIVO), Valencia, Spain

ABSTRACT

The potential of gas chromatography coupled to tandem mass spectrometry (GC/MS/MS) with a triple quadrupole analyzer (QqQ) has been investigated for the accurate and sensitive determination of xenoestrogens in human breast tissues. Special emphasis has been given to the confirmation of the identity of compounds detected in the samples analyzed in order to avoid the reporting of false positives. The work has been focused on the determination of ~30 organochlorine compounds (PCBs and pesticides) and organobromine compounds (polybrominated diphenyl ethers) in adipose breast tissue and in tumoral fragment. Analytes were extracted by dissolving the samples in hexane, and the extracts were purified by automated normal-phase HPLC prior to GC/MS/MS analysis. Three isotopically labeled standards were added before extraction as surrogates for the quality control of the analyses. Accuracy and precision were evaluated by means of recovery experiments using adipose breast tissue spiked at three concentration levels, with satisfactory results for most analytes. The excellent selectivity and sensitity of QqQ in selected reaction monitoring mode allowed us satisfactory quantification and confirmation at levels as low as 5−25 ng/g, i.e., the lowest concentration level for which the method was fully validated. Two MS/MS

transitions were selected for each analyte, using the concentration ratio obtained from them as a confirmatory parameter. The developed methodology was applied to the analysis of 51 breast samples (26 adipose tissues and 25 tumoral fragments), giving as a result the detection and confirmation of several organochlorine compounds in both types of samples. Due to its adequate analytical characteristics, the optimized method fits with the requirements of accurate quantification and reliable confirmation of the identity of compounds detected according to the most recent European Guidelines. As an ultimate unequivocal confirmation, several selected samples were reanalyzed by gas chromatography coupled to mass spectrometry with a time-of-flight (TOF) analyzer. Confirmation of analytes present at higher concentrations was successful with mass error less than 5 mDa. However, confirmation by TOF MS was not possible al low concentrations (i.e., at the few ng/g level) as a consequence of its lower sensitivity compared with that of triple quadrupole in selected reaction monitoring mode.

INTRODUCTION

The wide use of organochlorine compounds (OCs) –including pesticides and polychlorinated byphenyls (PCBs)– in agriculture and their industrial applications for many years has led to the dispersion of these compounds in the biotic compartment of the environment worldwide. Polybrominated diphenyl ethers (PBDEs) are structurally similar to PCBs, differing only in the presence of an ether linkage, but mantaining the same nomenclature and number of congeners.[1] Over the past two decades, these compounds have been widely used as flame retardants in most types of polymers employed in electrical appliances including TV sets, computers, and other electronic household equipment.[1-3] The resistance of OCs and PBDEs to degradation and their high lipid solubility are the cause of their persistence and bioaccumulation along the food chain. Humans are exposed to these pollutants through many commonly eaten foods[4,5] but also through water, ambient and indoor air, dust, and soil.[5] As a result, OCs and PBDEs are usually detected in human serum,[6-8] adipose tissue,[1-3,7-14] and maternal milk,[7,15] as several authors have reported.

Many OCs and PCBs have shown endocrine-disrupting activity in some biological test systems.[11,13,16] They are exogenous chemicals that could evoke estrogenic

responses interfering with estrogen-controlled pathways[17] and also changing the development of endocrine systems.[18] Although PBDEs do not show high levels of toxicity, biological tests have proved that they could act as endocrine disruptors[3] as well.

Analytical methodology applied for biological monitoring of exposure to pesticides has been reviewed recently, presenting a critical assessment of this subject and exploring areas in which more research is needed.[19] Typically, the determination of OCs and PBDEs in fatty samples and, particularly, in biological samples involves several steps, including extraction and cleanup using adsortion chromatography with Florisil or silica gel columns,[5,13,20,21] gel permeation chromatography,[8,13] normal-phase HPLC,[9,10,22] and chemical treatment with H_2SO_4.[23-25]

Gas chromatography (GC) with electron capture detection has been widely used for OCs and PBDEs determination, but nowadays, mass spectrometry (MS) has become one of the most powerful tools for the acquisition of information on composition and structure of organic compounds in order to verify peak identity in a variety of environmental and food matrixes.[26,27] Although GC/MS, operating in selected ion monitoring mode, has been widely used in the determination of organic micropollutants in environmental samples, their trace analysis in complex matrixes, such as biological samples, becomes problematic due to the interferences by matrix components. The specificity of tandem mass spectrometry (MS/MS), using ion trap detectors (ITD) or triple quadrupole (QqQ) analyzers, allows us to minimize or even to eliminate many of these interferences, improving the selectivity, but it also leads to an improvement in sensitivity due to the possibility of adequate precursor and product ion selection, with much lower chemical noise in the chromatograms. The utility of product ion spectra for absolute identification at trace levels in complex matrixes together with the ease of use and low cost, compared with QqQ, has made ITD a powerful and widely used technique for the trace analysis of complex matrixes.[4,7,9-11,16,28] However, the use of two stages of mass analysis in MS/MS systems based on QqQ offers the possibility of applying selected reaction monitoring (SRM), one of the most selective and sensitive techniques for quantification and confirmation, especially in trace biomedical analysis, where normally there is high background chemical noise.

Relevant differences between ITD and QqQ can be mentioned when dealing with identification and quantification of organic compounds at low concentration levels.

Working in single MS mode, ion traps are more sensitive in full scan (allowing the identification of nontarget compounds), but normally QqQ are more sensitive in MS/MS mode, leading to lower limits of detection (LOD) for target compounds, with the confirmation of the analyte being attained by the use of full spectra of product ions (in the case of ITD) or by using several precursor ion−product ion transitions, each of them accounting for 2.5 identification points (IPs)[29] (in low-resolution QqQ instruments). The use of IPs is a recent approach to set up quality criteria for the identification of organic residues and contaminants: a laboratory is allowed to use any molecular spectrometric technique or combination of techniques in order to earn a minimum number of IPs to ensure the reliable confirmation of the identity.[29] In regard to quantification, ITD in MS/MS mode seems to be less powerful when compared with QqQ in SRM mode, which is very important in trace analysis of complex matrixes.[30-32] From a qualitative point of view, ITD might be superior as it presents as a unique feature the possibility of performing MS^n for deduction of fragmentation pathway facilitating identification of unknowns.[30] However, a limitation for ITD is that product ions of m/z values lower than 30% of m/z values for precursor ions cannot be detected, because is not possible to apply a stable trapping potential for both the precursor and the product ions.[33] In conclusion, ITD can be seen as being more powerful from the qualitative point of view, while QqQ is superior from the quantitative point of view, although the selection of an appropiate number of MS/MS transitions would result in a powerful confirmatory capability for QqQ as well.

Very little has been published until now on residue analysis by GC/MS/MS with QqQ despite its strong potential in this field. Recently, Patel et al.[34] have studied the potential of gas chromatography/tandem quadrupole mass spectrometry for the multiresidue analysis of organochlorine pesticides in fats and oils, emphasizing the excellent selectivity and limits of detection. Other examples are the determination of bacterial compounds, drugs. and anabolic steroids in hair samples[35,36] and markers for bacteria (e.g., muramic acid) in complex clinical and environmental matrixes, such as organic dust and body fluids.[31,32] Another recent and powerful technique is GC coupled to time-of-flight (TOF) mass spectrometry that can provide conclusive information on the identity of unknowns and on confirmation of known (target) compounds from their accurate mass measurements (in the order of 5 ppm) achievable due to the characteristics of high resolution. Some applications of this technique consisted on the

determination of PBDEs in human milk samples,[37] pesticide residues in peaches,[38] confirmation and identification of impurities of the herbicide metolachlor,[39] and flavor research in seafood.[40]

The aim of this work is to investigate the potential of GC coupled to triple quadrupole analyzers for the sensitive determination and reliable confirmation of OC and organobromine (OBr) compounds in human breast tissues and the development of improved analytical methodology with the new generation of instruments available nowadays. Besides, preliminary results are also obtained with GC/TOF MS in order to show the excellent performance of this technique for qualitative analysis.

EXPERIMENTAL SECTION

Reagents

Reference materials supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) with a purity 97−99.7% were used for standard preparation: pentachlorobenzene, HCB, ß-HCH, lindane, *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE, dieldrin, methoxychlor, mirex, vinclozolin, heptachlor, heptachlor epoxide A, endosulfan-ether, and endosulfan-sulfate. PCB mix 3 from Dr. Ehrenstorfer (100 µg/nL in isooctane) was used for single quantification of PCB congeners IUPAC 28, 52, 101, 118, 138, 153, and 180. Standard solutions from Dr. Ehrenstorfer (100 µg/mL in cychlohexane or isooctane) were used for single quantification of *cis*- and *trans*-chlordane, *cis*- and *trans*-nonachlor, and oxychlordane. Standards of PBDEs, BDE-100 and BDE-99 (50 µg/mL in nonane), were obtained from Wellington Laboratories (Guelph, ON, Canada).

Stock solutions (~500 µg/mL) were prepared by dissolving reference standards in acetone and stored in a freezer at −20 °C. Working solutions for sample fortification and for injection in the LC and the GC systems were prepared by diluting stock solutions in *n*-hexane. Before injection into the LC cleanup system, residues of acetone were removed using a gentle N_2 stream.

Ethyl acetate and *n*-hexane were ultratrace quality and purchased from Scharlab (Barcelona, Spain). Anhydrous sodium sulfate of pesticide residue quality (Baker, Deventer, Holland) was dried for 18 h at 300 °C before use.

Three surrogates were used: hexachlorobenzene-$^{13}C_6$ (Cambridge Isotope Labs, Inc. Andover, MA), *p,p'*-DDE-d_8, and ß-endosulfan-d_4 (Dr. Ehrenstorfer). Working solutions of labeled standards (~1 µg/mL) were prepared by dilution of stock solution with hexane and stored at 4 °C.

Sample Material

Human breast tissues were obtained from women with breast cancer with the exception of only one sample that corresponded to a healthy woman (sample 1). Two different samples were collected from each woman: breast tissue (labeled as sample a) and tumoral fragment (sample b). After collecting the samples, they were frozen at ≤18 °C until analysis.

A pooled sample obtained by mixing several breast tissue samples was used as a "blank" to optimize the analytical procedure.

LC Instrumentation. The LC system used for sample extracts cleanup was based on our previous works.[9,10,22] It consisted on a LC Pump Master 305 (Gilson), two six-way high-pressure valves VICI Valco (Europe Instruments, Schenkon, Switzerland), a sampler injector valve Rheodyne (Cotati, CA) with 1.0-mL loop, a silica column Novapack 150 × 3.9 mm i.d., 4 µm (Waters, Milford, MA), and a fraction collector Gilson FC 203B. Mobile phases used were hexane and hexane/ethyl acetate mixtures (95:5, v/v) at a flow rate of 1 mL/min.

GC Instrumentation. Two GC systems (Agilent 6890N, Palo Alto, CA) equipped with an autosampler (Agilent 7683) were coupled to (1) a triple quadrupole mass spectrometer, Quattro Micro GC (Micromass, Boston, MA), and (2) a time-of-flight mass spectrometer, GCT (Micromass), both operating in electron impact ionization mode (EI). The GC separation was performed using a fused-silica HP-5MS capillary column with a length of 30 m × 0.35 mm i.d. and a film thickness of 0.25 µm (J&W Scientific, Folson, CA). The oven temperature was programmed as follows: 90 °C (1 min); 30 °C/s to 180 °C; 3 °C/min to 250 °C, 30 °C/s to 300 °C (2 min). Splitless injections of a 1-µL sample were carried out. Helium was used as carrier gas at 1.2 mL/min.

The interface and source temperatures were set to 250 °C for all analytes studied in both systems, and a solvent delay of 3 min was selected. To operate in

MS/MS mode in the triple quadrupole system, argon 99.995% (Carburos Metálicos, Valencia, Spain) was applied as collision gas at a pressure of 2.8×10^{-3} mbar in the collision cell, and dwell times/channel of 0.1 s were chosen. The time-of-flight mass spectrometer was operated at 0.3 scan/s scanning the mass range *m*/*z* 50−650 and using a multichannel plate voltage of 2500 V. TOF-MS resolution was ~7000 (fwhm). Heptacosa, used for the daily mass calibration and as lock mass, was injected via syringe in the reference reservoir for this purpose.

Analytical Procedure. Sample Preparation and Extraction

Samples were thawed at room temperature. Between 0.1 and 0.5 g of the tissue was chopped, and 0.5 mL of surrogate solution was added. The mixture was homogenized with 5−10 g of anhydrous sodium sulfate and extracted three times at room temperature with 5 mL of *n*-hexane each time, shaking in a vortex. After filtration, the extract was preconcentrated under a gentle nitrogen stream at 40 °C, and the final residue was adjusted to 5 mL with *n*-hexane.

Cleanup Procedure

The general cleanup procedure was based on previous work carried out in our laboratory.[9,10] Each hexanic extract was purified by two complementary cleanup procedures (named as A and B), by injecting 1 mL of hexanic extract each time into the LC system. The mobile phase was *n*-hexane (procedure A) or *n*-hexane/ethyl acetate (95:5 v/v) (procedure B), in both cases at a flow rate of 1 mL/min. In both procedures, after 16 min of injecting the sample extract, a pulse of 4 mL of modifier solvent (ethyl acetate) was introduced. The fraction eluting between minutes 1 and 17 (procedure A), and between minutes 4 and 17 (procedure B), was collected and preconcentrated under a gentle nitrogen stream at 40 °C until 1 mL.

GC Analysis

The two final extracts obtained after cleanup A and B were injected directly into the Quattro Micro GC system in MS/MS mode under the experimental conditions

shown in Table 1. Quantification of the samples was carried out by using calibration curves with standards in solvent, using relative responses to three internal labeled standards added as surrogates to the samples: hexachlorobenzene-$^{13}C_6$ for pentachlorobenzene and hexachlorobenzene; *p,p'*-DDE-d_8 for ß-HCH, PBCs, heptachlor, heptachlor epoxide A, oxychlordane, *trans*/*cis*-chlordane, *trans*/*cis*-nonachlor, *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, mirex, and BDEs; and ß-endosulfan-d_4 for lindane, endosulfan-ether, vinclozolin, dieldrin, endosulfan-sulfate, and methoxychlor. The selection of each internal standard was made according to its elution behavior in the cleanup procedure and its gas chromatographic retention time. The application manager Quanlynx was used to process the quantitative data obtained from calibration standards and from the samples

Validation Study.

Statistical validation of the method developed was performed evaluating the following parameters:

Linearity. The calibration curves were obtained by analyzing reference standard solutions in triplicate. The range of concentration studied was 0.2−200 ng/mL (7 concentration points). Linearity was assumed when regression coefficient was >0.99.

Accuracy. The accuracy was estimated by means of recovery experiments, analyzing blank breast tissue samples (n = 5) spiked at three concentrations levels (5, 25, and 250 ng/g). Previously, the blank sample was analyzed to determine the content of analytes in sextuplicate. Recoveries were obtained as the ratio (in %) between the calculated concentration of spiked samples and the theoretical concentration added.

Precision. The precision, expressed as repeatibility of the method, was determined in terms of relative standard deviation (RSD, in %) from the recovery experiments (n = 5) at each fortification level.

Selectivity. The selectivity was based on monitoring the appropiate MS/MS transitions for each analyte by selecting the adequate precursor and product ions.

Limit of Quantification (LOQ). The LOQ was established as the lowest concentration level validated with satisfactory values of recovery (70−110%) and precision (RSD < 20%).

Limit of Detection. The LOD was estimated as the analyte concentration that produced a peak signal of three times the background noise in the chromatogram at the lowest fortification level studied for each compound. For those analytes that were present in the blank breast tissue sample, the LODs were estimated from the chromatograms corresponding to the analyzed blank sample. In those cases where the concentration in the blank was high, making measurement of the noise manually unfeasible, the LOD was obtained using a software option for estimating the S/N ratio and referring/recounting this value to a S/N value of three.

Confirmation Ratio. The Q/q ratio, defined as the ratio between the intensity of the quantification ion (Q) and the confirmation ion (q), was used to confirm peak identity in real and spiked samples. The experimental average Q/q value for each compound was calculated as the mean value obtained from four standard solutions injected in triplicate (n = 12) (see Table 1). Confirmation of analytes detected in samples was considered positive when the Q/q ratio was within ±20% of the average Q/q value calculated from standards.

Table 1. Conditions of the Optimized SRM Method

t_R	Window (min)	Compounds	Precursor ion (m/z)[a]		Product ion (m/z)[b]	Q/q	Collision energy (eV)	Q/q ratio[b]
4.77	3–5.4	pentachlorobenzene	248	(M)	142	*Q*	30	1.09 (2)
			250	(M+2)	142	*q*	30	
6.1	5.4–6.25	HCB	284	(M+2)	249	*Q*	20	1.48 (3)
			284	(M+2)	214	*q*	20	
		HCB-$^{13}C_6$	292	(M+4)	257		20	
6.48/6.54	6.25–6.9	ß-HCH	217	(F)	181	*Q*	10	1.05 (2)
		lindane	219	(F)	183	*q*	10	1.06 (3)
7.31	6.9–7.45	endosulfan-ether	239	(F)	204	*Q*	10	1.08 (2)
			272	(F)	237	*q*	10	
7.67	7.45–8.2	PCB 28	256	(M)	186	*Q*	20	1.61 (2)
			258	(M+2)	186	*q*	20	
7.82		vinclozolin	212	(F)	172	*Q*	10	1.64 (5)
			285	(M)	212	*q*	10	
8		heptachlor	272	(F)	237	*Q*	10	1.58 (2)
			274	(F)	239	*q*	10	
8.51	8.2–9.2	PCB 52	290	(M)	220	*Q*	20	1.48 (2)
			290	(M)	255	*q*	10	
10.09	9.2–10.45	oxychlordane	185	(F)	121	*Q*	20	1.40 (11)
			235	(F)	141	*q*	20	
10.2		heptachlor epox A	183	(F)	119	*Q*	20	1.04 (4)
			185	(F)	157	*q*	10	
10.84/11.35	10.45–11.8	*trans*/*cis*-chlordane	373	(F)	266	*Q*	20	1.35 (4)
			373	(F)	264	*q*	20	
11.18		PCB 101	324	(M)	254	*Q*	20	1.21 (3)
			326	(M+2)	256	*q*	20	
11.53		*trans*-nonachlor	409	(F)	300	*Q*	20	1.09 (5)
			407	(F)	300	*q*	20	
12.23	11.8–12.7	dieldrin	263	(F)	193	*Q*	30	1.11 (5)
			261	(F)	191	*q*	20	
12.24		*p*,*p*'-DDE-d_8	324	(M)	254		20	

Table 1. Conditions of the Optimized SRM Method

t_R	Window (min)	Compounds	Precursor ion (m/z)[a]		Product ion (m/z)[b]	Q/q	Collision energy (eV)	Q/q ratio[b]
12.26		*p,p'*-DDE	316	(M)	246	*Q*	20	1.58 (2)
			318	(M+2)	246	*q*	20	
13.28	12.7–13.75	ß-endosulfan-d_4	235	(F)	141		20	
13.53		PCB 118	326	(M+2)	256	*Q*	20	1.14 (3)
			324	(M)	254	*q*	20	
13.93	13.75–14.1	*p,p'*-DDD	235	(F)	165	*Q*	20	1.86 (2)
			237	(F)	165	*q*	20	
13.93		*cis*-nonachlor	409	(F)	300	*Q*	20	1.12 (3)
			407	(F)	300	*q*	20	
14.48	14.1–14.85	PCB 153	360	(M+2)	290	*Q*	20	1.54 (1)
			358	(M)	288	*q*	20	
15.1	14.85–15.35	endosulfan-sulfate	272	(F)	237	*Q*	20	1.13 (5)
			274	(F)	239	*q*	10	
15.5	15.35–17.1	*p,p'*-DDT	235	(F)	165	*Q*	30	2.52 (5)
			237	(F)	165	*q*	10	
15.65		PCB 138	360	(M+2)	290	*Q*	20	1.53 (2)
			358	(M)	288	*q*	20	
19.57	17.1–18.6	methoxychlor	227	(F)	169	*Q*	30	1.02 (3)
			227	(F)	141	*q*	20	
18.8	18.6–19.2	PCB 180	394	(M+2)	324	*Q*	20	1.70 (2)
			392	(M)	322	*q*	30	
19.56	19.2–21	mirex	272	(F)	239	*Q*	10	1.60 (1)
			274	(F)	237	*q*	10	
24.53	21–34	BDE-100	404	(F)	297	*Q*	20	1.59 (2)
26.16		BDE-99	564	(M)	404	*q*	20	1.61 (4)

[a] M, molecular ion; F, fragment ion. [b] Average value calculated from 12 injections of standard solutions (3 replicates, 4 concentration levels) and RSD in parentheses.

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RESULTS AND DISCUSSION

GC/MS/MS Optimization.

Optimization of the MS/MS method was performed with hexanic standard solutions using triple quadrupole MS operating in EI ionization mode. After obtaining the full scan spectra, the precursor ions for every analyte were selected as base peak of the spectra (Table 1). The presence of several chlorine atoms in the majority of compounds investigated allowed us to use different precursor ions accordingly to their isotopic chlorine pattern (pentachlorobenzene, ß-HCH, lindane, heptachlor, *cis*- and *trans*-nonachlor, dieldrin, *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, endosulfan-sulfate, mirex, and PCB congeners 28, 101, 118, 153, 138, and 180). Once the precursor ions were selected, different values of collision energy (between 10 and 30 eV) were tested to perform the fragmentation. The final purpose was to develop a SRM method with at least two transitions, normally the most sensitive ones, for each compound in order to avoid the reporting of false positives.

According to the manufacturer's information, eq 1 can be used to obtain the maximum

$$n_{srm} = \frac{W_b}{n_{dpap} t_{cycle}} = \frac{W_b}{n_{dpap}(t_{dwell} + t_{icd})} \qquad (1)$$

number of transitions in every SRM acquisition. A good chromatographic peak should be drawn with at least 10 data points across the peak (n_{dpap}). Considering an interchannel delay time (t_{icd}) of 0.01 s and a dwell time (t_{dwell}) of 0.1 s, it was feasible to calculate the maximum number of transitions (n_{srm}) that should be acquired simultaneously using eq 1. As the peaks obtained showed peak widths at base (W_b) of around 5−6 s, every SRM experiment should contain no more than six transitions.

The dwell time parameter was modified (between 0.01 and 0.5 s) in order to investigate its influence on sensitivity. A value of 0.1 s was eventually selected as a compromise between sensitivity and number of points per peak required to obtain satisfactory peak shapes.

As a summary, Table 1 shows both the quantitative (*Q*) and confirmative (*q*) transitions selected for every compound (with the exceptions of surrogates, with only one transition). Using a dwell time of 0.1 s, no function (between drawn lines) acquired

more than six transitions which was in accordance with that mentioned above. The optimized collision energy and average values of the *Q*/*q* ratios are also shown in Table 1. Average *Q*/*q* ratios, used for confirmation, were calculated for every analyte from standard solutions at four concentration levels (10, 50, 100, and 200 ng/mL) injected each in triplicate (n = 12), obtaining excellent RSDs (lower than 5%), except for oxychlordane (11%). For further experiments in samples, a ratio tolerance of ±20% was accepted to confirm a finding as positive, except for *p,p'*-DDT, where a tolerance of ±25% was admitted, because of the intensity of the confirmative transition for this analyte was notably lower than that of the quantitative one (~40%). This criterion is in line with the European Comission Decision (2002/657/CE). This decision was originally defined for the determination of organic contaminants in food samples, although it is being increasingly used for the confirmation of positive findings in other matrixes such as environmental and biological samples.[41-43]

Linearity of relative response of analytes was established by analyzing standard solutions, in triplicate, in the range 0.2−200 (for HCB, *p,p'*-DDE, *p,p'*-DDD, mirex, PCB congeners 28, 52, 118, 153, and 138), 0.5−200 (for pentachlorobenzene, ß-HCH, lindane, endosulfan-ether, heptachlor, PCBs 101 and 180), 1−200 (for vinclozolin, chlordanes, nonachlors, heptachlor epoxide A, *p,p'*-DDT), and 10−200 ng/mL (for oxychlordane, dieldrin, vinclozolin, endosulfan-sulfate, methoxychlor, PBDEs). The values of r were higher than 0.99 for all compounds over the whole range tested, with residuals lower than 15% except for *cis*-nonachlor (18%) and methoxychlor (21%) with no tendencies observed and errors of slope better than 3%.

Cleanup Optimization.

Based on our previous work on analysis of human adipose tissue,[9,10] two complementary cleanup procedures A and B (see Experimental Section) were optimized by studying separately lipids and analyte elution patterns in the normal-phase LC system (Figure 1). After injecting 1 mL of hexanic extract (0.1 g/mL, expressed as fresh breast adipose tissue weight), every 1-mL LC fraction was analyzed in order to determine presence and concentration of the selected analytes as well as the amount of fat calculated by applying a conventional colorimetric method used for total lipids determination, based on sulfophosphovainilline reactivity.[44]

Some additional experiments were carried out by injection of more concentrated hexanic extracts (0.2 g/mL) spiked with the analytes studied. The GC/MS/MS analysis of the LC fractions collected demonstrated that the content of lipids under these conditions was too high for GC/MS/MS analysis, affecting the peak shape and damaging the column. Finally, sample extracts with a maximum lipid concentration of 0.1 g/mL were selected for analysis.

As Figure 1 shows, using the LC cleanup procedure B with a mobile-phase hexane/ethyl acetate (95:5), the most polar compounds, lindane, endosulfan-ether, vinclozolin, dieldrin, endosulfan-sulfate, methoxychlor, and ß-endosulfan-d_4 eluted in a large fraction collected between minutes 4 and 17. The rest of analytes (less polar) and the corresponding surrogate compounds were collected in a fraction between minutes 1 and 17 using hexane as mobile phase (procedure A). The amount of fat in the two fractions analyzed was sufficiently low to obtain satisfactory results in the GC capillary columns used in this work.

In every set of analysis, a hexanic extract spiked with the studied compounds was purified using the two cleanup procedures, and it was used as a quality control to check the robustness of HPLC system

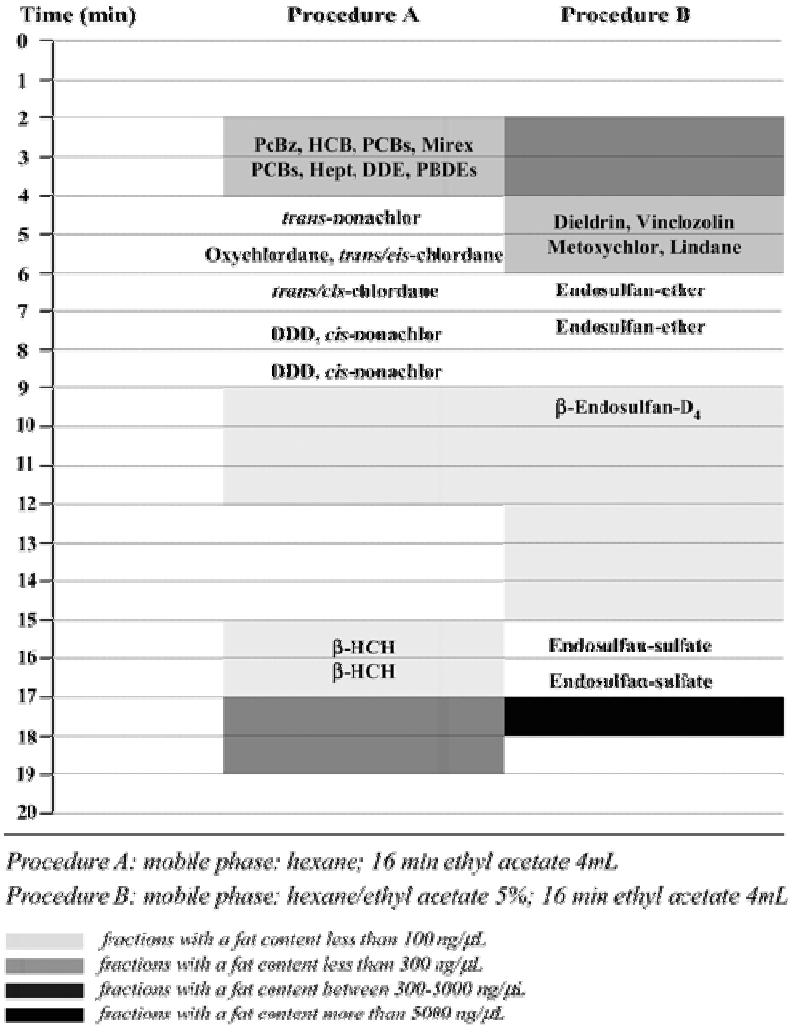

Figure 1. Elution pattern of OCs, PBDEs and lipids contained in human adipose breast tissue after applying the recommended HPLC cleanup procedures.

Matrix Effect

Matrix effects were checked by determining two different response factors (R) from standard solutions at 10 ng/mL: standards prepared in hexane (R1) and standards added into a hexanic adipose breast tissue extract after applying the cleanup procedure (R2). Then, matrix effects were quantified by determining the R2/R1 ratio.[45] As can be seen in Figure 2, most of studied compounds exhibited a relative response

factor between 0.8 and 1.2, which means that no severe matrix effects affected the response of the analytes after application of the overall analytical procedure. Only methoxychlor showed an evident signal enhancement in matrix with a response

In the particular case of HCB, ß-HCH, and *p,p'*-DDE, matrix effects were more properly evaluated using the standard additions procedure, due to the high concentration levels of these compounds in the blank. Four points (0, 12.5, 25, and 37.5 ng added, for HCB and ß-HCH; 0, 50, 100, and 150 ng added, for *p,p'*-DDE) were prepared for this study. Relative errors of the slopes of the calibration curves obtained with standards in matrix relative to those in solvent were 7−13%, proving that no important matrix effect occurred for these three compounds.

Data obtained show that quantification with standards in solvent leads to satisfactory results for all the compounds, except for methoxychlor, which presented an important matrix effect, which would lead to an overestimation of the concentration levels present in samples.

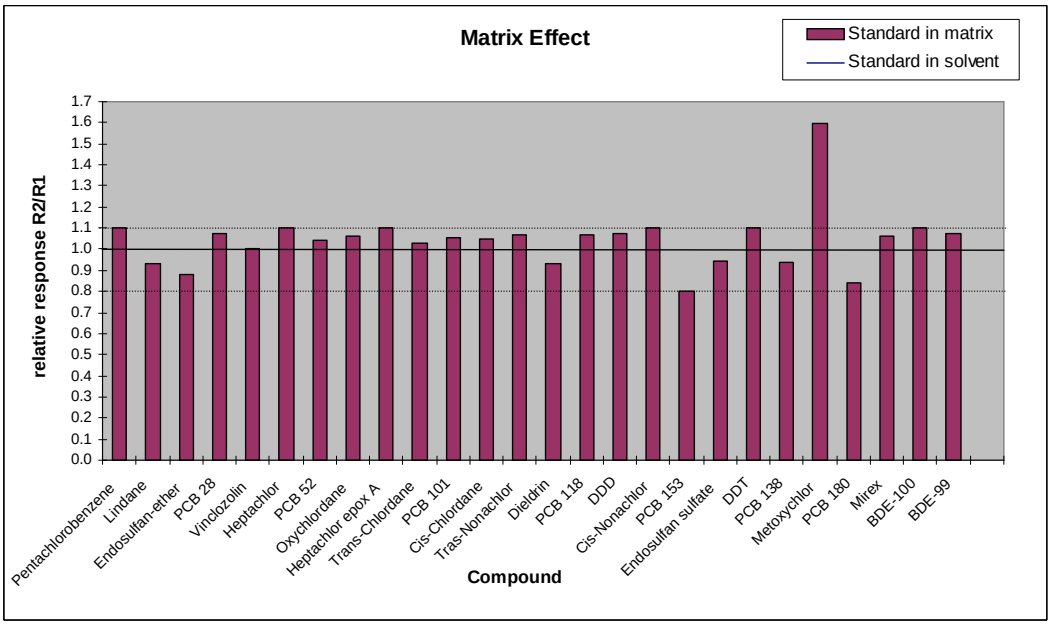

Figure 2. Matrix effects for the compounds investigated, measured by the response factor of standards added into a fatty cleaned-up extract (R2) relative to the pure standard solution in solvent (R1). Matrix effects for HCB, ß-HCH, and p,p'-DDE (see text).

Analytical Parameters

The sample used in the validation process consisted of a pool of several human adipose tissue samples. This blank sample was previously analyzed (n = 6), and it was found to contain several OCs (HCB, ß-HCH, *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, and PCB congeners 28, 118, 153, 138, and 180), compounds that are usually detected in human population. Therefore, and due to the unavailability of real blank samples of adipose breast tissue, it was necessary to correct the quantitative results along the validation process for these analytes, by subtracting the blank concentration values found for these compounds.

Validation of the overall analytical procedure was carried out as regards precision, accuracy, LODs, LOQs, and confirmation Q/q ratios. Three labeled standards were added at the initial stage of the procedure as quality control (surrogates) in order to correct for possible losses during the overall procedure and instrumental deviations.

Precision and accuracy of the developed procedure were calculated by analyzing five replicate blank samples spiked at three concentration levels each, 5, 25, and 250 ng/g (Table 2). In general, recoveries were satisfactory, with average values between 80 and 120% at the three levels assayed with a few exceptions, mainly some PCBs, which showed recoveries slightly higher than 120%. For some compounds (HCB, ß-HCH, *p,p'*-DDE, PCB congeners 153, 138, and 180) recoveries could not be accurately calculated at the lowest fortification level, due to the elevated concentrations found in the blank. RSDs were excellent at the highest concentration (better than 10%) and increased lightly at lower concentrations, but still being better than 15%, with only two exceptions: heptachlor (22%) and endosulfan-sulfate (24%).

Estimation of LOD was in some cases problematic due to the presence of several analytes in the blank sample. For those compounds found in the blank at low concentrations (PCB 28, PCB 118, *p,p'*-DDD, *p,p'*-DDT), LODs were calculated manually from the chromatogram corresponding to the analysis of this blank nonspiked sample. For the other analytes, found at higher concentrations (*p,p'*-DDE, HCB, ß-HCH, PCBs 153, 138, and 180), LODs were calculated using a software option that allowed us to obtain directly the signal-to-noise ratio from the chromatogram of the blank sample analysis.

As can be seen in Table 2, the majority of LODs varied between 1 and 20 ng/g for OCs (except dieldrin, 50 ng/g, and endosulfan-sulfate, 30 ng/g) while they were found to be 25 and 50 ng/g for BDE-100 and BDE-99, respectively. Table 2 also shows the LOQs of the method, established as the lowest concentration levels validated with satisfactory values of recovery and precision.

Moreover, the average intensity Q/q ratios calculated from reference standards prepared in solvent (see Table 1) were compared to those experimentally obtained from sample extracts spiked at the lowest level validated (i.e., the worst case), to test the robustness of the values and to check for matrix interferences that could affect the *Q/q* ratios and, consequently, the confirmation process. As Table 2 shows, average deviations were below ±20% (i.e., the tolerance value selected according to European Comission Decision (2002/657/CE)), with only the exception of *cis*-nonachlor, which confirmation seems to be more problematic at low concentration levels, close to the LOQ of the method. Similarly, in some of the replicates, the deviations for oxychlordane were found to be >20%, although the average deviation was 18%. The confirmation of this compound by using the two MS/MS transitions selected seems also to be in some way problematic at low concentration levels.

Results for methoxychlor were not satisfactory due to the above-mentioned matrix effects, which led to unnacceptable high recoveries at the medium level. Under these circumstancies, the GC/MS/MS method developed should be considered as a useful and sensitive screening for this compound, allowing us to confirm the presence of methoxychlor in the samples and roughly estimate its concentration level.

Table 2. Mean Recoveries (%) and Precision (RSD, %) for OCs and OBrs after Application of Overall Analytical Procedure to Human Breast Tissue Samples (n = 5), LODs and LOQs of the Method, and Deviations in the Q/q Values

	blank	Fortification level (ng/g)			LOD	LOQ	deviation in
	(ng/g)	250	25	5	(ng/g)	(ng/g)	Q/q ratio (%)[a]
pentachlorobenzene		87 (6)	86 (3)	103 (11)	5	5	13 (7–17)
HCB	293 (3)	91 (1)	b	b	2d	c	1 (0.3–2)
ß-HCH	181 (2)	90 (3)	102 (4)	b	5d	c	1 (0.2–3)
lindane		91 (6)	103 (10)	84 (9)	5	5	14 (9–20)
endosulfan-ether		97 (7)	90 (3)		10	25	8 (4–16)
PCB 28	3 (10)[f]	117 (2)	110 (2)	81 (5)	1e	5	9 (4–15)
vinclozolin		98 (7)	99 (10)		10	25	7.5 (4–16)
heptachlor		94 (2)	81 (22)		10	25	16 (10–20)
PCB 52		117 (1)	118 (1)	106 (14)	1	5	5 (0–12)
oxychlordane		96 (5)	110 (12)		20	25	18 (5–34)
heptachlor epox a		95 (7)	101 (15)		15	25	13 (5–19)
trans-chlordane		92 (3)	97 (14)		10	25	9 (0.4–13)
PCB 101		122 (1)	134 (4)	118 (10)	5	5	8 (3–15)
cis-chlordane		92 (4)	97 (12)		10	25	7 (2–14)
trans-nonachlor		96 (3)	100 (6)		10	25	7 (3–12)
dieldrin		98 (7)			50	250	4 (0.6–3)
p,p'-DDE	1130 (2)	94 (1)	b	b	2d	c	1 (0–3)
PCB 118	12 (6)	123 (2)	124 (3)	89 (7)	2d	5	8 (5–14)
p,p'-DDD	3 (9)	99 (1)	101 (9)	124 (4)	1d	5	2 (0–6)
cis-nonachlor		97 (2)	126 (15)		20	25	34 (3–84)
PCB 153	102 (3)	124 (1)	112 (4)	b	2d	c	3 (0.1–6)
endosulfan-sulfate		85 (24)			30	250	3 (0.5–7)
p,p'-DDT	13 (12)[f]	97 (6)	110 (15)		10e	25	10 (1–19)
PCB 138	67 (3)	124 (2)	124 (1)	b	2d	c	4 (1–7)
methoxychlor		104 (8)	225 (7)				
PCB 180	104 (3)	120 (2)	79 (4)	b	5d	c	3 (1–6)
Mirex		96 (2)	109 (6)	110 (7)	5	5	14 (1–20)
BDE-100		94 (2)	127 (10)		25	25	10 (3–20)
BDE-99		114 (4)			50	250	2 (0–5)

[a] Average deviation and range in (%) of the experimental Q/q ratios obtained from sample extracts spiked at the lowest level validated (n = 5) in relation to those theoretically calculated from standard solutions in solvent (see Table 1). [b]Not calculated due to the high concentration found in the blank. [c] Not estimated, as validation at the lowest concentration levels was unfeasible due to the high levels found in the blank sample. [d] Values obtained using a software option for estimating the S/N ratio due to the difficulties of measuring the noise manually. [e] Values obtained from the chromatogram corresponding to the analyzed blank sample. [f] Estimated concentration in the blank sample, lower to the LOQ of the method.

Application to Real Samples

The optimized procedure was applied to the analysis of 51 human adipose tissue samples: 26 adipose breast tissues and 25 tumoral fragments. In some cases, the amount of sample available was lower than 0.5 g (normally samples corresponding to tumoral fragment), and this fact was taken into account to recalculate the LOQ values in these samples.

The results obtained are shown in Table 3. As expected, the highest concentrations corresponded to the main *p,p'*-DDT metabolite, *p,p'*-DDE, which was detected in all the adipose tissue samples analyzed and in 80% of tumoral fragments, reaching levels up to 11.5 μg/g. However, parent *p,p'*-DDT was detected in only six adipose breast tissues and one tumoral fragment, at lower concentrations (between 27 and 128 ng/g), while *p,p'*-DDD was found in 70 and 30% of adipose tissue and tumoral fragment, respectively, at concentrations always lower than 50 ng/g. HCB and ß-HCH were also frequently detected, as ~90% of the adipose tissue samples contained both analytes, normally at concentrations above 100 ng/g (up to 936 ng/g HCB; up to 1386 ng/g ß-HCH). A total of 90% of tumoral fragments also contained HCB, while ß-HCH detection frequency decreased down to 70% in this type of sample. Mirex was detected in three adipose breast tissue samples, but it could not be quantified as its concentration was always below the LOQ.

Among chlordanes investigated (chlordanes, nonachlors, heptachlor), only two of these compounds were detected, and this occurred only in adipose tissue: *trans*-nonachlor was found in seven samples at concentration levels around or below the LOQ (25 ng/g), with a maximum value of 41 ng/g. Oxychlordane, the epoxide metabolite of chlordanes and nonachlors, was detected in only one sample (66 ng/g), the same sample that contained the highest concentration of *trans*-nonachlor (41 ng/g). These findings are in concordance with other authors who reported that oxychlordane and *trans*-nonachlor are the most plentiful chlordane-related residues in food and environmental samples.[46]

In relation to PCBs, the congeners 138, 153, and 180 were the majoritary compounds, as they were found in most of samples (above 90% of adipose tissue and ~70% of tumoral fragments), normally at concentration levels below 200 ng/g. PCBs 118, 101, and 28 were less abundantly detected, and at low concentration levels. Similar

findings have been reported in the literature,[47,48] and agree with the fact that the higher chlorinated PCB congeners are the most frequently detected in biological samples.

Finally, none of the PBDEs studied were detected in any of the 51 samples analyzed.

A larger number of samples should be analyzed in order to perform an adequate statistical analysis. Despite this, it seems that the concentration of the xenobiotics investigated in adipose breast tissue is higher than in tumoral fragment, with only two exceptions among all samples investigated. It can be pointed out that one of these samples presented the highest levels of *p,p'*-DDE (11584 ng/g), *p,p'*-DDD (47 ng/g), PCB 153 (385 ng/g), PCB 138 (271 ng/g), and PCB 180 (353 ng/g) found in this study.

No conclusions have been reached until now in order to correlate the presence and levels of xenoestrogens and the occurrence of breast cancer, waiting to obtain a higher number of data. From this point of view, more samples are being analyzed at present and the overall results will be presented and discussed in future publications.

As regards confirmation of positive findings, all the compounds quantified (≥ LOQ) as well as those detected at levels below the LOQ (between LOD and LOQ) were confirmed by the use of the two transitions selected and the compliance of the *Q*/*q* ratios.

As an example, Figure 3 shows the MS/MS chromatograms for several of the compounds detected in one of the samples of adipose breast tissue and tumoral fragment.

Table 3. Compounds Detected in Human Adipose Tissue Samples (Concentrations Expressed in ng/g)

	adipose breast tissue (n = 26)				tumoral fragment (n = 25)			
compound	frequency of detection (%)[a]	frequency of quantiftn (%)[b]	range min–max (ng/g)	concn percentile distribtn 90% (ng/g)	frequency of detection (%)	frequency of quantifitn (%)	range min–max (ng/g)	concn percentile distribtn 90% (ng/g)
hexachlorobenzene	96	96	13–936	77–848	92	92	19–995	30–798
β-HCH	88	88	14–1386	51–849	68	68	20–563	27–307
PCB28	31	12	5–11	5–11	4	0		
oxychlordane	4	4	66	66	0	0		
PCB 101	50	35	5–15	6–12	8	4	6	6
trans-nonachlor	27	8	27–41	27–40	0	0		
p,p'-DDE	100	100	90–2507	112–2384	84	84	30–11584	35–858
PCB 118	65	65	5–31	7–29	20	16	5–11	6–11
p,p'-DDD	69	27	5–20	5–17	28	8	5–47	7–45
PCB 153	92	92	24–241	36–178	68	68	11–385	16–163
p,p'-DDT	23	19	33–128	34–127	4	4	27	27
PCB 138	92	92	14–168	27–114	68	68	7–271	11–116
PCB 180	96	96	24–167	28–157	72	72	12–353	17–142
mirex	12	0			0	0		

[a] All the positive detections. [b] Samples above the LOQ values.

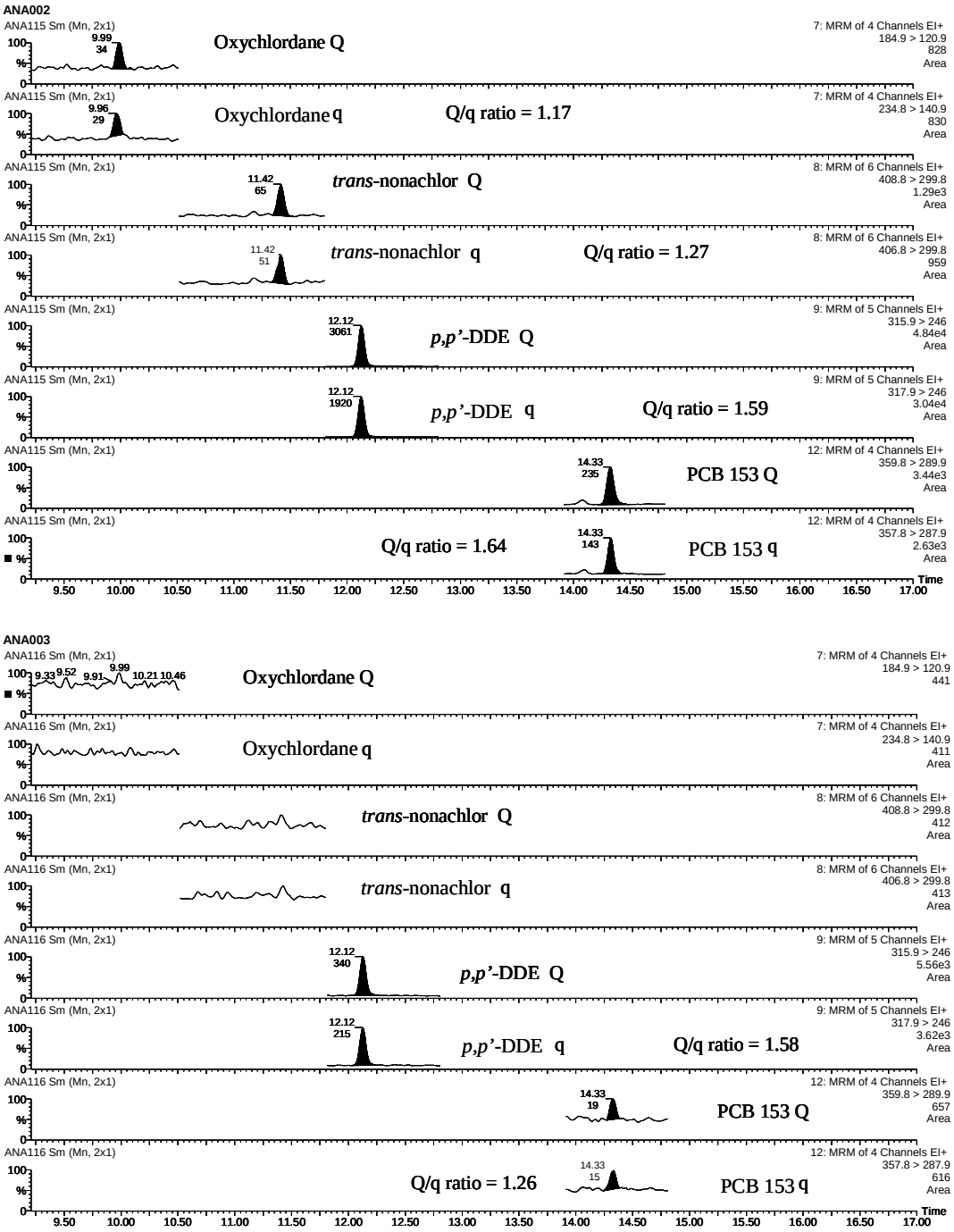

Figure 3. SRM chromatograms for several compounds detected in a sample of adipose breast tissue (sample 2a) (A) and tumoral fragment (sample 2b) (B). Q, quantification transition. q, confirmation transition. Concentrations found (in ng/g): see Table 4.

The *Q*/*q* ratios obtained for all positive samples were within the range of the tolerance accepted (±20%) around the experimental average *Q*/*q* value obtained from reference standards. Very few positives (peak obtained with the *Q* transition) could not be confirmed (1 out of 41 for ß-HCH, 8 out of 89 for DDTs, 12 out of 182 for PCBs) and, in all cases, corresponded to samples with very low concentrations, below or close to the limit of detection of the method. This fact evidences some difficulties for confirmation by using two MS/MS transitions at very low concentration levels.

The use of MS/MS in QqQ instruments working in SRM mode allows the rapid, efficient, and sensitive confirmation of xenobiotics by the selection of two MS/MS transitions, the most sensitive (*Q*) being used for quantification and the second one (*q*) used for confirmation. This approach has been previously applied by our group for pesticide residue analysis in the environmental and biomedical fields.[41,49] One of the limitations in this work has been the lack of a true negative blank control sample, due to the ubiquitous presence of OCs in lipid human matrixes. Other options, such as using adipose tissues from animals, have not been considered due to the differences in the lipid content and type between matrixes, which could affect the performance of the method.

Confirmation with GCT

Ten out of 51 samples analyzed, normally those with higher levels of analytes quantified by QqQ, were selected and reanalyzed by GCT for an additional confirmation of the compounds detected by triple quadrupole. MS data acquisition was performed in centroid mode using lock mass correction, which was continuously bled into the source, to measure the accurate mass. First, an extracted ion chromatogram with a 0.2-Da mass window at the exact *m*/*z* corresponding to the suspected analyte (molecular ion) or one of its main fragments was performed from the full scan data set. Second, if an appropiate chromatographic peak at the expected retention time appeared, the empirical *m*/*z* was obtained as a result of a combined spectrum, and it was then compared with the theoretical value. Accurate mass measurements and mass errors for the analytes found in the breast tissue samples are shown in Table 4.

Among all compounds detected and confirmed by triple quadrupole, only 70% were able to be confirmed by TOF. The rest of the positive findings could not be

confirmed by TOF, as there was no chromatographic peak present when an extracted ion chromatogram at *m/z* was carried out. This fact could be a consequence of the lower sensitivity of the TOF in comparison with the triple quadrupole in SRM mode. This was the case of some compounds such as *trans*-nonachlor, oxychlordane, PCB 101, *p,p'*-DDD, and mirex, all of them found by QqQ at low concentrations. However, the confirmation of other analytes present at higher concentrations was successful with mass error less than 5 mDa (e.g., *p,p'*-DDT, PCB 153, HCB, and ß-HCB). Among all the compounds confirmed by GC-TOF, 80% of them could be confirmed with mass error less than 3 mDa. The rest (20%) presented a mass error between 3 and 5 mDa except for only three cases: ß-HCH in sample 2b (8.9 mDa), *p,p'*-DDT in sample 3a (9.2 mDa), and PCB 138 in sample 3b (8.3 mDa).

In summary, the use of TOF allowed us to unequivocally confirm all the positives found by QqQ when the sensitivity was sufficient leading us to the conclusion that no false positives were reported by the use of two MS/MS transitions.

Table 4. Accurate Mass Measurements. Mass errors, in mDa, for Several Compounds Found in Breast Tissue Samples Using Time-of-Flight Mass Spectrometer.

sample[a]	HCB	β-HCH	PCB 28	oxychl	PCB 101	t-nonachlor	DDE	PCB 118	DDD	PCB 153	DDT	PCB 138	PCB 180	Mirex
	C_6Cl_6[b]	$C_6H_5Cl_4$	$C_{12}H_7Cl_3$	$C_{10}H_4Cl_8O$	$C_{12}H_5Cl_5$	$C_{10}H_5Cl_8$	$C_{14}H_8Cl_4$	$C_{12}H_5Cl_5$	$C_{13}H_9Cl_2$	$C_{12}H_4Cl_6$	$C_{13}H_9Cl_2$	$C_{12}H_4Cl_6$	$C_{12}H_3Cl_7$	C_5Cl_6
1a	2 (428)[c]	2.6 (152)	-0.7 (6)		nc[d] (8)	nc (d)[e]	-0.4(445)	2.2 (17)	nc (10)	0.4 (119)	nc (38)	1.1 (83)	0.6 (103)	d (nc)
2a	2 (158)	-0.2(550)	-2.7 (d)	nc (66)	nc (d)	nc (41)	0 (686)	2.4 (13)	nc (6)	4.1 (35)		0.9 (26)	-3.7 (24)	d (nc)
2b	3.8 (58)	8.9 (243)					-0.8 (352)			nc (17)		nc (20)	nc (12)	
3a	0.5 (936)	-0.2(882)			nc (15)	nc (d)	1.3 (2440)	5.5 (31)	nc (20)	1 (241)	-9.2 (51)	2.6 (168)	-0.1 (167)	
3b	1.3 (274)	-0.8(241)			nc (6)		-0.3 (746)	4.7 (11)	nc (5)	0.7 (80)	nc (27)	8.3 (52)	-1 (621)	
7a	0.8 (918)	1.8 (138)	nc (d)		nc (d)	nc (d)	-0.4 (804)	0.1 (15)	nc (10)	2.3 (115)	nc (33)	3.5 (87)	2.6 (118)	
7b	0.6 (111)	1.6 (159)					0 (122)			-3.5 (17)		-4.2 (13)	nc (18)	
9a	2 (259)	3.1 (180)					0.1 (1294)		nc (5)	-2 (166)		-2.9 (116)	-0.9 (138)	
9b	3 (76)	nc (60)					2.6 (214)						nc (31)	
10a	0.6 (432)	1.1 (380)	-3.6 (11)		nc (8)	nc (d)	0.6 (2216)	0.9 (28)	nc (12)	-1.4 (180)	-5.1 (125)	1.2 (106)	-2.4 (162)	d (nc)

[a] Samples a, adipose breast tissue; samples b, tumoral fragment. [b] *m/z* ion selected for the extracted ion chromatogram. [c] Mass error and concentration (in ng/g) found by QqQ (in parenthesis). [d] nc, not able to be confirmed due to the low sensitivity of the compound at the concentration level present in the sample. [e](d) detected but not quantified (concentration lower than the LOQ)

CONCLUSIONS

The potential of GC/MS/MS for quantification and confirmation of xenoestrogen compounds in human breast tissue has been discussed in this paper. A method based on GC/MS/MS with QqQ has been developed for the determination of ~30 organochlorine and organobromine compounds. The use of QqQ in selected reaction monitoring mode leads to excellent selectivity and sensitivity, allowing us to reach very low detection limits. The selectivity was improved by recording two transitions for each analyte, using the ratio obtained from them as a confirmatory parameter.

The application of this method allowed the quantification and confirmation of all selected compounds at the 5−25 ng/g level. The usefulness of the developed method was tested by the analysis of 51 samples (adipose breast tissue and tumoral fragment).

GC/MS with time-of-flight analyzer has allowed an unequivocal confirmation, but only at higher concentration levels due to the lower sensitivity compared with that of triple quadrupole in selected reaction monitoring mode. The result obtained by GC-TOF demonstrated that no false positives were reported after analysis by QqQ GC/MS.

ACKNOWLEDGMENT

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of the time-of-flight mass spectrometer GCT and to Dr. Juan Vicente Sancho for his valuable comments.

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CAPÍTULO 3

Desarrollo de métodos de screening basados en cromatografía de gases acoplada a espectrometría de masas con analizador de tiempo de vuelo

3.1 INTRODUCCIÓN

La mayoría de los métodos analíticos para contaminantes orgánicos que se aplican en la actualidad, tanto en el campo ambiental como biológico y de seguridad alimentaria, son métodos *target* (1-10). Estos métodos se desarrollan y validan en términos cuantitativos para un número determinado y finito de compuestos previamente seleccionados, normalmente en base a listas de compuestos prioritarios a controlar. La mayoría de métodos *target* no permiten detectar otros contaminantes que pudieran estar presentes en las muestras analizadas, pues requieren información específica sobre el analito. Este tipo de métodos están bastante consolidados, existiendo un amplio consenso en cuanto a los beneficios que las técnicas de MS en tándem aportan al respecto, sin olvidar el elevado uso del analizador de cuadrupolo simple en modo SIM en este tipo de aplicaciones basadas en GC-MS. Estos analizadores presentan innumerables ventajas desde el punto de vista de la sensibilidad y el poder de cuantificación y confirmación en el análisis *target*, en especial de los de triple cuadrupolo, tal y como se ha comentado en el capítulo anterior.

A pesar de que es bien conocida la necesidad de disponer de métodos de *screening* en los laboratorios, especialmente en el campo del medio ambiente y

seguridad alimentaria, apenas existen métodos de amplio rango de aplicación, tal como se desprende de la literatura científica. Idealmente, un método de *screening* debería ser capaz de detectar un elevado número de compuestos, algunos inesperados y no incluidos en los métodos habituales de control, de forma rápida, con poca manipulación de muestra, permitiendo además una identificación fiable del compuesto detectado. Esto facilitaría la aplicación posterior de métodos *target* únicamente a las muestras positivas, con el fin de realizar una cuantificación más exacta y precisa.

El alto nivel de multirresidualidad requerido en este tipo de análisis no se alcanza fácilmente con los analizadores de cuadrupolo (y triple cuadrupolo) y trampa de iones trabajando en modos SIM o MS/MS, debido a la necesidad de predefinir las masas adquiridas y a la dificultad de reducir el tiempo de monitorización de cada ion/transición por debajo de un determinado valor sin perder sensibilidad.

Los analizadores TOF MS abren un nuevo escenario en el desarrollo de métodos de *screening*. Las ventajas de la tecnología TOF en este campo derivan de la adquisición del espectro MS completo con datos de elevada exactitud de masa, gracias a su mayor poder de resolución, y con mayor sensibilidad que los analizadores convencionales. Por ello, no es necesario predefinir los iones a monitorizar para cada contaminante antes del análisis, con lo que los compuestos a investigar pueden ser seleccionados después de la adquisición de datos MS. Esta aproximación *post-target* podría permitir la detección de un número muy elevado de potenciales contaminantes, sin necesidad de reanalizar las muestras. Así, el número de analitos a investigar vendría limitado únicamente por las propias características del método de extracción y la técnica de análisis; es decir, sólo quedarían excluidos aquellos compuestos que se pierden en el proceso de extracción, que no tienen un adecuado comportamiento cromatográfico o adecuada ionización en MS. Esto implica que el tratamiento de muestra en los métodos con fines de *screening* debería ser lo más genérico posible, es decir, un procedimiento que fuera capaz de extraer y preconcentrar el mayor número de compuestos, sin necesidad de ser el más adecuado para cada uno de ellos individualmente. Por otro lado, la elevada resolución de los analizadores TOF permite reducir la ventana de extracción de masa, pudiendo ajustarla a una masa muy estrecha, con una reducción sustancial del ruido químico, facilitando la detección de los compuestos a bajos niveles de concentración en el cromatograma generado a partir de la extracción de un ion (nw-XIC) a su masa exacta (narrow window-XIC, nw-XIC).

Además de la detección de potenciales contaminantes, la confirmación de su identidad es fundamental por los efectos indeseables asociados a los falsos positivos y falsos negativos. Por ello, son necesarios métodos fiables que permitan la inequívoca confirmación de los posibles positivos. Idealmente, la confirmación debe ser objetiva y segura, requiriéndose criterios predefinidos, rigurosos y eficientes. Al igual que los métodos *target* con fines cuantitativos, los métodos de *screening* también deben ser sometidos a una validación, en este caso en términos cualitativos, con el fin de asegurar que el método de *screening* es fiable con fines de detección e identificación de los compuestos seleccionados a ciertos niveles de concentración pre-establecidos.

En este capítulo se explora el potencial del acoplamiento GC-TOF MS para el desarrollo de métodos rápidos y fiables para el *screening* y confirmación de un elevado número de contaminantes orgánicos en distintos tipos de muestras. En este estudio se abordan dos campos de aplicación donde la tecnología TOF MS tiene un gran potencial, como son el análisis medioambiental, representado por el análisis de aguas (**artículos científicos 4 y 5**), y el análisis biológico, en nuestro caso muestras de tejido adiposo humano (**artículo científico 7**). Adicionalmente, se contempla y discute la necesidad de validar cualitativamente los métodos de *screening* desarrollados con el fin de asegurar la calidad de los resultados obtenidos desde el punto de vista de la correcta identificación de los compuestos detectados (**artículo científico 6**). También se explora el potencial de la técnica GC-TOF MS para la elucidación de compuestos cuyo espectro experimental de EI no se encuentra en las librerías comerciales utilizadas (**artículo científico 8**). Finalmente, se exploran las capacidades de un nuevo prototipo de fuente de ionización ampliamente utilizada en combinación con LC: la fuente de ionización química a presión atmosférica (APCI), diseñada recientemente para su acoplamiento a sistemas de GC. El trabajo consiste en estudiar las ventajas del acoplamiento GC-(Q)TOF MS con la nueva fuente de APCI con fines de *screening* (**artículo científico 9**).

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3.2 POTENCIAL DE LA TÉCNICA GC-TOF MS PARA EL SCREENING DE CONTAMINANTES ORGÁNICOS EN AGUAS. ESTUDIO DE LAS APROXIMACIONES TARGET Y NON-TARGET

Como ya se ha comentado en la introducción general de este capítulo, la contaminación de las aguas es uno de los problemas medioambientales de mayor preocupación en la actualidad. Debido a la gran cantidad de potenciales contaminantes, de diferentes orígenes, que pueden llegar a estar presentes en las aguas (agricultura, industria, cosméticos, etc), parece insuficiente focalizar el análisis únicamente en una lista "prioritaria", generalmente bastante limitada, de contaminantes. Cada vez se hace más patente la necesidad de ampliar estas listas, lo cual implica el uso de técnicas que permitan la detección de contaminantes, sin expresa necesidad de pre-definirlos. Los llamados "contaminantes emergentes", muchos de ellos todavía poco conocidos, también deben ser investigados en las aguas. Una forma eficiente de abordar este problema es mediante el desarrollo de metodologías analíticas que incluyan el mayor número posible de compuestos. La tecnología TOF MS tiene un elevado potencial importante en este campo de aplicación, tal como se demuestra en esta Tesis.

Puesto que la inyección directa de agua en un sistema GC no es lo más adecuado, se suelen elegir tratamientos de muestra principalmente basados en SPE o SPME, por su carácter más universal. Tras la inyección de los extractos obtenidos (o la desorción de la fibra de SPME) en el sistema GC-TOF MS, la información sobre la muestra generada es inmensa, ya que el modo de adquisición proporciona espectros de iones completos (*full spectrum acquisition*), medidos con elevada exactitud de masa. Ante toda la información contenida en el cromatograma TIC adquirido mediante GC-TOF MS, el procesamiento de los datos obtenidos se convierte en una de las principales claves del éxito, sobretodo cuando se pretende investigar un número elevado de compuestos.

En este apartado se investiga el potencial de la técnica GC-TOF MS para el *screening* y confirmación de contaminantes orgánicos en aguas (**artículo científico 4**). Para ello, se han utilizado muestras de agua previamente analizadas por GC-(QqQ)MS/MS en modo SRM, los cuales se han reanalizado por GC-TOF MS. El tratamiento de muestra aplicado consiste en una extracción por SPE, que ha sido optimizada y validada en términos cuantitativos para alrededor de 50 contaminantes orgánicos

prioritarios en un trabajo previo, presentado en el capítulo anterior (**artículo científico 2**). Los extractos obtenidos se han inyectado en el GC-TOF MS bajo condiciones de adquisición de espectro de iones completo, investigando la presencia de los compuestos reportados como positivos mediante la técnica GC-(QqQ)MS/MS, en total 13 compuestos. Se han comparado dos estrategias de tratamiento de datos; una manual y otra automática (utilizando un software de tratamiento de datos, TargetLynx). Esta segunda ha resultado la más adecuada, no sólo por su carácter automatizado y rápido, imprescindible para un *screening* amplio de compuestos, sino por las ventajas aportadas ante la detección y confirmación de compuestos presentes a bajas concentraciones o en presencia de coeluciones. Como en todos los procesos automatizados, existen aspectos críticos a los que se debe prestar especial atención, para evitar falsos positivos/negativos. Así pues, se han estudiado algunos parámetros a tener en cuenta a la hora de procesar automáticamente los datos de MS obtenidos y que resultan críticos en el proceso de detección y confirmación de la identidad del compuesto detectado. Por ejemplo, cabe señalar la ventana de extracción de masa, la presencia de coeluciones, las posibles interferencias provocadas por componentes de la matriz o del *lock mass*, errores asociados a la saturación de la señal, etc. Asimismo, se comparan los resultados obtenidos por GC-TOF MS con los previamente conocidos mediante el análisis por GC-(QqQ)MS/MS con el fin de explorar la sensibilidad de la técnica GC-TOF MS en comparación con GC-(QqQ)MS/MS y establecer así ventajas y desventajas del método desarrollado.

La metodología de trabajo establecida en este trabajo se ha aplicado posteriormente a la determinación de un mayor número de compuestos en aguas, usando en este caso SPME para la extracción de la muestra (**artículo científico 5**). Se ha llevado a cabo una sencilla optimización de parámetros relevantes de la SPME (tipo de fibra y adición de sal).

En este trabajo se ha estudiado el potencial de la técnica GC-TOF MS desde varios puntos de vista. En primer lugar, se ha aplicado la metodología ya desarrollada para investigar la presencia de un amplio número de compuestos *target*, alrededor de 60, para los cuales el método se desarrolla desde un punto de vista semi-cuantitativo. En segundo lugar, aprovechando la ventaja de que se dispone de espectros de iones completos, medidos con elevada exactitud de masa, se ha estudiado el potencial del GC-TOF MS para la investigación de contaminantes orgánicos *a posteriori*. Así pues, se

seleccionan un total de 11 contaminantes y se investiga su presencia en las muestras previamente analizadas. Finalmente, se investiga el potencial de GC-TOF MS para el análisis non-*target*, para lo cual se utiliza un software de tratamiento de datos diseñados para ese fin. La posibilidad de llevar a cabo un análisis tipo *non-target* permite la detección de contaminantes que no estaban incluidos en la lista de compuestos *target* lo que supone un avance importante desde el punto de vista de la realidad medioambiental.

Una de las principales aportaciones de ambos trabajos ha sido establecer una metodología de trabajo con GC-TOF MS para la determinación de un amplio número de contaminantes orgánicos combinando estrategias *target* y *non-target*.

Como ya se ha mencionado, el análisis *non-target* supone una importante ventaja de GC-TOF MS para el *screening* de contaminantes orgánicos en aguas. La aplicación de esta metodología al análisis de muestras de agua de diferentes orígenes por el método de SPME combinado con GC-TOF MS ha permitido la detección y confirmación de diferentes contaminantes orgánicos no predefinidos como, por ejemplo, Bisfenol A, el antioxidante 3,5-di-tert-butil-4-hidroxi-tolueno (BHT) y su metabolito 3,5-di-tert-butil-4-hidroxibenzaldehido (BHT−CHO), la *polycyclic musk galaxolide*, y el filtro de U.V. benzofenona, entre otros.

Algunas de estas sustancias son consideradas como "*personal care product ingredients*" (PCPIs), compuestos que representan un grupo de creciente interés desde que se conoce el carácter disruptor endocrino de algunos de ellos. Además, son compuestos con elevado carácter lipofílico que tienden a bioacumularse. Son considerados como contaminantes emergentes y abarcan un elevado número de productos ampliamente utilizados en la vida diaria, como fragancias sintéticas, filtros U.V, antisépticos, antioxidantes, repelentes de insectos… (1-3)

Para la determinación de PCPIs la técnica de elección suele ser GC-MS, ya que son compuestos lipofílicos, tal y como se observa en la mayoría de métodos desarrollados para aguas superficiales, subterráneas y residuales (4-10).

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3.2.2 Artículo científico 4

J. Mass Spectrom. 42, 1175-1185, 2007

METHODICAL APPROACH FOR THE USE OF GC-TOF MS FOR SCREENING AND CONFIRMATION OF ORGANIC POLLUTANTS IN ENVIRONMENTAL WATER

Tania Portolés, Elena Pitarch, Francisco J. López, Juan V. Sancho, Félix Hernández

Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain,

ABSTRACT

The potential of gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF MS) for the screening of organic pollutants in water was explored. After a conventional SPE step with C_{18} cartridges, the comparison of spectra with available libraries together with an evaluation of the mass accuracy was the first approach used for the screening and confirmation of target analytes. However, at low analyte concentrations (i.e. below 0.1 µg/l), this procedure was not feasible and the use of the application manager TargetLynx was evaluated. This application allows the selection of up to five representative ions per analyte, measured with high mass accuracy, and their intensity ratio evaluation. Ion selection, extraction mass window and concentration levels were found to be the critical parameters. The reference compound used as lock mass was also found to affect to the quality of information obtained in some particular cases.

Full spectral acquisition data generated by the TOF MS analyzer allowed investigation of the presence of several analytes in samples in a post-target style, without the need of reanalyze the water samples.

Finally, a methodical approach was established for the reliable screening and confirmation of organic pollutants (PAHs, pesticides, octyl/nonyl phenols) in real-world samples, which led to satisfactory results of 0.1 µg/l.

KEYWORDS

high-resolution time-of-flight mass spectrometer, gas chromatography, screening and confirmation, organic pollutants, water

INTRODUCTION

Hyphenation of gas chromatography (GC) to mass spectrometry (MS) with quadrupole or ion trap analyzers has become a routine analytical tool for the determination of organic contaminants in food, environmental and biological samples because of their relatively low cost and ease of use [1-8]. Both type of instruments provide unit mass resolution (R < 1000 FWHM), moderate scan speeds of 5-15 scan/s and limits of detection (LODs) in the low-picogram range [9]. To achieve these low LODs, quadrupole instruments must operate in the Selected Ion Monitoring (SIM) mode, while ion trap instruments normally operate in the tandem MS mode (MS/MS). Under these circumstances, the spectral information is sacrificed, which decreases the potential of these techniques in qualitative analysis, i.e. for identification purposes. Thus, the analytical methods based on these approaches are appropriate for quantification of target analytes, but further investigations of other analytes in samples requiring additional analysis and identification of nontarget (unknown) analytes become troublesome, specially at the sub-ppb levels.

Contrary to these analyzers, in which electrical fields are employed for separation of ions with different *m*/*z* values, ions generated in a time-of-flight mass spectrometer (TOF MS) ion source are in the first phase accelerated from the source to 40 eV to get constant kinetic energy, and discrete packets of ions are then accelerated orthogonally into the time-of-flight mass analyzer with a pulse repetition rate of 20-30 kHz. The flight times of the ions separated are proportional to the square root of the *m*/*z* ratio [9-11]. Each stored spectrum is the sum of thousands of individual spectra; typically 1-100 spectra/s are stored in the computer system. Owing to the high repetition rate, a large fraction of the ions generated in the ion source is pulsed into the flight tube followed by their simultaneous detection according to their flight times (which does not occur with instruments such as quadrupole and ion traps where ions are ejected and detected sequentially). Consequently, mass analyzer efficiency of a TOF MS is 20-30%, as against 0.1-1% for other scanning instruments [9], such as

quadrupole, generating high-sensitivity full spectral acquisition data and recording all quantitative and confirmatory ions simultaneously.

Two main approaches are commercially available at present in GC-TOF MS instruments: those that provide high resolution (with mass accuracy of ± 5-10 ppm) but have moderate spectral acquisition rates (up to 20 full spectra stored per second), and instruments that feature a high spectral acquisition rate of, typically, 100-500 spectra/s but provide only unit mass resolution.

High-speed TOF MS has been investigated for the determination of organophosphorus pesticides, triazine herbicides and polycyclic aromatic hydrocarbons (PAHs) in several types of samples such as surface water, tea and sediments [12]. These instruments offer very fast spectral acquisition rates, allowing the separation of overlapping peaks using automated mass spectral deconvolution of overlapping signals. An interesting application of these mass spectrometers is their use as detectors for comprehensive two-dimensional gas chromatography (GC × GC) [13,14]. The fast chromatographic separation in the second-dimension results in very narrow peaks with widths of 50-600 ms at the baseline, which require fast detectors to reconstruct the second-dimension chromatogram correctly. Until recently, detection in GC × GC was limited to the use of fast analog detectors such as flame ionization detectors (FIDs) or electron capture detectors (ECDs). However, the commercialization of TOF MS instruments providing very fast acquisition rates has considerably enlarged the application potential of the GC × GC technique [13].

High-resolution TOF MS, capable of routine operation at over 7000 full width at half-maximum (FWHM) are becoming an attractive and alternative technique for accurate mass measurements against traditional instruments such as double-focusing magnetic sectors [15] which are highly expensive and need highly skilled operators. Owing to the higher mass resolution of TOF MS, matrix components yielding ions with the same nominal mass as that of the target analyte can often be partially or completely resolved, decreasing the possibility of mass interferences with coeluting matrix components. Moreover, it gives the possibility of performing an extracted-ion chromatogram using a narrow mass window (mw-XIC), excluding a large amount of chemical background and consequently improving signal-to-noise ratios. For those peaks with sufficient intensity and using an internal reference mass (lock mass)

introduced via a heated reference inlet, a mass accuracy of 1 mDa can be obtained (e.g. 5 ppm for measurements at *m/z* 200) [11].

Very little work has been published until now on organic contaminant analysis by high-resolution GC-TOF MS and its potential in this field also remains unexplored. Some applications have been described in environmental samples, such as the determination of polybrominated diphenyl ethers (PBDEs) and other halogenated persistent organic pollutants in fish muscle and river sediments [16] or the determination of pesticides, PAHs and polychlorinated biphenyls (PCBs) in waste water and eel samples [9]. Other applications are in biological samples such as the determination of PBDEs in human milk samples [17], flavor research in seafood [18] or xenoestrogens in human breast tissues [19]. Recently, Čajka and Hajšlovà [20] reviewed the application of GC-TOF MS in food analysis.

The aim of this work is to explore the potential of GC-TOF MS using a high-resolution instrument and to establish a methodical approach for the screening and confirmation of organic pollutants in water samples. For this purpose, positive water samples, previously analyzed by GC-MS/MS with triple-quadrupole (QqQ) mass spectrometry [21], were reanalyzed by GC-TOF MS. The identification of target analytes was made taking advantage of the high mass resolution and mass accuracy provided by GC-TOF MS, which allowed us the use of mw-XIC at the selected *m/z* ions together with the measurement of the ion intensity ratios. Additional non(pre)target analytes were also investigated in the samples making use of acquired data without any need for reanalyzing the samples.

EXPERIMENTAL

Reagents and chemicals

All reference standards were purchased from Dr Ehrenstorfer (Augsburg, Germany), Wellington Laboratories (Guelph, Ontario, Canada), Fluka (Buchs, Switzerland) and Riedel de Haen (Seelze, Germany) with purity between 97 and 99.7%. Stock solutions (around 500 µg/ml) were prepared by dissolving reference standards in acetone, and stored in a freezer at - 20 °C. Working solutions were prepared by

diluting stock solutions in acetone for sample fortification and in hexane for GC injection.

Acetone (pesticide residue analysis), ethyl acetate, dichloromethane (DCM) and hexane (ultra-trace quality) were purchased from Scharlab (Barcelona, Spain). Five hundred milligrams of Bond Elut cartridges C_{18} (Varian, Harbor City, CA, USA) was used for solid-phase extraction.

Sample material

Water samples were collected through 2005 from different sites at the Valencia Mediterranean area (Spain) and stored at 4 °C until analysis. Three surface water samples were collected from the Castellón province: Borriana (Clot), Vila-Real (Mijares River) and Alcora (Ma Cristina Dam). Two ground water samples were collected from wells located at Puerto de Sagunto (Valencia) and Almassora (Castellón). Two samples - before and after treatment - were obtained from an urban waste-water treatment plant sited at Vila-Real. For all these cases, two different samplings were carried out at different dates. Three more samples corresponded to urban solid-waste leachates and were collected from a municipal treatment plant sited at Onda: two corresponded to treated water (reversed osmosis) and the third one was raw leachate. This raw sample was diluted 50 times with deionized water before being subjected to SPE. Those samples with suspended solids or turbidity were filtered through a Nylon filter (0.45 μm, under vacuum) before extraction.

GC instrumentation

An Agilent 6890N GC system (Paloalto, CA, USA) equipped with an Agilent 7683 autosampler was coupled to a time-of-flight mass spectrometer, GCT (Waters Corporation, Manchester, UK), operating in the electron ionization (EI) mode. The GC separation was performed using a fused silica HP-5MS capillary column with a length of 30 m × 0.35 mm i.d. and a film thickness of 0.25 μm (J&W Scientific, Folson, CA, USA). The oven temperature was programmed as follows: 90 °C (1 min); 5 °C/min to 300 °C (2 min). Splitless injections of 1 μl sample were carried out. Helium was used as carrier gas at 1.2 ml/min.

The interface and source temperatures were both set to 250 °C and a solvent delay of 3 min was selected. The time-of-flight mass spectrometer was operated with 1 spectrum/s acquiring the mass range *m/z* 50-650 and using a multichannel plate voltage of 2700 V. The TOF MS resolution was about 8500 (FWHM) at *m/z* 612. Heptacosa, used for daily mass calibration as well as the lock mass, was injected via a syringe in the reference reservoir at 30 °C for this purpose. The *m/z* ion monitored was 218.9856. The application manager TargetLynx was used to process the data obtained.

Sampling procedure

The SPE sampling procedure has been described in our previous work [21]. Briefly, 100 ml of water sample was passed through a 500 mg C_{18} cartridge, and the analytes were eluted with 5 ml of ethyl acetate : DCM (50 : 50). The eluate was evaporated under a gentle nitrogen stream at 40 °C and redissolved in 1 ml of hexane. The extract obtained was injected into the GC-TOF MS instrument under the conditions given above. Chromatographic conditions for the selected compounds were taken from our previous paper [21].

RESULTS AND DISCUSSION

A multiresidue GC-MS/MS method, using a triple -quadrupole (QqQ) analyzer, for the determination of around 50 organic micropollutants in water was reported previously [21]. The majority of the target analytes is included in the list of priority substances in Annex X of the Directive 2000/60/EC [22], and correspond to different chemical families: pentachlorobenzene, organochlorine and organophosphorus insecticides, herbicides, PCBs, PAHs, PBDEs and octyl/nonyl phenols. The method was applied to different types of water samples, leading to the detection and quantification of low levels of several of the selected analytes. In the present work, we have reanalyzed by GC-TOF MS all those samples as well as others collected from a landfill leachate treatment plant. Thus, the potential of TOF MS has been investigated for those target analytes that were detected in water samples previously analyzed by GC (QqQ) MS/MS.

The analytical strategy developed in this paper was focused first on the confirmation of positive findings for the target analytes. Second, the potential of GC-TOF MS in post-target analysis was also explored (i.e. searching for an analyte selected after MS data acquisition without any additional analysis) [23], which was feasible as a consequence of the availability of full spectral information. This possibility is not available when using single- or triple-quadrupole analyzers in the SIM or SRM mode, respectively, or using ion traps in the MS/MS mode where the selection of analytes must be done before the acquisition.

Screening/confirmation method development

The procedure applied to confirm a target compound detected in a real-world sample consisted of two steps: (1) searching the spectrum in the NIST library and evaluation of mass accuracy measured for the most characteristic ions, and (2) obtaining up to five micro-windows eXtracted Ion Chromatograms (mw-XIC) at selected *m/z* ions and evaluating their Q/q intensity ratios.

The procedure to search the spectrum in the library was as follows: first, an mw-XIC at the exact *m/z* corresponding to the analyte (molecular ion), or one of its main fragments, was performed on the data set. Then, if a chromatographic peak appeared at the expected retention time, a background-subtracted combined spectrum for this peak was obtained and compared with the library. Additionally, accurate masses from the spectrum were entered into an Elemental Composition program to calculate elemental compositions and to compare the experimental mass with the theoretical one, for five representative ions.

Regarding the second step, use was made of the TargetLynx application manager, a module of the Masslynx Software, which allows automated data processing and reporting of all quantitative and qualitative results for target compounds resulting from GC-TOF MS analysis. The huge amount of information generated by TOF instruments makes the management of data a highly time-consuming step. For this reason, a processing software that allows managing and simplifying all available data is an important requirement.

Initially a TargetLynx processing method was created for selected analytes using reference standard solutions in the solvent. The spectrum for each compound was obtained and then up to five ions (the maximum allowed in the software) were selected for each analyte taking into account the sensitivity and selectivity obtained (Table 1). In order to investigate the selectivity of the fragments, accurate mass measurements of the different ions were obtained from the spectra of solvent standard solutions and were subsequently used for elemental composition calculation using a software option designed for this purpose. Mass windows with a value of 0.02 Da were chosen as a compromise between sensitivity, peak shape and accurate mass measurements.

Q/q intensity ratios were used as the confirmation parameter. Theoretical Q/q intensity ratios were calculated from standard solvent solutions as the ratio between the most sensitive ion (Q, quantitative) and each of the other measured ions (q, confirmative). Thus, the selection of five ions provides up to four Q/q intensity ratio values, which can be used for the reliable confirmation of compounds in samples.

To confirm a finding, the software allows user-defined fixing of a maximum ratio tolerance. For the majority of target analytes, a Q/q tolerance of ± 20% was accepted because the intensity of the confirmative ion (q) was higher than 50% of the quantitative one (Q) (e.g. Q/q ratio < 2). For higher Q/q ratios, tolerances were increased up to ± 25% (relative intensity 20-50%, Q/q ratio 2-5), ± 30% (relative intensity 10-20%, Q/q ratio 5-10) and ± 50% (relative intensity 10%, Q/q ratio > 10). These criteria are in line with those of the European Commission Decision (2002/657/EC) [24], originally defined for the determination of pharmaceutical compounds and other organic contaminants in food samples, although it is being increasingly used in environmental and biological samples [19,25,26]. The agreement in the retention time of a compound in the sample and standard was also required to confirm a positive result (relative error 0.5% when compared with standard reference).

This strategy was applied to 17 water samples that had been previously analyzed by GC-MS/MS QqQ with a total of 130 positive findings. In the following sections, the most relevant parameters that affect the confirmation process by GC-TOF MS are discussed, showing some representative examples in order to better illustrate this process.

Table 1. *m/z* ions selected for the identification of each compound

Compound	tR	Molecular Formula	Molecular Mass	Ion 1 (Q)	m/z 1	Ion 2	m/z 2	Ion 3	m/z 3	Ion 4	m/z 4	Ion 5	m/z 5
Naphtalene	5.12	$C_{10}H_8$	128.0626	$C_{10}H_8$	128.0626	$C_{10}H_7$	127.0548	$C_{10}H_6$	126.0470	C_8H_6	102.0470	C_8H_5	101.0391
Acenaphtylene	10.57	$C_{12}H_8$	152.0626	$C_{12}H_8$	152.0626	$C_{12}H_7$	151.0548	$C_{12}H_6$	150.047	$C_{10}H_6$	126.047	C_6H_4	76.0313
4-t-Octylphenol	14.08	$C_{14}H_{22}O$	206.1671	$C_9H_{11}O$	135.081	C_7H_7O	107.0497	C_6H_7O	95.0497	$C_{14}H_{22}O$	206.1671	-	-
Simazine	17.00	$C_7H_{12}N_5Cl$	201.0781	$C_7H_{12}N_5Cl$	201.0781	$C_6H_9N_5Cl$	186.0546	$C_5H_8N_5Cl$	173.0468	$C_5H_7N_4Cl$	158.0359	$C_5H_8N_5$	138.0780
Atrazine	17.27	$C_8H_{14}N_5Cl$	215.0938	$C_7H_{11}N_5Cl$	200.0703	$C_7H_{11}N_5\ ^{37}Cl$	202.0674	$C_8H_{14}N_5Cl$	215.0938	$C_5H_8N_5Cl$	173.0468	$C_4H_5N_5Cl$	158.0233
Lindane	17.35	$C_6H_6Cl_6$	287.8601	$C_6H_4Cl_3$	180.9379	$C_6H_4{}^{35}Cl_2{}^{37}Cl$	182.9349	$C_6H_5Cl_4$	216.9145	$C_6H_4Cl_2$	145.9690	C_6H_5Cl	112.0080
Phenanthrene	17.63	$C_{14}H_{10}$	178.0783	$C_{14}H_{10}$	178.0783	$C_{14}H_8$	176.0626	$C_{12}H_8$	152.0626	$C_{12}H_7$	151.0548	C_7H_5	89.0391
Terbuthylazine	17.83	$C_9H_{16}N_5Cl$	229.1094	$C_8H_{13}N_5Cl$	214.0859	$C_8H_{13}N_5\ ^{37}Cl$	216.0831	$C_9H_{16}N_5Cl$	229.1094	$C_5H_8N_5Cl$	173.0468	$C_5H_8N_5$	138.0780
Alachlor	20.40	$C_{14}H_{20}NO_2Cl$	269.1183	$C_{10}H_{12}N$	146.097	$C_{11}H_{14}N$	160.1126	C_8H_8N	118.0657	$C_{13}H_{16}NOCl$	237.0920	$C_{14}H_{20}NO_2Cl$	269.1183
Chlorpyriphos	22.00	$C_9H_{11}NO_3SCl_3P$	348.9263	$C_5H_2NOCl_3$	196.9202	$C_5H_2NO^{35}Cl_2{}^{37}Cl$	198.9172	$C_5H_3NO_3SCl_2P$	257.8948	$C_9H_{11}NO_3S^{37}Cl_2P$	315.9544	H_2O_3SP	96.9513
Chlorfenvinphos	23.58	$C_{12}H_{14}O_4\ Cl_3P$	357.9695	$C_8H_6O_4Cl_2P$	266.9381	$C_8H_6O_4{}^{35}Cl{}^{37}ClP$	268.9353	$C_{12}H_{14}O_4Cl_2P$	323.0007	$C_{10}H_{10}O_4Cl_2P$	294.9694	$C_{12}H_{14}O_4{}^{35}Cl{}^{37}ClP$	324.9980
Pyrene	23.93	$C_{16}H_{10}$	202.0783	$C_{16}H_{10}$	202.0783	$C_{16}H_9$	201.0704	$C_{16}H_8$	200.0626	$C_{14}H_6$	174.0470	C_8H_5	101.0391
Benzo(g,h,l)perylene	40.32	$C_{22}H_{12}$	276.0939	$C_{22}H_{12}$	276.0939	$C_{22}H_{10}$	274.0783	$C_{22}H_8$	276.0626	$C_{11}H_6$	138.047	$C_{10}H_5$	125.0391

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Concentration level

The general procedure mentioned above based on two different approaches, (1) spectral library search and (2) mw-XICs and Q/q ratio evaluation, was feasible for positive findings at relatively high analyte concentrations (above 50 ng/l). However, the success of the first approach depended on the analyte concentration level and coeluting matrix interfering compound. A notable decrease of library match was observed when lowering the concentration level. As an example, Fig. 1 shows the confirmation process for simazine detected at a concentration around 1000 ng/l in a surface water sample. Simazine was confirmed by a library search (forward match 90%) and also by the evaluation of mass accuracy for five representative ions, obtaining mass error values below 4 mDa (four of them were within 1 mDa) (Fig. 1(A)).

Regarding the second approach, the use of five mw-XICs allowed an efficient confirmation of simazine by fulfilling the predefined criteria (four Q/q ratios) (Fig. 1(B)).

However, at analyte concentrations < 50 ng/l the confirmation of a finding was feasible only with the second approach, as the purity of the spectrum was lower owing to the high background noise, which made the library search infeasible in practice.

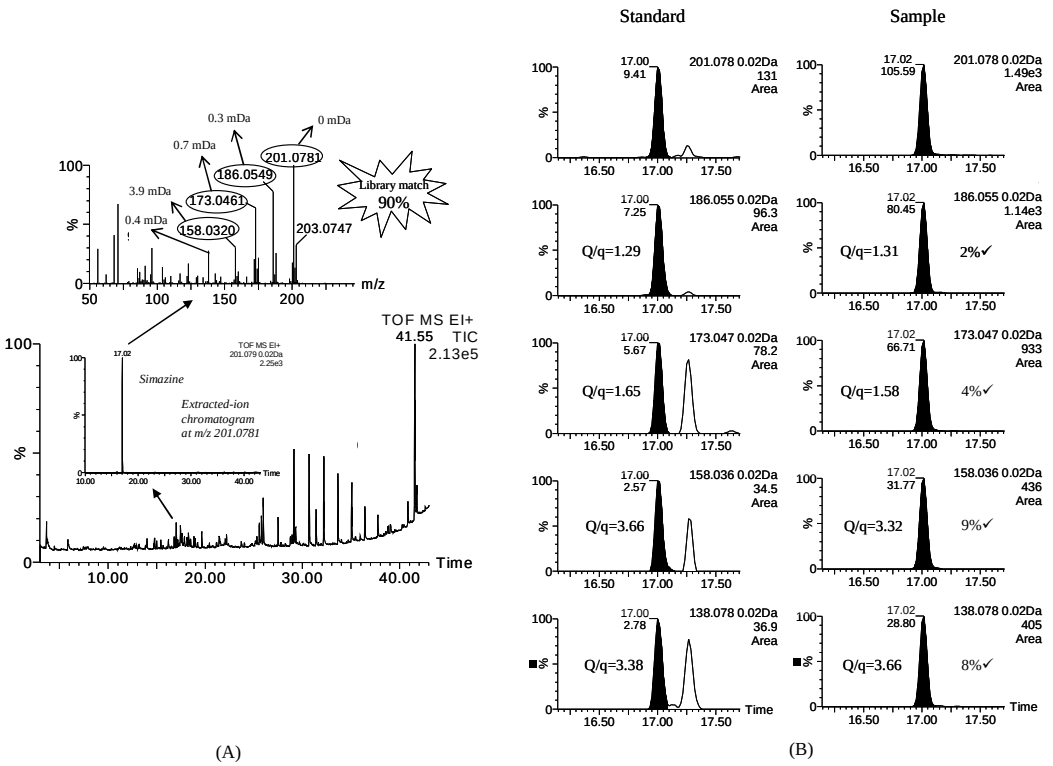

Figure 1. (A) Total ion chromatogram and extracted-ion chromatogram at *m/z* 201.0781 for a positive finding of simazine in a surface water (1000 ng/l) sample with its corresponding experimental EI spectrum. Inset: Mass accuracy evaluation for five representative ions. (B) Extracted-ion chromatograms (micro-window 0.02 Da) at different *m/z* for a solvent standard of simazine (50 µg/l) and the positive finding in surface water. Peaks present in the standard solution at 17.27 min correspond to atrazine (a pesticide-mixed standard solution was used) as some fragment ions are common for both compounds. ✓: Q/q ratio within allowed limits.

Coeluting/interfering compounds

Library search was also unsuccessful when interferences from the matrix or column coelute with the analyte of interest. A ground water sample analyzed in our laboratory by SPME using Carbowax/divinilbenzene (65 µm) fiber (within another research project) was used to illustrate this aspect. Figure 2 shows a positive finding of terbuthylazine for which library search identification was infeasible as a consequence of chromatographic coelution of an interference coming from the SPME fiber used for

sample extraction. This interfering compound was present in the sample chromatogram, giving a signal 100 times higher than that of the analyte. However, the use of 5 mw-XICs made its confirmation reliable by the accomplishment of all the Q/q ratios evaluated. Nowadays, deconvolution software is commercially available, which may allow the generation of clean mass spectra of coeluting peaks when the chromatographic resolution is not good. However, this approach has not been investigated in this paper, and it will be considered in future work.

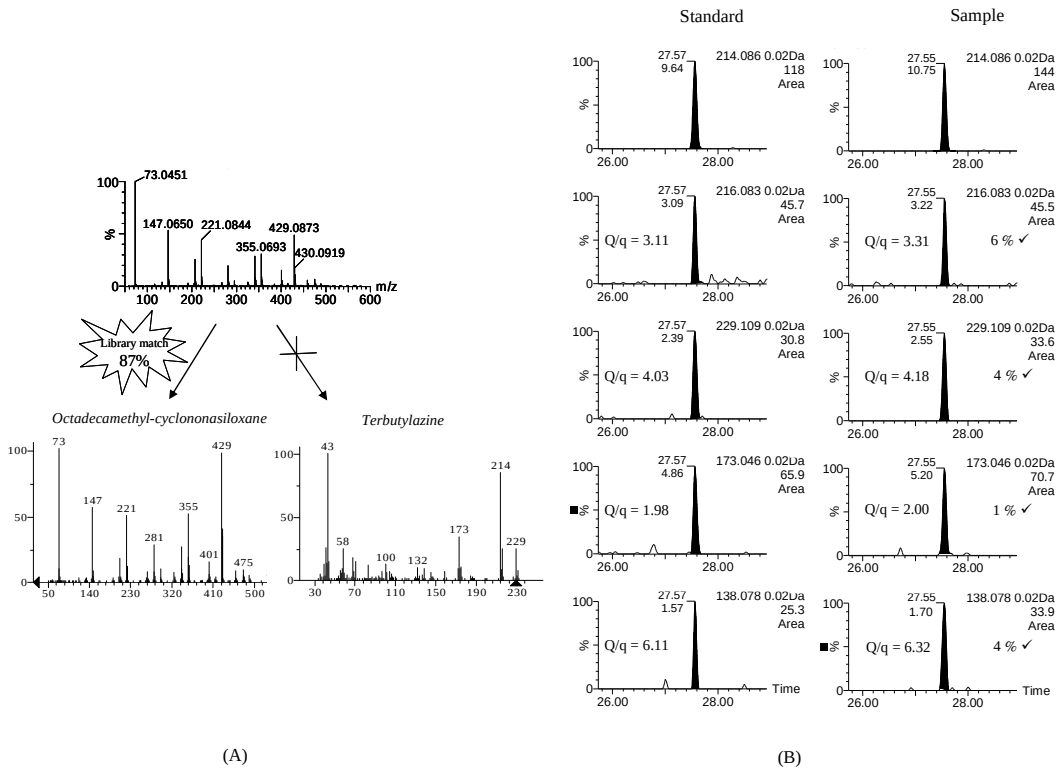

Figure 2. (A) EI spectrum for a positive finding of terbuthylazine in ground water (top) compared to the library spectra (bottom). (B) Extracted-ion chromatograms (micro-window 0.02 Da) at different *m/z* for a standard of terbuthylazine and the positive sample. ✓: Q/q ratio within allowed limits.

Mass window/resolution

The use of high-resolution TOF MS and its capability of obtaining accurate mass measurements allowed confirmation of some positive findings that would not have been satisfactorily confirmed by using mass spectrometers with lower resolution. The influence of the mass extraction window was studied, selecting values between 0.01 and 1 Da.

This case was illustrated in a positive finding of alachlor in surface water (400 ng/l), where different mass extraction windows were used: 1, 0.05, 0.02 and 0.01 Da. For a mass window of 1 Da, alachlor could be confirmed only with 1 out of 4 Q/q ratios available. When decreasing the mass window down to 0.05 Da and 0.02 Da, some chemical background was removed and the compound could be confirmed with three out of four Q/q ratios. Q/q ratio for one of the most abundant ions (*m/z* 118.0657) was still out of limits, but it was acceptable when the mass window was lowered to 0.01 Da. This fact illustrates that selectivity can be notably improved by combination of elevated resolution and high mass accuracy measurements, enabling target compounds to be identified in samples and eliminating the contribution from background interferences close to the measured ion. However, a mw-XIC of 0.01 Da is sometimes too narrow, especially for less abundant ions, as peak shape can be notably affected resulting in failure to meet the Q/q confirmatory ratio. As a compromise between an acceptable peak shape and a mass window narrow enough for minimizing interferences, a mw-XIC of 0.02 Da was selected for further experiments.

Lock mass

When searching for the ions originating from perhalogenated analytes at low concentrations, as well as the other ions that may come from the sample matrix, it is important to take into account also interferences that may appear from heptacosa, which is continuously introduced into the ion source during the analyses and is used as a single-point correction of the base mass calibration. It should be noticed that these ions can affect the confirmation process regardless of operating the instrument in a high-resolution mode since the exact masses of both lock mass and target analyte can be very similar. This fact is illustrated in Fig. 3, which shows a positive finding of 100 ng/l chlorpyriphos in surface water. The XIC at *m/z* 313.9574 (0.05 Da mass window

width) did not show any peak owing to the high background noise produced by one of the heptacosa ions (*m/z* 313.9839). In order to solve this problem, another ion, considering the presence of two Cl in above chlorpyriphos fragment, was chosen (A + 2: 315.9544), making now feasible its confirmation with five ions (4 Q/q ratios). This problem could also be solved by lowering the mass window to 0.02 Da mw-XIC for the 313.9574 ion, supporting the preliminary proposal of using 0.02 Da mass window width.

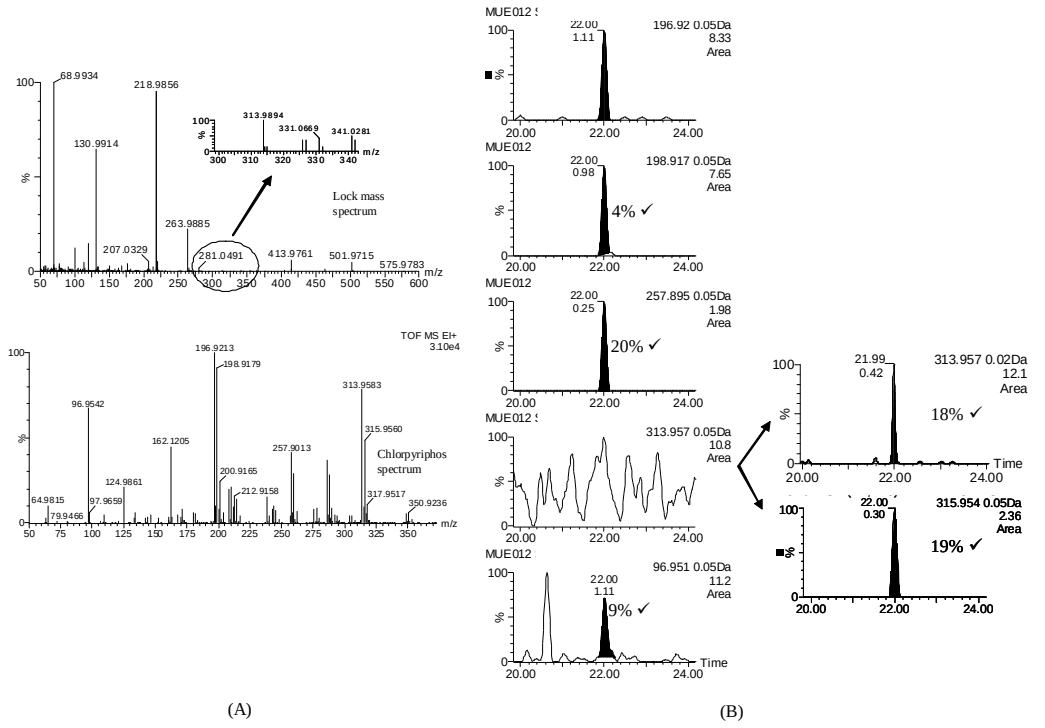

Figure 3. (A) EI spectra of heptacosa, used as lock mass, and of chlorpyriphos. (B) Extracted-ion chromatograms for a positive finding of chorpyriphos in surface water (100 ng/l). ✓: Q/q ratio within allowed limits.

Saturated peaks

Confirmation of analytes by evaluating the Q/q ratios of selected ions can be limiting at high analyte concentrations because of the possibility of detector saturation. As a consequence, the intensity for the measured ion can be lower than expected,

resulting in incorrect Q/q ratio values. This occurred, for example, in a positive finding of 80 µg/l terbuthylazine in highly polluted surface water. The spectrum presented two saturated ions (at the experimental *m/z* 173.0374 and 214.0676), which should not be used when evaluating the Q/q intensity ratios. The detector saturation is evidenced specially in the double peak observed when a mw-XIC of the Q ion at its theoretical exact mass (214.0859) is performed. Obviously, the experimental Q/q ratios using this quantitation ion did not fit with the theoretical ones. However, the use of nonsaturated ions, instead, made feasible the confirmation of this positive finding with the accomplishment of three out of four Q/q ratios preestablished.

Investigating the presence of organic pollutants using post-targeted data analysis

The complete spectral information available for each sample after analysis by GC-TOF MS allows a post-target screening to be performed where the analytes can be selected after MS data acquisition. Consequently, any compound might be investigated with the obvious restrictions derived from sample treatment and chromatographic requirements [23]. Although this approach is very attractive, it has practical limitations because the extraction process applied is optimized only for target analytes. In this context, the occurrence of nontargeted analytes in a sample could be taken as positive findings, although the whole sample preparation and extraction procedure would need to be validated afterwards. Several pesticides frequently used in the Mediterranean region [27], which had not been included in the TargetLynx method (terbumeton, terbacil, terbutryn, fenitrothion, malathion, methidation, buprofezin and azinfos-methyl), were investigated in a post-target way. Reference standards were used to obtain the spectral information. Malathion and terbutryn were detected in several surface and urban waste-water samples. Identification was confirmed by obtaining at least two Q/q ratios within the expected tolerances. As illustrative examples, Fig. 4 shows positive findings of terbutryn (estimated concentration of 200 ng/l in urban waste water) and malathion (estimated concentration of 400 ng/l in surface water), both established using external single-point calibration and confirmed with the four preselected Q/q ratios, although the library search gave a match of only around 40%.

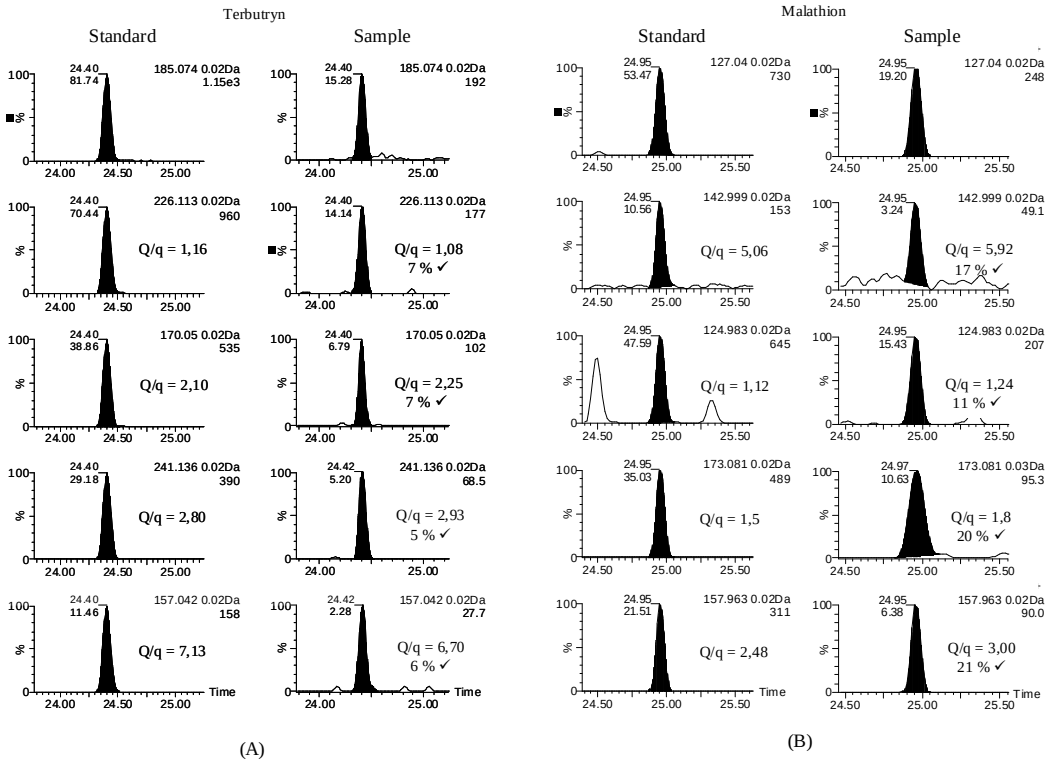

Figure 4. (A) Extracted-ion chromatograms of a terbutryn standard (100 µg/l) and a positive finding in urban waste water (around 200 ng/l). (B) Extracted-ion chromatograms of a malathion standard (100 µg/l) and a positive finding in surface water (around 400 ng/l). ✓: Q/q ratio within allowed limits.

In order to show other ways of confirming analytes in the samples (e.g. without using a reference standard for confirmation), the presence of diazinon was investigated. A mw-XIC at its *m/z* molecular ion (304.1010) was generated and the combined spectrum was searched against the library. Library match and experimental accurate masses for several ions were evaluated, with the result of diazinon being detected in seven samples (three surface water, two urban waste water and two urban solid-waste leachates). Considering the difficulty of using library search as the identification approach when the analyte is present in low concentrations, an optional methodology was developed on the basis of the spectral information available in commercial

libraries. Five fragment ions were selected and the predicted Q/q ratios were calculated from the library spectrum instead of from the reference standard injected in the GC-TOF. This information was used to create a TargetLynx method without the retention time information. All diazinon positives were identified after applying both approaches and with the attainment of at least two Q/q ratios. Figure 5 shows a positive finding of diazinon in a surface water sample by using both the approaches proposed. In the first approach, a library match of 80% and mass errors below 1.8 mDa were obtained. In the second approach, diazinon could be confirmed by the attainment of three out of four Q/q ratios preestablished.

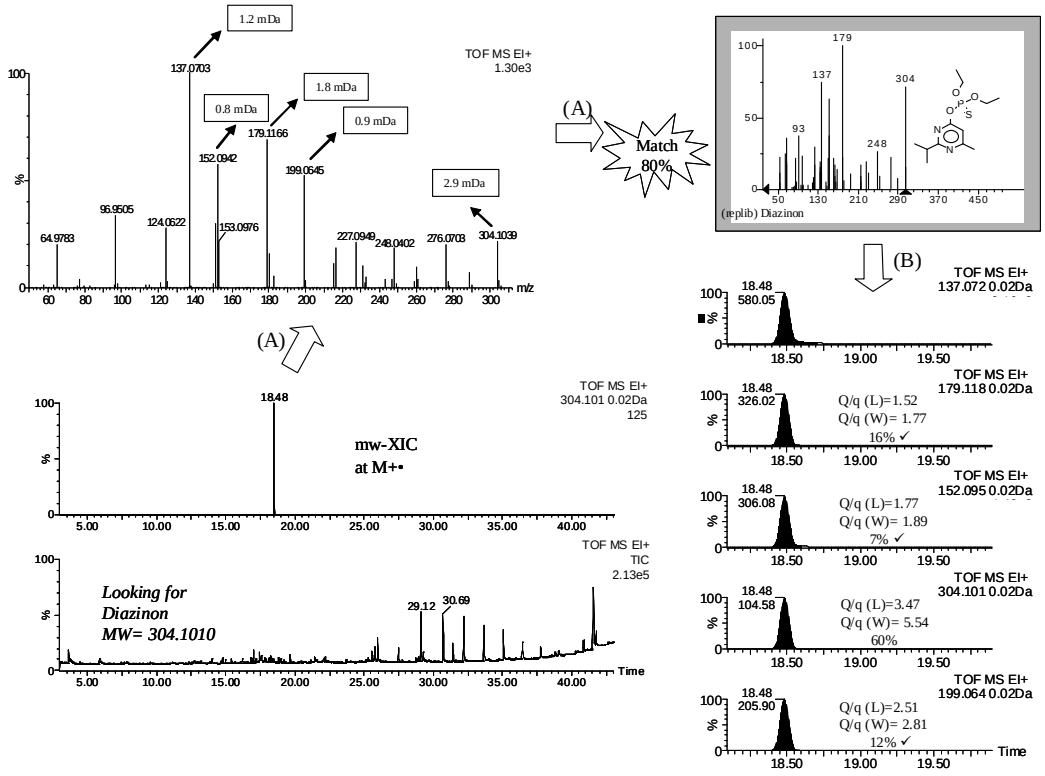

Figure 5. Total ion chromatogram and extracted-ion chromatogram at *m/z* 304.1010 for a positive finding of diazinon in surface water with its corresponding experimental and library EI spectrum. (A) Mass accuracy evaluation for five representative ions. (B) Extracted-ion chromatograms at different *m/z* for the positive finding. W: water sample. L: Library spectrum. ✓: Q/q ratio within allowed limits.

Confirmation proposal

In order to establish a final proposal for the confirmation of positive findings by GC-TOF MS, a comparison was carried out with positives found by GC-QqQ MS/MS using two transitions in the SRM mode.

Among the 130 positives previously detected by QqQ-MS, 25% were at concentration levels higher than 500 ng/l. Confirmation by TOF MS for all these cases was excellent, as most of them allowed the use of the five ions selected, with all four Q/q ratios measured falling within the specified tolerances. Searching the combined spectra in the MS library was also successful, resulting in unequivocal confirmation of the compounds detected, showing the advantage of using library search with improved sensitivity in comparison to quadrupole or ion trap analyzers.

For those compounds present in samples at lower concentrations (75% of positives), different situations were observed:

1. Twenty percent of the remaining positives could be confirmed by at least the presence of two ions and their Q/q ratio falling within the specified tolerances, although the library match was not satisfactory.

2. Fifty percent of the remaining positives presented only one ion, or the two most abundant ions, but the Q/q ratio was out of tolerances, so confirmation was not possible unless more experiments were performed in order to confirm the identity of the organic pollutants in samples. The majority of these cases corresponded to PAHs, whose EI fragmentation was poor and their confirmatory ions were lower than 10% of the base peak. Under these circumstances, higher analyte concentrations are required to be able to confirm its presence. Higher preconcentration factors during sample preparation (i.e. SPE step) than that used in our work (100 ml sample concentrated to 1 ml final extract) would help in the confirmation of these possible positives.

3. The 30% remaining positives were present at very low concentrations (below 50 ng/l) and could not be detected by GC-TOF MS because of its lower sensitivity compared to QqQ in SRM mode.

In the light of data obtained in this paper, the presence of at least two ions measured at their accurate mass and the attainment of their Q/q intensity ratio within specified tolerances seem a good compromise for a reliable confirmation of analytes by GC-TOF MS at low concentrations (around or below 100 ng/l). For higher concentrations, a high degree of safety can be obtained in the confirmation process by measuring up to five representative ions together with their Q/q intensity ratios. In terms of identification points (IPs) the number of IPs earned by each measured ion would be only one, as the TOF MS analyzer exhibits a mass resolving power of 7000 FWHM (approximately 3500 measured at 10% valley), lower than 10 000 (10% valley) required by the EU guidelines, according to the definition of high-resolution mass spectrometers.[24] Consequently, measuring two ions would lead to earn only two IPs (less than the three or four needed for the confirmation of authorized or banned compounds). However, the number of IPs (and thus the reliability of the confirmation process) should not only depend on mass resolution but even more on mass accuracy. According to this fact, 2 (high resolution), 1.5 and 1 (low resolution) IP have been proposed to be assigned to those ions that presented mass errors below 2 mDa, between 2-10 mDa or higher than 10 mDa, respectively.[28] In this way, an ion extracted with a mw-XIC of 20 mDa (mass error ± 10 mDa) would earn 1.5 IPs if the value of Q/q ratio is within tolerances, leading to earning three IPs when measuring two ions. Very recently, an interesting discussion about mass resolution versus mass accuracy, which was already introduced in environmental field confirmation by our group,[28] has also been initiated in hormone and veterinary drug residue analysis where the European Commission Decision (2002/657/EC)[24] really applies [29].

Regarding the first approach employed in this work, where the experimental spectrum is compared with the library and experimental masses are compared with the theoretical ones, the number of IPs can be higher, as the mass error obtained is normally below 2 mDa (2 IPs earned per ion). However, this approach must be done manually and it is a time-consuming procedure, together with the difficulty of getting a pure spectrum in some cases. By contrast, the second approach can be automatically performed using a TargetLynx processing method, which makes this procedure very attractive from a screening and confirmatory point of view.

CONCLUSIONS

The potential of GC-TOF MS for the reliable screening and confirmation of organic micro-pollutants in water has been explored. Two approaches have been applied to several types of water samples where around 130 positives have been investigated: (1) the spectrum library search together with the mass accuracy evaluation for representative ions, (2) the evaluation of the presence of up to five selected ions and the attainment of their Q/q intensity ratios from the corresponding mw-XIC at their *m/z* (typically using a mass window of 0.02 Da). Both procedures led to satisfactory data when the analytes were at concentration levels of 100 ng/l (concentration of the extract, 10 µg/l) or higher in water. However, when lower analyte concentrations and/or when matrix components were coeluting, the confirmation of the identity was successful only by applying the second approach.

Mass window was a critical parameter when performing an extracted-ion chromatogram. Selectivity was dramatically improved because mass chromatograms could be generated with a mass window as small as 0.01 Da centered on the exact mass of the target ion, enabling the correct identification of compounds in complex mixtures and minimizing the contribution from background ions.

To perform a reliable confirmation of positive findings, at least two ions together with the compliance of their Q/q ratio using a mw-XIC of 0.02 Da would be required. According to the results of this paper, using a sample preconcentration factor of 100, for analyte concentrations between 100 and 1000 ng/l, the number of ions measured at accurate mass could be enhanced by up to five in an automated and simple mode making use of the TargetLynx software of the instrument.

The acquisition of spectra by TOF analyzers offered the advantage of searching for selected analytes after MS data acquisition in a post-target mode, without the need of performing additional analysis, improving sensitivity and mass accuracy when compared to other MS analyzers working in the full-scan mode. This approach is not feasible when using the SIM (GC-MS) or SRM (GC-MS/MS) acquisition modes, which can be successfully applied for target analysis but do not allow detecting other pollutants for which no transitions have been previously acquired. Additionally, TOF MS allows the possibility to perform a nontarget analysis, which will be considered in our future works.

ACKNOWLEDGEMENTS

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of the time-of-flight mass spectrometer. This work forms a part of the project CTQ2006-07594 developed with the financial support of Ministerio de Ciencia y Tecnología. T. Portolés is very grateful to Ministerio de Educación y Ciencia for her predoctoral grant. The authors acknowledge the useful comments and the thorough revision of this paper by Timothy Jenkins, Peter Hancock and Cristiana Leandro from Waters Corporation.

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3.2.3 Artículo científico 5

Anal. Chem. 79, 9494-9504, 2007

TARGET AND NONTARGET SCREENING OF ORGANIC MICROPOLLUTANTS IN WATER BY SOLID-PHASE MICROEXTRACTION COMBINED WITH GAS CHROMATOGRAPHY/HIGH-RESOLUTION TIME-OF-FLIGHT MASS SPECTROMETRY

Félix Hernández, Tania Portolés, Elena Pitarch, Francisco J. López

Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain,

ABSTRACT

The potential of gas chromatography coupled to high-resolution time-of-flight mass spectrometry (GC/TOF-MS) for screening of organic pollutants in water has been explored. After optimization of the solid-phase microextraction (SPME) step, where parameters such as fiber selection and addition of salt were studied, this extraction technique was applied to the analysis of different types of water samples. Investigation of 60 target organic pollutants, including pesticides, octyl/nonyl phenols, pentachlorobenzene, and polycyclic aromatic hydrocarbons (PAHs) was carried out by evaluating the presence of up to five representative m/z ions per analyte, measured at high mass accuracy, and the attainment of their *Q*/*q* (Q, quantitative ion; q, confirmative ion) intensity ratio. This strategy led to the detection of 4-t-octylphenol, simazine, terbuthylazine, chlorpyrifos, terbumeton, and terbutryn in several water samples at low part-per-billion levels. Full spectrum acquisition data generated by the TOF-MS analyzer also allowed subsequent investigation of the presence of polybrominated diphenyl ethers and several fungicides in samples after MS data acquisition, without the need to reanalyze the water samples. In addition, nontarget analysis was also tested by application of a deconvolution software. Several organic pollutants that did not form a part of the list of contaminants investigated were identified in the water samples, thanks to the excellent sensitivity of TOF-MS in full spectrum acquisition mode and the valuable accurate mass information provided by

instrument. Bisphenol A, the antioxidant 3,5-di-tert-butyl-4-hydroxy-toluene (BHT), its metabolite 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT−CHO), the polycyclic musk galaxolide, and the UV filter benzophenone were some of the compounds present in the water samples analyzed. SPME in combination with GC/TOF-MS has been proved to be an attractive and powerful approach for the rapid screening of multiclass organic pollutants in water, with very little sample manipulation and no solvent consumption. This combination provides to the analyst with information-rich MS data that facilitates the reliable identification of many different organic compounds in samples.

INTRODUCTION

Gas chromatography coupled to mass spectrometry (GC/MS) has been widely used for identification and quantification of volatile and semivolatile organic pollutants in environmental samples, as it combines high selectivity and resolution, good precision, and satisfactory sensitivity. GC/MSs with quadrupole or ion trap analyzers have become a routine analytical tool for the determination of organic contaminants in food, environmental, and biological samples because of their relatively low cost and ease of use.[1-3] To achieve low detection limits (LODs), quadrupole instruments must operate in selected ion monitoring (SIM), whereas ion trap instruments normally operate in tandem MS (MS/MS). Under these circumstances, the determination of a finite number of target compounds can be successfully achieved, although most of the chemical information on sample composition is discarded, which makes these techniques unfeasible when searching for other analytes unless additional analysis are performed. Full spectrum techniques are required for this aim. Among these, high-resolution time-of-flight mass spectrometry (TOF-MS) allows a notable amount of chemical information to be obtained in a single experiment making this technique very attractive to the investigation of nontarget compounds present in samples and also for searching for analytes in a post-target way.[4,5] The TOF analyzer simultaneously samples and analyses all ions across the mass range in contrast to traditional scanning instruments (quadrupoles and ions traps), where masses are ejected and detected sequentially. As a consequence, GC coupled to TOF-MS has unrivalled full spectrum sensitivity, comparable to quadrupole instruments in SIM mode. Furthermore, this technique provides elevated mass resolution, of around 7000 fwhm (full width at half-maximum),

and also excellent mass accuracy (around 1 mDa, i.e., 5 ppm for measurements at m/z 200)[2] for those peaks with sufficient intensity and using an internal reference mass (lock mass) introduced via a heated reference inlet. Consequently, extracted ion chromatograms with a very narrow mass window (microwindow XICs; mw-XICs) can be used, enabling the removal of large amount of chemical background ions, dramatically improving selectivity in complex matrixes, and leading to improved detection limits. In addition, accurate mass measurements notably facilitate the elemental composition calculation of every peak in the spectrum, resulting in a reliable identification of target and nontarget compounds and also solving ambiguous results in library search. Very few papers have been published dealing with the determination of organic contaminants by GC/high-resolution-TOF-MS. All these articles appeared in the present decade and evidence the interest and the novelty of this subject. Several applications have been described on the determination of polybrominated diphenyl ethers (PBDEs), pesticides, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) in different environmental matrixes.[5-7] Other applications deal with the determination of PBDEs, xenoestrogens, or flavor research in biological samples.[8-10] Recently, Čajka and Hajšlova reviewed the application of GC/TOF-MS in food analysis.[11] As regards sample treatment, organic micropollutants are normally present in the environment at low concentrations, which forces the use of an extraction step able to concentrate the analytes prior to GC analysis. Solid-phase microextraction (SPME) has gained widespread acceptance for the determination of a wide spectrum of analytes in various fields[12-14] as it is a rapid, simply, easily automated, and solventless technique that allows the isolation, concentration, and purification of analytes from complex matrixes. SPME is widely used these days in combination with GC, and many papers can be found in the literature on its applications for the determination of organic compounds in different sample types. Therefore, SPME in combination with GC/TOF-MS seems to be a powerful approach for the rapid screening and confirmation of many organic pollutants in environmental samples, due to the inherent advantages of both techniques. However, few applications have been published until now based on SPME coupled to GC with high-resolution TOF-MS,[15,16] although some more papers have been found dealing with the use of the other type of instrument (nominal mass−high-speed TOF-MS analyzer), which has been applied to the determination of volatiles in apple fruit,[17] butter,[18] and olive oil,[19] mono- to octachlorobiphenyls in fish oil,[20] chloroanisoles in cork,[21] or pesticides in tea leaves.[22]

The aim of this work is to explore the potential of SPME in combination with GC/high-resolution-TOF-MS for the rapid screening and identification of organic micropollutants in water. For this purpose, 10 water samples of different types and origin were analyzed. Around 60 organic micropollutants, for which the SPME procedure had been optimized, were investigated as target analytes. Then, making use of the full spectral acquisition data acquired and without reanalyzing the samples, 11 PBDEs and 6 fungicides were selected to perform a post-target investigation of the samples. Finally, screening of unknown compounds present in water (nontarget analysis) was carried out making use of the deconvolution potential and the valuable accurate mass information provided by TOF-MS.

EXPERIMENTAL SECTION

Reagents

Reference standards of pesticides, octyl/nonyl phenols, pentachlorobenzene, PCBs (Mix 3, 100 µg/mL in cyclohexane), and PAHs (Mix 25, 100 µg/mL) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Acenaphthene and naphthalene (Fluka, Buchs, Switzerland) and fluoranthene (Riedel de Haen, Seelze, Germany) were also used. Standards of PBDEs (50 µg/mL in nonane) were obtained from Wellington Laboratories (Guelph, Ontario, Canada). In the case of solid reference standards, stock solutions (around 500 µg/mL) were prepared by dissolving reference standards in acetone and stored in a freezer at −20 °C. Working solutions were prepared by diluting stock solutions with acetone, for sample fortification, and with hexane, for GC injection. The list of all compounds investigated in this work is shown in Table 1.

Acetone (pesticide residue analysis) and hexane (ultratrace quality) were purchased from Scharlab (Barcelona, Spain). Sodium chloride from Sharlab (Barcelona, Spain) of analytical grade was used after purification by heating at 300 °C overnight.

Milli Q Gradient A10 (Millipore, Molsheim, France) water (18.2 MΩ cm) was used for SPME optimization purposes.

Five isotopically labeled surrogates were used: *p,p'*-DDE-d_8, lindane-d_6, benzo(a)anthracene-d_{12}, and terbuthylazine-d_5 (Dr. Ehrenstorfer) and hexachlorobenzene (HCB)-$^{13}C_6$ (Cambridge Isotope Labs, Inc. Andover, MA). The working

surrogate solution (0.04 µg/mL) consisted of a mix of individual labeled acetonic standards of 10 µg/mL (*p,p'*-DDE-d_8, terbuthylazine-d_5, (HCB)-$^{13}C_6$, lindane-d_6, and benzo(a)anthracene-d_{12}) and was prepared by dilution with acetone. This surrogate solution was added to both standards, used for calibration, and samples.

Table 1. m/z ions selected for the identification of target compounds

Compound	R_t[a]	Molecular formula	Molecular mass	Ion 1 (Q)	m/z 1	Ion 2	m/z 2	Ion 3	m/z 3	Ion 4	m/z 4	Ion 5	m/z 5
Naphthalene	12.33	$C_{10}H_8$	128.0626	$C_{10}H_8$	128.0626	$C_{10}H_7$	127.0548	$C_{10}H_6$	126.0470	C_8H_6	102.0470	C_8H_5	101.0391
Acenaphthylene	20.02	$C_{12}H_8$	152.0626	$C_{12}H_8$	152.0626	$C_{12}H_7$	151.0548	$C_{12}H_6$	150.0470	$C_{10}H_6$	126.0470	C_6H_4	76.0313
Acenaphthene	20.88	$C_{12}H_{10}$	154.0783	$C_{12}H_9$	153.0704	$C_{12}H_{10}$	154.0783	$C_{12}H_8$	152.0626	$C_{10}H_6$	126.0470	C_6H_4	76.0313
Pentachlorobenzene	21.48	C_6HCl_5	247.8521	$C_6H^{35}Cl_4{}^{37}Cl$	249.8492	C_6HCl_5	247.8521	$C_6H^{35}Cl_3{}^{37}Cl_2$	251.8462	$C_6H^{35}Cl_3{}^{37}Cl$	214.8803	C_6HCl	107.9760
Fluorene	23.38	$C_{13}H_{10}$	166.0783	$C_{13}H_9$	165.0704	$C_{13}H_{10}$	166.0783	$C_{13}H_8$	164.0626	$C_{11}H_7$	139.0548	C_9H_7	115.0548
4-t-Octylphenol	23.65	$C_{14}H_{22}O$	206.1671	$C_9H_{11}O$	135.0810	C_7H_7O	107.0497	C_6H_7O	95.0497	$C_{14}H_{22}O$	206.1671		
Trifluraline	25.08	$C_{13}H_{16}F_3N_3O_4$	335.1093	$C_{11}H_{11}F_3N_3O_4$	306.0702	$C_{11}H_{11}F_3N_3O_3$	290.0753	$C_8H_5F_3N_3O_3$	248.0283	$C_{13}H_{16}F_3N_3O_4$	335.1093		
Hexachlorobenzene	25.93	C_6Cl_6	281.8131	$C_6{}^{35}Cl_5{}^{37}Cl$	283.8102	C_6Cl_6	281.8131	$C_6{}^{35}Cl_4{}^{37}Cl$	248.8413	C_6Cl_4	211.8754	C_6Cl_2	141.9377
Simazine	26.80	$C_7H_{12}ClN_5$	201.0781	$C_7H_{12}ClN_5$	201.0781	$C_6H_9N_5Cl$	186.0546	$C_5H_8ClN_5$	173.0468	$C_5H_7ClN_4$	158.0359	$C_5H_8N_5$	138.0780
Atrazine	26.98	$C_8H_{14}ClN_5$	215.0938	$C_7H_{11}ClN_5$	200.0703	$C_7H_{11}{}^{37}ClN_5$	202.0674	$C_8H_{14}ClN_5$	215.0938	$C_5H_8ClN_5$	173.0468	$C_4H_5ClN_5$	158.0233
Terbumeton	27.27	$C_{10}H_{19}N_5O$	225.1590	$C_9H_{16}N_5O$	210.1355	$C_5H_{11}N_5O$	169.0964	$C_5H_8N_5O$	154.0729	$C_4H_7N_5O$	141.0651	$C_{10}H_{19}N_5O$	225.1590
Lindane	27.28	$C_6H_6Cl_6$	287.8601	$C_6H_4Cl_3$	180.9379	$C_6H_4{}^{35}Cl_2{}^{37}Cl$	182.9349	$C_6H_5Cl_4$	216.9145	$C_6H_4Cl_2$	145.9690	C_6H_5Cl	112.0080
4-n-Octylphenol	27.38	$C_{14}H_{22}O$	206.1671	C_7H_7O	107.0497	$C_{14}H_{22}O$	206.1671	C_7H_7	91.05480	C_6H_5	77.0391		
Terbuthylazine	27.57	$C_9H_{16}ClN_5$	229.1094	$C_8H_{13}ClN_5$	214.0859	$C_8H_{13}{}^{37}ClN_5$	216.0831	$C_9H_{16}ClN_5$	229.1094	$C_5H_8ClN_5$	173.0468	$C_5H_8N_5$	138.0780
Terbacil	27.58	$C_9H_{13}ClN_2O_2$	216.0666	$C_5H_6ClN_2O_2$	161.0118	$C_5H_5ClN_2O_2$	160.0040	C_4H_4ClNO	116.9981	$C_5H_6{}^{37}ClN_2O_2$	163.0090	$C_6H_5ClO_2$	143.9978
Phenanthrene	27.92	$C_{14}H_{10}$	178.0783	$C_{14}H_{10}$	178.0783	$C_{14}H_8$	176.0626	$C_{12}H_8$	152.0626	$C_{12}H_7$	151.0548	C_7H_5	89.0391
Anthracene	28.17	$C_{14}H_{10}$	178.0783	$C_{14}H_{10}$	178.0783	$C_{14}H_8$	176.0626	$C_{12}H_8$	152.0626	$C_{12}H_7$	151.0548	C_7H_5	89.0391
Endosulfan ether	29.18	$C_9H_6Cl_6O$	339.8550	$C_8H_4{}^{35}Cl_4{}^{37}Cl$	276.8727	$C_8H_4Cl_2$	169.9690	$C_5{}^{35}Cl_4{}^{37}Cl$	236.8413	$C_9H_6{}^{35}Cl_4{}^{37}ClO$	306.8832	$C_9H_6{}^{35}Cl_5{}^{37}ClO$	341.8521
4-n-nonylphenol	29.58	$C_{15}H_{24}O$	220.1827	C_7H_7O	107.0497	$C_{15}H_{24}O$	220.1827	C_7H_7	91.0548	C_6H_5	77.0391		
PCB 28	29.75	$C_{12}H_7Cl_3$	255.9613	$C_{12}H_7Cl_3$	255.9613	$C_{12}H_7{}^{35}Cl_2{}^{37}Cl$	257.9585	$C_{12}H_7{}^{35}Cl{}^{37}Cl_2$	259.9557	$C_{12}H_7Cl$	186.0236	$C_{12}H_6$	150.0470
Alachlor	30.02	$C_{14}H_{20}ClNO_2$	269.1183	$C_{10}H_{12}N$	146.0970	$C_{11}H_{14}N$	160.1126	C_8H_8N	118.0657	$C_{13}H_{16}ClNO$	237.0920	$C_{14}H_{20}ClNO_2$	269.1183
Heptachlor	30.28	$C_{10}H_5Cl_7$	369.8211	$C_5{}^{35}Cl_5{}^{37}Cl$	271.8102	$C_5{}^{35}Cl_4{}^{37}Cl$	236.8413	$C_{10}H_5Cl_3$	229.9457	$C_{10}H_5Cl$	160.0080	$C_{10}H_5{}^{35}Cl_5{}^{37}Cl$	336.8493
Terbutryn	30.98	$C_{10}H_{19}N_5S$	241.1361	$C_6H_{11}N_5S$	185.0735	$C_9H_{16}N_5S$	226.1126	$C_5H_8N_5S$	170.0500	$C_{10}H_{19}N_5S$	241.1361	$C_4H_7N_5S$	157.0422
PCB 52	31.12	$C_{12}H_6Cl_4$	289.9224	$C_{12}H_6{}^{35}Cl_3{}^{37}Cl$	291.9195	$C_{12}H_6Cl_4$	289.9224	$C_{12}H_6Cl_3$	254.9535	$C_{12}H_6Cl_2$	219.9846	$C_{12}H_6$	150.0470
Metolachlor	31.52	$C_{15}H_{22}ClNO_2$	283.1339	$C_{11}H_{16}N$	162.1283	$C_{13}H_{17}ClNO$	238.0999	$C_{13}H_{17}{}^{37}ClNO$	240.0973	$C_{10}H_{12}N$	146.0970		
Fenitrothion	31.62	$C_9H_{12}NO_5PS$	277.0174	$C_2H_6O_2PS$	124.9826	$C_2H_6O_3P$	109.0055	$C_9H_{12}NO_5PS$	277.0174	$C_9H_{11}NO_4PS$	260.0146	CH_4O_2P	78.9949
Chlorpyrifos	31.62	$C_9H_{11}Cl_3NO_3PS$	348.9263	$C_5H_2Cl_3NO$	196.9202	$C_5H_2{}^{35}Cl_2{}^{37}ClNO$	198.9173	$C_5H_3Cl_2NO_3PS$	257.8948	$C_7H_7Cl_2NO_3PS$	285.9261	$C_9H_{11}{}^{35}Cl{}^{37}ClNO_3PS$	315.9545
Aldrin	31.70	$C_{12}H_8Cl_6$	361.8757	$C_7H_2{}^{35}Cl_4{}^{37}Cl$	262.8570	$C_7H_2Cl_5$	260.8599	$C_{12}H_7{}^{35}Cl_3{}^{37}Cl$	292.9273	$C_7H_2{}^{35}Cl_5{}^{37}Cl$	297.8258	$C_{12}H_6Cl_3$	254.9535
Malathion	32.50	$C_{10}H_{19}O_6PS_2$	330.0361	$C_6H_7O_3$	127.0395	$C_2H_6O_2PS$	124.9826	$C_8H_{13}O_4$	173.0814	$C_2H_7O_2PS_2$	157.9625	$C_2H_6O_2P$	93.0105
Isodrin	32.85	$C_{12}H_8Cl_6$	361.8757	$C_7H_4Cl_3$	192.9379	$C_7H_4{}^{35}Cl_2{}^{37}Cl$	194.9349	$C_7H_2{}^{35}Cl_4{}^{37}Cl$	262.8570	$C_{12}H_7{}^{35}Cl_3{}^{37}Cl$	292.9273	$C_6H_5Cl_2$	146.9768
Heptachlor epoxide B	33.23	$C_{10}H_5Cl_7O$	385.8160	$C_{10}H_5{}^{35}Cl_5{}^{37}ClO$	352.8442	$C_{10}H_5Cl_6O$	350.8472	$C_{10}H_5{}^{35}Cl_4{}^{37}Cl_2O$	354.8413	$C_5{}^{35}Cl_4{}^{37}Cl$	236.8413	$C_{11}H_8{}^{35}Cl_4{}^{37}Cl$	316.8772
Chlorfenvinphos	33.32	$C_{12}H_{14}Cl_3O_4P$	357.9695	$C_8H_6Cl_2O_4P$	266.9381	$C_8H_6{}^{35}Cl{}^{37}ClO_4P$	268.9353	$C_{12}H_{14}Cl_2O_4P$	323.0007	$C_{10}H_{10}Cl_2O_4P$	294.9694	$C_{12}H_{14}{}^{35}Cl{}^{37}ClO_4P$	324.9980
Heptachlor epoxide A	33.40	$C_{10}H_5Cl_7O$	385.8160	$C_9H_4Cl_3$	216.9379	$C_9H_3Cl_4$	250.8989	$C_5{}^{35}Cl_4{}^{37}Cl$	236.8423	$C_9H_3{}^{35}Cl_3{}^{37}Cl$	252.8959	$C_{10}H_5{}^{35}Cl5{}^{37}ClO$	352.8442

Table 1. m/z ions selected for the identification of target compounds

Compound	R_t[a]	Molecular formula	Molecular mass	Ion 1 (Q)	m/z 1	Ion 2	m/z 2	Ion 3	m/z 3	Ion 4	m/z 4	Ion 5	m/z 5
Fluoranthene	33.57	$C_{16}H_{10}$	202.0783	$C_{16}H_{10}$	202.0783	$C_{16}H_9$	201.0704	$C_{16}H_8$	200.0626	$C_{14}H_6$	174.0470	C_8H_5	101.0391
Methidathion	33.92	$C_6H_{11}N_2O_4PS_3$	301.9619	$C_4H_5N_2O_2S$	145.0072	$C_3H_5N_2O$	85.0402	$C_2H_6O_2PS$	124.9826	CH_5N_2OS	93.0123	C_2H_3OS	74.9905
PCB 101	34.42	$C_{12}H_5Cl_5$	323.8834	$C_{12}H_5{}^{35}Cl_4{}^{37}Cl$	325.8805	$C_{12}H_5Cl_5$	323.8834	$C_{12}H_5{}^{35}Cl_3{}^{37}Cl$	290.9117	$C_{12}H_5Cl_3$	253.9457	$C_{12}H_5Cl$	184.0080
Pyrene	34.57	$C_{16}H_{10}$	202.0783	$C_{16}H_{10}$	202.0783	$C_{16}H_9$	201.0704	$C_{16}H_8$	200.0626	$C_{14}H_6$	174.0470	C_8H_5	101.0391
α-endosulfan	34.65	$C_9H_6Cl_6O_3S$	403.8169	$C_8H_4Cl_2$	169.9690	$C_9H_6{}^{35}Cl_4{}^{37}ClO$	306.8832	$C_9H_6Cl_5O_3$	336.8760	$C_9H_6{}^{35}Cl_4{}^{37}ClO_3$	338.8731	$C_7H_5Cl_2$	158.9768
p,p'-DDE	35.50	$C_{14}H_8Cl_4$	315.9380	$C_{14}H_8Cl_2$	246.0003	$C_{14}H_8{}^{35}Cl^{37}Cl$	247.9975	$C_{14}H_8{}^{35}Cl_3{}^{37}Cl$	317.9352	$C_{14}H_8Cl_4$	315.9380	$C_{14}H_8$	176.0626
Dieldrin	35.70	$C_{12}H_8Cl_6$	377.8706	$C_7H_2{}^{35}Cl_4{}^{37}Cl$	262.8570	$C_7H_2Cl_5$	260.8599	$C_8H_4Cl_5$	274.8755	$C_{12}H_8{}^{35}Cl_4{}^{37}ClO$	344.8989	$C_{12}H_8{}^{35}Cl_5{}^{37}ClO$	379.8677
Buprofezin	35.80	$C_{16}H_{23}N_3OS$	305.1562	C_7H_7N	105.0578	C_7H_6N	104.0500	$C_8H_{16}N_2S$	172.1034	$C_7H_{15}N_2OS$	175.0905	$C_{12}H_{23}N_3OS$	305.1562
PCB 118	36.75	$C_{12}H_5Cl_5$	323.8834	$C_{12}H_5{}^{35}Cl_4{}^{37}Cl$	325.8805	$C_{12}H_5Cl_5$	323.8834	$C_{12}H_5{}^{35}Cl_3{}^{37}Cl_2$	327.8774	$C_{12}H_5Cl_3$	253.9457	$C_{12}H_5Cl$	184.0080
ß-endosulfan	36.90	$C_9H_6Cl_6O_3S$	403.8169	$C_8H_4Cl_2$	169.9690	$C_9H_6{}^{35}Cl_4{}^{37}ClO$	306.8832	$C_9H_6Cl_5O_3$	336.8760	$C_9H_6{}^{35}Cl_4{}^{37}ClO_3$	338.8731	$C_7H_5Cl_2$	158.9768
p,p'-DDD	37.08	$C_{14}H_{10}Cl_4$	317.9537	$C_{13}H_9Cl_2$	235.0081	$C_{13}H_9{}^{35}Cl^{37}Cl$	237.0053	$C_{13}H_9$	165.0704	$C_{14}H_9Cl$	212.0393	$C_{13}H_8Cl$	199.0314
PCB 153	37.53	$C_{12}H_4Cl_6$	357.8444	$C_{12}H_4{}^{35}Cl_5{}^{37}Cl$	359.8415	$C_{12}H_4Cl_6$	357.8444	$C_{12}H_4{}^{35}Cl_4{}^{37}Cl$	324.8727	$C_{12}H_4{}^{35}Cl_3{}^{37}Cl$	289.9038	$C_{12}H_4Cl_2$	217.9690
Azinphos-methyl	37.89	$C_{10}H_{12}N_3O_3PS_2$	317.0058	$C_8H_6N_3O$	160.0511	C_8H_6NO	132.0449	C_7H_5O	105.034	C_7H_4O	104.0262		
Endosulfan sulfate	38.32	$C_9H_6Cl_6O_4S$	419.8118	$C_5{}^{35}Cl_5{}^{37}Cl$	271.8102	C_5Cl_6	269.8131	$C_9H_6{}^{35}Cl_4{}^{37}ClO_4S$	386.8400	$C_9H_6{}^{35}Cl_5{}^{37}ClO_4S$	421.8089	$C_7H_3{}^{35}Cl_3{}^{37}Cl$	228.8960
p,p'-DDT	38.49	$C_{14}H_9Cl_5$	351.9147	$C_{13}H_9Cl_2$	235.0081	$C_{14}H_8Cl_2$	246.0003	$C_{13}H_9{}^{35}Cl^{37}Cl$	237.0053	$C1_3H_9$	165.0704	$C_{14}H_9Cl$	212.0393
PCB 138	38.50	$C_{12}H_4Cl_6$	357.8444	$C_{12}H_4{}^{35}Cl_5{}^{37}Cl$	359.8415	$C_{12}H_4Cl_6$	357.8444	$C_{12}H_4{}^{35}Cl_4{}^{37}Cl$	324.8727	$C_{12}H_4{}^{35}Cl_3{}^{37}Cl$	289.9038	$C_{12}H_4Cl_2$	217.9690
Benzo(a)anthracene	40.32	$C_{18}H_{12}$	228.0939	$C_{18}H_{12}$	228.0939	$C_{18}H_{10}$	226.0783	$C_{16}H_8$	200.0626	C_9H_6	114.0470	C_8H_5	101.0391
Chrysene	40.47	$C_{18}H_{12}$	228.0939	$C_{18}H_{12}$	228.0939	$C_{18}H_{10}$	226.0783	$C_{16}H_8$	200.0626	C_9H_6	114.0470	C_8H_5	101.0391
Metoxychlor	40.54	$C_{16}H_{15}Cl_3O_2$	344.0138	$C_{15}H_{15}O_2$	227.1072	$C_{14}H_{12}O_2$	212.0837	$C_{16}H_{15}ClO_2$	274.0761	$C_{14}H_{12}O$	196.0888	$C_{16}H_{14}Cl_2O_2$	308.0371
PCB 180	40.92	$C_{12}H_3Cl_7$	391.8055	$C_{12}H_3{}^{35}Cl_6{}^{37}Cl$	393.8025	$C_{12}H_3{}^{35}Cl_5{}^{37}Cl_2$	395.7996	$C_{12}H_3Cl_7$	391.8055	$C_{12}H_3{}^{35}Cl_4{}^{37}Cl$	323.8648	$C_{12}H_3Cl_3$	251.9300
Mirex	42.37	$C_{10}Cl_{12}$	539.6262	$C_5{}^{35}Cl_5{}^{37}Cl$	271.8102	C_5Cl_6	269.8131	$C_5{}^{35}Cl_4{}^{37}Cl$	236.8413	$C_{10}{}^{35}Cl_5{}^{37}Cl$	331.8102	$C_{10}{}^{35}Cl_6{}^{37}Cl_2$	403.7450
Benzo(b)fluoranthene	45.05	$C_{20}H_{12}$	252.0939	$C_{20}H_{12}$	252.0939	$C_{20}H_{10}$	250.0783	$C_{20}H_8$	248.0626	$C_{10}H_6$	126.0470	C_9H_5	113.0391
Benzo(k)fluoranthene	45.17	$C_{20}H_{12}$	252.0939	$C_{20}H_{12}$	252.0939	$C_{20}H_{10}$	250.0783	$C_{20}H_8$	248.0626	$C_{10}H_6$	126.0470	C_9H_5	113.0391
Benzo(a)pyrene	46.32	$C_{20}H_{12}$	252.0939	$C_{20}H_{12}$	252.0939	$C_{20}H_{10}$	250.0783	$C_{20}H_8$	248.0626	$C_{10}H_6$	126.0470	C_9H_5	113.0391
Indeno(1,2,3,cd)pyrene	50.78	$C_{22}H_{12}$	276.0939	$C_{22}H_{12}$	276.0939	$C_{22}H_{10}$	274.0783	$C_{22}H_8$	272.0626	$C_{11}H_6$	138.0470	$C_{10}H_5$	125.0391
Dibenzo(a,h)anthracene	50.99	$C_{22}H_{14}$	278.1096	$C_{22}H_{14}$	278.1096	$C_{22}H_{12}$	276.0939	$C_{22}H_{11}$	274.0783	$C_{11}H_7$	139.0548	$C_{10}H_5$	125.0391
Benzo(g,h,l)perylene	51.97	$C_{22}H_{12}$	276.0939	$C_{22}H_{12}$	276.0939	$C_{22}H_{10}$	274.0783	$C_{22}H_8$	272.0626	$C_{11}H_6$	138.0470	$C_{10}H_5$	125.0391
BDE 28[b]	36.91	$C_{12}H_7OBr_3$	403.8047	$C_{12}H_7O^{79}Br_2{}^{81}Br$	405.8027	$C_{12}H_7O^{79}Br^{81}Br_2$	407.8007	$C_{12}H_7OBr$	245.9680	$C_{12}H_7O^{81}Br$	247.9661	$C_{12}H_7OBr_3$	403.8047
BDE 71[b]	40.59	$C_{12}H_6OBr_4$	481.7152	$C_{12}H_6O^{79}Br^{81}Br$	325.8765	$C_{12}H_6OBr_2$	323.8785	$C_{12}H_6O^{79}Br_3{}^{81}Br$	483.7132	$C_{12}H_6O^{79}Br_2{}^{81}Br_2$	485.7112	$C_{12}H_6O^{79}Br^{81}Br_3$	487.7092
BDE 47[b]	41.02	$C_{12}H_6OBr_4$	481.7152	$C_{12}H_6O^{79}Br^{81}Br$	325.8765	$C_{12}H_6OBr_2$	323.8785	$C_{12}H_6O^{79}Br_3{}^{81}Br$	483.7132	$C_{12}H_6O^{79}Br_2{}^{81}Br_2$	485.7112	$C_{12}H_6O^{79}Br^{81}Br_3$	487.7092
BDE 66[b]	38.04	$C_{12}H_6OBr_4$	481.7152	$C_{12}H_6O^{79}Br^{81}Br$	325.8765	$C_{12}H_6OBr_2$	323.8785	$C_{12}H_6O^{79}Br_3{}^{81}Br$	483.7132	$C_{12}H_6O^{79}Br_2{}^{81}Br_2$	485.7112	$C_{12}H_6O^{79}Br^{81}Br_3$	487.7092
BDE 100[b]	44.17	$C_{12}H_5OBr_5$	559.6257	$C_{12}H_5O^{79}Br_2{}^{81}Br$	403.7870	$C_{12}H_5O^{79}Br^{81}Br_2$	405.7850	$C_{12}H_5O^{79}Br_3{}^{81}Br_2$	563.6216	$C_{12}H_5O^{79}Br_2{}^{81}Br_3$	565.6197	$C_{12}H_5O^{79}Br_4{}^{81}Br$	561.6237
BDE 99[b]	45.12	$C_{12}H_5OBr_5$	559.6257	$C_{12}H_5O^{79}Br_2{}^{81}Br$	403.7870	$C_{12}H_5O^{79}Br^{81}Br_2$	405.7850	$C_{12}H_5O^{79}Br_3{}^{81}Br_2$	563.6216	$C_{12}H_5O^{79}Br_2{}^{81}Br_3$	565.6197	$C_{12}H_5O^{79}Br_4{}^{81}Br$	561.6237

Table 1. m/z ions selected for the identification of target compounds

Compound	R_t[a]	Molecular formula	Molecular mass	Ion 1 (Q)	m/z 1	Ion 2	m/z 2	Ion 3	m/z 3	Ion 4	m/z 4	Ion 5	m/z 5
BDE 85[b]	46.60	$C_{12}H_5OBr_5$	559.6257	$C_{12}H_5O^{79}Br_2{}^{81}Br$	403.7870	$C_{12}H_5O^{79}Br^{81}Br_2$	405.7850	$C_{12}H_5O^{79}Br_3{}^{81}Br_2$	563.6216	$C_{12}H_5O^{79}Br_2{}^{81}Br_3$	565.6197	$C_{12}H_5O^{79}Br_4{}^{81}Br$	561.6237
BDE 154[b]	45.59	$C_{12}H_4OBr_6$	637.5362	$C_{12}H_4O^{79}Br_2{}^{81}Br_2$	483.6955	$C_{12}H_4O^{79}Br_3{}^{81}Br$	481.6975	$C_{12}H_4O^{79}Br^{81}Br_3$	485.6935	$C_{12}H_4O^{79}Br_3{}^{81}Br_3$	641.5322	$C_{12}H_4O^{79}Br_2{}^{81}Br_4$	643.5302
BDE 153[b]	50.05	$C_{12}H_4OBr_6$	637.5362	$C_{12}H_4O^{79}Br_2{}^{81}Br_2$	483.6955	$C_{12}H_4O^{79}Br_3{}^{81}Br$	481.6975	$C_{12}H_4O^{79}Br^{81}Br_3$	485.6935	$C_{12}H_4O^{79}Br_3{}^{81}Br_3$	641.5322	$C_{12}H_4O^{79}Br_2{}^{81}Br_4$	643.5302
BDE 138[b]	50.75	$C_{12}H_4OBr_6$	637.5362	$C_{12}H_4O^{79}Br_2{}^{81}Br_2$	483.6955	$C_{12}H_4O^{79}Br_3{}^{81}Br$	481.6975	$C_{12}H_4O^{79}Br^{81}Br_3$	485.6935	$C_{12}H_4O^{79}Br_3{}^{81}Br_3$	641.5322	$C_{12}H_4O^{79}Br_2{}^{81}Br_4$	643.5302
BDE 183[b]	51.25	$C_{12}H_3OBr_7$	715.4467	$C_{12}H_3O^{79}Br_3{}^{81}Br_2$	561.6060	$C_{12}H_3O^{79}Br_2{}^{81}Br_3$	563.6040	$C_{12}H_3O^{79}Br_4{}^{81}Br$	559.6080	$C_{12}H_3O^{79}Br^{81}Br_4$	565.6021	$C_{12}H_3OBr_5$	557.6100
Diphenylamine[b]	19.57	$C_{12}H_{11}N$	169.0891	$C_{12}H_{11}N$	169.0891	$C_{12}H_{10}N$	168.0813	$C_{12}H_9N$	167.0735				
Cyprodinil[b]	28.28	$C_{14}H_{15}N_3$	225.1266	$C_{14}H_{14}N_3$	224.1188	$C_{14}H_{15}N_3$	225.1266	$C_{12}H_{12}N_3$	210.1031				
Thiabendazole[b]	28.83	$C_{10}H_7N_3S$	201.0361	$C_{10}H_7N_3S$	201.0361	$C_9H_6N_2S$	174.0252						
Metalaxyl[b]	30.32	$C_{15}H_{21}NO_4$	279.1471	$C_{12}H_{16}NO_2$	206.1181	$C_{12}H_{18}NO_2$	220.1388	$C_{13}H_{16}NO_3$	234.113	$C_{14}H_{19}NO_3$	249.1365	$C_{10}H_{12}N$	146.097
Imazalil[b]	30.63	$C_{14}H_{14}Cl_2N_2O$	296.0483	$C_7H_3Cl_2O$	172.9561	$C_{10}H_9Cl_2O$	215.003	$C_7H_3{}^{35}Cl^{37}ClO$	172.9561	$C_{10}H_9{}^{35}Cl^{37}ClO$	217.0002	$C_7H_5Cl_2$	158.9768
Oxadixyl[b]	37.33	$C_{14}H_{18}N_2O_4$	278.1267	$C_{10}H_{13}NO$	163.0997	$C_9H_{10}N$	132.0813	C_8H_9	105.0704	$C_8H_{10}N$	120.0813	$C_{12}H_{13}N_2O_3$	233.0926

[a] Retention time (min). [b] All PBDEs and fungicides were investigated in sample after MS data acquisition (post-target analysis)

Samples

Water samples of different types were collected from July 2003 to September 2005 from different sites in the Valencian area (Spain) and stored at less than −18 °C until analysis. Two samples corresponded to surface water and were collected at Borriana and Alcora locations. Four were groundwater samples and were collected from wells located at Carcaixent and Cabanes. Two samplesbefore and after treatmentwere obtained from a wastewater treatment plant sited at Vila-Real. Two more samples corresponded to urban solid waste leachates and were collected from a municipal treatment plant sited at Onda: one was treated water (reversed osmosis), and the other was raw leachate. This raw sample was diluted 2.5 times with deionized water before being subjected to SPME.

Instrumentation

SPME Setup

The SPME device used for manual extraction, consisting of a holder assembly and several replaceable fibers, was purchased from Supelco (Madrid, Spain). Four different fiber types were compared: nonpolar poly(dimethylsiloxane) (PDMS, 100 μm) and more polar, such as polyacrylate (PA, 85 μm), poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB, 65 μm), and Carbowax/divinylbenzene (CW/DVB 65 μm). The fibers were conditioned as recommended by the manufacturer by heating them in the injection port of the chromatographic system during 0.5−2 h at 220−300 °C depending on the fiber coating.

GC Equipment

An Agilent 6890N GC system (Palo Alto, CA), equipped with an Agilent 7683 autosampler, was coupled to a GCT time-of-flight mass spectrometer (Waters Corporation, Manchester, U.K.), operating in electron impact ionization mode (EI). The GC separation was performed using a fused-silica HP-5MS capillary column with a length of 30 m × 0.25 mm i.d. and a film thickness of 0.25 μm (J&W Scientific, Folson, CA). The oven temperature was programmed as follows: 90 °C (6 min); 5 °C/min to 300 °C (2 min). Helium was used as carrier gas at 1 mL/min.

The interface and source temperatures were set to 250 °C for all analytes studied, and a solvent delay of 3 min was selected. The time-of-flight mass spectrometer was operated at 1 spectrum/s, acquisition rate over the mass range m/z 50−650, using a multichannel plate voltage of 2500 V. TOF-MS resolution was approximately 7000 (fwhm). Heptacosa, used for the daily mass calibration and as lock mass, was injected via syringe in the reference reservoir at 30 °C for this purpose; the m/z ion monitored was 218.9856. The application manager TargetLynx, a module of MassLynx software, was used to process the qualitative and quantitative data obtained from standards and samples for target compounds. The application manager ChromaLynx, also a module of MassLynx software, was used to investigate the presence of nontarget compounds in samples. Library search was performed using the commercial NIST library.

Analytical Procedure

Extraction of water samples was carried out by direct immersion of the CW/DVB fiber into the sample (4 mL, 10% NaCl, 100 μL of 0.04 μg/mL surrogate solution), contained in a 5 mL clear glass vial, under magnetic stirring for 45 min at room temperature. The fiber was situated off center in the vial, so the sample flowed perpendicularly to the fiber axis. Thermal desorption was carried out at 250 °C for 5 min in the split−splitless injector of the gas chromatograph. Quantitative data on samples were obtained from calibration curves in the concentration range of 0.01−5 μg/L, which were prepared spiking ultrapure water with selected compounds and applying the overall SPME procedure. Several isotopically labeled compounds were added to both standards and samples before being subjected to SPME and were used as surrogate/internal standards: (HCB)-$^{13}C_6$ was used for pentachorobenzene, HCB and trifluraline; lindane-d6 for lindane; *p,p'*-DDE-d_8 for the rest of organochlorine insecticides and PCBs; benzo(a)anthracene-d_{12} for PAHs; and terbuthylazine-d_5 for herbicides, octyl/nonyl phenols, and organophosphorus insecticides.

RESULTS AND DISCUSSION

SPME Study

SPME was selected in this work as a simple, modern, and solventless technique to perform rapid screening of organic contaminants in water samples. Many papers can be found in the literature describing multiple applications of SPME for a wide variety of organic pollutants in water. Thus, the aim of this paper was not a detailed and thorough study of the SPME step but to investigate the potential of this technique in combination to GC/TOF-MS in environmental analysis; only the relevant parameters of fiber selection and addition of salt were studied, whereas the rest of variables affecting the extracting equilibrium, (extraction temperature, extraction time, desorption time, desorption temperature, amount of sample) were established according to our own experience [12,23,24] and the manufacturer's recommendation. Four fiber coatings, PDMS (100 µm), PDMS/DVB (85 µm), PA (65 µm), and CW/DVB (65 µm), were selected to evaluate their suitability for the extraction of target analytes, by analyzing 4 mL of a ultrapure water sample spiked at 1 µg/L with each organic contaminant. Moreover, the effect of adding 10% (0.4 g) of NaCl to the sample was also tested. The extraction time was set at 45 min at room temperature. Desorption temperatures were different for each fiber according to manufacturer's recommendation: 280 °C (PDMS), 270 °C (PDMS/DVB), 300 °C (PA), and 250 °C (CW/DVB). Desorption time was set in all cases at 5 min. Figure 1 shows the TOF-MS absolute response for some representative analytes after SPME of a water sample spiked at 1µg/L (10% of NaCl) using the four fibers selected. As can be seen, the CW/DVB fiber led to the best overall results for the majority of pollutants. Besides, it was the only fiber that allowed all the analytes to be extracted in more of less extent. Therefore, CW/DVB fiber with 10% of NaCl was selected for further experiments.

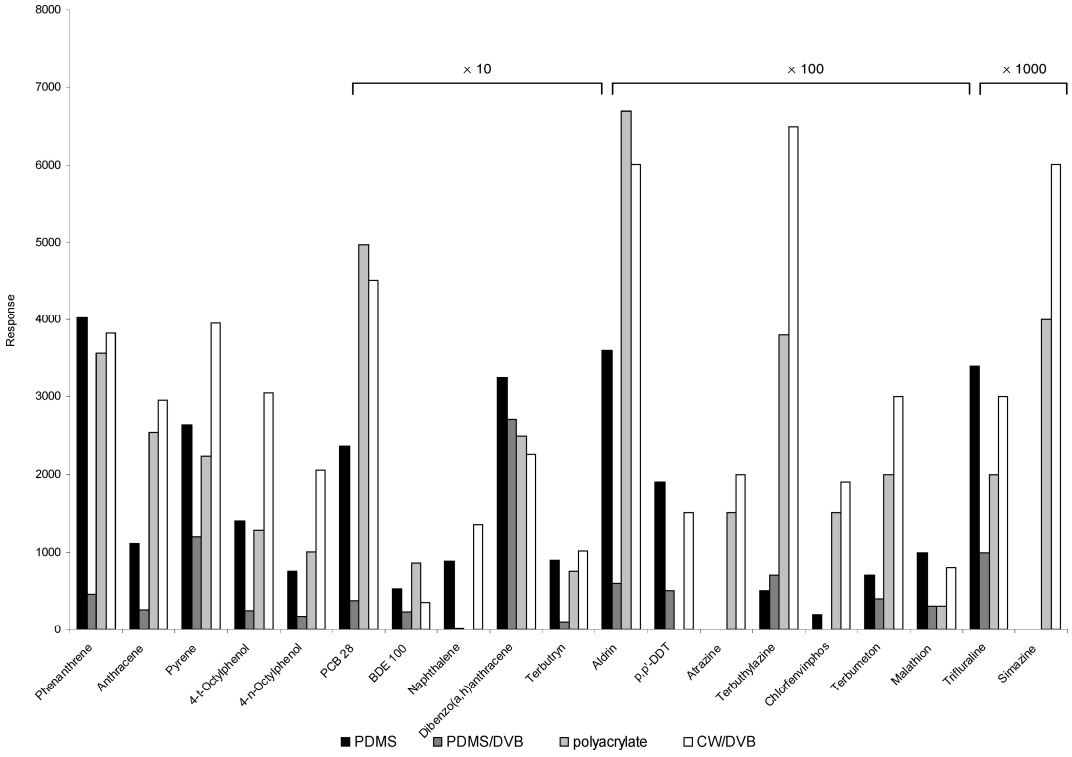


Figure 1 TOF-MS absolute response for some representative analytes after SPME of a water sample spiked at 1 µg/L (10% NaCl) using four different fibers

Linearity of relative response (versus IS) of the analytes was established by analyzing, in duplicate, ultrapure water spiked at seven concentration levels (0.01, 0.05, 0.1, 0.5, 1, 2.5, and 5 µg/L). The lowest calibration level (LCL) was the lowest concentration for which the correct identification of the compound was experimentally feasible by measuring, at least, two ions (Q, quantification; q, confirmation) with their *Q*/*q* ratio falling within specified tolerances. LCL was taken as the limit of quantification (LOQ), due to the difficulties for measuring signal-to-noise (S/N) ratio in TOF instruments when using a narrow mass window, as reported also by other authors.[25] As can be seen in Table 2, LOQ values were 0.01 or 0.05 µg/L for most of the analytes. As these low levels, not only the quantification but also the reliable identification of the analyte was feasible. Table 2 also shows the highest calibration level for which the linearity was acceptable (regression coefficient higher than 0.99).

Data fitted a linear regression for all the analytes, except for PCBs that were adjusted to quadratic calibration curves.

Table 2. Limit of Quantification for Each Compound after Application of the Overall SPME−GC/TOF-MS Procedure

0.01 µg/L	0.05 µg/L	0.1 µg/L	0.5 µg/L
acenaphthene (2.5)[a]	aldrin (2.5)	buprofezin (5)	alachlor (5)
acenaphthylene (0.5)	atrazine (5)	*p*,*p*-DDT (5)	azinphos-methyl (5)
anthracene (0.5)	chlorfenvinphos (5)	α-endosulfan (5)	methidathion (5)
benzo(*a*)anthracene (1)	dieldrin (2.5)	ß-endosulfan (5)	
benzo(*a*)pyrene (2.5)	endosulfan ether (5)	metoxychlor (2.5)	
benzo(*b*)fluoranthene (2.5)	endosulfan sulfate (5)	terbacil (5)	
benzo(*g*,*h*,*l*)perylene (2.5)	fenitrothion (5)	terbumeton (5)	
benzo(*k*)fluoranthene (2.5)	heptachlor (5)		
chlorpyrifos (2.5)	heptachlor epoxide A (1)		
chrysene (1)	isodrin (5)		
p,*p*-DDD (2.5)	lindane (1)		
p,*p*-DDE (5)	malathion (5)		
dibenzo(*a*,*h*)anthracene (2.5)	metolachlor (5)		
fluoranthene (0.5)	mirex (5)		
fluorene (0.5)	4-*n*-nonylphenol (2.5)		
heptachlor epoxide B (0.5)	4-*n*-ocytlphenol (2.5)		
hexachlorobenzene (2.5)	PCB 138 (5)		
indeno(1,2,3,*cd*)pyrene (2.5)	PCB 180 (5)		
naphthalene (2.5)	simazine (5)		
4-*t*-octylphenol (0.5)	terbuthylazine (5)		
PCB 28 (2.5)	terbutryn (5)		
PCB 52 (5)	trifluraline (5)		
PCB 101 (5)			
PCB 118 (5)			
PCB 153 (5)			
pentachlorobenzene (2.5)			
phenanthrene (0.5)			
pyrene (0.5)			

[a] Linearity was satisfactory up to the highest calibration level, shown in parentheses, expressed in µg/L.

The estimation of LOD by extrapolation based on the data obtained for the lowest concentration level was not performed due to the difficulty of measuring chemical noise using narrow mass window, as stated before. Under this situation, radical elimination of chemical noise occurred resulting in a fictitious enhancement of the S/N parameter, as has been also pointed out by other authors.[25,26]

Water Analysis

Water samples of different types and origin were subjected to SPME and analyzed by GC/TOF-MS, performing first the screening and confirmation of target analytes. Taking advantage of the MS data acquired after sample analysis, the presence of other selected compounds was subsequently investigated in a post-target way (i.e., after MS data acquisition), as well as the presence of unknown compounds (nontarget analysis) without the need of reanalyzing the sample. For this purpose, the methodical approach for screening and confirmation of organic micropollutants in water described in our previous work5 was applied.

Target Analysis

The detection and identification of target analytes in the samples was carried out by obtaining up to five microwindow extracted ion chromatograms (mw-XIC) at selected m/z ions for every compound. The software application TargetLynx employed automatically processed data and reported quantitative and qualitative results. Mass windows of 0.02 Da were chosen as a compromise between sensitivity, peak shape, and accurate mass measurements.

Regarding analyte identification, *Q*/*q* intensity ratios in samples were obtained and compared with the theoretical ones, which were calculated from the injection of standards in solvent. *Q*/*q* was the ratio between the most abundant ion (Q, quantitative) and every one of the other measured ions (q, confirmative) (i.e., *Q*/*q* ratio was always ≥1). Maximum deviations accepted were based on the European Commission Decision (2002/657/EC).[27] This approach has been widely applied by our own research group for the identification and confirmation of organic contaminants in environmental [4,28,29] and biological samples.[10,30]

The presence of, at least, two ions measured at their accurate mass and the attainment of their *Q*/*q* intensity ratio within specified tolerances was required for the reliable identification of target analytes in water samples.5 In the present work, 60 organic micropollutants were targeted, for which the SPME procedure was previously tested. As these compounds were selected before analysis; we call this approach a pretargeted analysis. Once the samples were analyzed, 11 PBDEs and 6 fungicides were also searched after MS acquisition (a post-target analysis). Table 1 shows the m/z ions selected for every compound investigated in a pretarget or post-target way.

When searching for the 60 pretarget compounds, calibration curves prepared described in "analytical procedure" (concentration range of 0.01−5 μg/L) were included in the sample sequence, so quantitation of positive findings could be performed. Table 3 shows the compounds detected in water samples. Four herbicides (simazine, terbuthylazine, terbumeton, and terbutryn), one insecticide (chlorpyrifos), and 4-t-octylphenol were detected in several samples. Analyte concentrations were normally in the range of 0.01−1.5 μg/mL, terbuthylazine being the compound most frequently detected. As an example Figure 2 shows the extracted ion chromatograms of a surface water sample, where terbuthylazine (0.2 μg/L), chlorpyrifos (0.4 μg/L), and terbutryn (0.1 μg/L) were detected.

Table 3. Target Compounds Detected in the Water Samples Analyzed[a]

compd	no. positives[b]	concn range (μg/L)	no. *Q*/*q* ratios[c]	IPs[d]
4-*t*-octylphenol	1	0.01	1	3
simazine	2	1; 1.5	3	6
terbuthylazine	6	0.1−0.3	3−4	6−7.5
chlorpyrifos	1	0.4	4	7.5
terbumeton	2	0.7; 1.1	2	4.5
terbutryn	4	0.1−1.5	3−4	6−7.5

[a] *Q*/*q* ratios and number of identification points assigned. [b] Ten water samples of different types were analyzed. [c] Number of experimental *Q*/*q* ratios that were within specified tolerances (*Q*, intensity of the most abundant, quantitative ion; *q*, intensity of the ion/s used for confirmation). [d] Identification points.

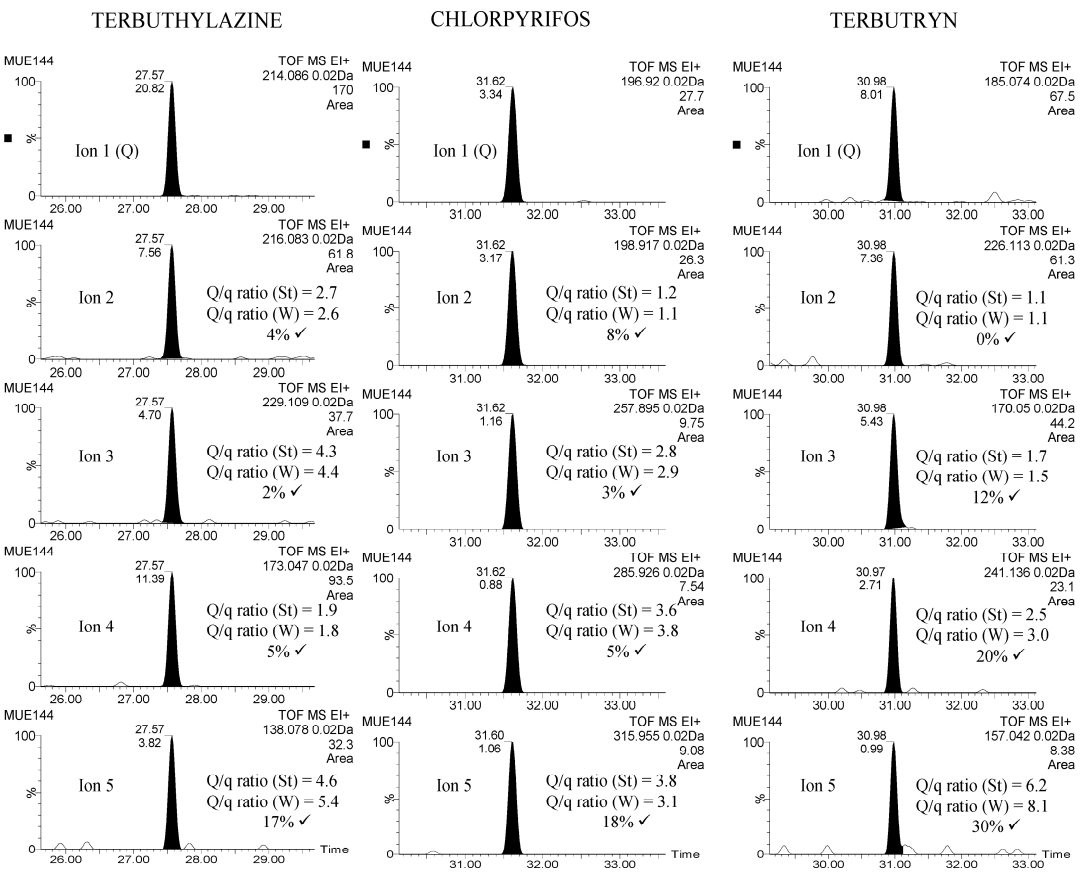


Figure 2 Extracted ion chromatograms at different m/z (mass window 0.02 Da) for positive findings of target terbuthylazine, chlorpyrifos, and terbutryn in surface water sample. Q, quantitative ion; q, confirmative ion; St, reference standard; W, water sample; ✓, *Q*/*q* ratio within tolerance limits; ×, *Q*/*q* ratio out of tolerance limits.

The use of identification points (IPs)[27] is of interest as a simple and standardized way to give a value to the reliability of the identification process. Although it may be questionable in some specific cases, this approach is useful. The spirit of using IPs is that the higher the number of IPs the higher the confidence in the confirmation of the identity of the compound detected. As we discussed in our previous paper,[5] each ion extracted with a mw-XIC of 20 mDa (mass error ±10 mDa) would earn 1.5 IPs given that the *Q*/*q* ratio is within tolerances, leading to earning 3 IPs when measuring two ions. The agreement in the retention time of the compound in sample and in the reference standard was also required to confirm a finding (maximum relative error ±0.5%).

In summary, among the 16 positives found, 13 could be confirmed by the attainment of at least three out of four *Q*/*q* ratios pre-established, obtaining in this way a minimum of 6 IPs (four ions measured, three *Q*/*q* ratios within tolerances). The positive finding of 4-t-octylphenol could only be confirmed by the accomplishment of one *Q*/*q* ratio as this compound presents a poor fragmentation spectrum with the confirmatory ions being less than 10% of the peak base. The two positive findings of terbumeton (around 0.5 µg/L) were confirmed by the attainment of two out of four *Q*/*q* ratios established in advance. In this case, the concentration level in samples was close to the LCL and only the three most abundant ions showed a chromatographic peak when a mw-XIC at the exact mass was performed, so only two *Q*/*q* ratios could be evaluated for confirmatory purposes.

The complete spectral information acquired by GC/TOF-MS allowed us to perform a searching of analytes selected in a post-targeted style. PBDEs, persistent halogenated contaminants, were chosen for this search due to concern regarding the occurrence of these compounds in the environment. This has started a growing interest in recent years in developing analytical methods to determine these persistent and liphophilic pollutants in different matrixes.[31,32] Six fungicides (diphenylamine, metalaxyl, cyprodinil, thiabendazole, imazalil, and oxadixyl) were also selected for post-target analysis as some of these compounds have been recently detected in environmental water at our area.[33] After applying the TargetLynx approach to these post-target analytes, three fungicides (diphenylamine, thiabendazole, and imazalil) were detected in several samples. In order to give support to the post-target approach, the SPME procedure applied in this work was subsequently tested for PBDEs and the six

fungicides investigated. Calibration curves (0.01−5 μg/L) were prepared, as mentioned above, and analyzed in duplicate. Linearity was acceptable (regression coefficient higher than 0.99) in the concentration range tested for all the PBDEs except for BDE 183 (0.1−5 μg/L). Diphenylamine and cyprodinil showed linear behavior in the range of 0.01−2.5 μg/L and thiabendazole and imazalil in the range of 0.1−5 μg/L. Metalaxyl and oxadixyl did not give satisfactory results. Thus, after testing the overall SPME−GC/TOF-MS procedure we could quantify the three fungicides detected in samples. Concentrations found were in the range of 0.1−1 μg/L, with diphenylamine being the compound most frequently detected.

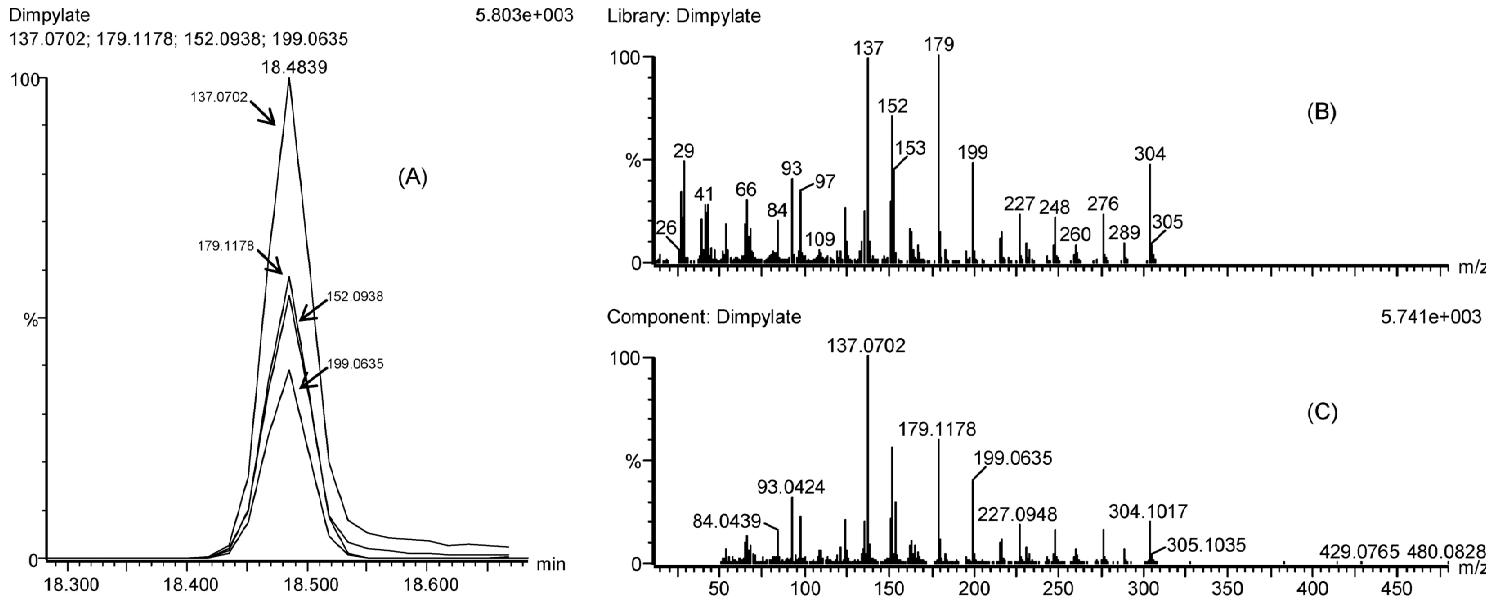
Nontarget Analysis

The investigation of nontarget compounds in water, or in any type of sample matrixes, is a laborious, hard, and time-consuming task that is rarely successful due to the huge amount of peaks coming from the matrix, column bleed, etc., masking and sometimes coeluting with compounds being investigated. This fact makes it difficult, and sometimes unfeasible, to get a pure spectrum to be searched in the library. In target GC/TOF-MS screening, this is not very important unless coeluting peaks have the same exact masses, but in nontarget screening, the ability to obtain a "clean" spectrum for each unknown component investigated is one of the keys to this process. Under these circumstances it is necessary to use powerful software options in order to identify first the presence of multiple components, and then the application of deconvolution software is required to produce pure spectra for each of the individual components. In this work, the deconvolution package ChromaLynx Application Manager was used to automatically process data in nontarget analysis. Although it can plot the reconstructed ion chromatograms of up to eight ions, only four abundant ions were selected in the present study, as it was considered sufficient for a reliable identification. When a peak was found to satisfy user-defined parameters (such as scan width, spectra rejection factor, peak width at 5% height, etc.) the software displayed its deconvoluted mass spectrum, which was submitted to an automatic library search routine. Components were reduced to a list of possible candidates by using the fit factor from the mass library search. A library match >70% was required for nontarget compounds identification. After that, the accurate mass confirmation of the library

search was automatically performed. The formula from the library hit was submitted to an elemental composition calculator, and then the accurate mass measurements of up to five most intense ions were evaluated for the confirmation/rejection of the finding.

All the water samples analyzed were processed using the above-described software, and several contaminants, not included in the target method, were identified. The organophosphorus insecticide dimpylate, also called diazinon, was detected in one surface water, in two samples from a wastewater treatment plantbefore and after treatmentand in the treated water from urban solid waste leachates. Figure 3 shows the positive finding of diazinon in treated water when using the deconvolution process. Accurate mass confirmation automatically performed by the software for five representative ions led to the confirmation of the identity of diazinon with mass errors for every ion always below 1.3 mDa.

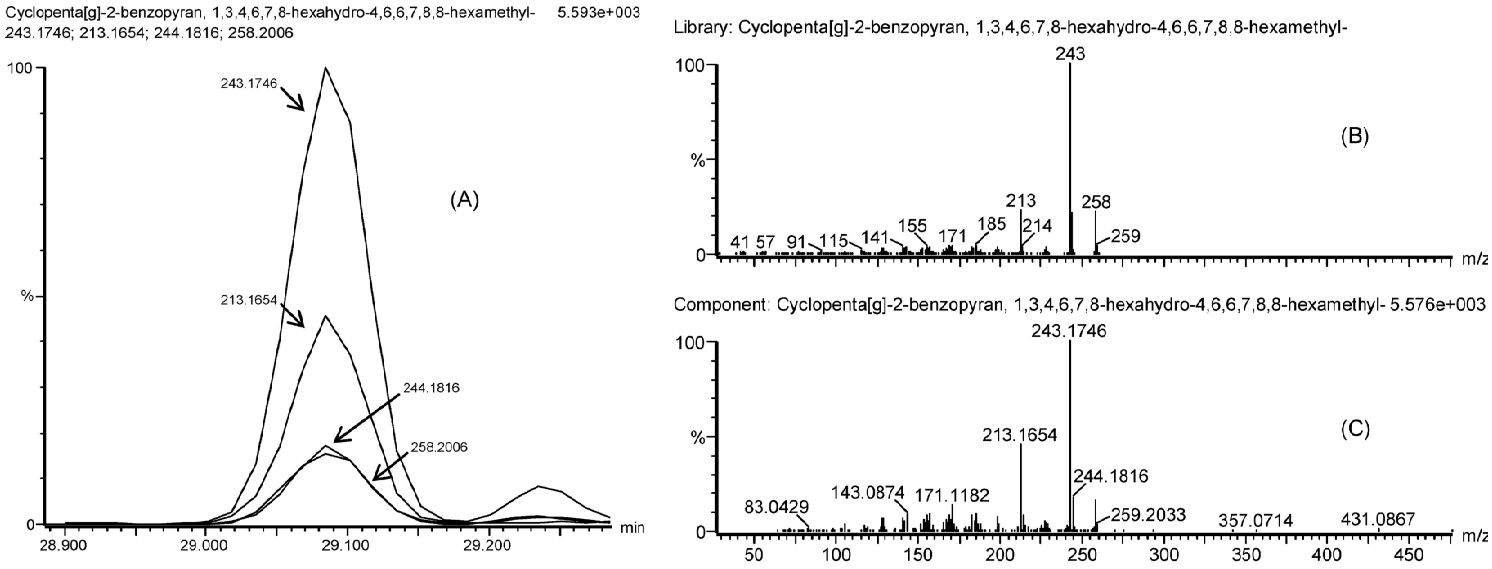
Another compound of interest detected in the samples was 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8,-hexamethyl-cyclopenta[g]-2-benzopyran (galaxolide), a polycyclic musk (PCM) compound. PCMs are used as fragrances in laundry detergents, cosmetics, perfumes, and personal care products. The major source of PCMs is municipal wastewater effluent that is discharged directly into receiving waters. PCMs have been detected in sewage sludge, surface water, sediments, aquatic organisms, and other biota, as well as in fat tissue of marine organism and human milk, indicating their widespread presence in the environment.[34,35] In this work, galaxolide was detected in all the samples. Figure 4 shows the identification of galaxolide in a surface water. Accurate mass confirmation automatically performed by the software for five ions led to the safe identification of galaxolide with mass errors below 2.2 mDa.



(D)

Compound	Molecular Formula	Molecular Mass	Forward Fit	m/z_1	m/z_2	m/z_3	m/z_4	m/z_5
Dimpylate	$C_{12}H_{21}N_2O_3PS$	304.1010	884	$C_7H_9N_2O$ 137.0702 (-1.3)	$C_{10}H_{15}N_2O$ 179.1178 (-0.6)	$C_8H_{12}N_2O$ 152.0938 (-1.2)	$C_8H_{12}N_2O_2P$ 199.0635 (-0.1)	$C_2H_7NO_3$ 93.0424 (-0.2)

Figure 3 (A) Identification of nontarget diazinon (dimpylate) in a treated water sample from a wastewater treatment plant. Extracted ion chromatograms for four diazinon ions used for deconvolution. (B) Library mass spectrum of diazinon at nominal masses. (C) Deconvoluted accurate mass spectrum of diazinon from the water sample. (D) Library forward fit and accurate mass confirmation of five fragments (mass errors in mDa, in brackets).



(D)

Compound	Molecular Formula	Molecular Mass	Forward Fit	m/z_1	m/z_2	m/z_3	m/z_4	m/z_5
Galaxolide	$C_{18}H_{26}O$	258.1984	797	$C_{17}H_{23}O$ 243.1746 (-0.3)	$C_{16}H_{21}$ 213.1654 (1.1)	$C_{17}H_{24}O$ 244.1816 (-1.1)	$C_{18}H_{26}O$ 258.2006 (2.2)	$C_{13}H_{15}$ 171.1182 (0.8)

Figure 4 Identification of nontarget galaxolide in a surface water sample. (A) Extracted ion chromatograms for four galaxolide ions used for deconvolution. (B) Library mass spectrum of galaxolide at nominal masses. (C) Deconvoluted mass spectrum of galaxolide from the water. (D) Library forward fit and accurate mass confirmation of five fragments (mass errors in mDa, in brackets).

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Benzophenone was also identified in all the water samples analyzed. Benzophenone and its derivatives are UV filters used primarily as photoinitiators, fragrance enhancers, ultraviolet curing agents, and occasionally, as flavor ingredients. They are also used in the manufacture of insecticides, agricultural chemicals, and pharmaceuticals and as additives for plastics, coatings, and adhesives. Furthermore, benzophenone has been listed as a chemical suspected of having endocrine-disrupting effects.[36] Some authors have monitored benzophenone and its derivatives in water and soil samples during the past decade.[36,37] Bisphenol A was also detected in all the water samples analyzed.

Bisphenol A is a chemical intermediate in the synthesis of polycarbonate and epoxy resins, unsaturated polyester−styrene resins, and flame retardants, and it is a well-known compound due to its ubiquity nature and its endocrine-disrupting potential. In the last years, some authors have monitored bisphenol A in different types of waters.[38,39]

3,5-Di-tert-butyl-4-hydroxy-toluene (BHT) was identified in four samples, which were collected from a wastewater treatment plant (raw and treated) and from urban solid waste leachates (raw and treated). BHT is a widely used antioxidant food and cosmetic and plastic additive. Several studies have already proved the presence of this compound in the aquatic environment.[40,41] Contrarily to BHT, which does not appear to pose a cancer risk to humans, some of its metabolites, such as 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT−CHO) and the dimer of BHT, 1,2-bis-(3,5-di-tert-butyl-4-hydroxyphenyl)ethane (2-BHT) may pose a human health risk.[40] In our work, the metabolite BHT−CHO, which has been studied extensively in terms of toxicity, was also identified in two surface water samples and in a sample from the wastewater treatment plant.

Caffeine was detected in the two samples (before and after treatment) collected from the wastewater urban treatment plant. Some authors have already studied the presence of caffeine in municipal wastewaters[42] and have shown that caffeine can be used as a chemical marker for surface water pollution by domestic wastewater.[43]

Finally, benzyl butyl phthalate was identified in two samples; one corresponded to surface water and the other to treated sample from urban solid waste leachates.

As an illustrative example Table 4 summarizes the nontarget compounds found in a surface water together with the parameters used for the identification of the findings.

Table 4. Nontarget Compounds Identified in a Surface Water Sample Analyzed

compd	forward fit[a]	m/z_1	m/z_2	m/z_3	m/z_4	m/z_5
benzophenone	935	C_7H_5O[b] 105.0319 (−2.1)[c]	$C_{13}H_{10}O$ 182.0710 (−2.2)	C_6H_5 77.0380 (−1.1)		
galaxolide	867	$C_{17}H_{23}O$ 243.1734 (−1.5)	$C_{16}H_{21}$ 213.1655 (1.2)	$C_{17}H_{24}O$ 244.1799 (−2.8)	$C_{18}H_{26}O$ 258.1994 (1.0)	$C_{13}H_{15}$ 171.1177 (0.3)
bisphenol A	821	$C_{14}H_{13}O_2$ 213.0920 (0.4)	C_8H_7O 119.0517 (2.0)	$C_{15}H_{16}O_2$ 228.1174 (2.4)	$C_{14}H_{14}O_2$ 214.0968 (−2.6)	C_7H_7 91.0554 (0.6)
BHT	673	$C_{14}H_{21}O$ 205.1590 (−0.2)	$C_{15}H_{24}O$ 220.1840 (1.3)	$C_{11}H_{13}$ 145.1002 (−1.4)	$C_{11}H_{13}O$ 161.0977 (1.1)	
diazinon	884	$C_7H_9N_2O$ 137.0702 (−1.3)	$C_{10}H_{15}N_2O$ 179.1178 (−0.6)	$C_8H_{12}N_2O$ 152.0938 (−1.2)	$C_8H_{12}N_2O_2P$ 199.0635 (−0.1)	$C_2H_7NO_3$ 93.0424 (−0.2)
benzyl butyl phthalate	704	$C_8H_5O_3$ 149.0247 (0.8)	C_7H_7 91.0550 (0.2)	C_7H_4O 104.0276 (1.4)	C_3HO_2 68.9955 (−2.2)	C_6H_4 76.0320 (0.7)

[a] Assigned in the nominal mass library searching. [b] Suggested formula, given by the Elemental Composition program, for the experimental mass. [c] Experimental mass (mass error in mDa in parentheses).

It is worth it to mention that among the 16 positives (target analytes) found using TargetLynx, only 2 of them, terbutryn and terbuthylazine in raw urban leachate water, were also found using ChromaLynx when the samples were considered as "unknown". This fact shows that ChromaLynx has some limitations when searching nontarget compounds that are present at relatively low concentrations. Under this situation, the algorithm used by ChromaLynx for discovering the presence of ions that could be related to a certain sample component fails, being unable to discriminate those ions from the background. Consequently, this component is missed. So, these

days the screening of organic contaminants in the environment at trace concentration levels cannot be only performed with the use of a deconvolution software, although ChromaLynx has resulted an advantageous application when searching for nontarget compounds. Target analysis is indispensable at present, and it is the simplest way for rapid screening and quantification of a wide number of analytes at trace concentration levels. Therefore both, target and nontarget analysis, are complementary approaches when searching for organic (micro)pollutants in the environment. GC/TOF-MS seems to be a powerful and attractive technique that allows both approaches to be performed from a single injection mainly for identification and confirmation purposes.

CONCLUSIONS

Combination of SPME and GC/TOF-MS resulted in a rapid, solventless, and efficient approach for the screening and identification of organic (micro)pollutants in water. The evaluation of up to five microwindow-extracted ion chromatograms (0.02 Da) at selected m/z ions and the attainment of their Q/q intensity ratios allowed the detection of several target compounds (4-t-octylphenol, simazine, terbuthylazine, chlorpyrifos, terbumeton, and terbutryn) within a group of 60 selected analytes. Moreover, after MS data acquisition, the presence of several (post-target) analytes was investigated without the need of reanalyzing the samples. In this way, PBDEs and several fungicides were searched in the water samples. In both cases, (pre) and (post) target analysis, the investigation of the presence of the ions, measured at accurate mass, was performed in an automated and simple way using an adequate commercial software.

The acquisition of full spectra by TOF analyzers offered the possibility of searching for nontarget contaminants (unknowns) by the application of a deconvolution software. This resulted in the identification of several compounds that were not included in the list of target analytes, like diazinon, galaxolide, benzophenone, bisphenol A, BHT, BHT−CHO, caffeine, and benzyl butyl phthalate, in the water samples analyzed. Most of these pollutants were detected in samples from urban wastes, although some of them (benzophenone and bisphenol A) were found in all water samples analyzed, including surface and groundwater.

The huge amount of useful information provided by TOF-MS together with the measurements of accurate masses for several representative ions using a powerful software was proved to be an efficient approach for nontarget screening in water. Obviously, some limitations were observed, which prevented the investigations of nontarget compounds, especially at low levels (i.e., sub-part-per-billion). Thus, the screening of organic contaminants in the environment cannot be efficiently performed only with the use of a deconvolution software treating samples and analytes as unknown, as it can fail when trying to discriminate ions from the background when they are present at low concentrations. In addition, still some compounds present in samples might not be unequivocally identified due to library searching limitations. Therefore, both approaches, target and nontarget analysis, are complementary and both are required these days when searching for organic (micro) pollutants in the environment.

ACKNOWLEDGMENT

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of the time-of-flight mass spectrometer. This work forms a part of the project CTQ2006-07594 developed with the financial support of Ministerio de Ciencia y Tecnología. T. Portolés is very pleased to Ministerio de Educación y Ciencia for her Predoctoral Grant. The authors acknowledge the useful comments and support given by Timothy Jenkins, Peter Hancock, and Cristiana Leandro (Waters Corporation) when writing this article.

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3.3 DESARROLLO Y VALIDACIÓN DE UN MÉTODO DE SCREENING AMPLIO Y RÁPIDO PARA CONTAMINANTES ORGÁNICOS EN AGUAS NATURALES Y RESIDUALES MEDIANTE GC-TOF MS

La información química sobre la composición de una muestra puede ser muy diversa: qué analitos están presente en una muestra, en cuánta cantidad, de qué forma, etc. En función de su objetivo, los métodos analíticos se clasifican en dos grandes grupos: métodos de análisis cualitativo y métodos de análisis cuantitativo. Si la información requerida sobre la muestra se refiere a los compuestos que contiene, será más adecuado escoger un método cualitativo, mientras que si el interés radica en la cantidad, deberemos optar por un método cuantitativo. Los métodos con fines cualitativos (generalmente métodos de *screening)* son cada vez de mayor interés en distintos ámbitos de aplicación, por ejemplo, en análisis de alimentos, donde es muy conveniente aplicar un *screening* rápido a las muestras para determinar si un analito se encuentra presente por encima de una determinada concentración. Así, en los casos en los que esta concentración pueda superar los límites permitidos, se aplicaría un segundo método con fines cuantitativos. Esta metodología de trabajo, también aplicable a otros campos de análisis, ahorra tiempo, esfuerzo y costes, ya que solamente se deben cuantificar aquellas muestras que sobrepasen un nivel de concentración establecido.

Además de la importancia de escoger un método analítico adecuado a cada problemática, cabe destacar que es necesario tener fiabilidad sobre el resultado obtenido. Esto implica que cualquier método analítico debe tener definidos sus requisitos y cualidades analíticas y se debe comprobar que estos parámetros tienen realmente el valor que se les ha asignado. En otras palabras, aunque no se persigan fines de cuantificación, los métodos de *screening* deben ser también sometidos a un proceso de validación, condición indispensable para la utilización con garantías de cualquier método analítico.

En la validación de métodos de *screening* se debe asegurar la correcta identificación de los compuestos investigados a un nivel de concentración establecido. Es decir, se debe establecer un nivel de concentración mínimo a partir del cual se pueda asegurar que si el compuesto se encuentra en las muestras a dicho nivel, el método utilizado será capaz de identificarlo y confirmarlo correctamente. Esto implica

asegurar la calidad de los resultados obtenidos desde un punto de vista cualitativo y de identificación. Para ello, se deben llevar a cabo ensayos a diferentes niveles de concentración en las matrices que se pretendan validar y estudiar los criterios para una correcta identificación. Actualmente, existe un amplio consenso con respecto a los parámetros a validar en los métodos cuantitativos. Sin embargo, ante un método de *screening*, los parámetros a evaluar no están tan claros y no existe unanimidad. Se han publicado algunos artículos sobre validaciones cualitativas de métodos de *screening* en el campo del análisis de plaguicidas (1) y de anti-doping (2), y en los últimos años, algunas organizaciones han propuestos algunas guías sobre validación de métodos analíticos cualitativos (3, 3-8).

En el trabajo que se presenta a continuación, se ha validado cualitativamente un método de *screening* para la detección e identificación de un amplio número de contaminantes orgánicos en muestras de agua de diferente complejidad mediante GC-TOF MS. El tratamiento de muestra aplicado consiste en una SPE con cartuchos C_{18}. El método se valida en términos cualitativos para distintos tipos de aguas: naturales (superficiales y subterráneas) y aguas residuales. En el caso de las aguas naturales, los niveles de validación han sido 0.02, 0.1 y 1 µg/L, mientras que en las aguas residuales han sido 0.1 y 1 µg/L. En todos los casos se han realizado seis réplicas.

En la validación se han incluido alrededor de 150 contaminantes orgánicos de diferentes familias, incluyendo PAHs, octil/nonil fenoles, PCBs, PBDEs y un gran número de plaguicidas, como insecticidas (OCs, OPs, carbamatos y piretroides), herbicidas (triazinas, cloroacetanilidas), fungicidas y algunos metabolitos relevantes. La mayoría de los compuestos investigados han sido correctamente validados en términos cualitativos al nivel de 1 µg/L. Al nivel de concentración de 0.1 µg/L la identificación fue más problemática para algunos compuestos, sobretodo en las matrices más complejas. Sin embargo, en las matrices más simples, se pudo llevar a cabo la validación satisfactoriamente incluso a 0.02 µg/L. La especificidad/selectividad del método de *screening* viene determinada por la elevada exactitud de masa proporcionada por el TOF MS, la cual permite usar ventanas de extracción de masa estrechas (0.01 Da), así como proceder a una identificación fiable del compuesto detectado.

El procedimiento desarrollado se aplicó al análisis de contaminantes orgánicos en aguas y permitió la detección e identificación de PAHs (naftaleno y pireno), herbicidas triazinas (simazina, terbumetona, terbutilazina y terbutrina), insecticidas OPs (malation, clorpirifos, diazinon) y otros herbicidas y fungicidas como difenilamina y cloropropam. Los positivos encontrados pudieron ser correctamente identificados según el criterio establecido, consistente en la presencia de, al menos, dos iones medidos en masa exacta y el cumplimiento de sus relaciones iónicas de intensidad.

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3.3.2 Artículo científico 6

Journal of Chromatography A (submitted)

DEVELOPMENT AND VALIDATION OF A RAPID AND WIDE-SCOPE QUALITATIVE SCREENING FOR DETECTION OF ORGANIC POLLUTANTS IN NATURAL AND WASTEWATER BY GAS CHROMATOGRAPHY TIME-OF-FLIGHT MASS SPECTROMETRY

Tania Portolés, Elena Pitarch, Francisco J. López,Félix Hernández

Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain,

ABSTRACT

Oppositely to other applied fields, like toxicology or anti-doping analysis, there is a lack of wide-scope screening methods in environment focused on qualitative purposes that are conveniently validated following a widely accepted methodical approach. The objective of these methods is to report a sample as positive or negative to a given contaminant, at a given concentration relevant from an environmental point of view. In this work, a multiclass screening of organic contaminants in natural and waste water has been developed and validated for qualitative purposes, i.e. the reliable and sensitive identification of compounds detected in samples at a certain level of concentration. The screening is based on the use of gas chromatography coupled to high-resolution time-of-flight mass spectrometry (GC-TOF MS), and sample procedure involves solid phase extraction (SPE) with C_{18} cartridges. The method has been applied to water samples of different origin and matrix composition (surface and ground water, raw leachate from a municipal solid waste treatment plant, influent and effluent urban wastewater). Around 150 organic contaminants from different chemical families were investigated, including polycyclic hydrocarbons (PAHs), octyl/nonyl phenols, polychlorinated byphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and a notable number of pesticides, such us insecticides (organochlorines, organophosphorus, carbamates and pyretroids), herbicides (triazines and chloroacetanilides), fungicides and several relevant metabolites. Natural water samples were spiked with a standard mixture of all target analytes at three concentration levels

(0.02, 0.1 and 1µg/L) and wastewater samples were spiked at two levels (0.1 and 1µg/L). All samples were analyzed by sixtuplicate.

After SPE extraction, sample extracts were analyzed by GC-TOF MS processing the full spectrum acquisition data generated. The presence of up to five ions measured at their accurate mass at the expected retention time was evaluated in every spiked sample at all the levels tested. Additionally, their intensity ion ratios were compared to those obtained from reference standards in solvent. This methodology together with the use of narrow-mass windows (0.02 Da) eXtracted Ion Chromatograms much improved the selectivity and sensitivity of the screening methods. The presence of at least two ions and the attainment of their Q/q_i intensity ratio within specified tolerances (European Commission Decision 2002/657/EC) was required for the reliable confirmation of target analytes. Most of compounds investigated were correctly identified in water samples spiked at 1µg/L. When the analyte concentration was lowered down to 0.1µg/L the identification was more problematic for some compounds, especially in complex-matrix samples like influent wastewater. On the contrary, many contaminants could be properly identified at the lowest level 0.02 µg/L in cleaner matrices (ground and surface water, wastewater effluent).

The procedure was applied to the screening of different water samples and allowed the detection and identification of several PAHs (naphthalene and pyrene), triazine herbicides (simazine, terbumeton, terbuthylazine and terbutryn), organophosphorus insecticides (malathion, chlorpyriphos, diazinon), and other herbicides and fungicides like diphenylamine and chlorpropham. Positive findings could be correctly identified following the established criteria, in some cases at concentrations even below the lowest concentration validated, which illustrates the strong potential and excellent sensitivity of the screening approach developed in the present work.

KEYWORDS

Screening, water samples, gas chromatography, time-of-flight mass spectrometry, qualitative validation.

INTRODUCTION

The number of potentially hazardous chemicals that can reach the environment is continuously increasing and new chemical substances are constantly being synthesized and released. Water pollution is one of the main consequences and one of the most prominent environmental concerns. Modern analytical chemistry has to give an answer to this problem by developing advanced multi-analyte (multi-class) methodologies that can be applied in monitoring programs, providing a broad and realistic knowledge about water pollution in a rapid, sensitive and selective way. Additionally, it is crucial that these methodologies can be easily updated, as "emerging contaminants" are continuously appearing and being a new reason of concern [1, 2].

The number of papers related to multi-residue, multi-analyte methodologies in water samples have much increased over recent years [3-8]. Most of these methods are focused on target analysis with quantitative purposes and their scope rarely exceeds several tens of analytes, being quite unusual to find analytical methods for the determination of more than 100 organic pollutants. The most widely applied techniques are gas chromatography (GC) or liquid-chromatography (LC) coupled to mass spectrometry (MS) with different analyzers, mainly single quadrupole in selected ion monitoring (SIM), or triple quadrupole and ion trap working under tandem MS (MS/MS) conditions. The sensitivity and selectivity of these techniques, especially when using tandem MS, are unquestionable, as demonstrated by the large number of applications reported in different fields. Using these configurations, identification and quantification of pre-defined contaminants (those for which MS data have been acquired) can be successfully carried out at low analyte concentrations. However, the number of compounds to be included in the scope of the method is restricted, and other compounds potentially harmful that might be present in the samples would not be detected under these conditions. This is an important drawback of most quantitative methods reported, as the knowledge of aquatic environment pollution requires as much information as possible on the presence of as many pollutants as possible, not only on a group of selected compounds. In addition, from a practical point of view, it would be useful to reconsider whether quantitative results are always necessary. Thus, instead of pursuing the quantification of pollutants as the first goal, it would be better in many occasions to assure if they are present above or below the permitted concentration level in the samples[9]. Qualitative methods, used for screening purposes before

quantification with the routine method, allow to select positive samples and considerably reduce time and cost of confirmatory/quantification analysis [10]. This strategy is in the line of modern trends such us increasing demands for yes/no binary responses about samples and analytes [11].

In the last decade there has been a notable increase in the use of full spectrum acquisition techniques, as time-of-flight mass spectrometry (TOF MS), which allows acquiring huge amount of chemical information on the sample in a single analysis. This fact facilitates widening the number of analytes that can be searched in a single experiment, with the additional advantage that data can be re-examined at any time to search for other compounds not included in the first screening, without the need of additional analysis. TOF MS and hybrid quadrupole-TOF MS have been successfully applied for screening purposes in combination with GC or LC in different applied fields, like environmental analysis, food safety or toxycology [12-19]. This analyzer provides the selectivity and sensitivity required for wide-scope screening, as it combines high full-spectral sensitivity with high mass resolution. Accurate mass data obtained can be processed in both "post-target" and/or non-target way, which gives high versatility to the instrument allowing the user to face up an analytical problem in different ways, depending on the aim of the analysis [12-14, 20].

The aim of a qualitative screening applied to environmental samples is to detect and identify a large number of analytes; therefore, the sample treatment applied should be as universal as possible in order to include the maximum number of compounds, even if they have quite different physicochemical characteristics. In principle, recoveries of the overall analytical procedures should not be the key point, as quantification is not the main objective of the screening. However, it would be necessary to test that analytical methodology applied is robust and adequately detects the target contaminants included in the screening. The analytical requirements must be defined and the values of the performance parameters assessed before they are used as routine methods in the laboratory, i.e., qualitative methods must be validated as occurs in quantitative applications [9]. The wide majority of validation processes described in the literature are addressed to quantitative methods and it is easy to find well-established protocols and international guidelines [21]. By contrast, the issue of qualitative methods has received less attention. Although there are some guidelines and documents available at present, there is no general, widely-accepted, guideline to

be applied, for example, in the field of environmental analysis [9, 10, 22-25]. In the validation of qualitative methods, selectivity/specificity and limit of detection (LOD) are the most important parameters [26].

The objective of this work is to develope and qualitatively validate a wide-scope screening for around 150 organic contaminants in natural water and wastewater based on the use of gas chromatography coupled to high-resolution time-of-flight mass spectrometry (GC-TOF MS). Critical parameters of the identification and confirmation process of detected compounds are discussed. Once validated, the screening method has been applied to the analysis of different water matrices, including ground, surface water and wastewater, in order to test its applicability. Also, a brief discussion about the state-of-the art in qualitative methods validation is made.

EXPERIMENTAL

Reagents

Reference standards of pesticides, octyl/nonyl phenols, PCBs (Mix 3, 100 µg/mL in cyclohexane; Mix 41, 10 µg/mL in cyclohexane) and PAHs (Mix 9, 100 µg/mL) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). PBDE standard mixture "Lake Michigan Study", containing BDE 28, 47, 66, 85, 99, 100, 138, 153 and 154 (50 µg/mL in isooctane) and two individual standards of BDE 71 and 183 (50 µg/mL in isooctane) were purchased from Chiron (Trondheim, Norway). Stock solutions (around 500 µg/mL) were prepared by dissolving solid reference standards in acetone and stored in a freezer at –20ºC. Working solutions were prepared by diluting stock solutions in acetone for sample fortification and diluting in hexane for injection in the chromatographic system.

Acetone (residue analysis), ethyl acetate, dichloromethane (DCM) and hexane (ultra-trace quality) were purchased from Scharlab (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralised water in a Milli-Q Gradient A10 (Millipore, Bedford, MA. USA). About 500 mg Bond Elut cartridges C_{18} (Varian, Harbor City, CA, USA) were used for solid-phase extraction.

Samples

Water samples of different types and origin were collected from different sites of the Castellón province (Spain). Concretely, two surface water (SW) (Villarreal and Burriana), two ground water (GW) (Almassora and Castellón), and two effluent water samples (EWW) from a wastewater treatment plant (WWTP) of Castellón were collected for method validation in less-complex sample matrices. Additionally, three influent water samples (IWW) from the WWTP (Castellón) and three raw leachate water samples (RLW) from a municipal solid waste treatment plant sited at Onda were selected for validation in highly-complex sample matrices. RLW samples were diluted 2.5 times with HPLC-grade water before SPE treatment due to its high organic matter content and density.

In addition to the samples used for validation purposes, the developed procedure was applied to some other water samples. Six SW samples were collected at different sites from the Comunidad Valenciana and from Ebro River surroundings (Tarragona). Five GW samples were also collected from wells in the Comunidad Valenciana. GW sampling points corresponded to high vulnerability aquifers within areas with intensive agriculture practices. All samples were collected in high-density polyethylene bottles and stored in the dark at a temperature below -18ºC until analysis.

Instrumentation

GC instrumentation consisted on an Agilent 6890N GC system (Paloalto, CA, USA), equipped with an Agilent 7683 autosampler, coupled to a time-of-flight mass spectrometer, GCT (Waters Corporation, Manchester, UK), operating in electron ionization (EI) mode. The GC separation was performed using a fused silica HP-5MS capillary column of 30 m x 0.25 mm i.d. and a film thickness of 0.25 µm (J&W Scientific, Folson, CA, USA). The oven temperature was programmed as follows: 90ºC (1 min); 5ºC/min to 300ºC (2 min). Splitless injections of 1 µL sample were carried out. Helium was used as carrier gas at 1 mL/min.

The interface and source temperatures were both set to 250ºC and a solvent delay of 3 minutes was selected. TOF MS was operated at 1 spectrum/s acquiring the mass range *m/z* 50-650 and using a multi-channel plate voltage of 2800V. TOF-MS

resolution was about 8500 (FWHM) at *m/z* 614. Heptacosa, used for the daily mass calibration as well as lock mass, was injected via syringe in the reference reservoir at 30ºC. The *m/z* ion monitored was 218.9856. The application manager TargetLynx, a module of MassLynx software, was used to process data obtained from standards and samples for target compounds. The application manager ChromaLynx, also a module of MassLynx software, was used to investigate the presence of non-target compounds in samples. Library searching was performed using the commercial NIST library.

Analytical procedure

The procedure applied was based on our previous work for determination of priority organic pollutants in water 7 with a few modifications. 250 mL of water sample (RLW were previously diluted 2.5 times) were passed through a 500 mg C18 SPE cartridge previously conditioned by passing 6 mL methanol, 6 mL ethyl acetate:DCM (50:50), 6 mL methanol and 6 mL water avoiding dryness. After loading the sample, cartridges were washed with 3 mL water. The cartridge was air-dried, using vacuum for at least 15 min, and then eluted with 5 mL ethyl acetate:DCM (50:50). The extract collected was evaporated to dryness under a gentle nitrogen stream at 40ºC and redissolved in 0.5 mL hexane. The final extract obtained was injected into the GC-TOF MS.

Qualitative validation protocol

Validation of the screening method was mainly based on Eurachem guidelines for qualitative validation [22]. Two SW, two GW and two EWW samples were spiked at three concentration levels (0.02, 0.1 and 1 µg/L) and analyzed together with their respective blanks for qualitative validation of less-complex sample matrices. In addition, six wastewater samples (three IWW and three RLW) were spiked at two levels (0.1 and 1 µg/L) and analyzed together with their respective blanks for qualitative validation of highly-complex water samples. The limit of identification (LOI) was established as the lowest concentration for which a compound was satisfactorily identified in all spiked samples tested. The identification criterion was the presence of, at least, two *m/z* ions at the expected retention time, measured at their accurate mass (two peaks in the

respective narrow-window eXtracted Ion Chromatograms, nw-XIC) and the attainment of their Q/q_i intensity ratio within specified tolerances [23]. Q/q_i was the ratio between the most abundant ion (Q) and every one of the other measured ions (qi).

Selectivity, considered as the ability of the method to discriminate between the analyte and other compounds [27], was tested by determining every analyte in the presence of the rest of compounds included in the screening. It was based on the presence of characteristics *m/z* ions, measured at accurate mass, for each compound in the EI spectrum. The elevated mass resolution of the TOF instrument allowed us using narrow mass windows (0.02 Da) to perform the XICs, which highly improved the selectivity required for this application. In addition, the use of a narrow mass window also led to a notable improvement of sensitivity due to the decrease in the background noise in the chromatogram and the improvement of the signal-to-noise ratio. Specificity, considered as the ability of the detector (supported by the selectivity of the extraction, clean-up, derivatization or separation, if necessary) to provide signals that effectively identify the analyte [27], was checked by analyzing six "blank" natural water samples and six "blank" wastewater samples. Specifity could not be demonstrated for a few compounds that were present in the "blank" samples.

RESULTS AND DISCUSSION

General aspects of qualitative validation protocols

The output of a qualitative analysis is the yes/no binary response depending on the presence of a given analyte in the sample [28]. The extent of the validation depends on the aim of the analytical method, and the first step is to decide which performance parameters must be studied and then design the validation procedure accordingly [26]. Some papers have been reported on qualitative validation of screening methods in the field of pesticide residue analysis [29] or anti-doping analysis [30]. In recent years, several organizations have published guidances or proposals about the validation of qualitative analytical methods. One of the recommendations is the participation in collaborative studies as AOAC suggests [9, 25]. In "The Fitness for Purpose of Analytical Methods" document [22], Eurachem specifies that the qualitative performance parameters that should be evaluated are: confirmation of identity, sensitivity, selectivity/specificity and precision. In addition to the limit of detection and selectivity/specificity, the

European Union (EU) proposes the evaluation of other parameters like stability, applicability and robustness [23]. The European Cooperation for Accreditation (EAL) in the guide entitled "Validation of Test Methods" states that the uncertainty associated with the method is the most important quality parameter [24]. Although performance parameters are normally well defined, it is still necessary to establish the methodology for their evaluation. Moreover, the nomenclature related to qualitative analysis as well as the classification of qualitative methods is still confusing for the users [9]. Recently, European Union has proposed some performance criteria for qualitative validation of screening methods in food and feed pesticide residue analysis. For these methods, validation is focused on detectability at the lowest spiking level for which has been demonstrated that a certain analyte can be detected in at least 95 % of the samples; so a false-negative rate of 5% is accepted.[27]

In this work, the screening method validation was performed based on Eurachem guidelines for qualitative validation [22, 30] with a few modifications. The qualitative validation protocol has been described above (see Experimental section). Because the main purpose of the qualitative screening is to distinguish between negative and positive samples at a determined level, the method proposed in this work was considered as satisfactorily validated at certain concentration level only when the target analyte was detected and correctly identified in all different-matrix spiked samples tested, independently on their recovery and precision [30].

GC-TOF MS screening measurement

The final extracts obtained after application of the analytical procedure were injected in the GC-TOF MS system. Full-spectrum acquisition data were treated using an automated processing method, which consisted of automatically obtain between 2 and 5 nw-XICs, (mass window 0.02 Da), at pre-selected characteristic *m/z* ions for every compound. The screening method was validated for a total number of 150 organic pollutants. Table 1 shows the exact masses for the three main *m/z* ions of each compound. For some analytes, it was feasible to use up to 5 ions giving the extraordinary power to the identification process. Analyte identification was performed by comparing the experimental Q/q_i intensity ratios in samples with the theoretical ones, which were calculated from injection of standards in solvent. The presence of at

least two ions at the expected retention time, measured at their accurate mass (nw-XIC, 0.02 Da), was required together with the attainment of the Q/q_i ratio within specified tolerances to give the identification of the target analytes as positive. Maximum deviations accepted in Q/q_i ratios were based on the European Commission Decision 2002/657/EC [23], as applied in our previous works [12, 13, 31].

Table 1. m/z ions selected for the identification of target compounds*

Compound	R_t (min)	Molecular mass	Molecular formula	m/z 1	Ion 1 (Q)	m/z 2	Ion 2 (q_1)	m/z 3	Ion 3 (q_2)
Naphthalene	6.50	128.0626	$C_{10}H_8$	128.0626	$C_{10}H_8$	127.0548	$C_{10}H_7$	126.0470	$C_{10}H_6$
Methamidophos	7.35	141.0013	$C_2H_8NO_2PS$	94.0058	CH_5NO_2P	95.0136	CH_6NO_2P	141.0013	$C_2H_8NO_2PS$
Diclorvos	7.85	219.9459	$C_4H_7Cl_2O_4P$	109.0055	$C_2H_6O_3P$	184.9770	$C_4H_7ClO_4P$	186.9743	$C_4H_7{}^{37}ClO_4P$
Mevinfos	12.08	224.0450	$C_7H_{13}O_6P$	127.0160	$C_2H_8O_4P$	164.0238	$C_5H_9O_4P$	192.0188	$C_6H_9O_5P$
Acenaphthylene	12.43	152.0626	$C_{12}H_8$	152.0626	$C_{12}H_8$	151.0548	$C_{12}H_7$	150.0470	$C_{12}H_6$
Acenaphthene	13.25	154.0783	$C_{12}H_{10}$	153.0704	$C_{12}H_9$	154.0783	$C_{12}H_{10}$	152.0626	$C_{12}H_8$
Methacrifos	13.80	240.0221	$C_7H_{13}O_5PS$	124.9826	$C_2H_6O_2PS$	93.0099	$C_2H_6O_2P$	207.9959	$C_6H_9O_4PS$
Pentachlorobenzene	14.09	247.8521	C_6HCl_5	249.8492	$C_6H^{35}Cl_4{}^{37}Cl$	247.8521	C_6HCl_5	251.8462	$C_6H^{35}Cl_3{}^{37}Cl_2$
Molinate	15.38	187.1031	$C_9H_{17}NOS$	126.0919	$C_7H_{12}NO$	187.1031	$C_9H_{17}NOS$	98.0970	$C_6H_{12}N$
Heptenofos	15.45	250.0162	$C_9H_{12}ClO_4P$	124.0080	C_7H_5Cl	126.0051	$C_7H_5{}^{37}Cl$	109.0055	$C_2H_6O_3P$
Fluorene	15.47	166.0783	$C_{13}H_{10}$	165.0704	$C_{13}H_9$	166.0783	$C_{13}H_{10}$	164.0626	$C_{13}H_8$
Omethoate	15.72	213.0225	$C_5H_{12}NO_4PS$	110.0133	$C_2H_7O_3P$	109.0055	$C_2H_6O_3P$	156.0010	$C_3H_9O_3PS$
Tecnazene	15.95	258.8761	$C_6HCl_4NO_2$	200.8832	C_5HCl_4	212.8832	C_6HCl_4	258.8761	$C_6HCl_4NO_2$
4-t-Octylphenol	15.99	206.1671	$C_{14}H_{22}O$	135.0810	$C_9H_{11}O$	107.0497	C_7H_7O	95.0497	C_6H_7O
Diphenylamine	16.33	169.0891	$C_{12}H_{11}N$	169.0891	$C_{12}H_{11}N$	168.0813	$C_{12}H_{10}N$	167.0735	$C_{12}H_9N$
Atrazine desisopropyl	16.98	173.0468	$C_5H_8ClN_5$	158.0233	$C_4H_5ClN_5$	173.0468	$C_5H_8ClN_5$	145.0155	$C_3H_4N_5Cl$
Chlorpropham	17.08	213.0557	$C_{10}H_{12}ClNO_2$	127.0189	C_6H_6ClN	152.9981	C_7H_4NClO	213.0557	$C_{10}H_{12}ClNO_2$
Terbumeton desethyl	17.18	197.1277	$C_8H_{15}N_5O$	182.1042	$C_7H_{12}N_5O$	141.0651	$C_4H_7N_5O$	197.1277	$C_8H_{15}N_5O$
Atrazine desethyl	17.28	187.0625	$C_6H_{10}ClN_5$	172.0390	$C_5H_7ClN_5$	174.0361	$C_5H_7{}^{37}ClN_5$	187.0625	$C_6H_{10}ClN_5$
Terbuthylazine desethyl	17.68	201.0781	$C_7H_{12}ClN_5$	186.0546	$C_6H_9ClN_5$	188.0518	$C_6H_9{}^{37}ClN_5$	201.0781	$C_7H_{12}ClN_5$
Trifluralina	17.79	335.1093	$C_{13}H_{16}F_3N_3O_4$	306.0702	$C_{11}H_{11}F_3N_3O_4$	290.0753	$C_{11}H_{11}F_3N_3O_3$	248.0283	$C_8H_5F_3N_3O_3$
Forate	17.97	260.0128	$C_7H_{17}O_2PS_3$	121.0418	$C_4H_{10}O_2P$	230.9737	$C_5H_{15}O_2PS_3$	260.0128	$C_7H_{17}O_2PS_3$
Hexachlorobenzene	18.30	281.8131	C_6Cl_6	283.8102	$C_6{}^{35}Cl_5{}^{37}Cl$	281.8131	C_6Cl_6	248.8413	$C_6{}^{35}Cl_4{}^{37}Cl$
Dimethoate	18.68	228.9996	$C_5H_{12}NO_3PS_2$	87.0143	C_3H_5NS	93.0105	$C_2H_6O_2P$	124.9826	$C_2H_6O_2PS$
Simazine	18.95	201.0781	$C_7H_{12}ClN_5$	201.0781	$C_7H_{12}ClN_5$	186.0546	$C_6H_9N_5Cl$	173.0468	$C_5H_8ClN_5$
Atrazine	19.20	215.0938	$C_8H_{14}ClN_5$	200.0703	$C_7H_{11}ClN_5$	202.0674	$C_7H_{11}{}^{37}ClN_5$	215.0938	$C_8H_{14}ClN_5$
Lindane	19.39	287.8601	$C_6H_6Cl_6$	180.9379	$C_6H_4Cl_3$	182.9349	$C_6H_4{}^{35}Cl_2{}^{37}Cl$	216.9145	$C_6H_5Cl_4$
4-n-Octylphenol	19.44	206.1671	$C_{14}H_{22}O$	107.0497	C_7H_7O	206.1671	$C_{14}H_{22}O$	91.0548	C_7H_7

Table 1. m/z ions selected for the identification of target compounds*

Compound	R_t (min)	Molecular mass	Molecular formula	m/z 1	Ion 1 (Q)	m/z 2	Ion 2 (q_1)	m/z 3	Ion 3 (q_2)
Terbumeton	19.47	225.1590	$C_{10}H_{19}N_5O$	210.1355	$C_9H_{16}N_5O$	169.0964	$C_5H_{11}N_5O$	154.0729	$C_5H_8N_5O$
Phenanthrene	19.72	178.0783	$C_{14}H_{10}$	178.0783	$C_{14}H_{10}$	176.0626	$C_{14}H_8$	152.0626	$C_{12}H_8$
Terbuthylazine	19.77	229.1094	$C_9H_{16}ClN_5$	214.0859	$C_8H_{13}ClN_5$	216.0831	$C_8H_{13}{}^{37}ClN_5$	229.1094	$C_9H_{16}ClN_5$
Fonofos	19.80	246.0302	$C_{10}H_{15}OPS_2$	108.9877	C_2H_6OPS	137.0190	$C_4H_{10}OPS$	246.0302	$C_{10}H_{15}OPS_2$
Propyzamide	19.92	255.0218	$C_{12}H_{11}Cl_2NO$	172.9561	$C_7H_3OCl_2$	174.9532	$C_7H_3O^{35}Cl^{37}Cl$	144.9612	$C_6H_3Cl_2$
Anthracene	19.92	178.0783	$C_{14}H_{10}$	178.0783	$C_{14}H_{10}$	176.0626	$C_{14}H_8$	152.0626	$C_{12}H_8$
Diazinon	20.37	304.1010	$C_{12}H_{21}N_2O_3PS$	137.0715	$C_7H_9N_2O$	152.0950	$C_8H_{12}N_2O$	179.1184	$C_{10}H_{15}N_2O$
Terbacil	20.54	216.0666	$C_9H_{13}ClN_2O_2$	161.0118	$C_5H_6ClN_2O_2$	160.0040	$C_5H_5ClN_2O_2$	116.9981	C_4H_4ClNO
Etrimfos	20.92	292.0647	$C_{10}H_{17}N_2O_4PS$	181.0977	$C_9H_{13}N_2O_2$	292.0647	$C_{10}H_{17}N_2O_4PS$	277.0412	$C_9H_{14}N_2O_4PS$
Endosulfan ether	21.04	339.8550	$C_9H_6Cl_6O$	276.8727	$C_8H_4{}^{35}Cl_4{}^{37}Cl$	169.9690	$C_8H_4Cl_2$	236.8413	$C_5{}^{35}Cl_4{}^{37}Cl$
Pirimicarb	21.35	238.1430	$C_{11}H_{18}N_4O_2$	166.0980	$C_8H_{12}N_3O$	72.0449	C_3H_6NO	238.1430	$C_{11}H_{18}N_4O_2$
4-n-nonylphenol	21.57	220.1827	$C_{15}H_{24}O$	107.0497	C_7H_7O	220.1827	$C_{15}H_{24}O$	91.0548	C_7H_7
PCB 28	21.69	255.9613	$C_{12}H_7Cl_3$	255.9613	$C_{12}H_7Cl_3$	257.9585	$C_{12}H_7{}^{35}Cl_2{}^{37}Cl$	259.9557	$C_{12}H_7{}^{35}Cl{}^{37}Cl_2$
Fosfamidon	21.78	299.0689	$C_{10}H_{19}ClNO_5P$	127.0160	$C_2H_8O_4P$	138.0918	$C_8H_{12}NO$	264.1001	$C_{10}H_{19}NO_5P$
Chlorpyriphos methyl	21.95	320.8950	$C_7H_7Cl_3NO_3PS$	285.9261	$C_7H_7Cl_2NO_3PS$	124.9826	$C_2H_6O_2PS$	287.9232	$C_7H_7{}^{35}Cl{}^{37}ClNO_3PS$
Parathion methyl	22.05	263.0017	$C_8H_{10}NO_5PS$	124.9826	$C_2H_6O_2PS$	109.0055	$C_2H_6O_3P$	263.0017	$C_8H_{10}NO_5PS$
Chlozolinate	22.08	331.0014	$C_{13}H_{11}Cl_2NO_5$	186.9803	$C_4H_7NO_3Cl_2$	188.9774	$C_4H_7NO_3{}^{35}Cl{}^{37}Cl$	258.9803	$C_{10}H_7NO_3Cl_2$
Heptachlor	22.20	369.8211	$C_{10}H_5Cl_7$	271.8102	$C_5{}^{35}Cl_5{}^{37}Cl$	236.8413	$C_5{}^{35}Cl_4{}^{37}Cl$	229.9457	$C_{10}H_5Cl_3$
Carbaryl	22.22	201.0790	$C_{12}H_{11}NO_2$	144.0575	$C_{10}H_8O$	115.0548	C_9H_7	116.0626	C_9H_8
Alachlor	22.39	269.1183	$C_{14}H_{20}ClNO_2$	146.0970	$C_{10}H_{12}N$	160.1126	$C_{11}H_{14}N$	118.0657	C_8H_8N
Fenchlorfos	22.62	319.8997	$C_8H_8Cl_3O_3PS$	284.9309	$C_8H_8Cl_2O_3PS$	286.9280	$C_8H_8{}^{35}Cl{}^{37}ClO_3PS$	-	-
Metalaxyl	22.63	279.1471	$C_{15}H_{21}NO_4$	206.1181	$C_{12}H_{16}NO_2$	220.1388	$C_{12}H_{18}NO_2$	234.113	$C_{13}H_{16}NO_3$
Methiocarb sulfone	22.92	257.0722	$C_{11}H_{15}NO_4S$	121.0653	C_8H_9O	200.0507	$C_9H_{12}O_3S$	185.0272	$C_8H_9O_3S$
PCB 52	23.05	289.9224	$C_{12}H_6Cl_4$	291.9195	$C_{12}H_6{}^{35}Cl_3{}^{37}Cl$	289.9224	$C_{12}H_6Cl_4$	254.9535	$C_{12}H_6Cl_3$
Terbutryn	23.09	241.1361	$C_{10}H_{19}N_5S$	185.0735	$C_6H_{11}N_5S$	226.1126	$C_9H_{16}N_5S$	170.0500	$C_5H_8N_5S$
Methiocarb	23.14	225.0824	$C_{11}H_{15}NO_2S$	168.0609	$C_9H_{12}OS$	153.0374	C_8H_9OS	109.0653	C_7H_9O
Fenitrothion	23.17	277.0174	$C_9H_{12}NO_5PS$	124.9826	$C_2H_6O_2PS$	109.0055	$C_2H_6O_3P$	277.0174	$C_9H_{12}NO_5PS$
Pirimiphos methyl	23.32	305.0963	$C_{11}H_{20}N_3O_3PS$	290.0728	$C_{10}H_{17}N_3O_3PS$	276.0572	$C_9H_{15}N_3O_3PS$	305.0963	$C_{11}H_{20}N_3O_3PS$

Table 1. m/z ions selected for the identification of target compounds*

Compound	R_t (min)	Molecular mass	Molecular formula	m/z 1	Ion 1 (Q)	m/z 2	Ion 2 (q_1)	m/z 3	Ion 3 (q_2)
Aldrin	23.52	361.8757	$C_{12}H_8Cl_6$	262.8570	$C_7H_2{}^{35}Cl_4{}^{37}Cl$	260.8599	$C_7H_2Cl_5$	292.9273	$C_{12}H_7{}^{35}Cl_3{}^{37}Cl$
Dichlofluanide	23.42	331.9623	$C_9H_{11}Cl_2FN_2O_2S_2$	123.0143	C_6H_5NS	223.9504	$C_7H_5Cl_2FNS$	225.9474	$C_7H_5{}^{35}Cl^{37}ClFNS$
Malathion	23.67	330.0361	$C_{10}H_{19}O_6PS_2$	127.0395	$C_6H_7O_3$	124.9826	$C_2H_6O_2PS$	173.0814	$C_8H_{13}O_4$
Metolachlor	23.79	283.1339	$C_{15}H_{22}ClNO_2$	162.1283	$C_{11}H_{16}N$	238.0999	$C_{13}H_{17}ClNO$	240.0973	$C_{13}H_{17}{}^{37}ClNO$
Fenthion	23.92	278.0200	$C_{10}H_{15}O_3PS_2$	278.0200	$C_{10}H_{15}O_3PS_2$	169.0146	$C_8H_9S_2$	109.0055	$C_2H_6O_3P$
Chlorpyrifos	24.00	348.9263	$C_9H_{11}Cl_3NO_3PS$	196.9202	$C_5H_2Cl_3NO$	198.9173	$C_5H_2{}^{35}Cl_2{}^{37}ClNO$	257.8948	$C_5H_3Cl_2NO_3PS$
Parathion ethyl	24.02	291.0330	$C_{10}H_{14}NO_5PS$	109.0055	$C_2H_6O_3P$	291.0330	$C_{10}H_{14}NO_5PS$	96.9513	H_2O_2PS
Isodrin	24.62	361.8757	$C_{12}H_8Cl_6$	192.9379	$C_7H_4Cl_3$	194.9349	$C_7H_4{}^{35}Cl_2{}^{37}Cl$	262.8570	$C_7H_2{}^{35}Cl_4{}^{37}Cl$
Pirimiphos ethyl	24.90	333.1276	$C_{13}H_{24}N_3O_3PS$	318.1041	$C_{12}H_{21}N_3O_3PS$	333.1276	$C_{13}H_{24}N_3O_3PS$	304.0890	$C_{11}H_{19}N_3O_3PS$
Cyprodinil	24.95	225.1266	$C_{14}H_{15}N_3$	224.1188	$C_{14}H_{14}N_3$	225.1266	$C_{14}H_{15}N_3$	210.1031	$C_{13}H_{12}N_3$
Heptachlor epoxide B	25.09	385.8160	$C_{10}H_5Cl_7O$	352.8442	$C_{10}H_5{}^{35}Cl_5{}^{37}ClO$	350.8472	$C_{10}H_5Cl_6O$	354.8413	$C_{10}H_5{}^{35}Cl_4{}^{37}Cl_2O$
Fluoranthene	25.20	202.0783	$C_{16}H_{10}$	202.0783	$C_{16}H_{10}$	201.0704	$C_{16}H_9$	200.0626	$C_{16}H_8$
Penconazole	25.25	283.0643	$C_{13}H_{15}Cl_2N_3$	158.9768	$C_7H_5Cl_2$	160.9739	$C_7H_5{}^{35}Cl^{37}Cl$	248.0955	$C_{13}H_{15}ClN_3$
Heptachlor epoxide A	25.25	385.8160	$C_{10}H_5Cl_7O$	216.9379	$C_9H_4Cl_3$	250.8989	$C_9H_3Cl_4$	236.8423	$C_5{}^{35}Cl_4{}^{37}Cl$
Thiabendazole	25.30	201.0361	$C_{10}H_7N_3S$	201.0361	$C_{10}H_7N_3S$	174.0252	$C_9H_6N_2S$	-	-
Chlorfenvinphos	25.57	357.9695	$C_{12}H_{14}Cl_3O_4P$	266.9381	$C_8H_6Cl_2O_4P$	268.9353	$C_8H_6{}^{35}Cl^{37}ClO_4P$	323.0007	$C_{12}H_{14}Cl_2O_4P$
Isofenfos	25.60	345.1164	$C_{15}H_{24}NO_4PS$	213.0317	$C_9H_{10}O_4P$	121.0293	$C_7H_5O_2$	255.0786	$C_{12}H_{16}O_4P$
Quinalfos	25.65	298.0541	$C_{12}H_{15}N_2O_3PS$	146.0480	$C_8H_6N_2O$	157.0760	$C_{10}H_9N_2$	156.0682	$C_{10}H_8N_2$
Procymidone	25.85	283.0167	$C_{13}H_{11}Cl_2NO_2$	96.0575	C_6H_8O	283.0167	$C_{13}H_{11}Cl_2NO_2$	285.0139	$C_{13}H_{11}{}^{35}Cl^{37}ClNO_2$
Hexythiazox	26.04	352.1012	$C_{17}H_{21}ClN_2O_2S$	155.9800	C_7H_5ClS	184.0113	C_9H_9ClS	227.0172	$C_{10}H_{10}ClNOS$
Methidathion	26.12	301.9619	$C_6H_{11}N_2O_4PS_3$	145.0072	$C_4H_5N_2O_2S$	85.0402	$C_3H_5N_2O$	124.9826	$C_2H_6O_2PS$
Pyrene	26.15	202.0783	$C_{16}H_{10}$	202.0783	$C_{16}H_{10}$	201.0704	$C_{16}H_9$	200.0626	$C_{16}H_8$
PCB 101	26.35	323.8834	$C_{12}H_5Cl_5$	325.8805	$C_{12}H_5{}^{35}Cl_4{}^{37}Cl$	323.8834	$C_{12}H_5Cl_5$	290.9117	$C_{12}H_5{}^{35}Cl_3{}^{37}Cl$
Fenoxycarb	26.37	301.1334	$C_{17}H_{19}NO_4$	255.0895	$C_{15}H_{13}NO_3$	186.0681	$C_{12}H_{10}O_2$	185.0603	$C_{12}H_9O_2$
α-endosulfan	26.42	403.8169	$C_9H_6Cl_6O_3S$	169.9690	$C_8H_4Cl_2$	306.8832	$C_9H_6{}^{35}Cl_4{}^{37}ClO$	336.8760	$C_9H_6Cl_5O_3$
Imazalil	27.20	296.0483	$C_{14}H_{14}Cl_2N_2O$	172.9561	$C_7H_3Cl_2O$	215.003	$C_{10}H_9Cl_2O$	174.9532	$C_7H_3{}^{35}Cl^{37}ClO$
PCB 77	27.32	289.9224	$C_{12}H_6Cl_4$	291.9195	$C_{12}H_6{}^{35}Cl_3{}^{37}Cl$	289.9224	$C_{12}H_6Cl_4$	254.9535	$C_{12}H_6Cl_3$
Profenofos	27.35	371.9351	$C_{11}H_{15}BrClO_3PS$	138.9983	$C_3H_8O_2PS$	205.9134	C_6H_4BrClO	336.9663	$C_{11}H_{15}BrO_3PS$

Table 1. m/z ions selected for the identification of target compounds*

Compound	R_t (min)	Molecular mass	Molecular formula	m/z 1	Ion 1 (Q)	m/z 2	Ion 2 (q_1)	m/z 3	Ion 3 (q_2)
Dieldrin	27.39	377.8706	$C_{12}H_8Cl_6O$	262.8570	$C_7H_2{}^{35}Cl_4{}^{37}Cl$	260.8599	$C_7H_2Cl_5$	274.8755	$C_8H_4Cl_5$
p,p'-DDE	27.45	315.9380	$C_{14}H_8Cl_4$	246.0003	$C_{14}H_8Cl_2$	247.9975	$C_{14}H_8{}^{35}Cl{}^{37}Cl$	317.9352	$C_{14}H_8{}^{35}Cl_3{}^{37}Cl$
PCB 81	27.69	289.9224	$C_{12}H_6Cl_4$	291.9195	$C_{12}H_6{}^{35}Cl_3{}^{37}Cl$	289.9224	$C_{12}H_6Cl_4$	254.9535	$C_{12}H_6Cl_3$
Buprofezin	27.87	305.1562	$C_{16}H_{23}N_3OS$	105.0578	C_7H_7N	104.0500	C_7H_6N	172.1034	$C_8H_{16}N_2S$
Bupimirate	28.07	316.1569	$C_{13}H_{24}N_4O_3S$	273.1021	$C_{10}H_{17}N_4O_3S$	208.1450	$C_{11}H_{18}N_3O$	316.1569	$C_{13}H_{24}N_4O_3S$
ß-endosulfan	28.52	403.8169	$C_9H_6Cl_6O_3S$	169.9690	$C_8H_4Cl_2$	306.8832	$C_9H_6{}^{35}Cl_4{}^{37}ClO$	336.8760	$C_9H_6Cl_5O_3$
PCB 105	28.55	323.8834	$C_{12}H_5Cl_5$	325.8805	$C_{12}H_5{}^{35}Cl_4{}^{37}Cl$	323.8834	$C_{12}H_5Cl_5$	327.8774	$C_{12}H_5{}^{35}Cl_3{}^{37}Cl_2$
PCB 118	28.64	323.8834	$C_{12}H_5Cl_5$	325.8805	$C_{12}H_5{}^{35}Cl_4{}^{37}Cl$	323.8834	$C_{12}H_5Cl_5$	327.8774	$C_{12}H_5{}^{35}Cl_3{}^{37}Cl_2$
BDE 28	28.68	403.8047	$C_{12}H_7OBr_3$	405.8027	$C_{12}H_7O{}^{79}Br_2{}^{81}Br$	407.8007	$C_{12}H_7O{}^{79}Br{}^{81}Br_2$	245.9680	$C_{12}H_7OBr$
p,p'-DDD	28.97	317.9537	$C_{14}H_{10}Cl_4$	235.0081	$C_{13}H_9Cl_2$	237.0053	$C_{13}H_9{}^{35}Cl{}^{37}Cl$	165.0704	$C_{13}H_9$
PCB 114	29.04	323.8834	$C_{12}H_5Cl_5$	325.8805	$C_{12}H_5{}^{35}Cl_4{}^{37}Cl$	323.8834	$C_{12}H_5Cl_5$	327.8774	$C_{12}H_5{}^{35}Cl_3{}^{37}Cl_2$
Oxadixyl	29.15	278.1267	$C_{14}H_{18}N_2O_4$	163.0997	$C_{10}H_{13}NO$	132.0813	$C_9H_{10}N$	105.0704	C_8H_9
Ethion	29.24	383.9876	$C_9H_{22}O_4P_2S_4$	230.9737	$C_5H_{12}O_2PS_3$	153.0139	$C_4H_{10}O_2PS$	124.9826	$C_2H_6O_2PS$
PCB 153	29.47	357.8444	$C_{12}H_4Cl_6$	359.8415	$C_{12}H_4{}^{35}Cl_5{}^{37}Cl$	357.8444	$C_{12}H_4Cl_6$	324.8727	$C_{12}H_4{}^{35}Cl_4{}^{37}Cl$
PCB 123	29.59	323.8834	$C_{12}H_5Cl_5$	325.8805	$C_{12}H_5{}^{35}Cl_4{}^{37}Cl$	323.8834	$C_{12}H_5Cl_5$	327.8774	$C_{12}H_5{}^{35}Cl_3{}^{37}Cl_2$
Endosulfan sulfate	30.09	419.8118	$C_9H_6Cl_6O_4S$	271.8102	$C_5{}^{35}Cl_5{}^{37}Cl$	269.8131	C_5Cl_6	386.8400	$C_9H_6{}^{35}Cl_4{}^{37}ClO_4S$
p,p'-DDT	30.30	351.9147	$C_{14}H_9Cl_5$	235.0081	$C_{13}H_9Cl_2$	246.0003	$C_{14}H_8Cl_2$	237.0053	$C_{13}H_9{}^{35}Cl{}^{37}Cl$
PCB 138	30.45	357.8444	$C_{12}H_4Cl_6$	359.8415	$C_{12}H_4{}^{35}Cl_5{}^{37}Cl$	357.8444	$C_{12}H_4Cl_6$	324.8727	$C_{12}H_4{}^{35}Cl_4{}^{37}Cl$
PCB 126	30.75	323.8834	$C_{12}H_5Cl_5$	325.8805	$C_{12}H_5{}^{35}Cl_4{}^{37}Cl$	323.8834	$C_{12}H_5Cl_5$	327.8774	$C_{12}H_5{}^{35}Cl_3{}^{37}Cl_2$
Tebuconazole	30.80	307.1451	$C_{16}H_{22}ClN_3O$	125.0158	C_7H_6Cl	150.1031	$C_8H_{12}N_3$	250.0747	$C_{12}H_{13}N_3OCl$
Diflufenican	31.14	394.0741	$C_{19}H_{11}F_5N_2O_2$	266.0429	$C_{13}H_7F_3NO_2$	394.0741	$C_{19}H_{11}F_5N_2O_2$	267.0461	$^{12}C_{12}{}^{13}CH_7F_3NO_2$
PCB 156	31.45	357.8444	$C_{12}H_4Cl_6$	359.8415	$C_{12}H_4{}^{35}Cl_5{}^{37}Cl$	357.8444	$C_{12}H_4Cl_6$	324.8727	$C_{12}H_4{}^{35}Cl_4{}^{37}Cl$
Benzo(a)anthracene	31.84	228.0939	$C_{18}H_{12}$	228.0939	$C_{18}H_{12}$	226.0783	$C_{18}H_{10}$	200.0626	$C_{16}H_8$
Iprodione	31.89	329.0334	$C_{13}H_{13}Cl_2N_3O_3$	314.0099	$C_{12}H_{10}N_3O_3Cl_2$	316.0072	$C_{12}H_{10}N_3O_3{}^{35}Cl{}^{37}Cl$	186.9592	$C_7H_3NOCl_2$
Chrysene	32.02	228.0939	$C_{18}H_{12}$	228.0939	$C_{18}H_{12}$	226.0783	$C_{18}H_{10}$	200.0626	$C_{16}H_8$
Phosmet	32.08	316.9945	$C_{11}H_{12}NO_4PS_2$	160.0399	$C_9H_6NO_2$	161.0430	$^{12}C_8{}^{13}CH_6NO_2$	316.9945	$C_{11}H_{12}NO_4PS_2$
PCB 157	32.24	357.8444	$C_{12}H_4Cl_6$	359.8415	$C_{12}H_4{}^{35}Cl_5{}^{37}Cl$	357.8444	$C_{12}H_4Cl_6$	324.8727	$C_{12}H_4{}^{35}Cl_4{}^{37}Cl$
Bifentrin	32.39	422.1260	$C_{23}H_{22}ClF_3O_2$	181.1017	$C_{14}H_{13}$	166.0783	$C_{13}H_{10}$	165.0704	$C_{13}H_{12}$

Table 1. m/z ions selected for the identification of target compounds*

Compound	R_t (min)	Molecular mass	Molecular formula	m/z 1	Ion 1 (Q)	m/z 2	Ion 2 (q_1)	m/z 3	Ion 3 (q_2)
BDE 71	32.40	481.7152	$C_{12}H_6OBr_4$	325.8765	$C_{12}H_6O^{79}Br^{81}Br$	323.8785	$C_{12}H_6OBr_2$	483.7132	$C_{12}H_6O^{79}Br_3{}^{81}Br$
Metoxychlor	32.42	344.0138	$C_{16}H_{15}Cl_3O_2$	227.1072	$C_{15}H_{15}O_2$	212.0837	$C_{14}H_{12}O_2$	274.0761	$C_{16}H_{15}ClO_2$
PCB 167	32.44	357.8444	$C_{12}H_4Cl_6$	359.8415	$C_{12}H_4{}^{35}Cl_5{}^{37}Cl$	357.8444	$C_{12}H_4Cl_6$	324.8727	$C_{12}H_4{}^{35}Cl_4{}^{37}Cl$
PCB 180	32.84	391.8055	$C_{12}H_3Cl_7$	393.8025	$C_{12}H_3{}^{35}Cl_6{}^{37}Cl$	395.7996	$C_{12}H_3{}^{35}Cl_5{}^{37}Cl_2$	391.8055	$C_{12}H_3Cl_7$
BDE 47	32.92	481.7152	$C_{12}H_6OBr_4$	325.8765	$C_{12}H_6O^{79}Br^{81}Br$	323.8785	$C_{12}H_6OBr_2$	483.7132	$C_{12}H_6O^{79}Br_3{}^{81}Br$
Tetradifon	33.07	353.8843	$C_{12}H_6Cl_4O_2S$	158.9665	C_6H_4ClOS	226.8892	$C_6H_2OSCl_3$	353.8843	$C_{12}H_6Cl_4O_2S$
Fosalone	33.44	366.9869	$C_{12}H_{15}ClNO_4PS_2$	182.0009	$C_8H_5NO_2Cl$	183.9981	$C_8H_5NO_2{}^{37}Cl$	366.9869	$C_{12}H_{15}ClNO_4PS_2$
BDE 66	33.47	481.7152	$C_{12}H_6OBr_4$	325.8765	$C_{12}H_6O^{79}Br^{81}Br$	323.8785	$C_{12}H_6OBr_2$	483.7132	$C_{12}H_6O^{79}Br_3{}^{81}Br$
PCB 169	33.55	357.8444	$C_{12}H_4Cl_6$	359.8415	$C_{12}H_4{}^{35}Cl_5{}^{37}Cl$	357.8444	$C_{12}H_4Cl_6$	324.8727	$C_{12}H_4{}^{35}Cl_4{}^{37}Cl$
Mirex	33.62	539.6262	$C_{10}Cl_{12}$	271.8102	$C_5{}^{35}Cl_5{}^{37}Cl$	269.8131	C_5Cl_6	236.8413	$C_5{}^{35}Cl_4{}^{37}Cl$
λ-cyhalothrin	34.34	449.1006	$C_{23}H_{19}ClF_3NO_3$	181.0653	$C_{13}H_9O$	197.0345	$C_8H_9ClF_3$	-	-
Fenarimol	34.39	330.0327	$C_{17}H_{12}Cl_2N_2O$	138.9951	C_7H_4OCl	251.0030	$C_{13}H_9Cl_2O$	313.0299	$C_{17}H_{11}Cl_2N_2$
Pyrazofos	34.74	373.0861	$C_{14}H_{20}N_3O_5PS$	221.0800	$C_{10}H_{11}N_3O_3$	232.1080	$C_{12}H_{14}N_3O_2$	373.0861	$C_{14}H_{20}N_3O_5PS$
PCB 189	34.82	391.8055	$C_{12}H_3Cl_7$	393.8025	$C_{12}H_3{}^{35}Cl_6{}^{37}Cl$	395.7996	$C_{12}H_3{}^{35}Cl_5{}^{37}Cl_2$	391.8055	$C_{12}H_3Cl_7$
Permethrin I	35.65	390.0790	$C_{21}H_{20}Cl_2O_3$	183.0810	$C_{13}H_{11}O$	163.0081	$C_9H_9Cl_2$	184.0844	$^{12}C_{12}{}^{13}CH_{11}O$
Permethrin II	35.90	390.0790	$C_{21}H_{20}Cl_2O_3$	183.0810	$C_{13}H_{11}O$	163.0081	$C_9H_9Cl_2$	184.0844	$^{12}C_{12}{}^{13}CH_{11}O$
BDE 100	35.95	559.6257	$C_{12}H_5OBr_5$	403.7870	$C_{12}H_5O^{79}Br_2{}^{81}Br$	405.7850	$C_{12}H_5O^{79}Br^{81}Br_2$	563.6216	$C_{12}H_5O^{79}Br_3{}^{81}Br_2$
Coumaphos	36.02	362.0145	$C_{14}H_{16}ClO_5PS$	362.0145	$C_{14}H_{16}ClO_5PS$	225.9855	$C_{10}H_7O_2SCl$	333.9832	$C_{12}H_{12}O_5SClP$
Benzo(b)fluoranthene	36.55	252.0939	$C_{20}H_{12}$	252.0939	$C_{20}H_{12}$	250.0783	$C_{20}H_{10}$	248.0626	$C_{20}H_8$
Benzo(k)fluoranthene	36.65	252.0939	$C_{20}H_{12}$	252.0939	$C_{20}H_{12}$	250.0783	$C_{20}H_{10}$	248.0626	$C_{20}H_8$
BDE 99	36.80	559.6257	$C_{12}H_5OBr_5$	403.7870	$C_{12}H_5O^{79}Br_2{}^{81}Br$	405.7850	$C_{12}H_5O^{79}Br^{81}Br_2$	563.6216	$C_{12}H_5O^{79}Br_3{}^{81}Br_2$
Cypermethrin I	37.42	415.0742	$C_{22}H_{19}Cl_2NO_3$	181.0653	$C_{13}H_9O$	163.0081	$C_7H_9Cl_2$	209.0841	$C_{14}H_{11}NO$
Cypermethrin II	37.62	415.0742	$C_{22}H_{19}Cl_2NO_3$	181.0653	$C_{13}H_9O$	163.0081	$C_7H_9Cl_2$	209.0841	$C_{14}H_{11}NO$
Cypermethrin III	37.79	415.0742	$C_{22}H_{19}Cl_2NO_3$	181.0653	$C_{13}H_9O$	163.0081	$C_7H_9Cl_2$	209.0841	$C_{14}H_{11}NO$
Cypermethrin IV	37.79	415.0742	$C_{22}H_{19}Cl_2NO_3$	181.0653	$C_{13}H_9O$	163.0081	$C_7H_9Cl_2$	209.0841	$C_{14}H_{11}NO$
Benzo(a)pyrene	37.81	252.0939	$C_{20}H_{12}$	252.0939	$C_{20}H_{12}$	250.0783	$C_{20}H_{10}$	248.0626	$C_{20}H_8$
BDE 85	38.35	559.6257	$C_{12}H_5OBr_5$	403.7870	$C_{12}H_5O^{79}Br_2{}^{81}Br$	405.7850	$C_{12}H_5O^{79}Br^{81}Br_2$	563.6216	$C_{12}H_5O^{79}Br_3{}^{81}Br_2$
Fenvalerate I	39.15	419.1288	$C_{25}H_{22}ClNO_3$	125.0158	C_7H_6Cl	181.0653	$C_{13}H_9O$	167.0628	$C_{10}H_{12}Cl$

Table 1. m/z ions selected for the identification of target compounds*

Compound	R_t (min)	Molecular mass	Molecular formula	m/z 1	Ion 1 (Q)	m/z 2	Ion 2 (q_1)	m/z 3	Ion 3 (q_2)
BDE 154	39.17	637.5362	$C_{12}H_4OBr_6$	483.6955	$C_{12}H_4O^{79}Br_2{}^{81}Br_2$	481.6975	$C_{12}H_4O^{79}Br_3{}^{81}Br$	485.6935	$C_{12}H_4O^{79}Br^{81}Br_3$
Fenvalerate II	39.55	419.1288	$C_{25}H_{22}ClNO_3$	125.0158	C_7H_6Cl	181.0653	$C_{13}H_9O$	167.0628	$C_{10}H_{12}Cl$
Tau-fluvalinate I	39.57	502.1271	$C_{26}H_{22}ClF_3N_2O_3$	250.0610	$C_{11}H_{12}ClF_3N$	252.0583	$C_{11}H_{12}{}^{37}ClF_3N$	181.0653	$C_{13}H_9O$
Tau-fluvalinate II	39.70	502.1271	$C_{26}H_{22}ClF_3N_2O_3$	250.0610	$C_{11}H_{12}ClF_3N$	252.0583	$C_{11}H_{12}{}^{37}ClF_3N$	181.0653	$C_{13}H_9O$
BDE 153	40.30	637.5362	$C_{12}H_4OBr_6$	483.6955	$C_{12}H_4O^{79}Br_2{}^{81}Br_2$	481.6975	$C_{12}H_4O^{79}Br_3{}^{81}Br$	485.6935	$C_{12}H_4O^{79}Br^{81}Br_3$
Deltamethrin	40.55	502.9732	$C_{22}H_{19}Br_2NO_3$	181.0653	$C_{13}H_9O$	252.9051	$C_7H_9{}^{81}Br$	250.9071	C_7H_9Br
BDE 138	41.85	637.5362	$C_{12}H_4OBr_6$	483.6955	$C_{12}H_4O^{79}Br_2{}^{81}Br_2$	481.6975	$C_{12}H_4O^{79}Br_3{}^{81}Br$	485.6935	$C_{12}H_4O^{79}Br^{81}Br_3$
Indeno(1,2,3,cd)pyrene	41.89	276.0939	$C_{22}H_{12}$	276.0939	$C_{22}H_{12}$	274.0783	$C_{22}H_{10}$	272.0626	$C_{22}H_8$
Dibenzo(a,h)anthracene	42.07	278.1096	$C_{22}H_{14}$	278.1096	$C_{22}H_{14}$	276.0939	$C_{22}H_{12}$	274.0783	$C_{22}H_{11}$
Benzo(g,h,l)perylene	42.69	276.0939	$C_{22}H_{12}$	276.0939	$C_{22}H_{12}$	274.0783	$C_{22}H_{10}$	272.0626	$C_{22}H_8$
BDE 183	43.65	715.4467	$C_{12}H_3OBr_7$	561.6060	$C_{12}H_3O^{79}Br_3{}^{81}Br_2$	563.6040	$C_{12}H_3O^{79}Br_2{}^{81}Br_3$	559.6080	$C_{12}H_3O^{79}Br_4{}^{81}Br$

* data for some of these compounds were taken from our previous work[14].

Q: most abundant ion; q_i: other m/z ions

Validation results

Qualitative validation was carried out considering two different groups of water samples according to their matrix complexity: "clean" and wastewater. Samples with less complex matrix (surface and ground water, and effluent urban wastewater) were considered as "clean" water. Six of these samples were used for validation (2 SW, 2 GW and 2 EWW). Another six samples with higher matrix complexity (3 IWW and 3 RLW) were selected as wastewater. These world-life samples used for qualitative validation could not be considered as a blank actually, as several target analytes were present (see Table 2). Taking into account the different complexity of the waters tested, two values of LOI were proposed for each analyte, one for each type of water matrix ("clean" and waste). The LOI was estimated for each analyte as the lower concentration tested where a 6/6 positive score was obtained in the spiked samples (Table 2). Consequently, a compound was considered as satisfactorily identified and the screening method qualitatively validated, at a certain concentration level, only when the six samples spiked at this level were positive by the accomplishment of the identification criterion defined above.

Qualitative validation in "clean" water was successfully carried out in all different samples, and most of the compounds could be identified in a reliable way at the lowest fortification level tested (0.02 µg/L). For example, PCBs and most PBDEs, PAHs and OC insecticides achieved the established identification criteria at 0.02 µg/L. As regards OP insecticides, most of them showed a LOI of 0.1 µg/L, although four of them (chlorpyriphos, chlorpyriphos methyl, dichlorvos and parathion methyl) could be satisfactorily validated at 0.02 µg/L. Moreover, seven OP insecticides could be only identified at the highest level studied (1 µg/L) and other two (methamidophos and omethoate) could not be identified at any concentration level probably due to its high polarity which difficults retention in C_{18} cartridges [32]. LOIs for triazine herbicides were 0.02 or 0.1 µg/L, and for most of alkylphenols, chloroacetanilide herbicides and fungicides was 0.1 µg/L. Carbamate and pyretroid insecticides could be mostly validated at 1 µg/L. No LOI value could be established for cypermethrin, probably due to the low sensitivity observed for this compound.

Regarding validation in wastewater samples, LOIs for most compounds were normally higher than those for "clean" water samples. This was in part due to that 0.02

µg/L spiking level was not assayed in wastewater (so it could not be set-up as LOI), and also because the higher complexity of the matrix made more complicated the identification analytes. In spite of this, a large number of PCBs, PBDEs, PAHs and OC insecticides achieved the established identification criteria at the lowest concentration tested (0.1 µg/L). Surely, for these compounds, it would have been possible to decrease the LOI as the peak intensity obtained at 0.1 µg/L was rather high. However, no additional experiments were carried out at lower concentrations, as we considered that 0.1 µg/L was satisfactory LOI for wastewater. As regards OP insecticides, 13 out of 30 could be validated at 0.1 µg/L, and 15 out of 30 at 1 µg/L. Similarly to "clean" water, methamidophos and omethoate could not be validated at any concentration level. As regards other target insecticides, seven could be validated at 1 µg/L and hexythiazox at 0.1 µg/L. Alkylphenols, herbicides and fungicides, LOIs were set-up at 0.1 or 1 µg/L, with the exception of dichlofluanid, for which the identification criterion was not accomplished even at the higher level tested.

It is worth to mention that 12 compounds with LOI of 1 µg/L (methacrifos, thiabendazole, isofenfos, bupirimate, ethion, iprodione, fenarimol, diazinon, pirimicarb, methiocarb, pirimiphos methyl and fenthion) (see Table 2), were satisfactorily detected (i.e. chromatographic peaks were observed for at least two *m/z* ions in the corresponding nw-XIC) in the six wastewater samples spiked at 0.1 µg/L. However, they could not be reported as satisfactorily validated at this level because the Q/q_i ratio was out of specified tolerances. This fact made us to realize on the strict criteria established regarding Q/q_i ratio deviation tolerances, especially when dealing with highly complex matrices. Although theoretical Q/q_i ratios, calculated from standards in solvent, were updated in every sequence/day by injection of reference standards within the sample sequence, in some cases the variations observed along a sequence/day together with the effect of the matrix made difficult to accomplish the Q/q_i ratio, mainly at low analyte concentration. At present this interesting topic is under study in our group.

It should be mentioned at this point that in cases of high sensitivity (compounds like OC insecticides, PCBs, PAHs and PBDEs) it was necessary to discard the most abundant ion when validating at the highest level (1 µg/L) due to detector saturation. The selection of other *m/z* ions from the EI spectrum for these compounds (see Table 1) helped us to solve this problem. This aspect has to be taken into account; otherwise, when

saturation occurs the analyte would not be satisfactorily identified at high concentrations because of the no accomplishment of the identification criteria. So, special care should be taken in the analysis of those real samples where the presence of high analyte concentrations might lead to detector saturation (for some *m*/*z* ions) with the risk of reporting false negatives.

Table 2. Positive findings score after analysis of six different spiked samples at different concentration levels

Compound	Family	SURFACE, GROUND AND EFFLUENT WATER					WASTEWATER			
		n*	0.02 µg/L	0.1 µg/L	1 µg/L	LOI (µg/L)	n*	0.1 µg/L	1 µg/L	LOI (µg/L)
Bupimirate	FG		0/6	6/6	6/6	0.1		1/6	6/6	1
Chlozolinate	FG		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Cyprodinil	FG		4/6	6/6	6/6	0.1		6/6	6/6	0.1
Dichlofluanid	FG		0/6	6/6	6/6	0.1		0/6	1/6	-
Diphenylamine	FG	3/6	6/6	6/6	6/6	0.02	2/6	6/6	6/6	0.1
Fenarimol	FG		0/6	6/6	6/6	0.1		0/6	6/6	1
Imazalil	FG		0/6	4/6	6/6	1		6/6	6/6	0.1
Iprodione	FG		0/6	6/6	6/6	0.1		0/6	6/6	1
Metalaxyl	FG		0/6	4/6	6/6	1		4/6	6/6	1
Oxadixyl	FG		0/6	0/6	6/6	1		0/6	6/6	1
Penconazole	FG		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Pirazofos	FG		0/6	6/6	6/6	0.1		0/6	6/6	1
Procymidone	FG		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Tebuconazole	FG		0/6	6/6	6/6	0.1		0/6	6/6	1
Tecnazene	FG		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Thiabendazole	FG		0/6	6/6	6/6	0.1	2/6	5/6	6/6	1
Pentachlorobenzene	FG		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Hexachlorobenzene	FG		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Chlorpropham	HB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Diflufenican	HB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Molinate	HB		1/6	6/6	6/6	0.1		0/6	6/6	1

Table 2. Positive findings score after analysis of six different spiked samples at different concentration levels

Compound	Family	SURFACE, GROUND AND EFFLUENT WATER					WASTEWATER			
		n*	0.02 µg/L	0.1 µg/L	1 µg/L	LOI (µg/L)	n*	0.1 µg/L	1 µg/L	LOI (µg/L)
Propizamide	HB		0/6	6/6	6/6	0.1		3/6	6/6	1
Terbacil	HB		0/6	6/6	6/6	0.1	3/6	3/6	6/6	1
Trifluraline	HB		2/6	6/6	6/6	0.1		6/6	6/6	0.1
Alachlor	HB CA		5/6	6/6	6/6	0.1		3/6	6/6	1
Metolachlor	HB CA		3/6	6/6	6/6	0.1		6/6	6/6	0.1
Atrazine	HB TZ		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Atrazine desethyl	HB TZ		2/6	0/6	6/6	1		6/6	6/6	0.1
Atrazine desisopropyl	HB TZ		0/6	0/6	6/6	1		0/6	6/6	1
Simazine	HB TZ		6/6	6/6	6/6	0.02	3/6	3/6	6/6	1
Terbumeton	HB TZ		2/6	6/6	6/6	0.1		0/6	6/6	1
Terbumeton desethyl	HB TZ		0/6	6/6	6/6	0.1		0/6	6/6	1
Terbuthylazine	HB TZ		6/6	6/6	6/6	0.02	3/6	6/6	6/6	0.1
Terbuthylazine desethyl	HB TZ		6/6	6/6	6/6	0.02	1/6	6/6	6/6	0.1
Terbutryn	HB TZ		1/6	6/6	6/6	0.1		4/6	6/6	1
Buprofezin	INS		0/6	6/6	6/6	0.1		0/6	6/6	1
Fenoxycarb	INS		0/6	6/6	6/6	0.1		0/6	1/6	-
Hexythiazox	INS		0/6	0/6	6/6	1		6/6	6/6	0.1
Carbaryl	INS CAR		0/6	0/6	6/6	1		0/6	3/6	-
Pirimicarb	INS CAR		0/6	6/6	6/6	0.1		0/6	6/6	1
Methiocarb	INS CAR		0/6	4/6	6/6	1		1/6	6/6	1
Methiocarb sulfone	INS CAR		0/6	0/6	6/6	1		0/6	6/6	1

Table 2. Positive findings score after analysis of six different spiked samples at different concentration levels

Compound	Family	SURFACE, GROUND AND EFFLUENT WATER					WASTEWATER			
		n*	0.02 µg/L	0.1 µg/L	1 µg/L	LOI (µg/L)	n*	0.1 µg/L	1 µg/L	LOI (µg/L)
Aldrin	INS OC		6/6	6/6	6/6	0.02		6/6	6/6	0.1
α-endosulfan	INS OC		3/6	6/6	6/6	0.1		6/6	6/6	0.1
β-endosulfan	INS OC		1/6	6/6	6/6	0.1		6/6	6/6	0.1
Dieldrin	INS OC		3/6	6/6	6/6	0.1		6/6	6/6	0.1
Endosulfan ether	INS OC		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Endosulfan sulfate	INS OC		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Heptachlor	INS OC		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Heptachlor epoxide B	INS OC		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Heptachlor epoxide A	INS OC		3/6	6/6	6/6	0.1		6/6	6/6	0.1
Isodrin	INS OC		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Lindane	INS OC		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Mirex	INS OC		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Metoxychlor	INS OC		6/6	6/6	6/6	0.02		3/6	6/6	1
p,p'-DDE	INS OC		6/6	6/6	6/6	0.02		6/6	6/6	0.1
p,p'-DDD	INS OC		6/6	6/6	6/6	0.02		6/6	6/6	0.1
p,p'-DDT	INS OC		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Chlorfenvinphos	INS OP		2/6	6/6	6/6	0.1	3/6	6/6	6/6	0.1
Chlorpyriphos	INS OP	3/6	6/6	6/6	6/6	0.02	6/6	6/6	6/6	0.1
Chlropyriphos methyl	INS OP		6/6	6/6	6/6	0.02		5/6	6/6	1
Coumaphos	INS OP		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Diazinon	INS OP	2/6	3/6	6/6	6/6	0.1	1/6	0/6	6/6	1

Table 2. Positive findings score after analysis of six different spiked samples at different concentration levels

Compound	Family	SURFACE, GROUND AND EFFLUENT WATER					WASTEWATER			
		n*	0.02 µg/L	0.1 µg/L	1 µg/L	LOI (µg/L)	n*	0.1 µg/L	1 µg/L	LOI (µg/L)
Diclorvos	INS OP		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Dimethoate	INS OP		0/6	6/6	6/6	0.1	5/6	3/6	6/6	1
Ethion	INS OP		0/6	6/6	6/6	0.1		0/6	6/6	1
Etrimfos	INS OP		0/6	6/6	6/6	0.1		0/6	6/6	1
Fenchlorfos	INS OP		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Fenitrothion	INS OP		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Fenthion	INS OP		0/6	0/6	6/6	1		3/6	6/6	1
Fonofos	INS OP		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Forate	INS OP		0/6	0/6	6/6	1		0/6	6/6	1
Fosalone	INS OP		0/6	4/6	6/6	1		6/6	6/6	0.1
Heptenofos	INS OP		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Isofenfos	INS OP		0/6	6/6	6/6	0.1		3/6	6/6	1
Malathion	INS OP		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Metacrifos	INS OP		0/6	0/6	6/6	1		0/6	6/6	1
Metamidophos	INS OP		0/6	0/6	0/6	-		0/6	0/6	-
Methidathion	INS OP		0/6	6/6	6/6	0.1		0/6	6/6	1
Mevinfos	INS OP		0/6	6/6	6/6	0.1		0/6	6/6	1
Omethoate	INS OP		0/6	0/6	0/6	-		0/6	0/6	-
Parathion-ethyl	INS OP		0/6	0/6	6/6	1		0/6	6/6	1
Parathion-methyl	INS OP		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Pirimiphos ethil	INS OP		0/6	6/6	6/6	0.1		6/6	6/6	0.1

Table 2. Positive findings score after analysis of six different spiked samples at different concentration levels

Compound	Family	SURFACE, GROUND AND EFFLUENT WATER					WASTEWATER			
		n*	0.02 µg/L	0.1 µg/L	1 µg/L	LOI (µg/L)	n*	0.1 µg/L	1 µg/L	LOI (µg/L)
Profenofos	INS OP		0/6	6/6	6/6	0.1		6/6	6/6	0.1
Pirimiphos methyl	INS OP		3/6	6/6	6/6	0.1		0/6	6/6	1
Phosmet	INS OP		0/6	0/6	6/6	1		1/6	6/6	1
Quinalfos	INS OP		0/6	4/6	6/6	1		0/6	6/6	1
Bifentrin	INS PY		1/6	6/6	6/6	0.1		3/6	6/6	1
Cypermethrin I	INS PY		0/6	0/6	0/6	-		0/6	0/6	-
Cypermethrin II	INS PY		0/6	0/6	0/6	-		0/6	0/6	-
Cypermethrin III	INS PY		0/6	0/6	0/6	-		0/6	0/6	-
Cypermethrin IV	INS PY		0/6	0/6	0/6	-		0/6	0/6	-
λ-cyhalothrin	INS PY		0/6	0/6	6/6	1		0/6	6/6	1
Deltamethrin	INS PY		0/6	0/6	6/6	1		0/6	0/6	-
Fenvalerate I	INS PY		0/6	0/6	6/6	1		0/6	1/6	-
Fenvalerate II	INS PY		0/6	0/6	6/6	1		0/6	0/6	-
Permethrin I	INS PY		0/6	0/6	6/6	1		0/6	6/6	1
Permethrin II	INS PY		0/6	0/6	6/6	1		0/6	6/6	1
tau-fluvalinate I	INS PY		0/6	0/6	6/6	1		0/6	0/6	-
tau-fluvalinate II	INS PY		0/6	0/6	6/6	1		0/6	0/6	-
4-t-Octylphenol	ONP		2/6	6/6	6/6	0.1		0/6	6/6	0.1
4-n-Octylphenol	ONP		6/6	6/6	6/6	0.02		4/6	6/6	1
4-n-Nonylphenol	ONP		5/6	6/6	6/6	0.1		3/6	6/6	1
Acenaphthene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1

Table 2. Positive findings score after analysis of six different spiked samples at different concentration levels

Compound	Family	SURFACE, GROUND AND EFFLUENT WATER					WASTEWATER			
		n*	0.02 µg/L	0.1 µg/L	1 µg/L	LOI (µg/L)	n*	0.1 µg/L	1 µg/L	LOI (µg/L)
Acenaphthylene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Anthracene	PAH	2/6	5/6	6/6	6/6	0.1		6/6	6/6	0.1
Benzo(a)anthracene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Benzo(b)fluoranthene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Benzo(k)fluoranthene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Benzo(a)pyrene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Benzo(g,h,l)perylene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Chrysene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Dibenzo(a,h)anthracene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Fluoranthene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Fluorene	PAH	1/6	6/6	6/6	6/6	0.02		6/6	6/6	0.1
Indeno(1,2,3,cd)pyrene	PAH		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Naphthalene	PAH	3/6	6/6	6/6	6/6	0.02	5/6	6/6	6/6	0.1
Phenanthrene	PAH		5/6	6/6	6/6	0.1		6/6	6/6	0.1
Pyrene	PAH	2/6	3/6	6/6	6/6	0.1		6/6	6/6	0.1
BDE 28	PBDE		6/6	6/6	6/6	0.02		6/6	6/6	0.1
BDE 47	PBDE		6/6	6/6	6/6	0.02		6/6	6/6	0.1
BDE 66	PBDE		6/6	6/6	6/6	0.02		6/6	6/6	0.1
BDE 71	PBDE		6/6	6/6	6/6	0.02		6/6	6/6	0.1
BDE 85	PBDE		6/6	6/6	6/6	0.02		6/6	6/6	0.1
BDE 99	PBDE		6/6	6/6	6/6	0.02		6/6	6/6	0.1

Table 2. Positive findings score after analysis of six different spiked samples at different concentration levels

Compound	Family	SURFACE, GROUND AND EFFLUENT WATER					WASTEWATER			
		n*	0.02 µg/L	0.1 µg/L	1 µg/L	LOI (µg/L)	n*	0.1 µg/L	1 µg/L	LOI (µg/L)
BDE 100	PBDE		6/6	6/6	6/6	0.02		6/6	6/6	0.1
BDE 138	PBDE		4/6	6/6	6/6	0.1		6/6	6/6	0.1
BDE 153	PBDE		6/6	6/6	6/6	0.02		6/6	6/6	0.1
BDE 154	PBDE		6/6	6/6	6/6	0.02		6/6	6/6	0.1
BDE 183	PBDE		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 28	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 52	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 77	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 81	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 101	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 105	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 118	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 114	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 123	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 126	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 138	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 153	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 156	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 157	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 167	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 169	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1

Table 2. Positive findings score after analysis of six different spiked samples at different concentration levels

Compound	Family	SURFACE, GROUND AND EFFLUENT WATER					WASTEWATER			
		n*	0.02 µg/L	0.1 µg/L	1 µg/L	LOI (µg/L)	n*	0.1 µg/L	1 µg/L	LOI (µg/L)
PCB 180	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
PCB 189	PCB		6/6	6/6	6/6	0.02		6/6	6/6	0.1
Fosfamidon	-		0/6	4/6	6/6	1		0/6	6/6	1
Tetradifon	AC		6/6	6/6	6/6	0.02		6/6	6/6	0.1

* n/6 means that n out of 6 "blank" samples analyzed were positive for the target analyte.

LOI: limit of identification

FG: fungicide; HB: herbicide; INS: insecticide; ONP: octyl/nonyl phenol; PAH: polyaromatic hydrocarbon; PBDE: polybrominated diphenyl ether; PCB: polychlorinated byphenil; CA: chloroacetanilide; TZ: triazine; CAR: carbamate; OC: organochlorine; OP: organophosphorus; PY: pyretroid; AC: acaricide

Application to routine samples

A total of 23 water samples (8SW, 7GW, 2EWW, 3IWW, 3RLW) were analyzed following the developed procedure. Up to 24 pollutants were detected and properly identified in surface and ground water (see Figure 1). The compounds more frequently detected in surface water were atrazine (6 out of 8 samples), and 4-n-octylphenol, chlorpyriphos, naphthalene and terbuthylazine (5 out of 8 samples). As regards ground water, the most frequently detected were chlorpyriphos (6 out of 7 samples), followed by alachlor, atrazine, fenitrothion, naphthalene, simazine, terbumeton and terbuthylazine (5 out of 7 samples). In the two effluent water samples collected from the WWTP of Castellón, only one positive finding of naphthalene, two of chlorpyiriphos and two of diazinon were found.

In wastewater samples, the compound most frequently detected was chlorpyriphos (6 out of 6 samples), compound widely used as insecticide in citric crops in our area, followed by naphthalene and dimethoate (5 out of 6 samples) and chlorfenvinphos (4 out of 6). Also, positive findings of simazine and terbacil (3 out of 6), terbuthylazine, thiabendazole and diphenylamine (2 out of 6) were found.

In every sequence of analysis, two quality control samples (QCs), i.e. a “blank” water sample (previously analyzed) fortified at LOI, were also analyzed. The correct identification of target analytes peaks in the QC samples was tested for quality control analysis in every batch of samples analyzed.

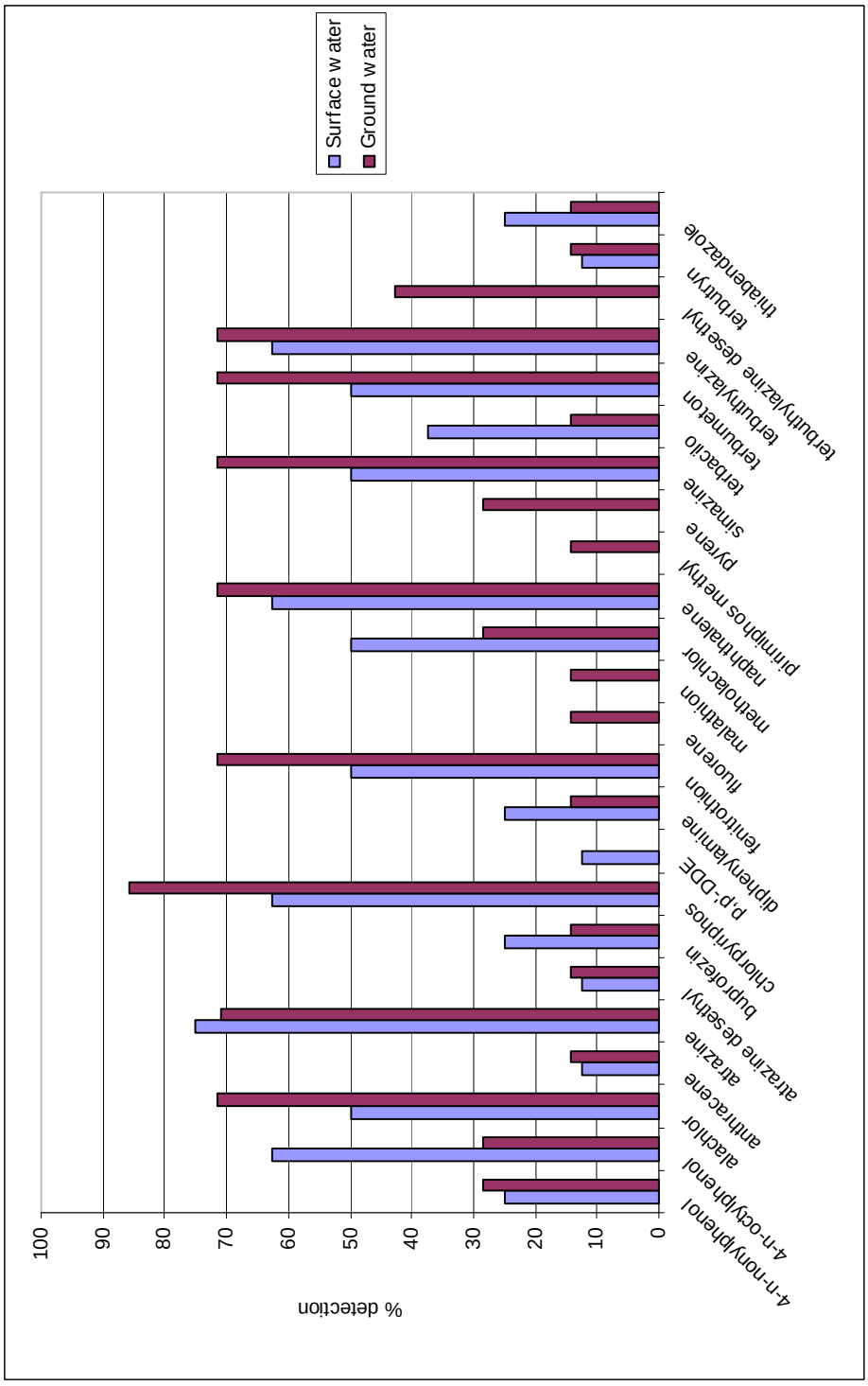

Figure 1. Frequency of detection (%) of organic contaminants in the surface and ground water samples analyzed.

As an illustrative example, Figure 2 shows a positive finding of the herbicide atrazine in surface water from Ebro river. In this case, we observed 5 characteristic ions at the expected retention time in the nw-XIC. The attainment of all 4 Q/q_i ratios within accepted tolerances led to the unequivocal confirmation of this compound. The accurate mass spectrum of the sample peak is shown together with mass errors for the five ions, which were below 2.3 mDa (except for *m/z* 158, with 4.9 mDa). Also, chemical structures for the most abundant EI fragment ions were suggested based on the elemental compositions proposed for those ions accordingly to the accurate mass measurements given by the instrument in the target methodology applied (see Table 1).

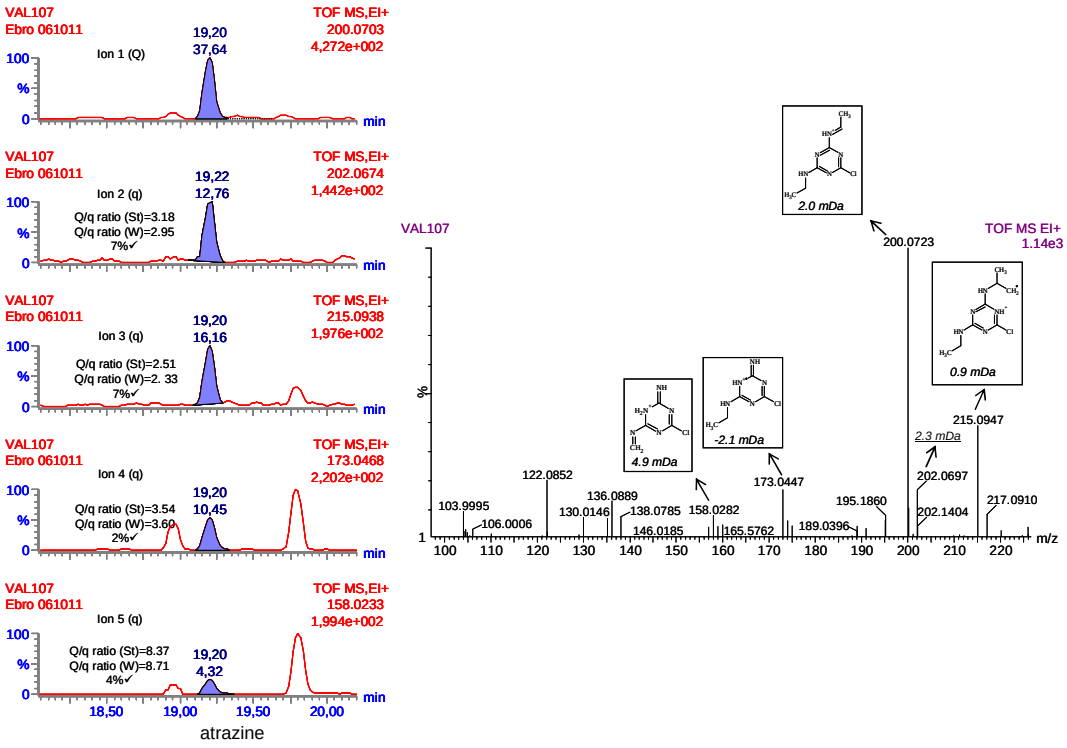

Figure 2. Extracted-ion chromatograms (mass window 0.02 Da) showing a positive finding of atrazine in surface water. Experimental EI accurate mass spectrum. Chemical structures proposed for the most abundant EI fragment ions.

Q: quantitative ion; qi: confirmative ion; St: reference standard; W: water sample; ✓: Q/q_i ratio within tolerance limits; x: Q/q_i ratio out of tolerance limits.

Figure 3 shows another example, the detection and identification of the OP insecticide chlorfenvinphos in influent wastewater. The detection was confirmed by the presence of 5 *m/z* ion at expected retention time in the nw-XIC. However, only 2 out of 4 available Q/q_i ratios fulfilled the specified tolerances, possibly due to the low analyte concentration and to the complexity of the influent water matrix. For this reason, the attainment of at least 1 Q/q_i ratio was established as a criterion for identification considering that, even under this situation, there is relevant information available to support the identification process (the presence of several ions, their accurate mass and agreement in Q/q ratio). The accurate mass spectrum of the sample peak shows the mass errors for the five ions monitored that were below 3.3 mDa.

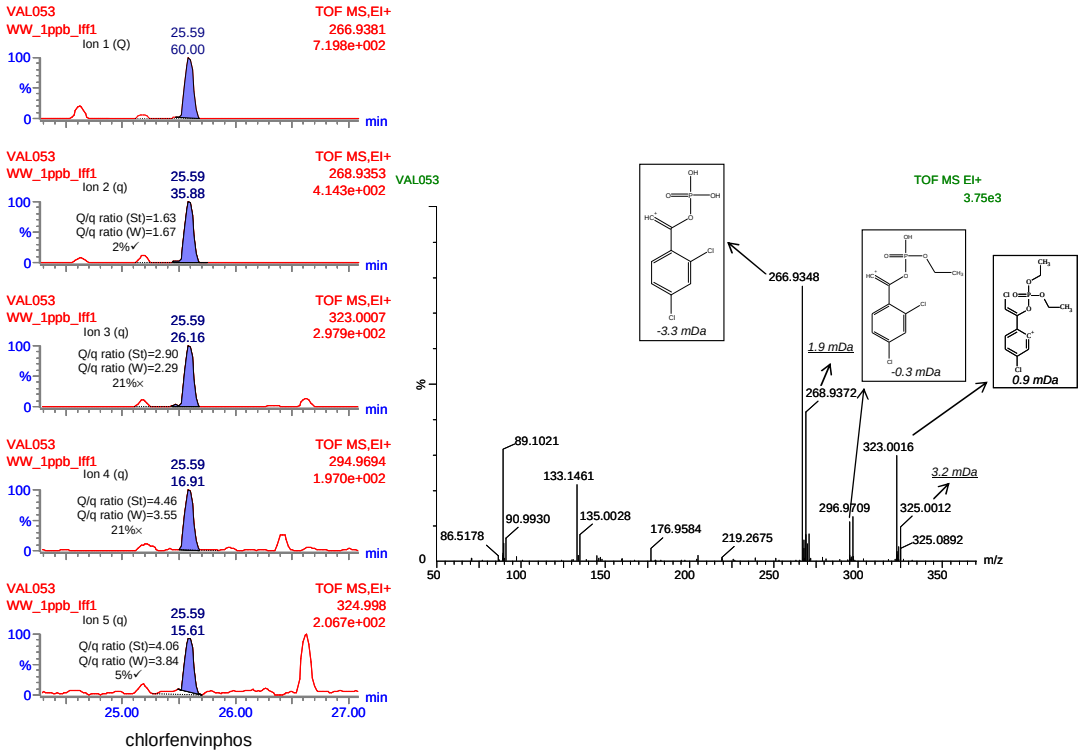

Figure 3. Extracted-ion chromatograms (mass window 0.02 Da) showing a positive finding of chlorfenvinphos in influent wastewater sample. Experimental EI accurate mass spectrum. Chemical structures proposed for the most abundant EI fragment ions.

Q: quantitative ion; qi: confirmative ion; St: reference standard; W: water sample; ✓: Q/q_i ratio within tolerance limits; ×: Q/q_i ratio out of tolerance limits.

Accurate mass measurements are, of course, of much relevance in the confirmation process. However, mass errors in a great deal depend on the ion abundance. Therefore, mass errors higher than usual could be expected when measuring low intensity ions. This is illustrated in Figure 4, which shows the detection and confirmation of the identity of the herbicide terbacil in ground water. The detection was supported by the presence of 3 out of 5 ions monitored, at expected retention time in the nw-XIC, and the identity was confirmed by the accomplishment of one of the Q/q_i ratios. However, the remaining two ions were absent (see q ion 4 and q ion 5 in Figure 4). The reason was the high mass errors for these ions, which exceeded 10 mDa, explaining that no peak was present in the corresponding nw-XIC with a mass window of 0.02 Da ($\pm$ 10 mDa). In spite of this fact, sufficient evidences existed to give this finding as terbacil.

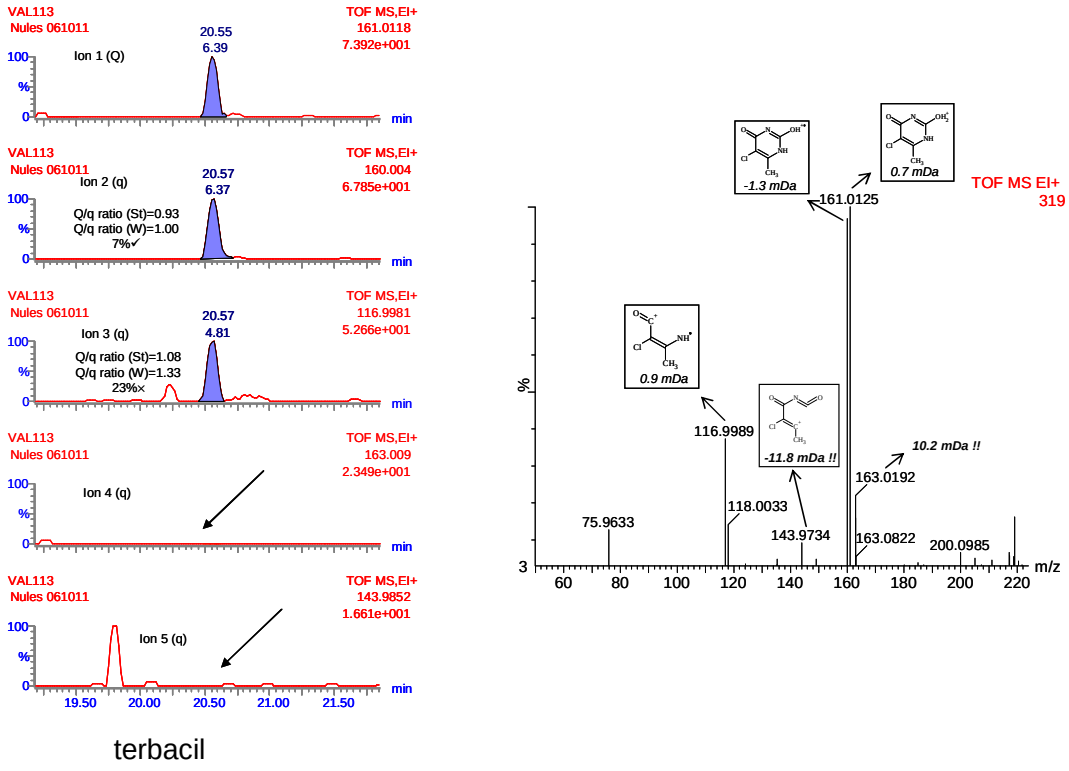

Figure 4. Extracted-ion chromatograms (mass window 0.02 Da) showing a positive finding of terbacil in ground water. Experimental EI accurate mass spectrum. Chemical structures proposed for the most abundant EI fragment ions. Q: quantitative ion; qi: confirmative ion; St: reference standard; W: water sample; ✓: Q/q_i ratio within tolerance limits; ×: Q/q_i ratio out of tolerance limits.

CONCLUSIONS

A multiclass wide-scope GC-TOF MS screening of organic contaminants in water has been developed and qualitatively validated. The screening method has been validated in several types of water matrices at different analyte concentrations. Specificity/selectivity of the screening was supported by accurate mass measurements provided by TOF MS, which allowed using narrow window-XIC (±0.01 Da) at selected *m/z* ions. The wide majority of the 150 compounds investigated were detected and

correctly identified in all surface, ground and wastewater samples tested spiked at 1µg/L. A large number of targeted analytes could also be satisfactorily identified at 0.1 µg/L level, although identification was more problematic for some compounds, especially in complex-matrix samples like influent wastewater or raw leachate from solid waste treatment plant, mainly because of the non-compliance of Q/q ratios. For a notable number of analytes, the method was validated at the lowest concentration level tested (0.02 µg/L) in less-complex matrices, like surface, ground or effluent wastewater.

The screening procedure was applied to around 20 water samples, with the result of detecting and correctly identifying several PAHs (naphthalene and pyrene), triazine herbicides (simazine, terbumeton, terbuthylazine and terbutryn), organophosphorus insecticides (malathion, chlorpyriphos, diazinon), and some herbicides and fungicides like diphenylamine and chlorpropham. Positive findings were correctly identified following the established criterion of monitoring up to 5 *m/z* ions at accurate mass and the compliance of Q/q_i intensity ratio. The analysis of QCs ("blank" samples spiked at the LOI level, i.e. the lowest concentration for which a compound was correctly identified in all spiked samples tested), included in every sample sequence, was used for quality control purposes and to test the robustness of the screening method. This allowed us to prove that some compounds detected in the samples were present at levels below the empirical LOI, which illustrates the strong potential and excellent sensitivity of the screening approach developed in the present work.

ACKNOWLEDGMENTS

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of GC-TOF MS (GCT). This work has received finnantial support from the Spanish Ministry of Science and Education (Project reference: CTQ2009-12347). The authors acknowledge the financial support of Generalitat Valenciana, as research group of excellence PROMETEO/2009/054. T. Portolés is very pleased to Spanish Ministry of Science and Education for her pre-doctoral grant.

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3.4 INVESTIGACIÓN DE CONTAMINANTES DE ORIGEN ANTROPOGÉNICO EN MUESTRAS DE TEJIDO ADIPOSO HUMANO MEDIANTE GC-TOF MS

En este apartado se estudia la aplicación al campo del análisis de muestras biológicas de la metodología analítica y de tratamiento de datos MS utilizando GC-TOF MS desarrollada en el apartado anterior para análisis de aguas. El objetivo es ampliar la información sobre los compuestos potencialmente peligrosos presentes en muestras de tejido adiposo humano.

En primer lugar, se han comparado los resultados obtenidos por GC-TOF MS y por GC-(QqQ)MS/MS con el fin de estudiar la complementariedad de ambas técnicas para la investigación de contaminantes de origen antropogénico en muestras de tejido adiposo mamario. Es estudio se ha centrado en 30 contaminantes orgánicos persistentes preseleccionados para los que, en un trabajo anterior, se optimizaron las condiciones de extracción, purificación y análisis mediante GC-(QqQ)MS/MS. De este modo, la mayoría de compuestos reportados como positivos en los análisis muestras en el análisis por GC-(QqQ)MS/MS también se han detectado y confirmado por GC-TOF MS. Esto ocurrió en aquellos casos en los que la concentración del analito era más alta. Sin embargo, para aquellos que se encontraban a concentraciones más bajas, la menor sensibilidad del GC-TOF MS ha impedido su correcta identificación y confirmación. Esto constituyó una de las principales desventajas del GC-TOF MS en comparación con el GC-(QqQ)MS/MS en modo SRM en análisis *target*.

En segundo lugar, se ha aprovechado toda la información adquirida sobre la muestra, al disponer de espectros de masas completos medidos con elevada exactitud de masa, para buscar otros compuestos que en su momento no se investigaron por triple cuadrupolo. Esta búsqueda *post-target* se centró en unos 100 contaminantes, seleccionados a partir e datos obtenidos en la bibliografía (otros congéneres de PCBs, PAHs, más PBDEs, algunos alquilfenoles, etc). Esto permitió encontrar compuestos en los análisis mediante GC-TOF MS, que no fueron detectados por GC-(QqQ)MS/MS, ya que, obviamente, no habían sido adquiridas las transiciones correspondientes, al no encontrarse en el listado *target* de analitos, como por ejemplo, algunos PAHs y PCBs.

Finalmente, se aplicó el software de deconvulación de datos seguido de una búsqueda de espectros en librería para investigar de un modo *non-target* la presencia de otros contaminantes en las muestras, que no hubieran sido incluidos en nuestros

métodos *target*. Siguiendo esta metodología se detectó la presencia de otros congeneres de PCBs (diferentes a los incluídos en las listas *target*), derivados del naphthalene, n-BBSA, el antioxidante BHT y su derivado BHT-CHO.

En base a los resultados obtenidos por el método *non-target*, se decidió adquirir patrones de referencia comerciales para algunos de los compuestos detectados con el fin de llevar a cabo una confirmación inequívoca de los mismos. Estos compuestos habían sido detectados e identificados en librería y a partir de la masa exacta de los principales fragmentos. Cabe resaltar que en todos los casos en los que se compró el patrón de referencia, se pudo confirmar inequívocamente la identidad de los compuestos detectados, lo que demuestra el potencial y la utilidad de los análisis de GC-TOF MS.

Los resultados obtenidos se recogen en la Tabla 1 del **artículo científico 7**, donde se puede observar el carácter complementario del análisis por GC-(QqQ)MS/MS y GC-TOF MS.

La presencia de los contaminantes encontrados en muestras de grasa ha sido ampliamente descrita en la literatura, sobretodo aquellos con mayor liposolubilidad como los OCs (*p,p'*-DDE, HCB, β-HCH, etc). Su continua presencia en muestras grasas sigue siendo objeto de estudio en la actualidad (1-8). Otros compuestos detectados, como los PAHs, no han sido tan ampliamente reportados en muestras biológicas de origen humano (9-11), pero sin embargo han sido objeto de estudio en otras matrices grasas como organismos marinos (12-18) y biota (19).

Con respecto a los compuestos encontrados en modo *non-target*, no hay mucha información sobre ellos en muestras de tejido adiposo, aunque sí han sido reportados en muestras medioambientales, sobretodo en aguas (20-22).

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3.4.2 Artículo científico 7

J. Mass Spectrom. 44, 1-11, 2009

SEARCHING FOR ANTHROPOGENIC CONTAMINANTS IN HUMAN BREAST ADIPOSE TISSUES USING GAS GROMATOGRAPHY-TIME-OF-FLIGHT MASS SPECTROMETRY

Félix Hernández, Tania Portolés, Elena Pitarch, Francisco J. López

Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain,

ABSTRACT

The potential of gas chromatography-time-of-flight mass spectrometry (GC-TOF MS) for screening anthropogenic organic contaminants in human breast adipose tissues has been investigated. Initially a target screening was performed for a list of 125 compounds which included persistent halogen pollutants [organochlorine (OC) pesticides, polychlorinated biphenylss (PCBs), polybrominated diphenyl ethers (PBDEs)], polyaromatic hydrocarbons (PAHs), alkylphenols, and a notable number of pesticides from the different fungicide, herbicide and insecticide families. Searching for target pollutants was done by evaluating the presence of up to five representative ions for every analyte, all measured at accurate mass (20-mDa mass window). The experimental ion abundance ratios were then compared to those of reference standards for confirmation. Sample treatment consisted of an extraction with hexane and subsequent normal-phase (NP) High performance liquid chromatography (HPLC) or SPE cleanup. The fat-free LC fractions were then investigated by GC-TOF MS.

Full-spectral acquisition and accurate mass data generated by GC-TOF MS also allowed the investigation of nontarget compounds using appropriate processing software to manage MS data. Identification was initially based on library fit using commercial nominal mass libraries. This was followed by comparing the experimental accurate masses of the most relevant ions with the theoretical exact masses with calculations made using the elemental composition calculator included in the software.

The application of both target and nontarget approaches to around 40 real samples allowed the detection and confirmation of several target pollutants including

p,p-DDE, hexachlorobenzene (HCB), and some polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs). Several nontarget compounds that could be considered anthropogenic pollutants were also detected. These included 3,5-di-tert-butyl-4-hydroxy-toluene (BHT) and its metabolite 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO), dibenzylamine, N-butyl benzenesulfonamide (N-BBSA), some naphthalene-related compounds and several PCBs isomers not included in the target list. As some of the compounds detected are xenoestrogens, the methodology developed in this paper could be useful in human breast cancer research.

KEYWORDS

high-resolution time-of-flight mass spectrometry, gas chromatography, adipose tissue, anthropogenic contaminants, screening and confirmation

INTRODUCTION

Human exposure to environmental contaminants has been widely reported in the literature in the last few decades. Many of these contaminants, e.g. those known as persistent organic pollutants (POPs), are lypophilic in nature and their presence in the environment and fatty food is well documented. Contaminants like organochlorine (OC) pesticides, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), polyaromatic hydrocarbons (PAHs) and alkylphenols, are frequently present in the environment and tend to bioaccumulate through the food chain. Consequently, these pollutants are commonly detected in human samples and, in some cases, their estrogenic potency has been reported.[1-4] As a result, human exposure to these pollutants is a public concern for the general population and occupationally exposed people.

Robust and advanced methodology becomes necessary to investigate and confirms the presence of these pollutants in biological samples. A wide number of analytical methodologies and instrumentations are available nowadays, in most cases making use of gas chromatography (GC) combined with mass spectrometry (MS). GC-MS, operating in selected ion monitoring (SIM) mode, has been the most widely used in the determination of organic micro pollutants in environmental, food and biological

samples. However, the analysis of complex matrices is still problematic due to interferences from matrix components, and the low selectivity of single quadrupole may not be sufficient for a reliable quantification and confirmation of the analyte. The special features of tandem mass spectrometry (MS/MS), using ion trap detectors (ITD) or triple quadrupole (QqQ) analyzers, have allowed the reduction and even the elimination of many of these interferences, with a notable improvement of selectivity. Tandem MS also offers better sensitivity and lower limits of detection by means of achieving appropriate precursor and product ion selection, thanks to the lower chemical noise in the chromatograms and much better signal-to-noise ratios. In recent years both analyzers (ITD and QqQ) have been applied to multiresidue determination of organic pollutants in biological samples.[5-9]

An inherent limitation of MS/MS techniques is their inability to detect untargeted compounds for which no data is acquired unless additional analysis is performed. Thus, it is not feasible to perform a search of compounds in a post-target way, i.e. investigating the presence of analytes (different to those pre-targeted) after MS data acquisition.[10,11] Contrary to this, full spectrum techniques offer the advantage of performing retrospective analysis. That is a careful examination of old raw data sets looking for ions of other residues without the need to reanalyze the samples, provided a residue has passed the sample preparation, chromatographic separations and ionization process with sufficient efficiency.[12] High-resolution time-of-flight mass spectrometry (TOF MS) is becoming an attractive and alternative full-spectrum technique for this purpose against traditional instruments such as double-focusing magnetic sectors. This technique which is highly expensive and needs highly skilled operators, has been employed for many years and is still being used to identify nontarget compounds in samples using ion composition elucidation.[13,14] In addition, the investigation of nontarget compounds using TOF MS is also possible using appropriate processing software making it feasible to manage the huge amount of data generated for samples. The unrivaled full spectrum sensitivity of this technique, together with its elevated mass resolution and excellent mass accuracy [15] make TOF MS very attractive for the rapid screening of target and nontarget compounds and for their reliable accurate mass confirmation.

Our own research group has recently shown that GC-TOF MS allows a rapid and automatic accurate mass screening of target analytes using extracted ion

chromatograms with narrow mass window [micro-window-extracted ion chromatograms (mw-XICs) or narrow-window-extracted ion chromatograms (nw-XICs)] to remove the chemical background and highly improve selectivity in the analysis of complex matrices.[16,17] For nontarget analysis, the possibility of discovering the presence of compounds that had not been included in the initial target list is undoubtedly an attractive and challenging application that becomes more and more necessary as the number and type of organic contaminants in the environment continually increases. The use of component detection algorithms (CODA) and deconvolution software is required to both identify the presence of multiple components, and to deconvolute mass spectra for each individual component to be subsequently searched for in a commercial or home-made library. Accurate mass measurements notably facilitate the elemental composition calculation for every component which reduces the list of candidates, and makes the elucidation process of nontarget compounds friendlier and helps to solve ambiguous results in the library search.

Until now very few papers have been published dealing with trace analysis by GC-high-resolution TOF MS, and all have appeared within the last decade, which provides evidence of the novelty of this subject. GC-TOF MS applications have been described for the determination of PBDEs, pesticides, PAHs and PCBs in different environmental matrices.[16-19] Other works deal with the determination of PBDEs, xenoestrogens, or flavor research in biological samples,[5,20,21] or pesticides in food.[22] Recently, Čajka and Hajšlova reviewed the application of GC-TOF MS in food analysis.[23]

The aim of this work is to investigate the capabilities of GC-TOF MS for screening a list of 125 target organic pollutants in human adipose breast tissues. Around 30 persistent halogen pollutants, for which the extraction and cleanup procedure had been optimized in a previous work,[5] have been investigated as pretarget analytes and the results have been compared to previous data obtained by GC-(QqQ)MS/MS. Then, making use of the full spectral acquisition data acquired, and without reanalyzing the samples, around 100 more contaminants, including other PCBs, PBDEs, PAHs, alkylphenols, and a notable number of pesticides, like insecticides (organophosphates, carbamates and pyretroids), herbicides (triazines and chloroacetanilides) and fungicides, have been selected to perform a posttarget screening of real-world samples. Finally, searching unknown compounds present in breast tissue (nontarget analysis) was

carried out using CODA, deconvolution potential and the valuable accurate mass information provided by TOF MS.

EXPERIMENTAL

Reagents

In relation to the list of target analytes, reference standards of pesticides, octyl/nonyl phenols, PCBs (Mix 3, 100 µg/ml in cyclohexane) and PAHs (Mix 25, 100 µg/ml) were purchased from Dr Ehrenstorfer (Augsburg, Germany). Acenaphthene and naphthalene (Fluka, Buchs, Switzerland) and fluoranthene (Riedel de Haen, Seelze, Germany) were also used. Standards of PBDEs (50 µg/ml in nonane) were obtained from Wellington Laboratories (Guelph, Ontario, Canada). In the case of solid reference standards, stock solutions (around 500 µg/ml) were prepared by dissolving reference standards in acetone and stored in a freezer at - 20 °C. Working solutions for sample fortification and for injection in the chromatographic systems were prepared by diluting stock solutions in n-hexane.

Ethyl acetate, acetone and n-hexane were ultra-trace quality and purchased from Scharlab (Barcelona, Spain). Anhydrous sodium sulfate of pesticide residue quality (Baker, Deventer, Holland) was dried for 18 h at 300 °C before use.

Three isotopically labeled surrogates were used: hexachlorobenzene (HCB)-$^{13}C_6$ (Cambridge Isotope Labs, Inc. Andover, MA, USA), *p,p*-DDE-d_8 and -endosulfan-d_4 (Dr Ehrenstorfer). Working solutions of labeled standards (1 µg/ml) were prepared by dilution of stock solution with hexane and stored at 4 °C.

Samples

Human breast tissues were obtained from women with breast cancer. Samples were collected from volunteer women at the Cancer Foundation's Oncology Institute in Valencia (FIVO). Adipose tissues were obtained from biopsies taken during breast surgery. For this study informed, written consents were obtained from the women beforehand. Samples were collected in sterilized polyethylene recipients, identified (devoid of personal identifiers) and immediately frozen. Two different samples were

collected from each woman: adipose breast tissue and tumor fragment; with a total of 42 samples being analyzed, corresponding to 21 patients.

Instrumentation

The normal-phase (NP) high performance liquid chromatography (HPLC) system used for sample cleanup was based on our previous work.[5] For the GC instrumentation, an Agilent 6890N GC system (Paloalto, CA, USA) equipped with an Agilent 7683 autosampler was coupled to a TOF MS, GCT (Waters Corporation, Manchester, UK), operating in electron ionization (EI) mode. The GC separation was performed using a fused silica HP-5MS capillary column with a length of 30 m × 0.25 mm i.d. and a film thickness of 0.25 μm (J & W Scientific, Folson, CA, USA). The oven temperature was programmed as follows: 90 °C (1 min); 5 °C/min to 300 °C (2 min). Splitless injections of 1-μl sample were carried out. Helium was used as a carrier gas at 1 ml/min.

The interface and source temperatures were both set to 250 °C and a solvent delay of 3 min was selected. TOF MS was operated at 1 spectrum/s acquiring the mass range m/z 50-650 and using a multichannel plate voltage of 2700 V. TOF MS resolution was about 8500 full width at half maximum (FWHM) at m/z 612. Heptacosa, used for the daily mass calibration as well as lock mass, was injected via syringe in the reference reservoir at 30 °C for this purpose. The m/z ion monitored was 218.9856. The application manager TargetLynx, a module of MassLynx software, was used to process the qualitative and quantitative data obtained from standards and samples for target compounds. The application manager ChromaLynx, also a module of MassLynx software, was used to investigate the presence of nontarget compounds in samples. Library searching was performed using the commercial US National Institute of Standards and Technology (NIST) library.

Analytical procedure

Sample preparation and extraction

Between 0.1 and 0.5 g of tissue sample was spiked with 0.5 ml isotopically labeled surrogate solution (500 ng/ml), then homogenized with 5- to 10-g anhydrous sodium sulfate and extracted three times with 5 ml of n-hexane each time, shaking in

vortex. After filtration, the extract was preconcentrated under a gentle nitrogen stream at 40 °C, and the final residue was adjusted to 5 ml (NP HPLC cleanup) or to 10 ml with hexane solid phase extraction (SPE) cleanup depending on the subsequent cleanup applied.

Cleanup procedure

Two cleanup methodologies were applied to the sample hexanic extracts, both based on our previous works.[5,8]

Twenty-eight samples (14 samples of adipose breast tissue and 14 of tumoral fragment from 14 patients) were submitted to HPLC cleanup with a silica column, using two complementary procedures, and injecting 1 ml of hexanic extract into the LC system in each case.[5] The mobile phase was n-hexane (procedure A) or n-hexane/ethyl acetate (95 : 5 v/v) (procedure B), at a flow rate of 1 ml/min. In both procedures, after 16 min of injecting the sample extract, a pulse of 4 ml of ethyl acetate was introduced. Fractions eluting between 1 and 17 min (procedure A) and between 4 and 17 min (procedure B) were collected and preconcentrated under a gentle nitrogen stream at 40 °C down to 1 ml. The two final cleaned-up extracts were injected separately into the GC-TOF MS instrument.

Fourteen samples (7 samples of adipose breast tissue and 7 of tumoral fragment from 7 patients) were submitted to SPE cleanup.[8] Ten milliliters of the sample hexanic extract was passed through the silica SPE cartridge previously conditioned by passing through 6 ml of hexane. The first 3 ml was discarded and the rest, approximately 7 ml, was collected together with an additional fraction eluted with another 3 ml of hexane. The cleaned-up extract was preconcentrated to dryness under a gentle nitrogen stream at 40 °C and redissolved in 0.5 ml of hexane before GC-TOF MS analysis. This SPE procedure led to more concentrated extracts (0.2-1 g sample/ml) in comparison with the HPLC cleaned-up extracts (0.02-0.1 g sample/ml).

RESULTS AND DISCUSSION

In our previous work, a number of human breast tissue samples were extracted with hexane and purified by the two complementary normal phase liquid chromatography (NPLC) cleanup procedures. Target analyses were performed by GC-(QqQ)MS/MS for around 30 organohalogen xenoestrogen compounds. The application of this methodology led to the detection and quantification of low levels of several analytes, mainly *p,p*-DDE, HCB, β-HCH and some PCBs.[5]

In the present work, we reanalyzed 28 of these samples by GC-TOF MS to confirm the presence of the analytes found by GC-(QqQ)MS/MS. Furthermore, taking advantage of the full spectrum acquisition in TOF MS, the presence of some other selected compounds was also investigated in a post-target way, without reanalyzing the samples.[10,24] In addition, the elucidation of several unknown compounds (nontarget analytes) was tested. The methodical approach previously developed for screening and confirmation of organic micropollutants in water [16,17] was applied in this paper for searching target and nontarget anthropogenic contaminants in human breast adipose tissues.

In addition, another 14 sample extracts were cleaned-up by SPE and analyzed by GC-TOF MS applying both target and nontarget approaches. Final extracts were more concentrated in these samples compared with those of HPLC clean-up, which facilitated the detection of nontarget contaminants as will be shown in the next sections.

Target screening

GC-TOF MS confirmation of target analytes in samples was carried out by obtaining up to 5 microwindow eXtracted Ion Chromatograms (mw-XIC), with a mass window of 0.02 Da, at selected m/z ions for every compound. The software application TargetLynx was employed to automatically process data and to confirm the identity of target compounds detected in samples. Analyte confirmation was performed by comparing the experimental Q/q intensity ratios in samples with the theoretical ones, calculated from injection of standards in solvent. Q/q was the ratio between the most abundant ion (Q, quantitative) and every one of the other measured ions (q,

confirmative). The presence of at least two ions measured at their accurate mass and the attainment of their Q/q intensity ratio within specified tolerances was required for the reliable confirmation of target analytes. Maximum deviations accepted were based on the European Commission Decision (2002/657/EC),[25] as it has been applied in previous works.[5,17,26-28]

As stated in the Introduction section, the sensitive full-spectrum acquisition is feasible together with accurate mass measurements when working with TOF MS. This makes the application of two different approaches possible when facing target analysis:[16,17] (1) pre-target analysis, where the compounds are selected before analysis, reference standards are normally injected, and the methods are fully validated making quantification feasible in most of cases; (b) post-target analysis, where the compounds are selected and searched after MS data acquisition. In this way, there is almost no limitation to the number of compounds that can be investigated, but obviously they have to achieve the GC and MS analysis requirements. Typically, a post-target analysis is focused on identification and/or confirmation of compounds detected, not on quantification, and reference standards are not necessarily injected as the abundant and rich information provided by the instrument is sufficient for the identification of the compound.

In the present work, around 30 organic micropollutants (OC pesticides, PCBs, and PBDEs), for which the extraction and cleanup procedures were previously validated using GC-(QqQ)MS/MS for measurements, were selected as pre-target analytes. Once the samples were analyzed, a notable number (around 100 compounds) of organic contaminants (other PCBs and PBDEs, PAHs, alkylphenols, and other pesticides) were also searched for after MS acquisition (post-target analysis). Table S1 (Supporting information) shows the list of target compounds selected in this work.

Regarding pre-target analysis, all reference standards were available because the sample procedure had previously been validated. Calibration standards were injected into the GC-TOF MS to evaluate the sensitivity of this technique and to estimate the lowest concentration for which the correct identification of the compound was experimentally feasible. This was done by measuring at least two ions with their Q/q ratio falling within specified tolerances.[17] In spite of the fact that TOF sensitivity in full acquisition is excellent, detection and confirmation of compounds were not

feasible at concentrations as low as is possible using QqQ working in selected reaction monitoring (SRM) mode (Table 1). As a consequence, the majority of the previous GC-(QqQ)MS/MS positive findings were confirmed by GC-TOF MS with the exception of those samples where analyte concentrations were below the detection capabilities of TOF MS. As Table 1 shows, all positives of *p,p*-DDE and 95% of the positives of HCB, β-HCH and PCB 153 were confirmed by TOF MS, as their concentration levels were high enough to be confirmed by this technique (>10 ng/g for *p,p*-DDE, HCB, and PCB 153; and > 50 ng/g for β-HCH). Contrary to this, the presence of oxychlordane, PCB 101, *p,p*-DDD, *p,p*-DDT and mirex could not be confirmed by TOF MS because of their low concentrations in samples (<10 ng/g for mirex and PCBs 101 and 153; < 50 ng/g for oxychlordane and *p,p*-DDD; and < 250 ng/g for *p,p*-DDT).

Table 1. Compounds detected in the GC-MS analysis of adipose human breast tissue samples

Pre-target compounds (28 samples analyzed)	**No. of positives by GC-(QqQ)MS/MS**	**No. of positives by GC-TOF MS (target screening)**	**No. of positives by GC-TOF MS (nontarget screening)[a]**
HCB	25	24	6
β-HCH	19	18	1
*p,p'*DDE	27	27	12
p,p'-DDD	12	0	0
p,p'-DDT	5	0	0
Oxychlordane	1	0	0
trans-nonachlor	4	1	0
Mirex	2	0	0
PCB 28	3	2	0
PCB 101	5	0	0
PCB 118	10	4	0
PCB 153	25	24	4
PCB 138	23	17	8
PCB 180	26	17	2
Post-target compounds[b] (42 samples analyzed)			
Naphthalene	-	10	4
Phenanthrene	-	8	0
Fluoranthene	-	8	0
Pyrene	-	19	0
PCB 114	-	9	0
PCB 123	-	12	0
PCB 156	-	8	0
PCB 157	-	16	0
PCB 167	-	6	0
PCB 189	-	3	1
Nontarget compounds[b] (42 samples analyzed)			
BHT	-	-	31
BHT-CHO[c]	-	-	1
PCB 4Cl	-	-	2
PCB 5Cl	-	-	1
PCB 7Cl (isomer 1)	-	-	11
PCB 7Cl (isomer 2)	-	-	2
PCB 7Cl (isomer 3)	-	-	7
PCB 8Cl	-	-	3
1,2-dimethylnaphthalene	-	-	1
2-methyl naphthalene	-	-	2
N-BBSA[c]	-	-	1

[a] Found when treating all compounds as unknown, i.e. applying the software for components detection and peak deconvolution.

[b] Not included in GC-QqQ analysis.

[c] Detected in the most polar HPLC-fraction (clean-up procedure B).

Among the remaining 66 positives (*trans*-nonachlor, PCBs 28, 118, 138, and 180), up to 41 were confirmed by TOF MS. In all cases where confirmation was not feasible, the reason was the lower sensitivity of this technique, as analyte concentrations were below 10 ng/g for PCBs 28, 118, 138, and 180 and below 50 ng/g for trans-nonachlor. As an illustrative example, Fig. 1 shows the XIC (GC-TOF MS) and the SRM chromatograms [GC-(QqQ)MS/MS] for two positives of *p,p*-DDE (445 ng/g) and HCB (428 ng/g) that were detected in an adipose tissue sample and could be confirmed by the two techniques. In both cases, the presence of chromatographic peaks at expected retention time and the attainment of all Q/q ratios when comparing with the reference standard allowed the confirmation of these findings in the samples. Additionally, the corresponding EI accurate mass spectra generated by TOF MS are shown. Mass errors for five representative ions were typically below 1.5 mDa, which gave more confidence to the confirmation process.

The complete spectral information acquired by GC-TOF MS allowed us to perform a post-target analysis. Thus, a strategy previously applied at our laboratory[17] was used for the determination of almost 100 compounds in the 42 samples processed. Among all the compounds investigated, four PAHs (naphthalene, phenanthrene, fluoranthene, and pyrene) and six PCB congeners were detected in several samples (Table 1). Figure 2 shows illustrative XICs for PCB 157, naphthalene, and pyrene detected in adipose tissue samples. In addition to the accurate mass measurements, reliable confirmation was feasible as all Q/q ratios were within specified tolerances. Experimental EI accurate mass spectra generated by TOF MS led to mass errors for five representative ions always below 1.9 mDa. None of these compounds were determined previously by GC-(QqQ)MS/MS, as their optimal SRM transitions had not been acquired when analyzing the samples.

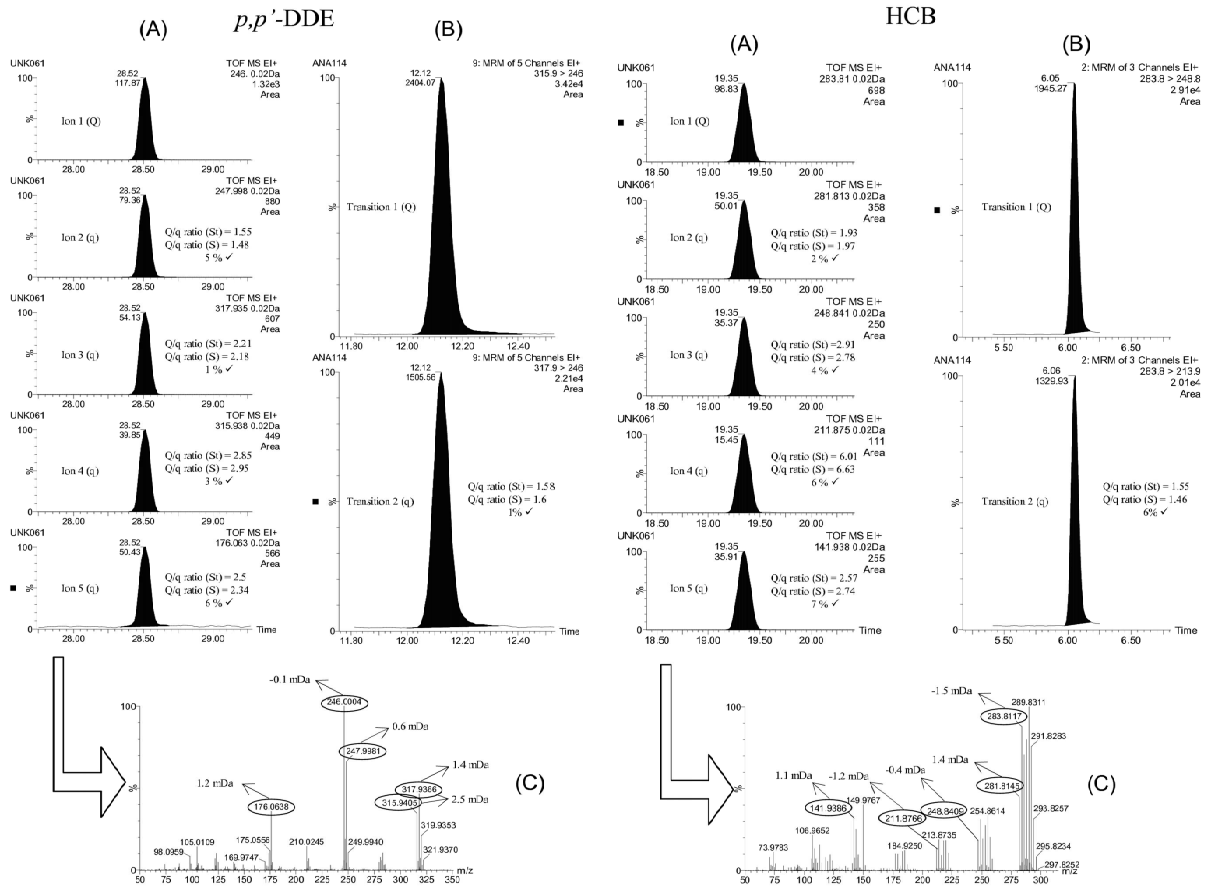


Figure 1. (A) GC-TOF MS extracted ion chromatograms at different m/z (mass window 0.02 Da) for "pre-target" *p,p'*-DDE and HCB detected in adipose breast tissue. (B) GC-(QqQ)MS/MS chromatograms for *p,p'*-DDE and HCB in the same sample as in (A). (C) Experimental EI accurate mass spectra.
Q, quantitative ion/transition; q, confirmative ion/transition; St, reference standard; S, sample; ✓, Q/q ratio within tolerance limits.

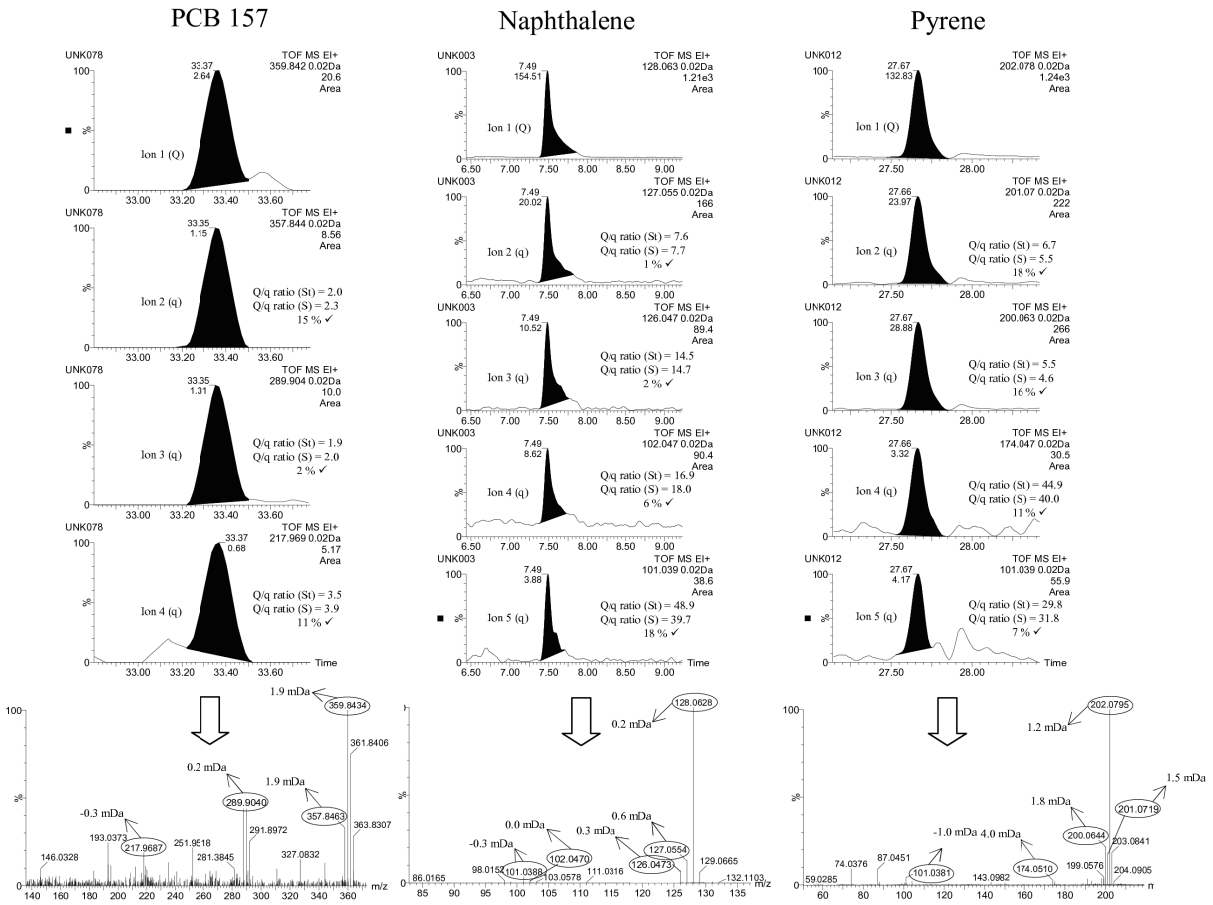


Figure 2. GC-TOF MS extracted ion chromatograms at different m/z (mass window 0.02 Da) for "post- target" PCB 157, naphthalene and pyrene detected in adipose breast tissue (top). Experimental EI accurate mass spectra (bottom).
Q, quantitative ion; q, confirmative ion; St, reference standard; S, sample; ✓, Q/q ratio within tolerance limits.

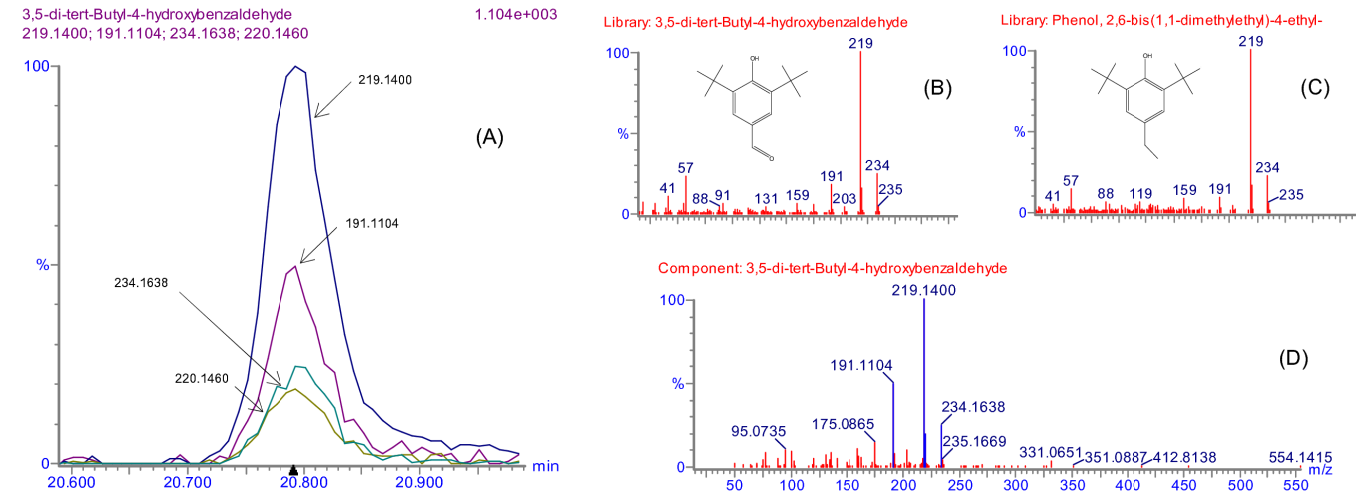
Nontarget screening

In this work, nontarget screening in human breast tissue extracts was carried out by applying the ChromaLynx Application Manager. This software automatically detected peaks with a response over user-defined parameters, displayed their deconvoluted mass spectra to be searched in the library, and produced a hit list with positive matches (library match > 700 was used as criterion). The formulas from the library hit were submitted to the elemental composition calculator and the five most intense ions were scored by exact mass measurement for the confirmation/rejection of the finding.[17]

All samples analyzed were processed using the described software. As shown in Table 1, several of the pre-target compounds were confirmed using this approach, in spite of the fact that no information or restriction was entered into the system, i.e. treating all compounds as actual unknowns. It is worth noting that the methodology employed was able to detect unknown components which were subsequently confirmed to be well-known OC contaminants. Most of detections corresponded to HCB, *p,p*-DDE and PCB 138, 153, and 180. The main limitation of this approach was its lower capability to discover the presence of components present at low concentrations in samples, only leading to satisfactory results in those cases where the components signal was significantly higher than background levels.[17] Obviously, this methodology is less powerful at low concentrations than target methods that have been purpose developed and validated, searching for the optimum analytical conditions for a limited number of target analytes. However, there are evident advantages for screening purposes because of much wider possibilities for detecting many other contaminants that would remain ignored in a target analysis.

The automated library search using extensive libraries (e.g. NIST) led to a large list of compounds identified using the nontarget approach. Within this large list, only a few, not included as target analytes, were considered as potential contaminants (Table 1). Thus, 3,5-di-tert-butyl-4-hydroxy-toluene (BHT) was identified in 31 out of 42 samples. BHT is a synthetic, highly lipid-soluble antioxidant added in rubber, petroleum products, and plastics, which is commonly used for preservation of food, cosmetics, and other lipid-containing products. This antioxidant has exhibited contradictory

actions on cancer growth as it has been shown to inhibit growth in some studies, and increase it in others. Therefore its toxicological implications are in permanent revision.[29] Several studies have already proved the presence of this compound in the aquatic environment,[30,31] food,[32-34] and in adipose tissue.[35] Some of its metabolites, such as 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO) and the dimer of BHT, 1,2-bis-(3,5-di-tert-butyl-4-hydroxyphenyl)ethane (2-BHT) seem to cause a human health risk. In our work, the metabolite BHT-CHO was also identified in one adipose tissue sample. Figure 3 shows the positive finding of BHT-CHO in adipose tissue sample. In this case, two library spectra fitted with the experimental spectrum (forward match of 724 for BHT-CHO, and 705 for 2,6-bis(1,14-dimethylethyl)-4-ethyl-phenol). However, the accurate mass scoring, automatically performed by the software for four representative ions led to the confirmation of the identity of BHT-CHO with mass errors below 2 mDa for three ions, and 3.2 mDa for the fourth. Mass errors were automatically calculated and they corresponded to the difference between the experimental accurate masses of the ions and the theoretical exact masses given by the elemental composition calculator.

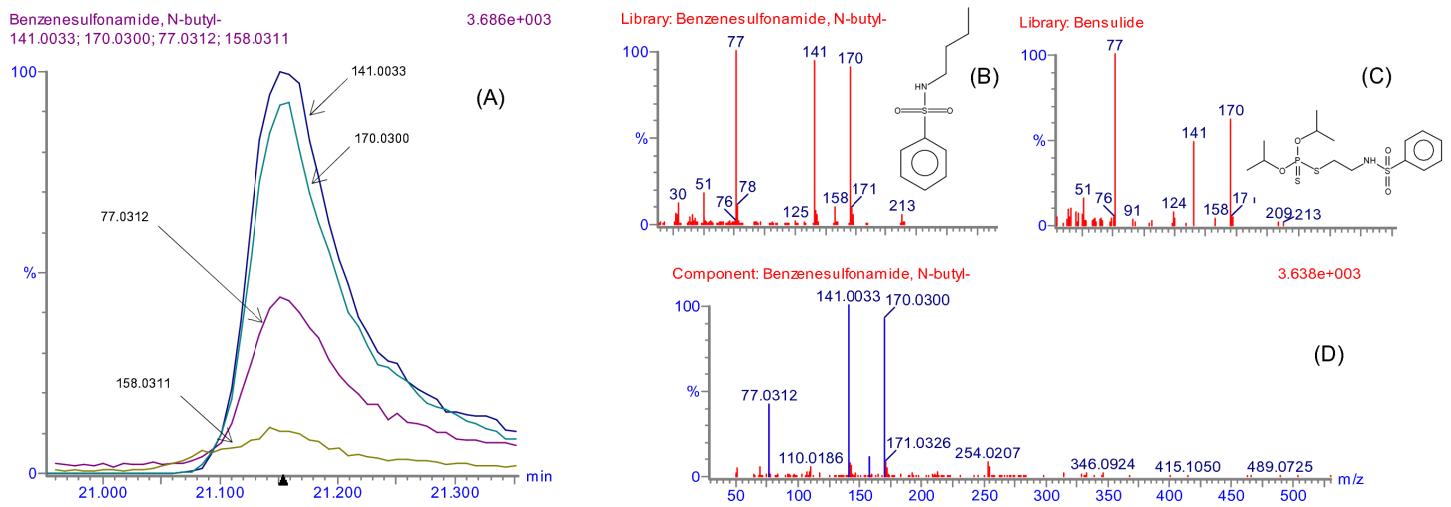


(E)

Compound	Molecular Formula	Molecular Mass	Forward Fit	m/z_1	m/z_2	m/z_3	m/z_4
3,5-di-tert-butyl-4-hydroxybenzaldehyde	$C_{15}H_{22}O_2$	234.1620	724	$C_{14}H_{19}O_2$ 219.1400 (1.5)	$C_{12}H_{15}O_2$ 191.1104 (3.2)	$C_{15}H_{22}O_2$ 234.1638 (1.8)	$C_{14}H_{20}O_2$ 220.1460 (-0.3)
2,6-bis(1,1-dimethylethyl)-4-ethyl-phenol	$C_{16}H_{26}O$	234.1984	705	$C_{15}H_{23}O$ 219.1400 (-34.9)	$C_{13}H_{19}O$ 191.1104 (-33.2)	$C_{16}H_{26}O$ 234.1638 (-34.6)	$C_{15}H_{24}O$ 220.1460 (-36.4)

Figure 3. Identification of non-target 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO) in an adipose tissue sample: (A) extracted-ion chromatograms for four BHT-CHO ions used for deconvolution. (B) Library mass spectrum of the candidate BHT-CHO at nominal mass. (C) Library mass spectrum of the candidate 2,6-bis(1,1-dimethylethyl)-4-ethyl-phenol at nominal mass. (D) Deconvoluted accurate mass spectrum of BHT-CHO in adipose tissue sample. (E) Library forward fit and accurate mass confirmation of 4 fragments; experimental accurate masses compared to theoretical exact masses (in brackets, mass errors in mDa) for the two possible candidates.

N-butyl benzenesulfonamide (N-BBSA) used in polyamide and copolyamide plastics and in the manufacturing of sulfonyl carbamate herbicides was found in one adipose tissue sample. Several authors have reported the presence of this compound, considered as neurotoxic to laboratory mammals, in the aquatic environment.[36-38] Fig. 4 shows the positive finding of N-BBSA in an adipose tissue sample using the nontarget approach. In this case, two library spectra fitted with the experimental one (forward match of 727 for N-BBSA, and 695 for bensulide), with low mass errors (2.4 mDa) for the three most abundant ions for both possible candidates, making the selection of the right structure troublesome. However, using a software option based on an isotope prediction filtering (i-FIT) of the sulfur atom, it was possible to discard the wrong structure. The filtering was carried out for two fragments with different number of sulfur atoms in the candidates (m/z1 141.0033 and m/z2 170.0300). As shown in Fig. 4, both selected fragments fitted better with the presence of only one sulfur atom in the molecule (the lower the i-FIT value the better the fit). Consequently, N-BBSA was selected as the appropriate structure which was subsequently confirmed by the retention time match when injecting a reference standard in solvent.



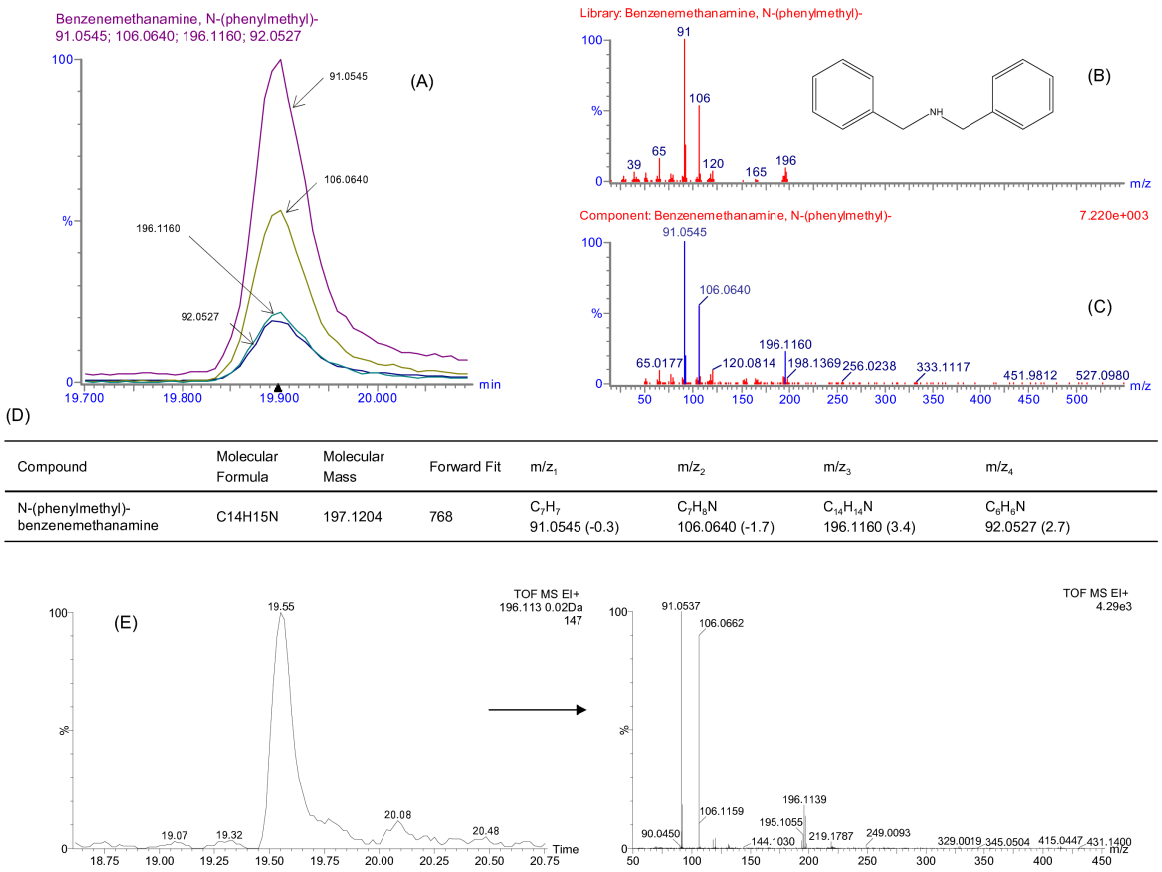
(E)

Compound	Molecular Formula	Molecular Mass	Forward Fit	m/z_1	i-FIT	m/z_2	i-FIT	m/z_3
N-butyl-benzenesulfonamide (N-BBSA)	$C_{10}H_{15}NO_2S$	213.0824	727	$C_6H_5O_2S$ 141.0033 (2.3)	4.0	$C_7H_8NO_2S$ 170.0300 (2.4)	2.3	C_2H_7NS 77.0312 (1.3)
Bensulide	$C_{14}H_{24}NO_4PS_3$	397.0305	695	$C_3H_9O_2S_2$ 141.0033 (-1.1)	57.5	$C_4H_{12}NO_2S_2$ 170.0300 (-0.9)	67.0	C_2H_7NS 77.0312 (1.3)

Figure 4. Identification of non-target N-butyl benzenesulfonamide (N-BBSA) in an adipose tissue sample: (A) extracted-ion chromatograms for four N-BBSA ions used for deconvolution; (B) Library mass spectrum N-BBSA at nominal masses; (C) Library mass spectrum of bensulide; (D) Deconvoluted accurate mass spectrum of N-BBSA from the adipose tissue sample; (E) Library forward fit and accurate mass confirmation of 4 fragments for experimental accurate masses compared to the theoretical exact masses (in brackets, mass errors in mDa) for two possible candidates.

Two naphthalene-related compounds (2-methylnaphthalene and 1,2-dimethylnaphthalene) were also identified in a few samples (Table 1). The bioaccumulation of these PAHs in mussels has been investigated by other authors.[39] Additionally, positive findings of other PCB congeners, not included in the pre-target and post-target list, were detected in several samples. As no standards of these PCB congeners were available in the laboratory, the identification of each individual congener was not feasible using retention times, but the library fit and the accurate mass confirmation of up to five ions allowed confirmation of these congeners which contained 4, 5, 7, and 8 chlorine atoms.

In addition, ten of the samples were submitted to a second extraction with 5-ml ethyl acetate after extraction with hexane. After preconcentration to 1 ml, the ethyl acetate extracts were directly injected into the GC-TOF MS for both target and nontarget analysis, with the objective of investigating the presence in the samples of more polar compounds. N-(phenylmethyl)-benzenemethanamine, also called dibenzylamine, a thermal decomposition product of the vulcanization agent zinc dibenzyldithiocarbamate and a possible precursor to the formation of N-nitrosodibenzylamine,[40] was discovered in four out of ten ethyl acetate extracts. Dibenzylamine has also been found by other authors in artificial saliva leachates from baby bottle teats[40] and in human-plasma and urine samples.[41] Fig. 5 shows a positive of dibenzylamine in an adipose tissue sample when using CODA and the deconvolution process. Accurate mass confirmation automatically performed by the software for four representative ions led to the confirmation of the identity of dibenzylamine with mass errors always below 3.4 mDa. For additional confirmation, a standard of this compound was acquired and injected in the system to check the retention time and spectrum and to unequivocally confirm the presence of this compound in the sample (Fig. 5).



(D)

Compound	Molecular Formula	Molecular Mass	Forward Fit	m/z_1	m/z_2	m/z_3	m/z_4
N-(phenylmethyl)-benzenemethanamine	C14H15N	197.1204	768	C_7H_7 91.0545 (-0.3)	C_7H_8N 106.0640 (-1.7)	$C_{14}H_{14}N$ 196.1160 (3.4)	C_6H_6N 92.0527 (2.7)

Figure 5. Identification of non-target N-(phenylmethyl)-benzenemethanamine, (dibenzylamine) in an adipose tissue sample by GC-TOF MS: (A) extracted-ion chromatograms for four dibenzylamine ions used for deconvolution. (B) Commercial library mass spectrum of dibenzylamine at nominal mass. (C) Deconvoluted accurate mass spectrum of dibenzylamine from the adipose tissue sample. (D) Library forward fit and accurate mass confirmation of 4 fragments; experimental accurate masses compared to theoretical exact masses (in brackets, mass errors in mDa). (E) Extracted ion chromatogram at m/z 196.1126 for a solvent standard of dibenzylamine (100µg/L) and its corresponding experimental EI TOF spectrum.

CONCLUSIONS

GC-TOF MS has been proved to be a rapid and efficient technique for the screening and confirmation of anthropogenic contaminants in human breast adipose tissues. The evaluation of up to 5 mw-XIC (0.02 Da) at selected m/z ions and the attainment of their Q/q intensity ratios allowed the detection of several target compounds (HCB, β-HCH, *p,p*-DDE, trans-nonachlor, and some PCBs). These were within a group of 30 selected analytes for which the extraction and cleanup procedures had been previously validated. Additionally, after MS data acquisition, the presence of around 100 additional compounds was investigated in a post-target way, without the need to reanalyze the samples. In this way, several PAHs (naphthalene, phenanthrene, fluoranthene, pyrene) and some PCB congeners (114, 123, 156, 157, 167 and 189) were found in several samples. In both cases, searching for the presence of the ions, measured at accurate mass, was performed in an automated and simple way using potent software. From the list of 30 pre-target analytes, a total number of 187 positives found in previous analyses performed in human breast tissues by GC-(QqQ)MS/MS with QqQ were investigated by GC-TOF MS. One hundred thirty four of these positives were confirmed by GC-TOF MS, the difference being due to the lower sensitivity of this technique compared to GC-(QqQ)MS/MS in SRM mode, which hampered some detections at low analyte concentrations. However, the possibility of performing a post-target screening as a consequence of the full spectrum acquisition in TOF MS allowed the identification of other selected contaminants, like some PAHs and other PCB congeners, which had not been included in the initial list of target analytes and consequently could not be investigated by GC-MS/MS, in this way illustrating the potential of GC-TOF MS for screening purposes.

The application of a (CODA) and subsequent deconvolution software has been found to be an attractive way to perform nontarget screening. This has allowed the discovery of several compounds that were not included in any of the lists of target analytes, like BHT, BHT-CHO, dibenzylamine, N-BBSA, 1,2-dimethylnaphthalene, 2-methylnapththalene and other PCB congeners. The methodology applied in nontarget analyses also allowed the detection and confirmation of a notable number of positives found in previous target analyses, i.e. the system was able to detect and confirm several compounds present in samples in spite of the fact that they were treated as

unknowns. These findings were only feasible when analyte concentration was relatively high. Thus, the screening of anthropogenic contaminants in biological samples, where samples and analytes are treated as unknowns using component detection algorithm and deconvolution software, may not be completely satisfactory at the moment as the success of this approach gets notably worse at low concentrations. Both target analysis, focused on priority contaminants, and nontarget analysis, is complementary and both are required to obtain the maximum sample composition information possible.

The use of nonspecific libraries (e.g. NIST) leads to a large list of possible components in the samples, the majority of them irrelevant for the screening purposes. This fact makes the selection of relevant compounds arduous for the analyst, as lists of many potential candidates have to be reviewed before the presence of anthropogenic contaminants can finally be reported. The availability of specific libraries purpose made for the type of research performed would make the work more user-friendly and would facilitate the discovery of contaminants in samples.

ACKNOWLEDGEMENTS

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of the time-of-flight mass spectrometer. This work forms a part of the project CTQ2006-07594 developed with the financial support of Ministerio de Ciencia y Tecnología and the project P1-1B2005-08 financed by Universitat Jaume I-Fundación Bancaixa. T. Portolés is very grateful to Ministerio de Educación y Ciencia for her predoctoral grant. The authors acknowledge Dr Carlos Vázquez (FIVO) for the supply of samples.

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SUPPORTING INFORMATION

Table S-1. List of target compounds

COMPOUNDS INVESTIGATED AS PRE-TARGET ANALYTES
BDE 99
BDE 100
β-HCH
cis-Chlordane
cis-Nonachlor
Dieldrin
Endosulfan ether
Endosulfan sulfate
Heptachlor
Heptachlor epoxide A
Hexachlorobenzene
Lindane
Metoxychlor
Mirex
Oxychlordane
p,p'-DDD
p,p'-DDE
p,p'-DDT
PCB 28
PCB 52
PCB 101
PCB 118
PCB 138
PCB 153
PCB 180
Pentachlorobenzene
trans-Chlordane
trans-Nonachlor
Vinclozolin

COMPOUNDS INVESTIGATED AS POST-TARGET ANALYTES

α-Endosulfan	Dimethoate	Simazine
4-n-Nonylphenol	Diphenylamina	*tau*-Fluvalinate
4-n-Octylphenol	Fenitrothion	Terbacil
4-t-Octylphenol	Fenoxycarb	Terbumeton
Acenaphthene	Fenthion	Terbumeton desethyl
Acenaphthylene	Fenvalerate	Terbuthylazine desethyl
Alachlor	Fluoranthene	Terbuthylazine
Aldrin	Fluorene	Terbutryn
Anthracene	Heptachlor epoxide B	Thiabendazole
Atrazine	Hexythiazox	Trifluraline
Atrazine desethyl	Imazalil	
Atrazine desisopropyl	Indeno(1,2,3,cd)pyrene	
Azinphos-methyl	Isodrin	
BDE 138	*lambda*-Cyhalothrin	
BDE 153	Malathion	
BDE 154	Metalaxyl	
BDE 183	Metamidophos	
BDE 28	Methidathion	
BDE 47	Methiocarb	
BDE 66	Methiocarb sulfone	
BDE 71	Metolachlor	
BDE 85	Molinate	
β-Endosulfan	Naphthalene	
Benzo(a)anthracene	Omethoate	
Benzo(a)pyrene	Oxadixyl	
Benzo(b)fluoranthene	Parathion-ethyl	
Benzo(g,h,l)perylene	Parathion-methyl	
Benzo(k)fluoranthene	PCB 105	
Bifentrin	PCB 114	
Buprofezin	PCB 123	
Carbaryl	PCB 126	
Chlorfenvinphos	PCB 156	
Chlorpropham	PCB 157	
Chlorpyriphos	PCB 167	
Chlropyriphos methyl	PCB 169	
Chrysene	PCB 189	
Cyfluthrin	PCB 77	
Cypermethrin	PCB 81	
Cyprodinil	Permethrin	
Deltamethrin	Phenanthrene	
Diazinon	Phosmet	
Dibenzo(a,h)anthracene	Pirimicarb	
Diclorvos	Pirimiphos methyl	
Diflufenican	Pyrene	

3.5 APLICACIÓN DE GC-TOF MS PARA LA ELUCIDACIÓN DE COMPUESTOS MEDIANTE EL USO COMBINADO DE FUENTES DE IONIZACIÓN ELECTRÓNICA E IONIZACIÓN QUÍMICA.

En este capítulo se estudia el poder del GC-TOF MS, mediante el uso combinado de las fuentes de ionización electrónica e ionización química, para la elucidación de compuestos que quedan sin identificar en los análisis *non-target* como consecuencia de no encontrarse sus espectros EI en las librerías comerciales utilizadas. Se utiliza la fuente de CI (tanto en modo de ionización positiva como negativa) como técnica que facilita la identificación del ion molecular, información que resulta imprescindible para elucidar un compuesto desconocido. Una vez identificado el ion molecular, se procede a la elucidación de la composición elemental para esa masa, que en el caso de utilizar la técnica GC-TOF MS, está medida con elevada exactitud.

Para una masa exacta concreta, el número de posibles composiciones elementales incrementa con el aumento en la masa y, en la mayoría de los casos, no es posible la asignación de una única composición elemental. Sin embargo, existen otras informaciones que también pueden utilizarse y que ayudan a limitar el número de posibles candidatos:

- Relaciones isotópicas: El estudio del patrón de distribución isotópica observado en el espectro experimental ofrece información acerca de la presencia de heteroátomos en la molécula, principalmente, cloro, bromo y azufre, y también ayuda a acotar el número de átomos de carbono presentes en la molécula.
- Regla del nitrógeno: Según esta regla, todas las sustancias orgánicas con peso molecular par deben contener un número par o ningún átomo de nitrógeno y las de número impar deben contener un número impar de átomos de nitrógeno. La fuente de ionización electrónica arranca un electrón de la estructura de la molécula y genera iones moleculares ($M^{+\cdot}$) variando la masa de la molécula mínimamente. Estos iones moleculares contienen un número impar de electrones (*odd-electron ions*, OE). Así pues, ante el ion molecular generado por la fuente de ionización electrónica ($M^{+\cdot}$), la regla del nitrógeno se aplica tal y como se ha descrito anteriormente para moléculas neutras. Por el contrario, la fuente de ionización química en modo positivo añade un protón a la estructura de la molécula ($M+H^+$), variando la masa de la molécula en una unidad. Estos iones contienen un número par de electrones (*even-electron ions*, EE). Así pues si el

ion observado corresponde al pico molecular generado por la fuente de CI en modo positivo ($M+H^{+\cdot}$), la regla del nitrógeno descrita anteriormente aplica a la inversa que para moléculas neutras y para iones OE. Es decir, si el ion M+1 observado en un espectro de CI positivo posee una masa par, el compuesto contendrá un número impar de átomos de nitrógeno, y viceversa. Con respecto a los fragmentos generados en la fuente, tanto en EI como en CI, estos conservaran la paridad del ion precursor si son fragmentos consecuencia de pérdidas neutras, mientras que si, por el contrario, son consecuencia de la pérdida de un radical, el ion fragmento cambiará la paridad con respecto al ion precursor. Estas consideraciones serán muy útiles a la hora de descartar posibles composiciones elementales durante el proceso de elucidación de un compuesto desconocido.

- Iones fragmentos: Estos proporcionan información estructural de gran valor para la elucidación de la molécula. Combinando la masa exacta correspondiente al peso molecular con la información de masas exactas de los fragmentos observados, es posible obtener importante información al respecto de la estructura de interés. La masa de los fragmentos será siempre menor que la masa del ion precursor y en consecuencia las posibles composiciones elementales serán menores. Los iones fragmento no solo ayudan a descartar posibles composiciones elementales para el compuesto desconocido, sino que son cruciales en el proceso de confirmación de la estructura finalmente propuesta.
- Grado de instauración: Corresponde al número de anillos y/o enlaces dobles que deben estar presentes para una composición elemental dada y es calculado automáticamente por el software del instrumento.

Así pues, mediante el uso de las masas exactas del ion molecular y de los iones fragmento y con las pautas indicadas anteriormente, en muchos casos, se puede llegar a la propuesta de una composición elemental. El siguiente paso consiste en buscar la composición elemental propuesta en bases de datos con el fin de proponer una estructura química para el compuesto. Una vez encontrada la estructura, se procede a la justificación de los fragmentos observados en el espectro de EI. Si todo encaja, el último paso sería comprar el patrón comercial para confirmar su identidad, mediante el espectro experimental y el tiempo de retención. En el **artículo científico 8** se aplica el uso combinado de las fuentes de EI y CI para la elucidación de la identidad de

contaminantes orgánicos en muestras de agua. Para ello se seleccionan plaguicidas modelo, escogidos debido a que sus espectros de ionización electrónica no estaban disponibles en la librería comercial utilizada. Se toma un extracto de agua subterránea, procesado por SPE, y se fortifica con una mezcla de plaguicidas seleccionados al nivel de 1µg/mL. El extracto resultante se inyecta por GC-TOF MS bajo tres modos de ionización diferentes (EI, CI positivo y CI negativo). Los datos obtenidos se procesan y se tratan como desconocidos. Se estudia las posibilidades de la metodología desarrollada para llegar a la identificación de los compuestos detectados.

Algunos autores han reportado aplicaciones en este campo mediante el uso de GC-TOF MS (1-3). En unos primero trabajos se utilizó GC-TOF MS para la identificación de compuestos desconocidos en extractos de agua. Estos primeros experimentos mostraron que la técnica GC-TOF MS no era tan poderosa para determinar composiciones elementales como lo era la técnica de "*double-focusing*". Seguramente, esto se debió a que la resolución de los primeros instrumentos TOF era alrededor de 5000 FWHM, muy inferior a la de los sectores magnéticos (generalmente > 10000) (4). Más recientemente, se ha utilizado la técnica GC-TOF MS con fuente de APCI para la identificación de más de más de 300 compuestos en muestras de fluido cerebroespinal humano. El uso de la masa exacta, junto con el estudio de distribución del patrón isotópico, permitió la identificación de algunos compuestos presentes en las muestras (5). Asímismo, la combinación de fuentes de ionización fuertes (como EI) y suaves (como CI, FI) ha sido reportada en unos pocos artículos científicos. La combinación de FI y EI resulta atractiva en el campo de la metabolomica, donde muchas estructuras no pueden ser fielmente identificadas por búsquedas en librerías (6).

3.5.1. Bibliografía

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3.5.2 Artículo científico 8

Journal of The American Society for Mass Spectrometry (submitted)

USE OF SOFT AND HARD MS IONIZATION TECHNIQUES FOR UNKNOWN COMPOUNDS ELUCIDATION BY GC-TOF MS

Tania Portolés[1], Elena Pitarch[1], Francisco J. López[1], W.M.A. Niessen[2], Félix Hernández[1]

[1]Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain

[2] hyphen MassSpec, de Wetstraat 8, 2332 XT Leiden, the Netherlands

Tel: 34-964-387366, Fax: 34-964-387368, E-mail: felix.hernandez@qfa.uji.es

ABSTRACT

Investigation of trace level non-target compounds by GC-MS is a challenging task that requires powerful software to detect the presence of unknown components and to obtain the deconvoluted MS spectra to be searched in standarized libraries. When no library match, or unacceptable match, is obtained the elucidation process becomes more difficult. One of the main problems is the absence of the molecular ion in many EI spectra. The use of soft-ionization techniques (CI, FI or APCI) facilitates the identification of the molecular ion. Thus, the elucidation process by using GC-MS normally requires the combined use of hard and soft ionization techniques, and/or performing tandem MS experiments, preferably by (Q)TOF MS. Based on accurate mass measurements of the molecular and fragment ions given by TOF MS, an empirical formula can be proposed. Isotopic patterns, carbon number prediction filter and nitrogen rule are helpful to reduce the number of possible formulae. Then, the candidate formulae can be searched in databases and a chemical structure can hopefully be proposed. Accurate masses of fragment ions are important in this process, and their structures should be compatible with the chemical structure assigned to the candidate.

In this paper, the complementary use of EI and CI is investigated in combination with GC-TOF MS for the elucidation of organic non-target (micro)contaminants in water samples. Several examples are shown to illustrate the methodology applied and the difficulties of this process when the MS spectra of the compounds investigated are not available in commercial libraries used in the laboratory.

INTRODUCTION

Gas chromatography coupled to mass spectrometry (GC-MS) is one of the most powerful techniques for detection, identification and quantification of volatile and semi-volatile contaminants and residues in environmental, biological and food matrices. In these applications, electron ionization (EI) is the most widely used ionization technique. The ability of an EI source to produce highly reproducible fragmentation spectra allows to obtain valuable structural information on the molecules and the generation of large spectral libraries highly useful for qualitative analysis. This allows compound identification based on matching experimental spectra to mass spectral databases libraries. The identification process gains power when the *m/z* values of the ions in the EI spectrum are measured with high mass accuracy, as occurs when using high resolution time-of-flight mass spectrometry (TOF MS) analyzers. Under these circumstances, the compounds identified by library matching can be confirmed by accurate mass measurements of the fragment ions and the molecular ion (if present in the EI spectrum) and can solve ambiguous results in library search [1]. The versatility of large libraries lies in the fact that EI mass spectra are comparable over a wide range of different types of mass spectrometers from different vendors, although quadrupoles may be tune to preferentially transmit high *m/z* ions, which thus result in slightly different ion abundance from those in TOF MS spectra [2]. This fact together with the high degree of fragmentation normally generated by this "hard" ionization technique can be a trouble when the EI experimental spectrum does not yield a conclusive library match. This situation may occur for many compounds (new emerging contaminants, transformation products, not regulated compounds, etc) that are not included in available libraries, which makes the identification process more difficult. Under these circumstances alternative ways of identification are needed.

The presence of the molecular ion in the MS spectrum, especially if measured at accurate mass, is a valuable tool as it provides indispensable information about the identity of the unknown compound. For these purpose, "soft" ionization techniques that produce spectra with less fragmentation and keep the molecular ion intact are required. Examples of 'soft' ionization techniques are chemical ionization (CI), negative ion chemical-ionization (NICI), field ionization (FI), and atmospheric pressure chemical ionization (APCI) [2-5]. Once the molecular ions have been identified, accurate mass and isotope data can be used to calculate formulae. The exact mass differences between ions can also be used to confirm the identity of fragmentation pathways. The difference in mass due to the loss of a specific functional group is often relatively small. Formulae based on these mass differences are very specific because of the reduced number of combinations of elements possible. This allows unambiguous assignments of the losses within the spectrum. Conversely, this information can be used to confidently determine the formula of the molecular ion in an unknown sample.

Only a few authors have reported examples on elucidation of compounds when their library mass spectra are not available. Thus, GC-TOF MS has been used for the identification of unknown compounds detected in extracts of well water. These first experiments showed that GC-TOF MS instrument was not as powerful for determining ion compositions as double-focusing mass spectrometers, perhaps due to the fact that resolution of early TOF instruments (~5000) was by far not as good as that of double focussing-sector instruments (generally >10.000) [3]. More recently, a method based on the use of GC-TOF MS with APCI source has been optimized for 31 compounds (amino acid, organic acids, alcohols, xantines, etc) for which the standard mixture was available. It was applied to human cerebrospinal fluid (CSF) samples for metabolic profiling. More than 300 compounds with different isotopic features were determined in the CSF samples. The identity of some of those peaks could be corroborated by the standards included in the mixture (comparing retention time, mass position, and isotopic pattern of standard an samples). When no standard was available, only mass position and isotopic distribute was used to achieve the identification of the analytes present in the CSF according to their molecular formulae [4]. The combination of hard and soft ionization techniques for elucidation purposes has been applied in a few papers. Despite the lower ion yields obtained by FI, the superiority of this method when coupled with TOFMS in producing molecular ions and chromatograms with good S/N was

impressive. When this is considered along with the ability to accurately measure the molecular mass to narrow down the possible formulae of an unknown, FI in combination with EI was very attractive. It showed a potential benefit in fields like metabolomics where structures that cannot be confidently identified by library matching or interpretation of EI spectra alone are regularly encountered [5].

In metabolomic applications that use GC-(EI)MS, the low abundance of the molecular ions normally impedes the calculation of formulae for the identification of unknowns. On changing the beam-steering voltage of the ion source, the relative abundances of molecular ions at 70 eV were increased up to ten-fold for alkanes, fatty acid methyl esters and trimethylsilylated metabolites, concomitant with 2-fold absolute increases in ion intensities [2]. Next, the abundance, mass accuracy and isotope ratio accuracy of molecular species in EI has been compared with those in CI with methane as reagent gas under high-mass tuning. When constraining lists of calculated elemental compositions by chemical and heuristic rules using the Seven Golden Rules algorithm and PubChem queries, the correct formula was retrieved as top hit in 60% of the cases and within the top-3 hits in 80% of the cases [2].

Other applications reported in the non-target field deal with the identification of impurities generated in organic synthesis or in flavor research using the accurate mass measurements provided by TOF MS. This allowed the elucidation of compounds that could not be identified when applying GC-quadrupole systems [6,7].

In this paper, EI and CI sources have been applied for the elucidation of the identity of organic contaminants in water samples. The model compounds investigated corresponded to pesticides, that have been chosen because their MS spectra are not registered in the commercial library available in our laboratory. The $[M+H]^+$ in methane positive CI spectrum was usually abundant and often represented the base peak of the spectrum. The degree of fragmentation of $[M+H]^+$ ions was much lower than under 70 eV EI conditions, as the extent of exothermicity of the protonation in CI is lower, resulting in internal energy minor than in EI.

EXPERIMENTAL

Reagents

Reference standards of pesticides were purchased from Dr. Ehrenstorfer (Augsburg, Germany). From solid reference standards, stock solutions (around 500 μg/mL) were prepared by dissolving reference standards in acetone and stored in a freezer at –20°C. Working solutions were prepared by diluting stock solutions in hexane for extract fortification and injection in the chromatographic system. Acetone (residue analysis), ethyl acetate, dichloromethane (DCM) and hexane (ultra-trace quality) were purchased from Scharlab (Barcelona, Spain). About 500 mg Bond Elut cartridges C_{18} (Varian, Harbor City, CA, USA) were used for solid-phase extraction.

Instrumentation

For the GC instrumentation, an Agilent 6890N GC system (Palo Alto, CA, USA) equipped with an Agilent 7683 autosampler was coupled to a GCT time-of-flight mass spectrometer (Waters Corporation, Manchester, UK), operating in EI and CI modes. The instrument was operated under MassLynx version 4.1 (Waters Corporation)

The GC separation was performed using a fused silica HP-5MS capillary column with 30 m x 0.25 mm i.d. and a film thickness of 0.25 μm (J&W Scientific, Folson, CA, USA). The oven temperature was programmed as follows: 90ºC (1 min); 5ºC/min to 300ºC (2 min). Splitless injections of 1 μL sample were carried out. Helium was used as carrier gas at 1 mL/min.

The interface temperature was set to 250ºC and the source temperatures were set to 250ºC and 100 ºC for EI and CI source, respectively. A solvent delay of 3 minutes was selected. TOF MS was operated at 1 spectrum/s acquiring the mass range *m/z* 50-650 and using a multi-channel plate voltage of 2800 V. TOF-MS resolution was about 8500 (FWHM) at *m/z* 614. Heptacosa, used for the daily mass calibration, was injected via syringe in the reference reservoir at 30ºC for this purpose. Additionally, heptacosa was used as a lock mass correction for EI experiments (monitoring the ion with *m/z* 218.9856); tris-(trifluoromethyl)-triazine for positive CI experiments (monitoring the ion with *m/z* 286.0027); and chloropentafluorobenzene for negative CI experiments

(monitoring the ion with *m/z* 201.9609). Methane was used as reagent gas in the CI source.

General methodology

250 mL of groundwater were passed through a 500 mg C_{18} SPE cartridge previously conditioned. After loading the sample, cartridges were washed with 3 mL water, air-dried using vacuum for at least 15 min, and then eluted with 5 mL ethyl acetate:DCM (50:50). The extract was evaporated to dryness under a gentle nitrogen stream at 40°C and redissolved in 0.5 mL hexane. The final extract obtained was spiked with a mixture of selected pesticides at a concentration of 1 µg/ml (adding 10µl of 50 µg/ml standard) and it was injected into the GC-TOF MS. Three different injections were carried out, one for each ionization mode employed (EI, positive CI, negative CI). Then, TOF MS full-acquisition data were processed, treating the sample as unknown, i.e., using non-target processing method [8-10].

Data processing

In the first place, EI data were processed in a non-target way by applying the ChromaLynx Application Manager, a module of MassLynx software. This software automatically detects peaks with a response over user-defined parameters, displays their deconvoluted mass spectra, searches them against the commercial nominal mass NIST library (2.0, US), and produces a hit list with positive matches (library match >700 was used as criterion). After that, an accurate-mass confirmation of the library search is automatically performed. The formulae from the top-five library hits is submitted to an Elemental Composition Calculator, and the accurate mass measurements of up to five most intense ions are evaluated to test whether they are in accordance with the proposed formula, with the aim of confirm or reject the finding. Components that showed a library match < 700, *e.g.* those that were probably not registered in the NIST library, were selected for elucidation purposes.

All the samples were re-injected into the GC-MS system using the CI source in positive and negative mode. These data were used to identify the intact molecule. Once the intact molecule was identified from the GC-CI-MS data, the accurate mass for

the protonated molecule was submitted to the calculation of all possible formulae with a maximum deviation of 5 mDa from the measured mass using the Elemental Composition Calculator. Parameter settings for all calculations were C: 0–30, H: 0–50, N: 0–10, O: 0–10, and P: 0–3. In principle, no F atoms were considered, as this would considerably increases the number of possible elemental compositions, which would complicate the elucidation. However, when consistent evidences on the presence of F atoms in the molecule were observed in the experimental MS data (loss of F• or HF), the presence of F (0–10) was considered during the elucidation step. In addition, from the characteristic isotopic patterns associated to ^{37}Cl (31.98% relative abundance), ^{81}Br (97.88%) and ^{34}S (4.44%), the appropriate number of Cl, Br and S atoms was evaluated and added. The number of Cl and Br atoms was easily adjusted. However, the lower relative abundance of ^{34}S made the adjustment of S atoms less precise, especially when halogens were also present. In these cases, an interval was given. The accepted deviations between the experimental and the theoretical values were set up in accordance with a previous work in our research group. Briefly, when the abundance of an isotopic peak was between 60 and 200 counts, the observed accepted deviation was 20%, and when the abundance was higher than 200 counts, the error decreased to below 10%.[11]

A carbon number prediction filter of ±5 was applied to reduce the number of possible elemental compositions for a particular mass if the intensity of the molecular ion in the spectrum was higher than 300 counts. The double-bond equivalent (DBE) parameter was set from -1.5 to 50, but was not used as an identification criterion, although information about aromaticity of the structure was obtained. Additionally, the option ‘‘even-electrons ions only’’ was selected for the (de)protonated molecule in CI ionization data. Fragment ions present in the CI spectrum were used to enable a further reduction in the number of possible molecular formulae; the option “odd and even-electron ions” was used for this purpose. Also, accurate mass EI fragmentation data were used to reduce the number of possible molecular formulae. The option “odd-electron ions only” was selected for the molecular ion in EI data (if it existed) and ‘‘odd- and even-electrons ions’’ was used for the fragment ions. Similarly, a carbon number prediction filter of ±5 was applied to reduce the number of possible formulae in the spectrum if the intensity of the ion in the spectrum was higher than 300 counts. It is worth to notice that, in the case of fragment ions, the carbon filter should applied

with care as an additional McLafferty rearrangement might occur during the fragmentation process and (apparently) disturb the expected isotopic pattern. Once a formula was elucidated, it was searched in databases. We have chosen the Reaxys database, a web-based search and retrieval system for chemical compounds, bibliographic data and chemical reactions, that contains more that 18.000.000 substances [12]. In some cases, EI spectra provide valuable information about a substructure of the unknown. Reaxys allows limiting the search of a formula taking into account a substructure. This notably reduces the number of possible structures for a given formula. Structures finally suggested were evaluated based on the fragmentation patterns observed in the EI and CI spectra.

RESULTS AND DISCUSSION

Accurate masses alone do not allow the retrieval of correct elemental formulae due to the large search space of chemically possible solutions. So, a combination of different rules that constrains and scores all chemically possible formulae based on accurate mass measurements, the formulae proposed, their isotopic patterns, carbon number prediction filter and nitrogen rule, among other, are necessary.

In the process of chemical identification of unknown compounds, it is important to obtain overall high signal intensities for molecular ions (or defined adducts or fragments of molecular ions) and therefore optimal signal-to-noise ratios for each peak. Higher signal intensities yield better ion statistics, thus improving accurate mass and isotopic abundance measurements, which subsequently lead to higher confidence in determining elemental compositions. Electrophilic addition in positive-ion CI fairly often gives rise to $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$ adduct ions next to $[M+H]^+$. Thus, [M+29.0391] and [M+41.0391] peaks may be observed in addition to the expected [M+1.0078].

Carbon filtering

The Elemental Composition Calculator within the MassLynx software allows calculating possible formulae using predefined parameter settings. Among these parameters, the element prediction filter applied to estimate the number of carbons of

the unknown structure reduces considerably the number of suggested formulae returned by the program. For this purpose, a carbon range must be defined by the user to exclude all suggestions that fall outside an estimated range of carbon atoms for the molecule of interest. The number of carbon atoms in a molecule can be estimated by considering the relative intensity of the "M+1" isotope peak which, in the absence of Si, is mainly due to the presence of $^{13}C_1$. With the carbon filtering, the Calculator returns only those results that include the estimated number of carbons, plus or minus the number of carbons entered by the user. An incorrect use of this option can unwittingly exclude the correct composition if the experimental data does not correctly reflect the mass and isotope pattern of the compound. In order to asses the most appropriate carbon range to be applied, a systematic study was carried out on the error in the estimation of the number of carbons in a molecule from the "M+1" peak using a series of standards in solvent. A mixture of several pesticides (dichlorvos, lindane, diazinon, chlorpyriphos methyl, pirimiphos-methyl, fenthion, simazine, terbutylazine, diphenylamine and molinate) at different concentration levels were injected into the GC-MS system under positive ion CI conditions. Considering the relative intensity of "M+1" isotope peaks, which under these conditions is the peak with *m/z* of $[M+H]^+$+1, the experimental number of carbons of the molecule was estimated and compared with real value for the target molecule. Experimental results showed that when the peak intensity was below 300 counts, no M+1 could be observed in the spectrum. In such cases, no carbon filter could be applied. When the intensity of the peak was higher than 300 counts, the estimated number of carbons generally did not differ by 3 or 4 from the true value. This led us to conclude that a carbon filter of ±5 would be a good choice (for peaks with intensities higher than 300 counts).

Selected examples

In this paper, we show selected examples for the elucidation of model compounds, which have been chosen because their MS spectra are not registered in the commercial library available in our laboratory. The pesticides selected were bifenazate, boscalid, epoxiconazole, and fenhexamid. A ground water extract was spiked with a mixture of these pesticides and injected into the GC-TOF MS, under EI and CI conditions. Then, TOF MS full-acquisition data were processed treating the sample as unknown, i.e.

a non-target processing method was applied without using any previous information on analyte identity.

Case 1

From the EI data and applying a non-target screening approach in the ChromaLynx software, a chromatographic peak with a retention time 31.6 min was found that returned a match of 670 in the NIST library search, indicating the compound is 1,1'-biphenyl, 4-methoxy (M 184,0875 Da). The accurate mass of the protonated molecule of this unknown compound was determined to be *m/z* 301.1563 from the CI+ spectrum (Figure 1A), indicating the match from the library search is not correct. Within the search limits outlined above, calculation of the possible elemental compositions resulted in 13 formulae. When applying the carbon filter, 5 formulae remained (Figure 1B). A fragment ion with *m/z* 259.1068 present in the positive CI spectrum corresponds to the loss of 42.0495 Da that could be due to the loss of C_3H_6 (42.0470).

Looking at the EI spectrum (Figure 1A, top), the major fragment is an ion with *m/z* 184.0881. For this *m/z*, 13 possible formulae are obtained, which number reduces to only 3, if the carbon filter is applied (Figure 1B). Other fragment ions present in the EI TOF MS spectrum could be considered as subsequent losses of $CH_3^{\bullet}$ (*m/z* 169.06578), CO (*m/z* 141.0708) and C_2H_2 (*m/z* 115.0551), which allowed us to discard 1 out of 3 formulae for the ion with *m/z* 184.0881 (the one without O) remaining $C_{13}H_{12}O$ or $C_9H_{15}NOP$. These two formulae allowed us to discard 1 out of 5 initial formulae calculated from the unknown protonated molecule, remaining $C_{13}H_{17}N_8O^+$, $C_{15}H_{26}O_4P^+$, $C_{17}H_{21}N_2O_3^+$, and $C_{14}H_{27}N_2OP_2^+$.

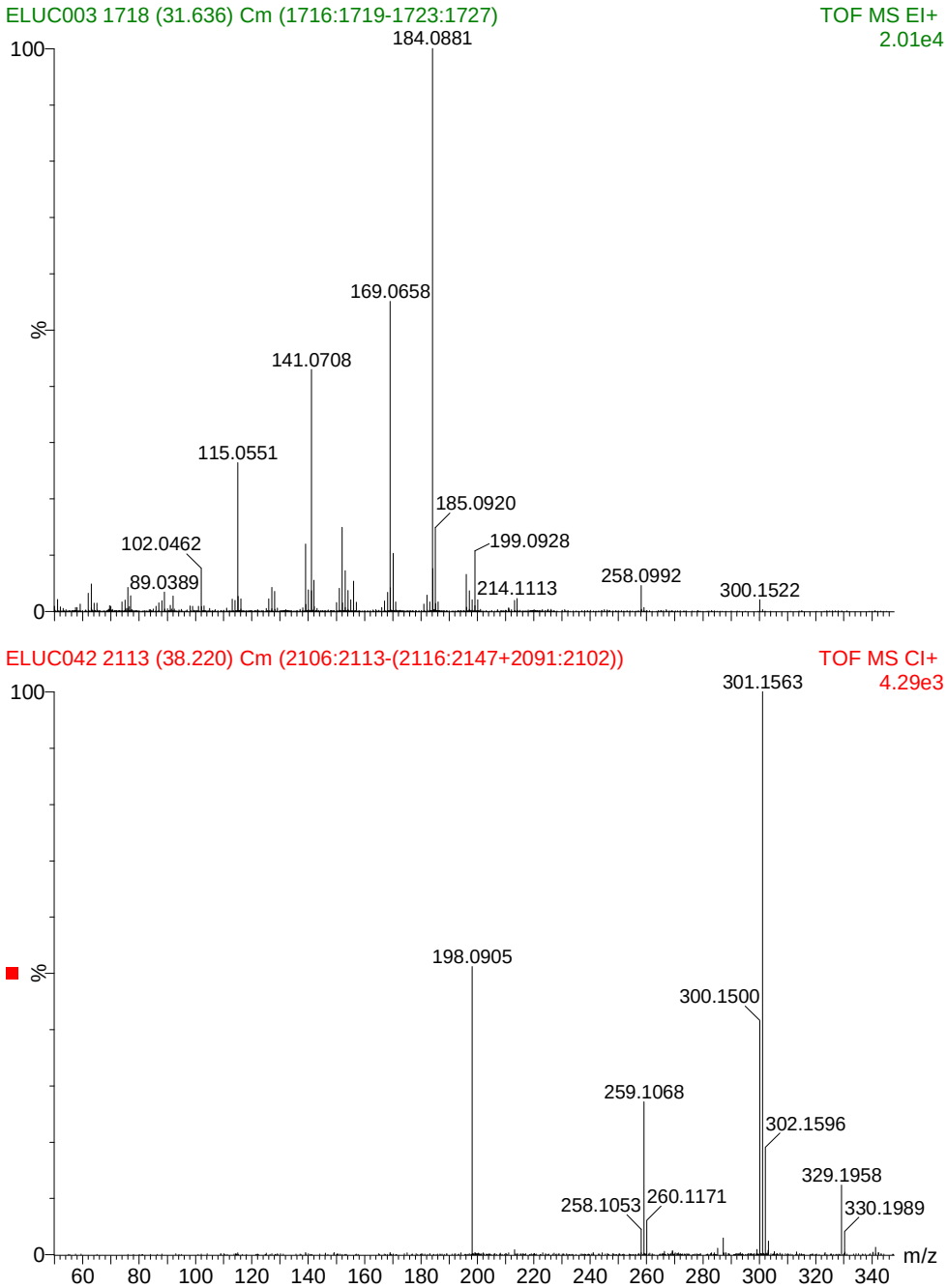
In this particular case, the EI spectrum possibly provides substructure information on the unknown, based on *m/z* 184 and its three fragment ions. The unknown would (most likely) contain a methoxy-substituted biphenyl, that is "for instance" 1,1'-biphenyl-4-methoxy. The methoxy-group could be at another position. By a search through the Wiley EI-MS Library, it appears both 2- and 4-methoxy-1,1'-biphenyl give about the same spectrum. This allowed us to discard again 2 out of 4 remaining formulae, as the unknown should have a DBE of at least 8 (due to the biphenyl group). At this point, still two formulae remained, $C_{17}H_{21}N_2O_3^+$ (DBE = 8.5) and

$C_{13}H_{17}N_8O^+$ (DBE = 9.5). Looking at the positive CI spectrum (Figure 1A, bottom), subsequent losses of C_3H_6 and CH_3NO_2 can be observed which only can be accomplished from $C_{17}H_{21}N_2O_3^+$.

A Reaxys database search was performed and the elemental composition $C_{17}H_{20}N_2O_3$ resulted in 1492 structures. Limiting the above search to the substructure revealed by EI spectrum (methoxy-substituted biphenyl), a total of only five structures were returned by the database (Figure 1C).

As commented above, in the CI spectrum we observed the loss of 42.0472 Da (C_3H_6) to an ion with *m/z* 259.1107. This loss allowed us to discard structures 2, 3 and 5.

From structure 1, both the formation of the odd-electron fragment ion with *m/z* 184 in EI and the fragment ions with *m/z* 259 and 198 are readily explained (see Figure 2) whereas the odd-electron fragment ion with *m/z* 184 in EI and the even-electron fragment ion with *m/z* 198 in CI are not expected to be formed from structure 4. In this case, in order to generate an ion with *m/z* 184, two different bonds to the ring should be cleaved. The NO_2-group would be lost as a radical (prior to or after the loss of propylene (C_3H_6). From the resulting even-electron structure, it would be highly unlikely to lose the other side chain in such away that an ion with *m/z* 184 is formed. In CI, the formation of the ion with *m/z* 198 would require the loss of two radicals: NO_2 and CH_3. Consequently, in the light of the results obtained, we proposed the structure 1 for this compound, which in fact corresponds to acaricide bifenazate, the pesticide already present in the water sample.



(A)

Figure 1. (A) Electron ionization (top) and positive chemical ionization (bottom) spectra of a detected compound.

(B)

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT (Norm)
301.1563	301.1569	-0.6	-2.0	3.5	C15 H26 O4 P	49.0	1.7
	301.1552	1.1	3.7	8.5	C17 H21 N2 O3	48.2	0.9
	301.1582	-1.9	-6.3	8.5	C16 H22 N4 P	48.2	1.0
	301.1599	-3.6	-12.0	3.5	C14 H27 N2 O P2	51.3	4.1
	301.1525	3.8	12.6	9.5	C13 H17 N8 O	50.7	3.5

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT (Nor
184.0881	184.0875	0.6	3.3	8.5	C11 H10 N3	201.1	5.1
	184.0888	-0.7	-3.8	8.0	C13 H12 O	196.0	0.0
	184.0891	-1.0	-5.4	3.5	C9 H15 N O P	203.5	7.5

(C)

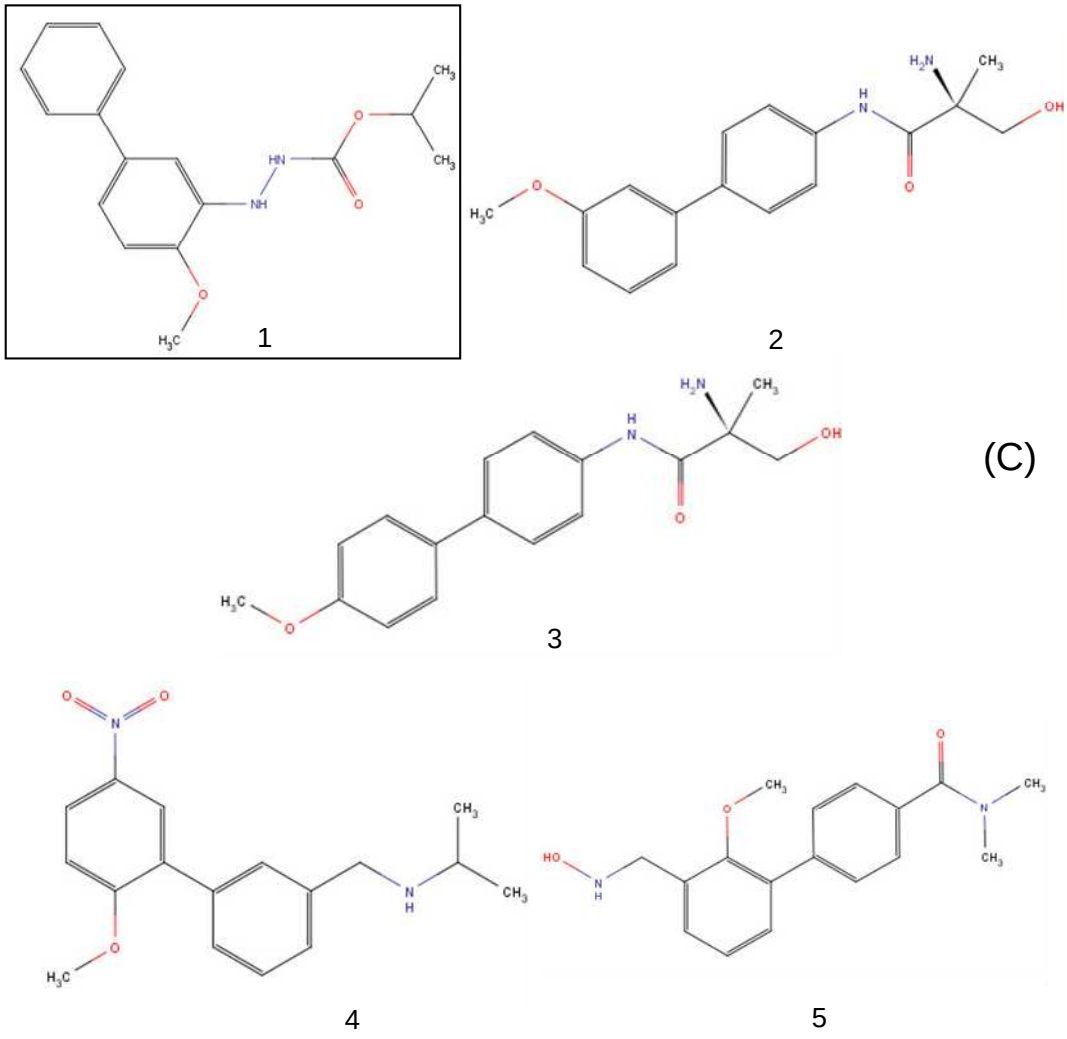


Figure 1. (B) Possible elemental compositions for different *m/z* ions after applying carbon filtering. (C) Possible structures for $C_{17}H_{20}N_2O_3$ limiting the Reaxys search to the methoxy-substuted biphenyl substructure.

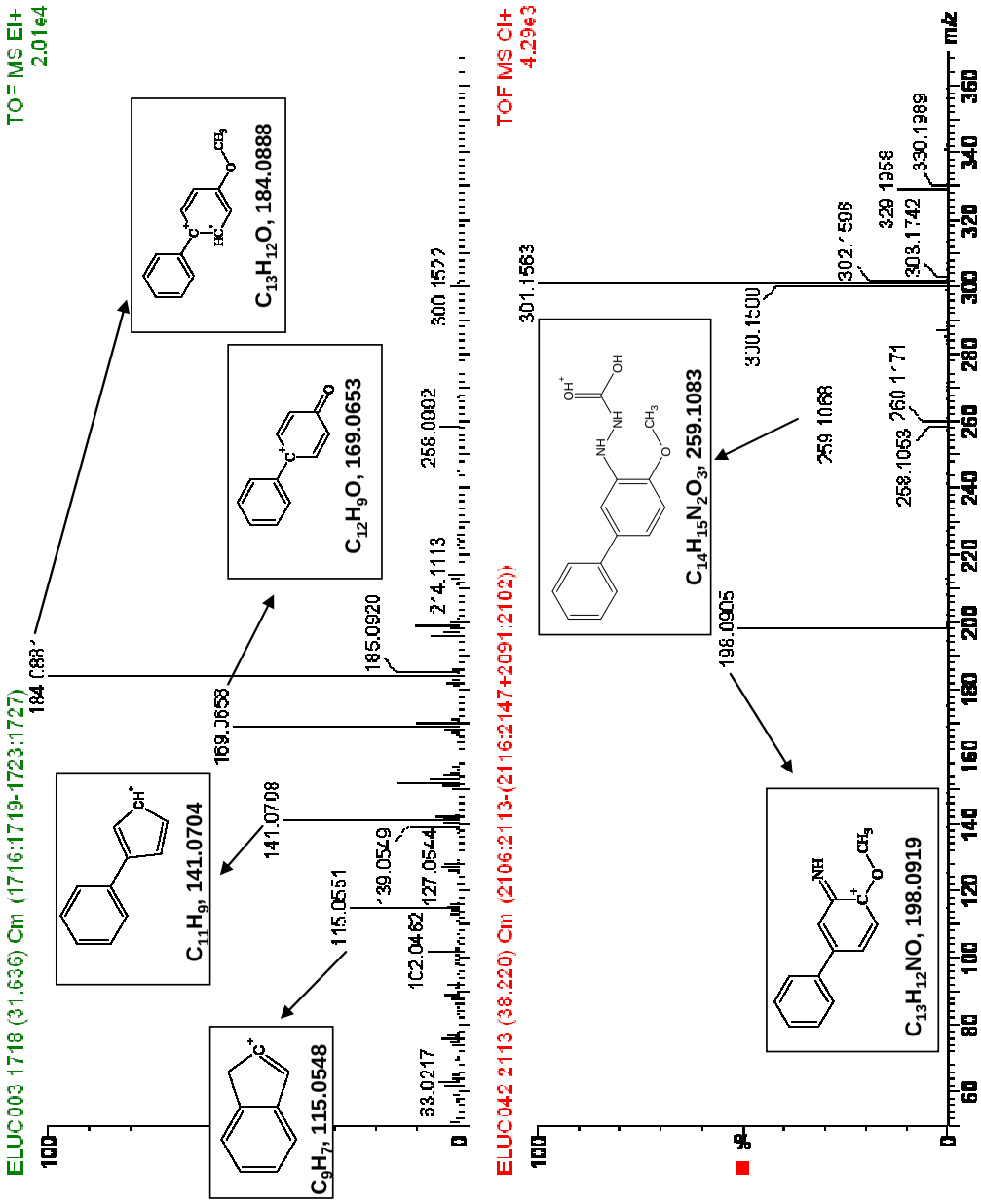


Figure 2. Possible structures for different m/z ions taking into account structure 1 (bifenazate). Electron ionization (top) and positive chemical ionization (bottom) spectra.

Case 2

The second example involves a compound with a retention time of 36.6 min and a protonated molecule with *m/z* 343.0443 (Figure 3, middle). Typical adducts with $C_2H_5^+$ and $C_3H_5^+$, consequence of the use of methane as a reagent gas in CI mode, were observed in the positive CI spectrum. The experimental M+2 abundance of 69.6%indicates the presence of two Cl atoms in the molecule, and nil to two S atoms, as deduced from the accepted tolerances in the M+2 percentage (±10%, i.e., 62.6-76.6%). Within the procedure outlined above, only one formula remained ($C_{18}H_{13}Cl_2N_2O^+$). A Reaxys database search was resulted in 81 structures. The EI spectrum provides useful substructure information (Figure 3, bottom). The unknown compound most likely is an ester/amide of monochloro-pyridine carboxylic acid, with an additional Cl in the other part of the molecule. Limiting the above search with this substructure ($C_6H_3ClNO^+$), only one structure is returned by the database corresponding to the pyridinecarboxamide fungicide boscalid ($[M+H]^+$ with *m/z* 343.0405). However, it is difficult to propose structures for the poor-abundance high-*m/z* fragments from this structure (loss of water, followed by loss of $Cl^\bullet$, followed by loss of HCl to *m/z* 253) (see figure 3, bottom). Obviously, the most logical next step would be to purchase the reference standard of the suggested candidate, boscalid, check its retention time and mass spectra in the various modes for definitive confirmation.

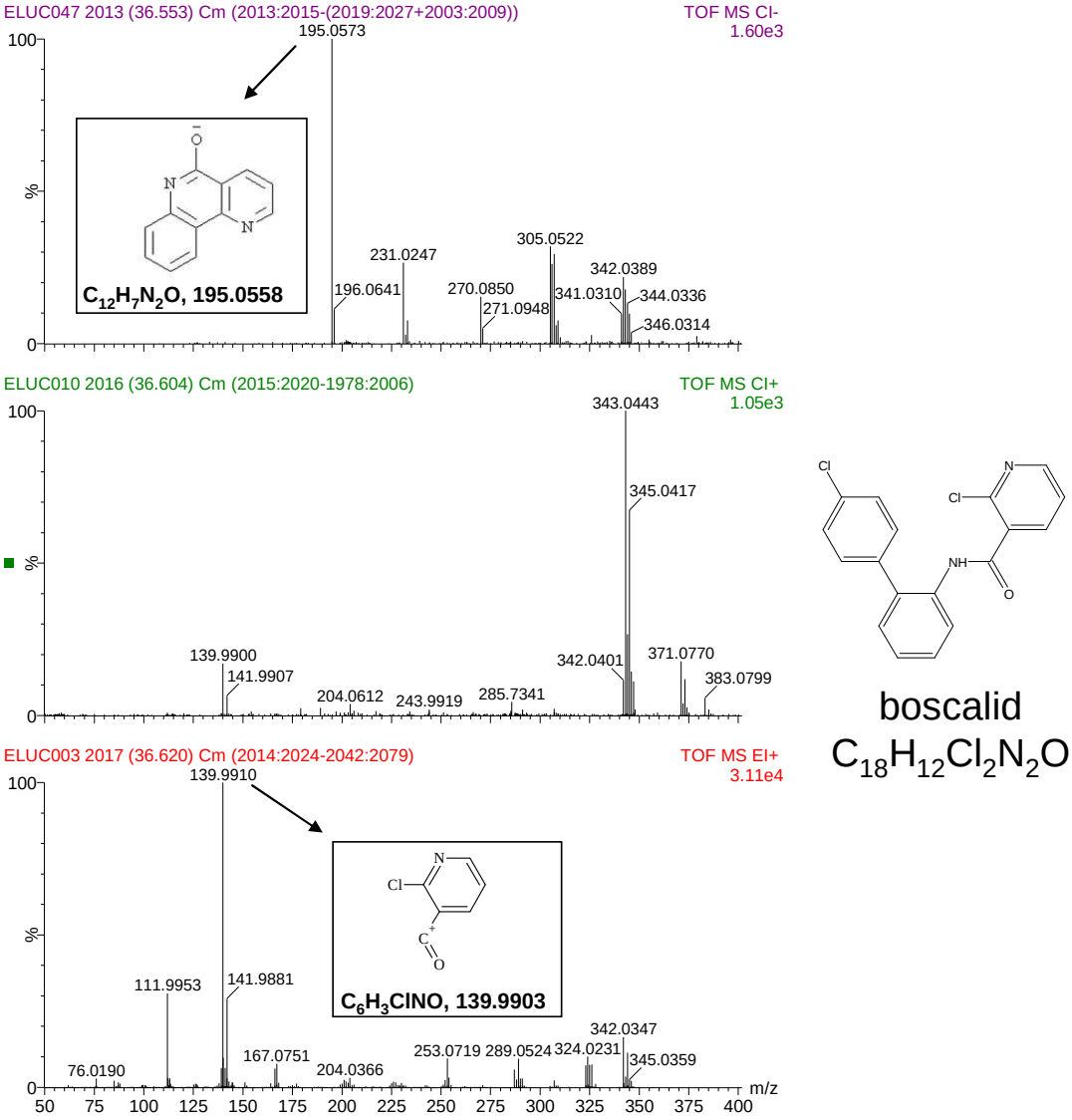


Figure 3. Structures suggested for different *m/z* ions taking into account the boscalid structure. Negative ion chemical ionization (top), positive ion chemical ionization (middle) and electron ionization (bottom) spectra.

Case 3

The accurate mass of a protonated unknown compound with retention time 30.67 min was determined to be *m/z* 330.0820; characteristic adducts with $C_2H_5^+$ and $C_3H_5^+$ were also observed in the CI+ spectrum (Figure 4A, middle). Given the experimental M+2 abundance of 34.2 %, we assumed the presence of one Cl atom in the molecule, and nil to one S atoms, as deduced from the accepted tolerances in the M+2 percentage (±10%, i.e., 30.8-37.6%). Calculation of the possible formulae yielded 5 results. The fragment ion with *m/z* 310.0743 present in the positive CI spectrum corresponded to the loss of 20.0077 Da that can only be due to the loss of HF (20.0062 Da). At that point, the possible presence of 1-10 F atoms was included in the calculations. Within the new limits, the calculation resulted in 7 possible formulae (Figure 4B). The EI fragment ion with *m/z* 313.0758 corresponded to the loss of 15.9937 Da that only can be the loss of O (15.9949 Da) (Figure 4A, bottom). This oxygen loss allowed us to discard the 3 molecular formulae that did not contain any atom of oxygen. At this point, 4 molecular formulae still remained. From calculation of the formula of the EI fragment ion with *m/z* 244.0457, only one possible formula was found ($C_{15}H_{10}FCl^{+\bullet}$) (Figure 4B). Using this information, two formulae containing less than 15 carbon atoms could be discarded from our list. The two remaining formulae were $C_{15}H_{19}ClFNO_2P^+$ and $C_{17}H_{14}ClFN_3O^+$ (protonated molecules). The first formula ($C_{15}H_{19}ClFNO_2P^+$) is highly unlikely, as the generation of the fragment ion with *m/z* 244 would require the loss of $H_8NO_2P^\bullet$. The rest of the CI and EI fragments did not help us to discard any of the two empirical formulae. So, a Reaxys database search was performed. The formula $C_{15}H_{18}ClFNO_2P$ did not result in a structure. However, the formula $C_{17}H_{13}ClFN_3O$ resulted in 44 possible structures in Reaxys, among which there are a number of stereoisomers that cannot be differentiated by MS. At this point, a substructure is needed to reduce the number of possibilities. A possible substructure may be derived from further interpretation of CI spectrum. Next to the loss of HF, the loss of 69.0305 Da is observed, which could be consistent with $C_2H_3N_3$ (69.0327 Da), a triazole substructure. The complementary fragment with *m/z* 70 is also observed (Figure 4A, middle). In fact, the fragment ion with *m/z* 244 in the EI spectrum is due to a combined loss of oxygen and the triazole ring. This triazole substructure is found in thirteen of the 44 possible structures found in the Reaxys database (Figure 4C). However, among these thirteen, there are 9 stereoisomers of the same structure

(structure 1). From three other structures, an easy loss of the triazole ring, as observed in both the EI and the CI spectrum, is not likely either because it requires the cleavage of too many bonds or a massive rearrangement (structures 2, 3 and 5). This means that only two isomeric structures are left (1 and 4). A choice between these two can (possibly) only be made from differences in retention time. Both these structure proposals enables us to explain the fragments in the negative-ion CI spectrum: the loss of $Cl^{\bullet}$ leads to the fragment ion with *m/z* 293, the loss of $C_3H_3N_3^{\bullet}$ to *m/z* 247, and the loss of $Cl^{\bullet}$ and $C_2H_3N_3$ to *m/z* 224.

At this stage, it must be admitted that this particular case also indicates one of the weak points of the current procedure. The recognition of relevant substructures seems to be an issue of experience and a bit of luck. In this case, the EI fragment ion with *m/z* 139 ($C_7H_4ClO^+$, that is most likely Cl–phenyl–C≡O^+) was considered as a relevant substructure (Figure 4A, bottom). This would indicate that the unknown most likely is an ester/amide of chlorobenzoic acid. Performing a Reaxys database search with $C_{17}H_{13}ClFN_3O$ and this substructure returned only one possible structure, from which the loss of the triazole ($C_2H_3N_3$) is not likely. Although not recognized by us, the formation of the Cl–phenyl–C≡O^+ fragment apparently is possible from the epoxide structure proposed.

Interestingly, this apparently possible substructure with *m/z* 139 allowed us to provisionally differentiate between the two possible structures left (1 and 4, see above). From structure 1, the formation of Cl–phenyl–C≡O^+ is readily expected (Figure 5), whereas with structure 4, the formation of F–phenyl– C≡O^+ would be less likely. Consequently, we propose the structure 1 for this compound, which in fact corresponds to epoxiconazole, the pesticide already present in the water sample.

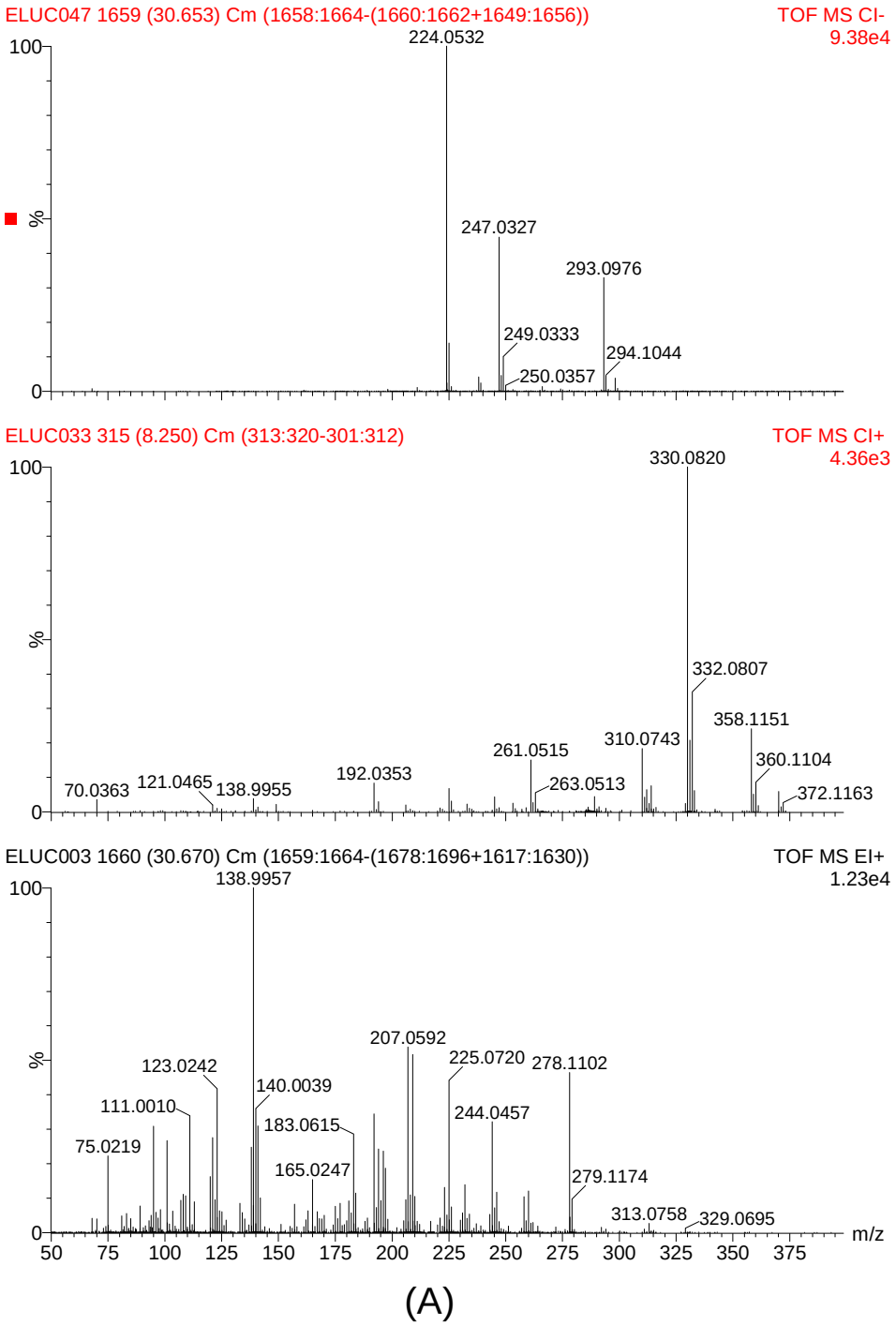
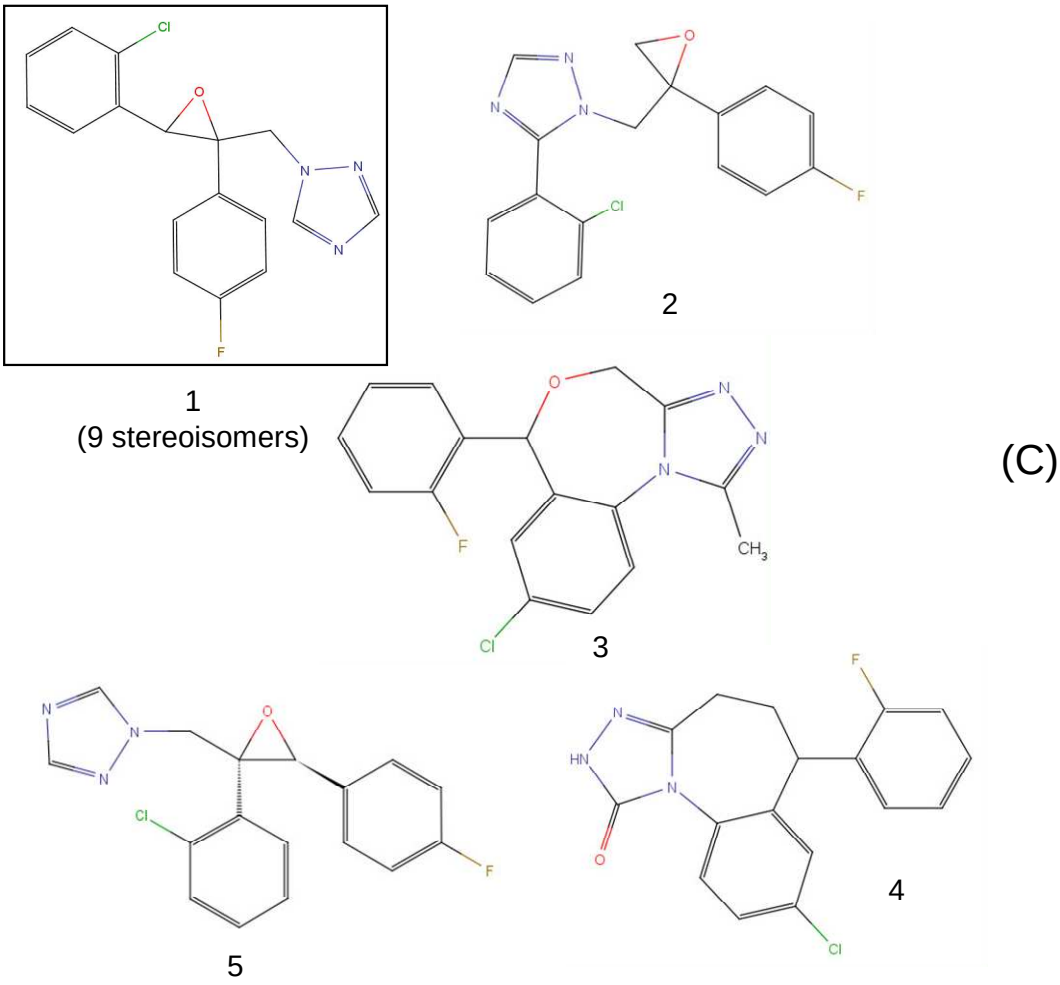


Figure 4. (A) Negative ion chemical ionization (top), positive ion chemical ionization (middle) and electron ionization (bottom) spectra of a detected compound.

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT (Norm)
330.0820	330.0821	-0.1	-0.3	7.5	C14 H15 N3 O2 F2 Cl	59.9	3.6
	330.0826	-0.6	-1.8	6.5	C15 H19 N O2 F P Cl	59.1	2.9
	330.0809	1.1	3.3	11.5	C17 H14 N3 O F Cl	56.6	0.3
	330.0837	-1.7	-5.2	2.5	C12 H20 N O3 F2 P Cl	61.6	5.3
	330.0802	1.8	5.5	3.5	C13 H18 N F4 P Cl	61.3	5.1
	330.0796	2.4	7.3	4.5	C12 H14 N3 F5 Cl	62.3	6.0
	330.0861	-4.1	-12.4	11.5	C19 H15 N F2 Cl	58.0	1.7

(B)

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT (Norm)
244.0457	244.0455	0.2	0.8	10.0	C15 H10 F Cl	131.3	0.0



(C)

Figure 4. (B) Possible elemental compositions for different m/z ions after applying carbon filtering. (C) Possible structures for $C_{17}H_{13}ClFN_3O$ limiting the Reaxys search to the triazole substructure.

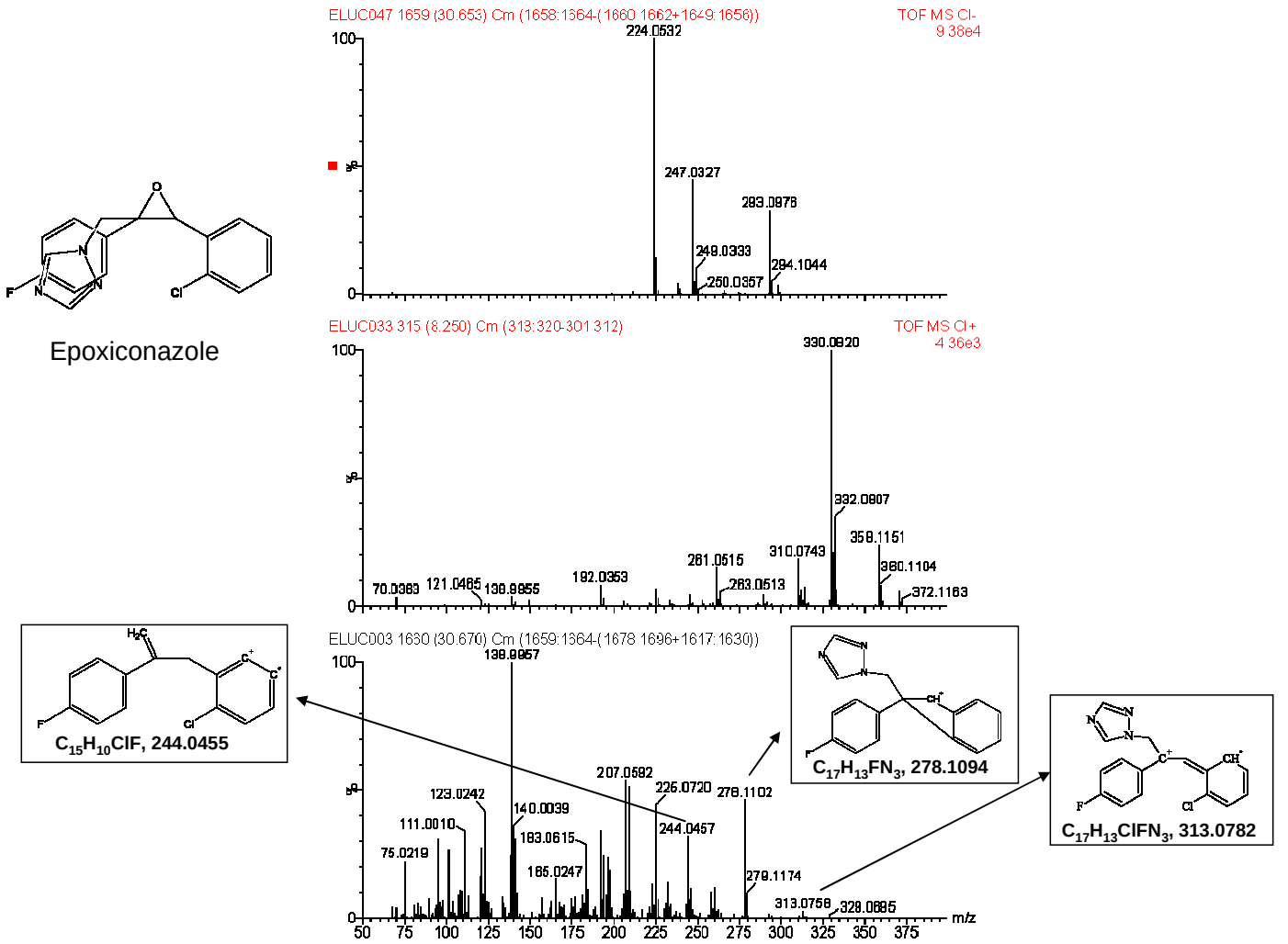


Figure 5. Possible structures for different *m/z* ions taking into account structure 1 (epoxiconazole). Negative ion chemical ionization (top), positive ion chemical ionization (middle) and electron ionization (bottom) spectra of a detected compound.

Case 4

In this case, a chromatographic peak was detected in the non-target screening with retention time 29.45 min that returned a match of 606 in the NIST library search, indicating the compound to be 1-methyl-cyclohexene. The accurate *m/z* of the protonated molecule of this unknown compound was *m/z* 302.0732 (Figure 6 middle). Within the search limits explained above, calculation of the possible elemental formulae resulted in only 1 formula ($C_{14}H_{18}Cl_2NO_2^+$). This formula is consistent with a DBE of 6. A Reaxys database search resulted in 171 possible structures. The positive-ion CI spectrum shows little fragmentation, whereas in negative-ion CI spectrum, only a loss of a $Cl^\bullet$ is observed (Figure 6 top). The data from the library search are rather non-informative, except that the possible presence of a methyl-substituted cyclohexane substructure is suggested. This is somewhat confirmed by the loss of C_7H_{14} from the molecular ion (*m/z* 301 to 203). The calculated formula for the resulting fragment ion with *m/z* 203 is $C_7H_3Cl_2NO_2^{+\bullet}$ (DBE=6), indicating most likely the presence of a dichloro-substituted benzene or pyridine ring next to the methyl-substituted cyclohexane (Figure 6 bottom). Three separate database searches were performed (one for each substructure). From the search results, those structures were selected, which showed both substructures, as both the aromatic and the non-aromatic rings are part of the structure. After that, only two isomeric structures remained (Figure 7)with the weak bond in the ester or amide link. Any MS fragmentation will lead to a 4-amino-2,3-dichlorophenol (*m/z* 177) and/or 2,3-dichloro-4-iminocyclohexa-2,5-dien-1-one (*m/z* 175) type of fragment (*m/z* 175, 177, 179), from which one never could be decided how this part is attached to the remainder of the molecule, that is via O or N. So, at this point, the only way to discriminate between the two is by checking the retention time after the injection of references standards. Structure (1) in Figure 7 is fenhexamide, the pesticide already present in the water sample.

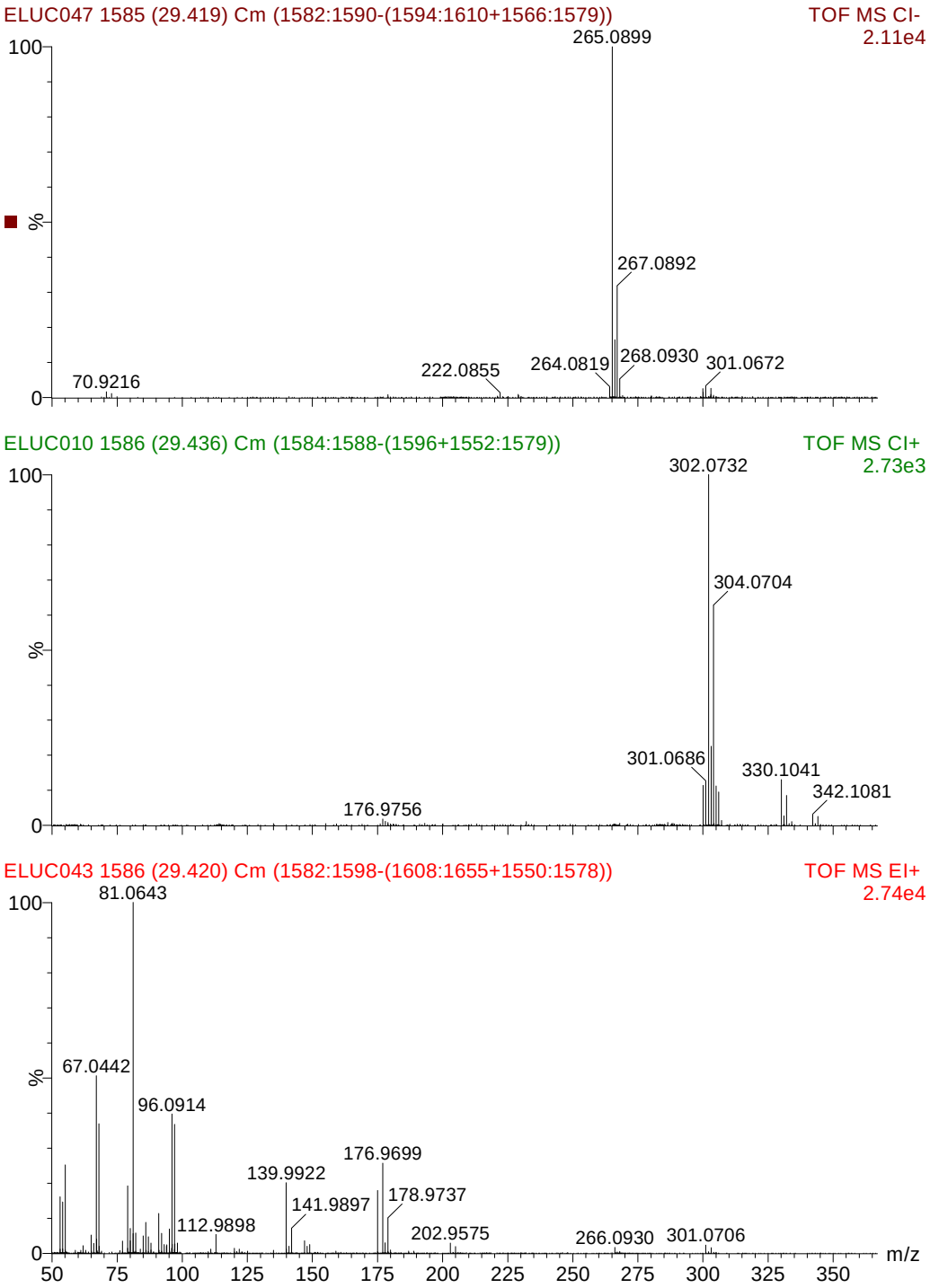
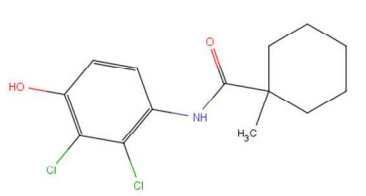
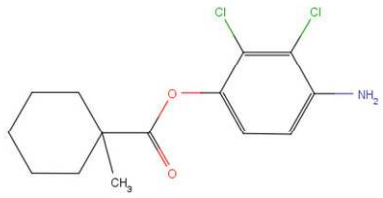


Figure 6. Negative ion chemical ionization (top), positive ion chemical ionization (middle) and electron ionization (bottom) spectra of a detected compound.



(1)



(2)

Figure 7 Possible structures for $C_{17}H_{13}ClFN_3O$ limiting the Reaxys search to different substructures

CONCLUSIONS

In this paper, the complementary use of EI and CI has been investigated in combination with GC-TOF MS for the elucidation of organic (micro)contaminants in water samples. Several model examples have been shown to illustrate the methodology applied and the difficulties of this process when the MS spectra of the compounds investigated are not available in the commercial library used in our laboratory. The use of the soft-ionization technique CI, has allowed the determination of the molecular mass. In addition, accurate mass measurement provided by TOF MS, together with the structure information generated by the accurate mass EI spectrum, has allowed the proposal of an appropriate formula for the unknown. The application of rules based on observed isotopic patterns, carbon number prediction filter and nitrogen rule, among others, has been crucial to reduce the number of possible formulae. Searching the candidate formulae in a database has allowed the proposal of chemical structures for the unknown. The recognition of relevant substructures on the unknown molecule has been of great help in order to reduce the number of possible structures given by the database search. At this stage, the recognition of relevant substructures seems to be an issue of experience, which reflects the difficulties of this challenging task. Accurate masses of fragment ions given by TOF MS are of outstanding importance. Their

structures should be compatible with the chemical structure assigned to the candidate. According with our own experience, the unknown compound could be identified in several cases, while in others two chemical possible (isomeric) structures remained as candidates. At this point, the unequivocal confirmation should be made by injecting the reference standard, if available, to test the retention time and experimentally confirm the presence of fragment ions generated by GC-TOF MS.

ACKOWLEDGMENTS

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of GC-TOF MS instrument. This work has received financial support from the Spanish Ministry of Science and Education (Project reference: CTQ2009-12347). The authors acknowledge the financial support of Generalitat Valenciana, as research group of excellence PROMETEO/2009/054. Tania Portolés is very grateful to University Jaume I for her post-doctoral grant.

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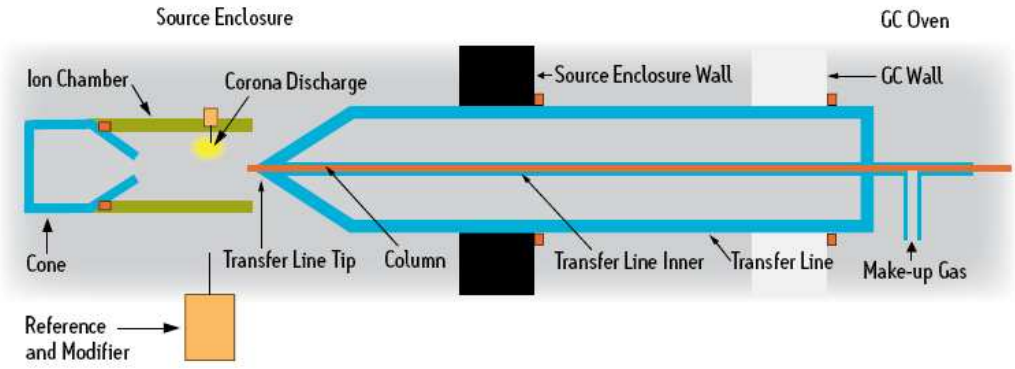
3.6 POTENCIAL DE LA NUEVA FUENTE DE IONIZACIÓN QUÍMICA A PRESIÓN ATMOSFÉRICA EN COMBINACIÓN CON GC-TOF MS PARA EL ANÁLISIS DE RESIDUOS DE PLAGUICIDAS

Como se ha descrito en los apartados anteriores, la técnica GC-TOF MS permite llevar a cabo métodos de *screening* eficientes y fiables tanto en modo *target* como *non-target*. Los métodos de *target screening* utilizando fuente de ionización electrónica presentan, como ya se ha indicado, muchas ventajas, sobretodo desde el punto de vista confirmativo, ya que en una única inyección se dispone, de un espectro de iones completo con fragmentos de la molécula medidos con elevada exactitud de masa, las cuales soportan la identidad de la estructura, juntamente con el *match* en librerías. Sin embargo, esta modalidad de *screening* viene condicionada considerablemente por la necesidad de disponer de los patrones de referencia de los analitos. Esto no implica que no se pueda llevar a cabo un *screening* con GC-(EI)TOF MS sin patrones (de hecho en esta tesis se muestran varios ejemplos de ello), pero cabe señalar que la disponibilidad de los mismos facilita enormemente la labor, principalmente durante la etapa inicial de desarrollo del método de procesamiento de datos. Además, la excesiva fragmentación para muchos compuestos, provoca que se pierda información muy valiosa relativa al ion molecular, sobretodo para fines de *screening* y confirmación. La situación es muy diferente a la del análisis mediante LC-TOF con fuentes ESI, donde la molécula protonada o desprotonada suele ser el ion base en el espectro de masas (1, 2). En este caso, la detección de los compuestos se puede realizar fácilmente extrayendo un cromatograma a partir del TIC con una ventana de masa muy estrecha (típicamente 0.01-0.02 Da), gracias a la elevada resolución y exactitud de masa ofrecida por los analizadores TOF. En el nw-XIC resultante resulta mucho más sencillo observar el pico cromatográfico del compuesto. Obviamente, este proceso de detección, también se ve simplificado cuando se dispone de los correspondientes patrones de referencia, ya que la búsqueda se limita a una ventana de tiempo de retención concreta.

En el caso de GC-TOF MS, la disponibilidad de fuentes de ionización más suaves que la ionización electrónica, pero más universales que la de ionización química, que promuevan la formación del ion molecular, ofrecería nuevas expectativas en *target screening*. La alta probabilidad de obtener el ion molecular en el espectro, en caso de que un compuesto estuviera presente en la muestra, facilitaría este *screening*, ya que

éste se podría realizar únicamente conociendo la masa exacta del compuesto, sin necesidad de conocer sus fragmentos *a priori*.

En este apartado se estudia un nuevo prototipo de fuente de ionización química a presión atmosférica (APCI) diseñada para su acoplamiento a GC (ver figura). Esta fuente ha sido ampliamente utilizada en combinación con LC, pero resulta novedosa en cuanto a su acoplamiento a sistemas de GC. El trabajo consiste en explorar las capacidades analíticas del acoplamiento GC-TOF MS con la fuente de APCI para fines de *screening*.



Principalmente, se persigue investigar las condiciones que promuevan la formación del ion molecular en la fuente, dadas las ventajas que ello aporta con respecto al *screening* amplio de contaminantes orgánicos en cualquier tipo de muestra, en comparación con los habituales espectros de elevada fragmentación normalmente obtenidos mediante la fuente de EI.

El trabajo se ha llevado a cabo para unos 100 plaguicidas, para los que se ha estudiado su comportamiento frente a la ionización/fragmentación en la fuente. El gas utilizado como "*make-up*" ha sido nitrógeno. Además, se ha estudiado la adición de agua como modificador. Los diferentes comportamientos mostrados por los plaguicidas estudiados ante la ionización y fragmentación en la fuente de APCI se muestran en la figura 4 del **artículo científico 9**. En general, la adición de agua como modificador ha mejorado la formación de ion molecular protonado, que es el objetivo perseguido en este trabajo.

La metodología desarrollada se ha aplicado posteriormente al *screening* de plaguicidas en frutas y vegetales. Para ello se utiliza un software de procesamiento de datos automático (Chromalynx XS en modo *target*) que posibilita la visualización del espectro de masas para cada compuesto detectado, aumentando la confianza en la identificación mediante comparación de la masa exacta del ión molecular y el *pattern* isotópico con los calculados teóricamente. A su vez, también permite comparar la masa experimental y *pattern* isotópico de posibles iones fragmentos con la masa teórica de la composición elemental más probable, aumentando así la fiabilidad de la confirmación. En una segunda etapa, los plaguicidas detectados fueron confirmados mediante experiencias adicionales en modo MS/MS al disponer de un QTOF.

La parte experimental del trabajo se realizó en las instalaciones de Waters Corporation, ya que al tratarse de un prototipo de fuente no estaba disponible comercialmente el equipo instrumental.

Cabe señalar que aunque la interfase APCI se introdujo hace más de 30 años (3, 4), hasta el momento han sido muy escasas sus aplicaciones en combinación con GC, principalmente por las dificultades técnicas y el elevado coste de este tipo de instrumentación. Se podría destacar una aplicación reciente donde se muestra que GC-(APCI)TOF MS es una técnica con un gran potencial para el análisis de metabolitos en muestras biológicas, como alternativa mejorada a los métodos clásicos basados en GC-MS con EI y CI (5).

Finalmente, cabe resaltar el gran impacto que seguramente esta fuente producirá en el análisis *target* con equipos de tandem MS, como triples cuadrupolos y trampas de iones. La ausencia de una técnica de ionización suave para GC, tan universal como la fuente de EI, capaz de proporcionar eficientemente iones moleculares con elevada sensibilidad, factibles de ser utilizados como iones precursores en experiencias de masas en tandem, ha supuesto hasta el momento que técnicas tan avanzadas como el triple cuadrupolo no hayan sido suficientemente explotadas en todos los campos. Así pues, se espera que esta técnica de ionización abra la puerta a nuevas aplicaciones donde la combinación de GC-MS/MS con fuentes de ionización suaves, como la APCI, proporcione los resultados esperados.

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3.6.2 Artículo científico 9

J. Mass Spectrom. 45, 926-936, 2010

POTENTIAL OF ATMOSPHERIC PRESSURE CHEMICAL IONIZATION SOURCE IN GC-QTOF MS FOR PESTICIDE RESIDUE ANALYSIS

Tania Portolés, Juan V. Sancho, Félix Hernández, Anthony Newton, Peter Hancock

[1]Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain

[2] Waters Corporation MS Technologies Centre, Atlas Park, Simonsway, Manchester, M22 5PP, United Kindom.

ABSTRACT

The potential applications of a new atmospheric pressure source for GC-MS analysis have been investigated in this work. A list of around 100 GC-amenable pesticides, which includes organochlorine, organophosphorus and organonitrogenated compounds, has been used to evaluate their behavior in the new source. Favoring themajor formation of the molecular ion in the source has been the main goal due to the wide-scope screening possibilities that this fact brings in comparison with the traditional, highly fragmented electron ionization spectra. Thus, the addition of water as modifier has been tested as a way to promote the generation of protonated molecules. Pesticides investigated have been classified into six groups according to their ionization/fragmentation behavior. Four of them are characterized by the abundant formation of the protonated molecule in the atmospheric pressure source,mostly being the base peak of the spectrum. These results show that wide-scope screening could be easily performed with this source by investigating the presence of the protonated molecule ion, MH+. The developed procedure has been applied to pesticide screening in different food samples (nectarine, orange and spinach) and it has allowed the presence of several pesticides to be confirmed such as chlorpyriphos ethyl, deltamethrin and endosulfan sulfate. The availability of a quadrupole time-of-flight instrument made it feasible to perform additional MS/MS experiments for both

standards and samples to go further in the confirmation of the identity of the detected compounds. Results shown in this paper have been obtained using a prototype source which exhibits promising features that could be applied to other analytical problems apart from those illustrated in this work.

KEYWORDS

Atmospheric pressure chemical ionization; gas chromatography; quadrupole time of flight mass spectrometry; pesticides; screening

INTRODUCTION

The increasing use of high-resolution full spectrum acquisition techniques in the last decade, such as time-of-flight mass spectrometry (TOF MS), has allowed a huge amount of chemical information on sample composition to be obtained, widening the number of analytes that can be investigated in a single experiment. This analyzer provides the selectivity and sensitivity required for efficient, wide-scope screening, as it combines high full-spectral sensitivity with high mass resolution. The useful and accurate mass data obtained can be processed in both target and/or non-target way, which gives the instrument an interesting versatility depending on the aim of the analysis and allows the user to look at an analytical problem from a different point of view.[1–5] Additionally, the full spectrum dataset remains and offers the analyst the possibility of performing a retrospective analysis, i.e. to make a careful examination of old raw data looking for the presence of any compound that becomes interesting later. All these characteristics make this technique ideal to perform screening analysis that provides greater analytical information and allows the user to efficiently discriminate samples with no detectable residues from those with contaminants or residues at a relevant level. In the pesticide residue analysis field, there is a clear trend toward liquid chromatography coupled to mass spectrometry (LC-MS) as new pesticides are more polar, less volatile and thermostable, and consequently, less GC amenable. However, high usage pesticides (in Europe or in developing countries) are still volatile and thermostable, therefore GC-MS methods cannot be abandoned yet.

Regarding the huge amount of full spectrum with accurate mass data generated by TOF instruments, choosing the right strategy to get the maximum information from the data is one of the major keys to success. The way to proceed is mainly driven by the kind of mass spectrum delivered by the system. Normally, in LC-TOF MS analysis, the ionization occurs at atmospheric pressure by electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photo ionization[3,6,7] or ambient ionization, e.g. direct analysis in real time or desorption ionization.[8,9] These types of ionization processes promote the formation of protonated or deprotonated molecules with very little fragmentation, yielding typically the [M+H]+ or [M−H]− ions as the base peak of the spectrum. Recent screening applications have been developed using LC-(ESI)TOFMS technique.[3,10,11] The predictable presence of the (de)protonated molecule in LC-TOF data has allowed an automatic and rapid 'post-target' searching[12] for many LC amenable compounds (including pesticides, antibiotics, veterinary drugs and banned dyes, among others, as well as several metabolites) by extracting chromatograms, with narrow-mass windows, at the exact mass of the protonated molecule. Standards of the most frequently detected compounds were also injected, so that the information of retention time and in-source fragmentation helped to increase the confidence in assigning the identity of the detected compound.

When using GC-TOF MS, ionization normally occurs under vacuum conditions either by EI or CI. EI is the most widely applied ionization technique as it is a robust source, which produces standardized mass spectra that can be compared with those from commercially available MS libraries. However, its extensive fragmentation can be a drawback for some applications when wide-scope screening is pursued. As we have recently shown, GC-(EI)TOF MS allows a rapid and automatic accurate mass screening of target analytes using extracted ion chromatograms with narrow-mass window (nw-XICs). In this way, the chemical background is notably reduced and the selectivity improved for the analysis of complex matrices.[1,2] However, the low probability of obtaining the molecular ion (M+·) in most of the EI spectra forces the user to know in advance the exact masses of the main analyte fragments in order to perform the nw-XIC at those m/z ions. To obtain this information it is common to rely on the injection of standards into the GC-MS system or retrieving EI spectra from commercial libraries. This is a time-consuming procedure especially when no reference standard is available and the information must be taken from commercial libraries where the data are

registered in nominal mass and the elucidation of the possible structure of the fragment ions becomes more difficult. Soft ionization techniques in GC-MS would overcome this issue, in those applications where the extensive EI fragmentation and the absence of the molecular ion in the spectrum could be considered a disadvantage. The high probability of the presence of the molecular ion would enhance the screening reliability, as the information on the molecular ion can be used to limit the number of potential candidates for a given compound, even more if accurate masses are acquired. Additionally, final identification would be supported by fragmentation experiments, which could be easily achieved using hybrid quadrupole TOF MS (QTOF MS). CI source is a long-term available soft ionization technique for GC instruments, although it has been less frequently used than EI. Positive or negative CI gives better selectivity for several pesticides compared to EI. This fact results in chromatograms with reduced matrix interference,[13-16] but with higher signal intensity variation of different pesticides compared to EI ionization. Preferentially, GC-MS with CI is focused on special substance classes only, e.g. organohalogen pesticides,[16-18] pyrethroids,[19] polybrominated diphenyl ethers[13,14] and organophosphates.[20] It is rarely used in multi-residue methods, because it is not as universal ionization technique as EI and requires several injections of the sample to cover a wider analytes range.[21,22]

Atmospheric pressure ionization has primarily been used to interface an MS with LC, as mentioned earlier. However, this attractive ionization interface can also be applied to GC. The ionization mechanism employed by the APCI source is low energy (soft), which generates spectral data typically rich in molecular or quasi-molecular ion information and ideal for compound confirmation. The first few developments with APCI were carried out by Horning et al.[23,24] in the 1970s who were also the first to interface a GC instrument to an APCI ion source. Since these initial publications, a series of papers in the late 1980s have been published by Korfmacher and coworkers[25-27] in which the effluent from a gas chromatograph is ionized at atmospheric pressure. However, GC/APCI was never fully commercialized, probably because of the high costs of the specialized instrumentation needed for these analyses at that time. Nowadays, new APCI sources are commercially available and capable to be interfaced with both GC and LC instruments.[28,29] This fact adds versatility and extends analytical capabilities providing flexibility to determine volatile and semivolatile compounds of low and intermediate polarity, traditionally analyzed by dedicated vacuum GC-MS

instruments. Very recently, GC/APCI in combination with TOF MS has been studied for its applicability to metabolic profiling.[30]

The aim of this work is to study the applicability of a new soft ionization APCI source, waters atmospheric pressure (APGC), using a GC-QTOF MS instrument to perform rapid and wide-scope screening and identification of pesticides residues in food samples. The predictable presence of the molecular ion/protonated molecule in the GC-(APCI)TOF MS spectrum allowed the rapid application of a screening method in post-target way, easily generating a list of compounds to be monitored making use of their molecular formulae, i.e. exact masses of their protonated molecules. The behavior of around 100 pesticides using GC-(APCI)QTOF MS instrument has been studied. The developed procedure has been applied to the screening of pesticides in fruit and vegetable samples such as nectarine, orange and spinach. MS/MS experiments have been performed to go further in the confirmation of the identity of the compounds detected; thanks to the product ion spectra at accurate mass provided by the QTOF MS instrument.

EXPERIMENTAL

Reagents

Reference standards of pesticides were purchased from Dr Ehrenstorfer (Augsburg,Germany).Fromsolid reference standards, stock solutions (around 500 µg/ml) were prepared by dissolving the standard in acetone and stored in a freezer at−20 ◦C. Working solutions were prepared by diluting stock solutions in hexane.

Instrumentation

GC-(EI)TOF MS

For the GC instrumentation, an Agilent 6890N GC system (Palo Alto, CA, USA) equipped with an Agilent 7683 autosampler was coupled to a time-of-flight mass spectrometer, GCT (Waters Corporation, Manchester, UK), operating in EI mode. The GC separation was performed using a fused silica HP-5MS capillary column with a length of 30 m x 0.25 mm i.d. and a film thickness of 0.25 µm (J&W Scientific, Folson, CA,

USA). The oven temperature was programmed as follows: 90ºC (1 min); 5ºC/min to 300ºC (2 min). Splitless injections of 1 µL sample were carried out. Helium was used as carrier gas at 1 mL/min.

The interface and source temperatures were both set to 250ºC and a solvent delay of 3 minutes was selected. TOF MS was operated at 1 spectrum/s acquiring the mass range m/z 50-650 using a multi-channel plate voltage of 2800V. TOF MS resolution was about 8500 (FWHM) at m/z 614.

The application manager ChromaLynx XS, a module of MassLynx software, was used to investigate the presence of target compounds in samples.

GC-(APCI)(Q)TOF MS

For the GC instrumentation, an Agilent 7890A GC system (Palo Alto, CA, USA) equipped with an Agilent 7683 autosampler was coupled to a quadrupole time-of-flight mass spectrometer, Xevo QTof (Waters Corporation, Manchester, UK), operating in APCI mode. The GC separation was performed using a fused silica DB-5MS capillary column with a length of 30 m x 0.25 mm i.d. and a film thickness of 0.25 µm (J&W Scientific, Folson, CA, USA). The oven temperature was programmed as follows: 70 ºC (1.5 min); 25 ºC/min to 180 ºC (3 min); 5 ºC/min to 310 ºC (5.1 min). Split injections (10:1) of 1 µL sample were carried out at 280 ºC. Helium was used as carrier gas at 1.2 mL/min.

In charge transfer mode, the interface temperature was set to 310 ºC using N_2 as an auxiliary gas at 400 L/hr, a make-up gas at 400 mL/min and a cone gas at 50 L/hr. The APCI corona pin was operated at 0.4 µA with a cone voltage of 30 V. In proton transfer mode using water, the interface temperature was set to 310 ºC using N_2 as an auxiliary gas at 400 L/hr, a make-up gas at 400 mL/min and a cone gas at 20 L/hr. The APCI corona pin was operated at 1.7 µA with a cone voltage of 20 V. The ionization process occurred within an enclosed ion volume, which enabled control over the protonation/charge transfer processes.

Xevo QTof MS was operated at 1 spectrum/s acquiring the mass range m/z 20-700 using a multi-channel plate voltage of 2300V. TOF MS resolution was approximately 10000 (FWHM) at m/z 614.

Sample treatment

Orange, nectarine and spinach samples were bought from local markets in Barcelona (Spain). The samples were chopped, homogenized and stored in a freezer at – 20ºC until sample treatment.

Sample treatment can be found elsewhere.[31] Briefly, 10 g of sample were subjected to accelerated solvent extraction (ASE) procedure with ethyl acetate. In the case of spinach samples, a gel-permeation chromatography (GPC) clean-up step was also applied before injection into the GC-MS system.

RESULTS AND DISCUSSION

Preliminary experiments

The 'soft' ionization behavior of the new interface was tested using standards in solvent on molecules with a well-known high degree of fragmentation in EI spectra. As illustrative example, we show the MS spectra for dieldrin (Fig. 1(A)). In this particular case, when an nw-XIC at the M+· was extracted, no signal was observed in the chromatogram, as the molecular ion was practically absent in the EI spectrum. However, very different results were obtained using the softer ionization produced by the APCI interface (Fig. 1(B)). In this case, an important peak was located (*t*R = 16.56 min) when extracting an nw-XIC at the M+· of dieldrin as can be seen in Fig. 1(B). When looking at the two MS spectra, very different fragmentation patterns were observed. In contrast with the EI spectrum, where the molecular ion was almost absent, in the APCI spectrum M+· has become the base peak.

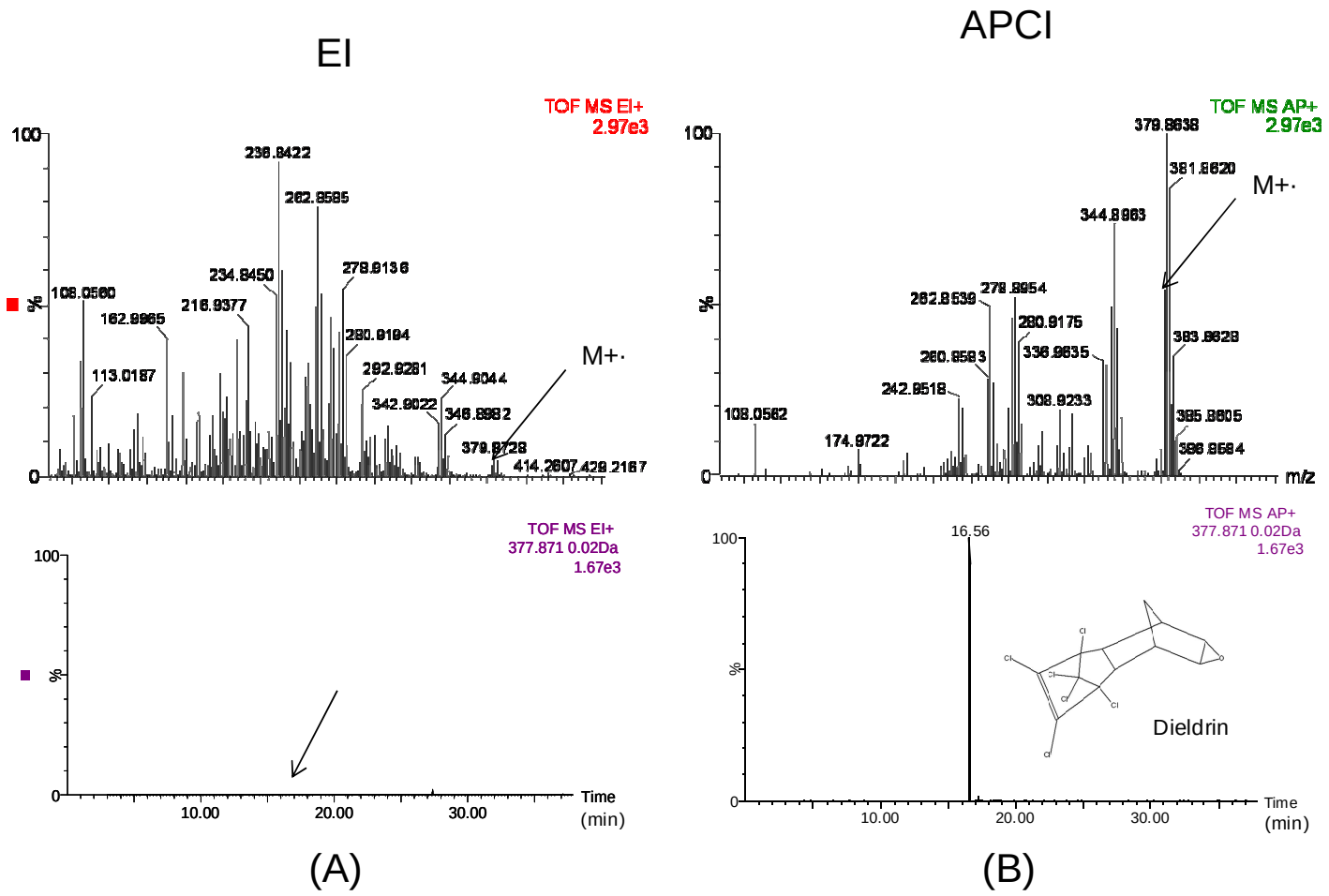

Figure 1. (A) Extracted ion chromatogram at M+· of dieldrin and TOF MS spectrum in EI source. (B) Extracted ion chromatogram at M+· of dieldrin and TOF MS spectrum in APCI source.

This behavior, when using N_2 as make-up gas, could be explained using the following molecule reactions where M represents the analyte. The corona discharge needle creates a nitrogen plasma, N_2^+ and N_4^+, which in the case of charge transfer reacts directly with analyte molecules (Fig.2(A)).

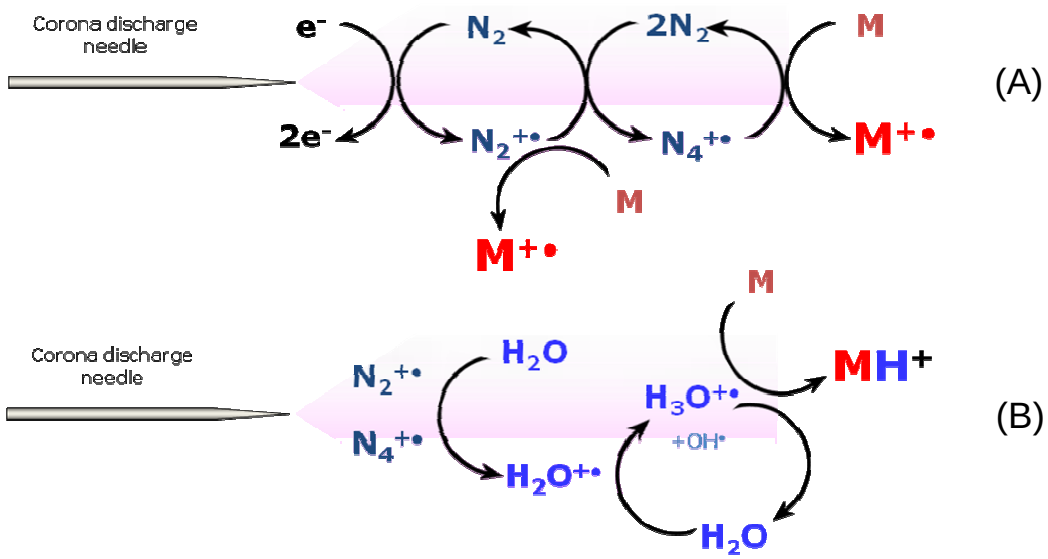

Figure 2. Molecule reactions when using APCI source. N_2 transfer conditions (A). Proton transfer conditions (B). M:analyte

These satisfactory results encouraged us to test this source on pesticides from the endosulfan family, which are known to have significantly fragmented EI spectra. In the case of α-endosulfan, no chromatographic peak (tR=15.69 min) was observed when doing a nw-XIC at its M+· on APCI data (Fig. 3(A)). However, when looking at the APCI spectrum, the expected isotopic pattern of hexachlorinated α-endosulfan was clearly observed at MH+ instead of M+·. An explanation of this fact might be the presence of water vapor traces in the source, which readily promote the formation of the protonated molecule instead of the molecular ion. This marked tendency of the molecule to be protonated led us to repeat the experiment but introducing water as a

modifier to facilitate the formation of the protonated molecule. The modifier was placed in an uncapped vial, which was located within a specially designed holder placed in the source door. In this case, the results obtained were even more satisfactory as fragmentation decreased and the protonated molecule was converted in the base peak of the spectrum (Fig. 3(B)).

This proton transfer behavior could be explained because of the nitrogen plasma reacts with water, or any proton source, and indirectly transfers protons to the analyte of interest (Fig. 2)B)).

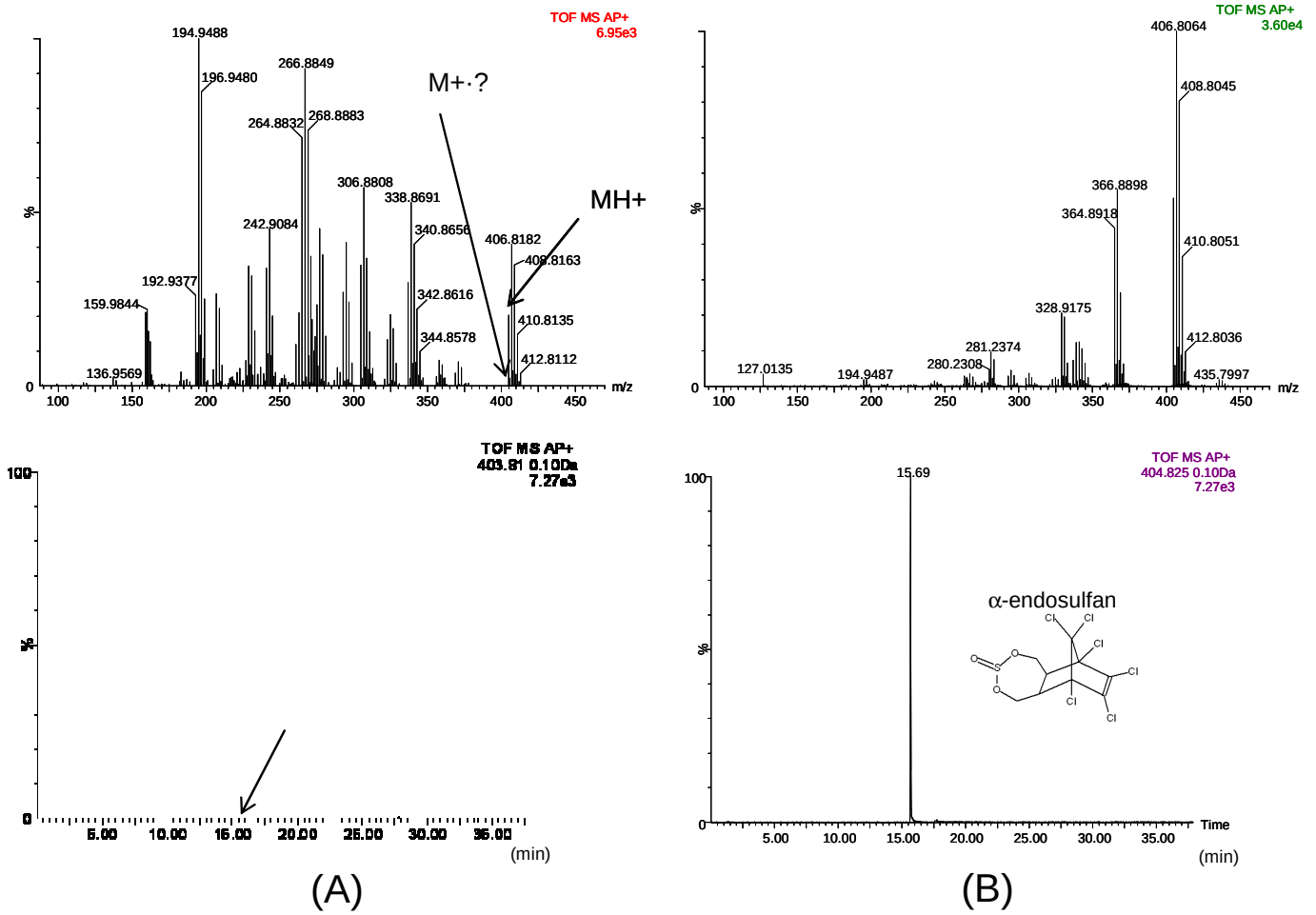

Figure 3. (A) Extracted ion chromatogram at M+• of α-endosulfan and TOF MS spectrum when using APCI source. (B) Extracted ion chromatogram at MH+• of α-endosulfan and spectrum in the APCI source using water as modifier.

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Systematic study

At this point a wider study of the APCI possibilities for screening purposes was carried out. Around 100 GC-amenable pesticides of different families (organochlorine, organophosphorus, organonitrogenated and others) were selected to study their APCI behavior. Standards in solvent were used for this purpose, which were injected into the GC(APCI)TOF MS instrument under generic chromatographic conditions. APCI-TOF MS data were evaluated as regards the presence/absence of the $M^{+\bullet}$ in the spectrum obtained under N_2 charged transfer conditions. The presence/absence of the MH^+ was also investigated as a consequence of the observed behavior for some compounds to be protonated during preliminary experiments due to water traces present in the source. In a second step, water was added on purpose as modifier and the presence/absence and/or improvement on the signal of the protonated molecule was evaluated. All gathered information was finally used to classify the compounds in six different groups according to their ionization behavior in the APCI source (Fig.4).

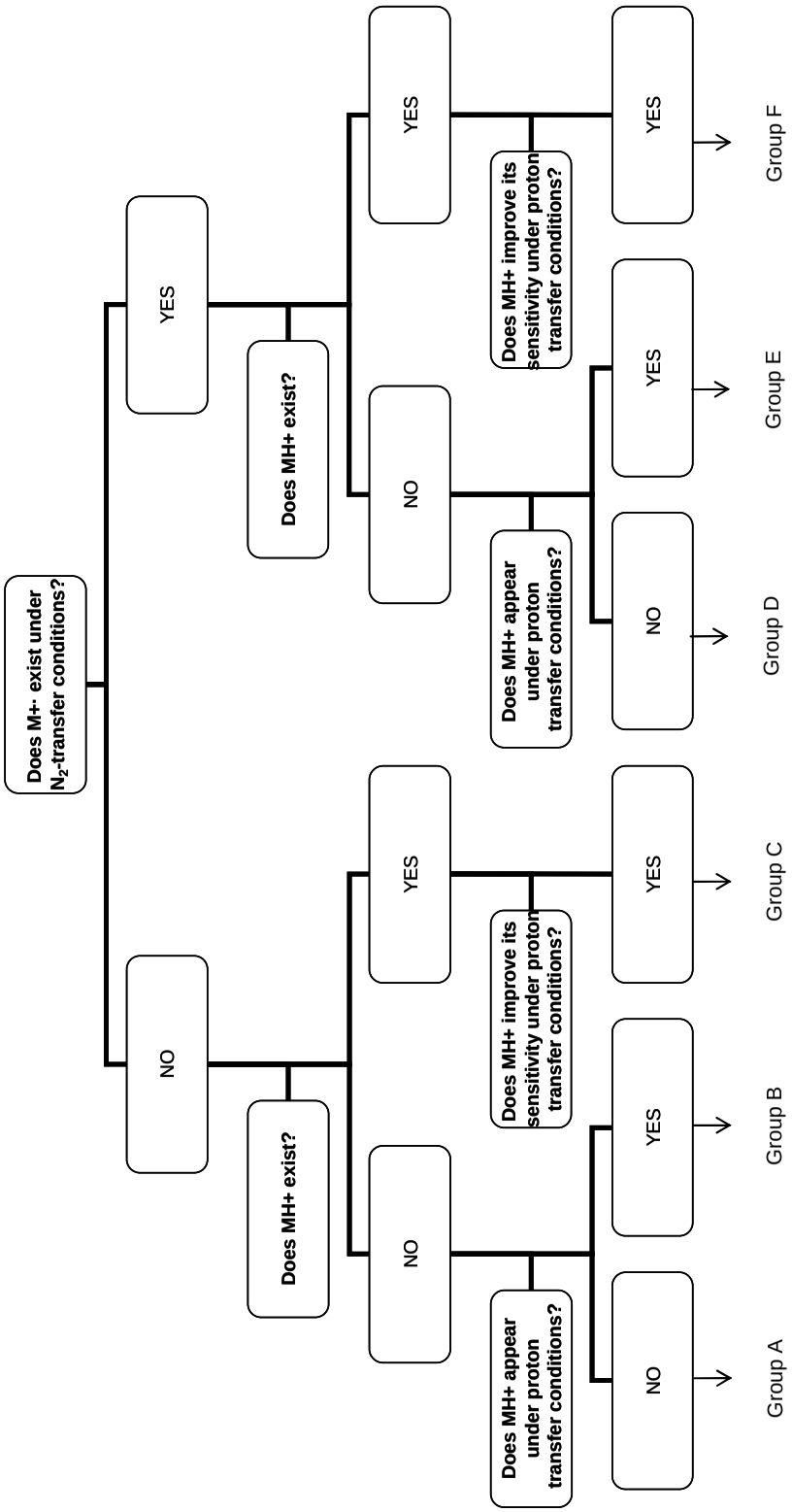

Figure 4. Scheme of the different behaviours for pesticides when using the APCI source.

The first group (A) included those compounds for which no presence of M+· or MH+ was observed in the APCI data under N_2- or proton transfer conditions (Fig. 5(A)). This behavior was observed for labile organochlorine insecticides that do not have an acidic group in their structure capable to be protonated (HCH isomers, *p*,*p*'-DDT and mirex). As regard Group B, this was formed by those compounds that, although no presence of M+· or MH+ was observed in the APCI data with N_2, showed an abundant formation of MH+ when introducing water as a modifier (Fig. 5(B) shows the MS spectra for phosmet, one of the pesticides included within Group B). For some compounds of this group, the addition of water as a modifier was so favorable that the MH+ ion was the base peak of the spectrum. Within the compounds that did not show the presence of M+· in the N_2-transfer APCI spectrum, we might consider another group (C) characterized because MH+ was present in their spectrum. We proved that the formation of the protonated ion was highly favored when adding water as a modifier with MH+ becoming the base peak of the spectrum (Fig. 5(C) shows propiconazole as an illustrative example).

The rest of the pesticides, which presented M+· ion in the N_2 APCI spectrum, were classified into another three groups. Group D was formed by those compounds that did not show MH+ in the N_2 APCI spectrum or after adding water. This happened for organochlorine insecticides where no acid group capable of being protonated was present in their structure (pentachlorobenzene, isodrin, HCB, *p*,*p*'-DDE, trans-chlordane, *p*,*p*'-DDD, heptachlor and fluvalinate). In these cases, when adding water as a modifier, little change was observed in the spectrum (Fig. 6(D) shows HCB as an example). Another group (E) included those compounds that did not show MH+ in the N2 APCI spectrum, but protonating after water addition (Fig. 6(E) shows mevinphos as an example). The last group of compounds (F) was the most numerous. Both M+· andMH+ were present in the N_2 APCI spectrum. Obviously, when adding water as a modifier, the formation of MH+ was favored and it finally became the base peak of the spectrum in most cases (Fig. 6(F) shows deltamethrin as an example). Groups of pesticides according to their ionization/fragmentation behavior in the APCI source are shown in Table 1.

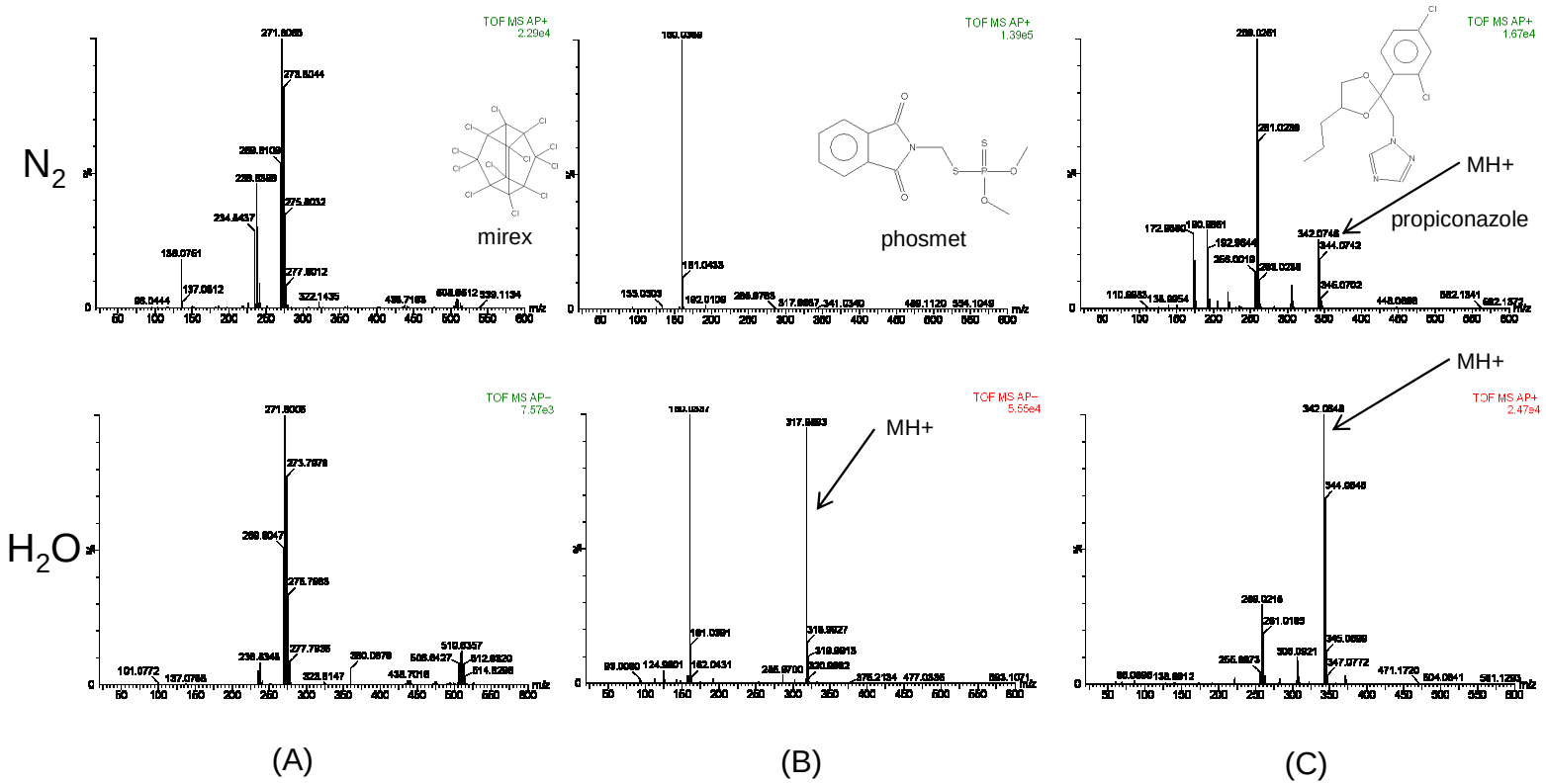

Figure 5. APCI spectra of selected pesticides, belonging to groups A, B and C: mirex (A), phosmet (B) and propiconazole (C). Spectra without adding water as modifier (top). Spectra using water as modifier (bottom).

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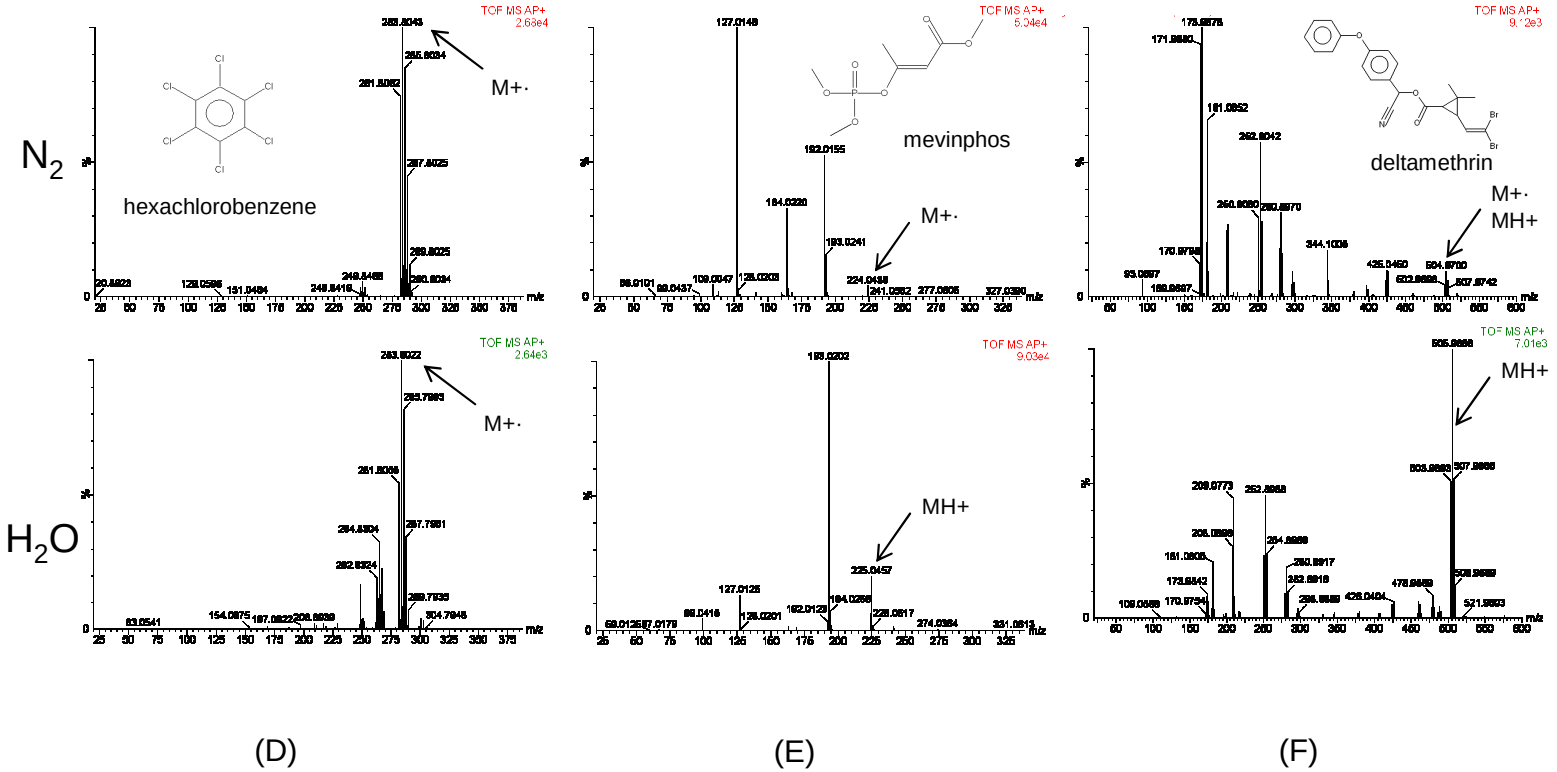

Figure 6. APCI spectra of selected pesticides belonging to groups D, E and F: hexachlorobenzene (D), mevinphos (E) and deltamethrin (F). Spectra without adding water as modifier (top). Spectra using water as modifier (bottom).

Table 1. Groups of pesticides according to its ionization/fragmentation behaviour in APCI source

Group A	Group B	Group C	Group D	Group E	Group F	
α-HCH	malathion	methamidophos	pentachlorobenzene	isodrin	tecnazene	quinalphos
γ-HCH	phosmet	dichlorvos	HCB	dieldrin	diphenylamine	procymidone
β-HCH	terbufos	ethoprophos	*p,p'*-DDE	endosulfan sulfate	trifluralin	profenofos
δ-HCH	disulfoton	dimethoate	trans-chlordane	mevinphos	simazine	myclobutanil
p,p'-DDT	methidathion	fonofos	*p,p'*-DDD	methacrifos	atrazine	bupirimate
mirex	azinphos methyl	tolclofos methyl	heptachlor	heptenophos	terbuthylazine	endrin
		fenchlorphos	fluvalinate	chlorpropham	propyzamide	oxadixyl
		chlorfenvinphos		phorate	pyrimethanil	ethion
		α-endosulfan		dichlofluanid	diazinon	triazophos
		tetrachlorvinphos		tolylfluanid	chlorthalonil	fosalone
		imazalil			etrimfos	lambda-cyhalothrin I
		β-endosulfan			endosulfan ether	fenarimol
		propiconazole i			pirimicarb	pyrazophos
		tebuconazole			fosfamidon	pyridaben
		pyriproxyfen			metribuzin	coumaphos
		iprodione			parathion methyl	cypermethrin
		metoxychlor			metalaxil	deltamethrin
					fenitrothion	azoxystrobin
					pirimiphos methyl	quintozene
					aldrin	chlorpyrifos methyl
					fenthion	alachlor
					chlorpyrifos ethyl	buprofezin
					parathion ethyl	bifenthrin
					pirimiphos ethyl	permethrin I
					cyprodinil	fenvalerate
					heptachlor epox A	esfenvalerate
					chlozolinate	

In light of our results, it seems that an APCI source using water as a modifier gives us confidence that the protonated molecule will be observed for the majority of the pesticides. Additionally, in most cases the protonated molecule is the base peak of the spectrum and very low fragmentation of the molecule occurs in APCI ionization compared to the well known EI spectra. This fact allows us to perform a wide-scope screening of pesticides in a different way to the traditional approach based on EI spectra. The low probability of observing the intact molecular ion (M+·) in conventional EI spectra forces the analyst to know in advance the main fragment ions in order to select the appropriate nw-XIC at their exact masses. This information could be taken from the injection of standards into the GC-MS system or from commercial EI spectra libraries, resulting in a time-consuming procedure especially when no standard is available and the information has to be taken from commercial nominal mass libraries. However, using the new APGC source, the predictable presence of the protonated molecule in GC-(APCI)TOF MS data would allow an automatic and rapid 'post-target' search of around 100 pesticides by only extracting the chromatogram at the exact mass of the MH+ ion. Then, we were able to use the same approach developed for UHPLC(ESI)QTOFMS using the ChromaLynx XS application manager, building a list of compound names and molecular formulae and performing fast, sensitive and wide-scope screening for GC-amenable pesticides. In addition, favoring the presence of the protonated molecule, the sensitivity improves due to the higher abundance of the ion monitored. Thus, the best option to perform wide-scope screening would be to use water as a modifier, and investigate the presence of the MH+ ion as it was present in the APCI spectra of 90% of the pesticides studied in this work. A second injection without using water would be carried out in order to investigate the presence of M+· for the rest of compounds, such as HCH isomers, *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, mirex, pentachlorobenzene,HCB, trans-chlordane, heptachlor and fluvalinate.

Application to real samples

The developed procedure was applied to the screening of pesticides in incurred food samples. The analysis of orange, nectarine and spinach samples was carried out in a 'post-target' way performing an accurate mass screen of around 100 pesticides after full MS acquisition using ChromaLynx XS application manager. Briefly, this application

manager automatically processes MS data and obtains XICs with a user-defined mass window (0.02 Da in this study) at selected *m/z* ions, typically those corresponding to the exact masses of the protonated molecules, based on a preselected list of exact masses and retention times, if available. Besides, the software allows visualization of the complete TOF MS spectrum of the positive findings. This facilitates a rapid and simple review by cataloging pollutants by colors, as a function of their attainment of retention time and mass errors. Following this approach, the insecticides chlorpyriphos ethyl and deltamethrin were detected and identified in a nectarine sample. As an example, Fig. 7 illustrates the detection and reliable identification of chlorpyriphos ethyl in incurred nectarine at a concentration level of 0.01mg/kg. A mass error of 0.9 mDa was obtained for the MH+ ion, and the expected isotopic pattern associated to the presence of three chlorine atoms was also observed.

Figure 7. Detection and identification of chlorpyriphos ethyl by GC-(APCI)TOF MS in a nectarine sample. (A) List of compounds investigated together with their molecular formulae, exact mass and retention time when available. (B) Extracted-ion chromatogram at m/z 349.9341 (MH+). (C) APCI mass spectrum of chlorpyriphos ehyl in the nectarine sample.

The availability of a QTOF instrument made it feasible to perform MS/MS experiments for both standards and samples to go further in the confirmation of the identity of the compounds detected. Collision energy of 10 eV was applied for this purpose. Thus, when comparing the relative ion abundances in the suspected positive sample of chlorpyriphos with those of a reference standard, all deviations were within the limits established by general guidelines in residue analysis, as the European Decision 2002/657/EC for contaminants and residues in food of animal origin legislation or Document SANCO/10684/2009 for pesticides control in food and feed.[32,33] Chemical structures for the most abundant product ions were suggested based on the elemental compositions proposed accordingly to the accurate mass measurements given by the instrument for these ions. All mass errors for chlopyriphos product ions in the nectarine sample were below 2.7 mDa as depicted in Fig. 8. Finally, retention times for the reference standard and sample peak were also compared, obtaining a deviation lower than 0.5%. Therefore, this sample was unequivocally confirmed by QTOF to be positive for chlorpyriphos ethyl. Similarly, positive findings of deltamethrin in nectarine and endosulfan sulfate in orange could be reported. In all cases, MS/MS experiments, selecting MH+ as precursor ion, led to the reliable confirmation of their identity.

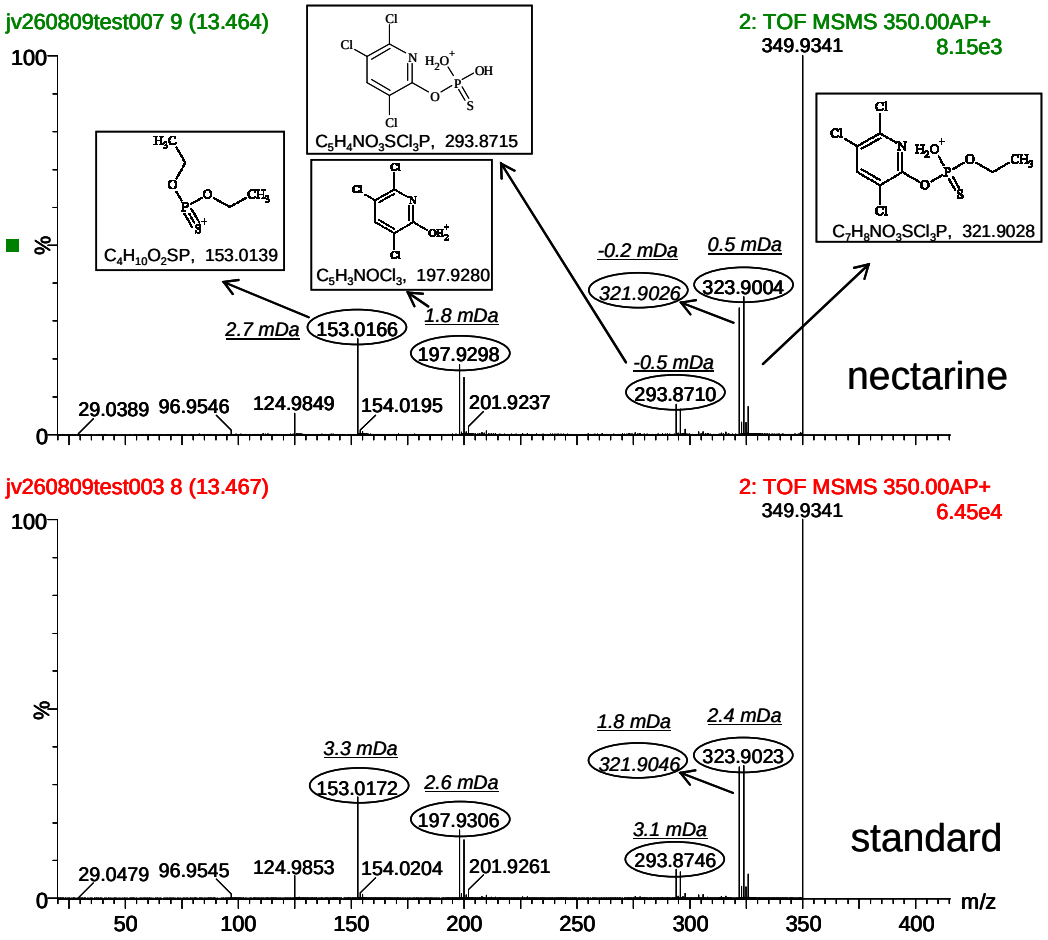

Figure 8. Product ion spectra (collision energy, 10eV) for chrlopyriphos ethyl (precursor ion, m/z 350) from a nectarine positive sample (top) and from the reference standard (bottom). Chemical structures proposed for the most abundant product ions.

CONCLUSIONS

The usefulness of a new atmospheric pressure source for GC-MS systems is reported in this work. The predictable presence of the protonated molecule when using this prototype APGC source in a GC-TOF MS system has allowed us to perform rapid and wide-scope screening of around 100 pesticides in food samples. The use of water as a modifier favored the formation of the protonated molecule, which was present in the TOF MS spectra of 90% of the pesticides investigated. Additionally, in most cases the protonated molecule was the base peak of the spectrum, and very low fragmentation was observed in APCI ionization when compared with the well-known EI spectra. This facilitates rapid and sensitive screening, searching for the protonated pesticide molecule, which is typically absent in the highly fragmented EI spectra. The ChromaLynx XS application manager allowed a list of compound names, molecular formulae, and exact masses to be built making it easier to perform fast and wide-scope screening of GC-amenable pesticides. The developed procedure has been applied to the analysis of fruit and vegetable samples with the result of detecting and identifying several pesticides. Additionally, the availability of a QTOF instrument made it feasible to perform MS/MS experiments to go further in the confirmation of the identity of the compounds detected in samples.

The results shown in this paper have been obtained using a prototype source, which exhibits promising features that might also be applied to other analytical approaches. For instance, to facilitate the detection and confirmation step in only one injection, a collision cell fragmentation might be performed. In this case, two acquisition functions would be monitored: the first one, at low collision energy, to detect the protonated molecule and the second one, at high collision energy, as a confirmatory function obtaining a similar fragmentation to that of MS/MS experiments. This acquisition mode, known as MSE, has shown excellent results in LC-QTOF MS applications investigated by our group,[34] and provides reproducible fragmented spectra without the need for ion pre-selection in the quadrupole.

The reduced fragmentation generated by the APGC source will surely have a significant impact on target analysis at trace levels. The high degree of fragmentation for many compounds when using EI constitutes a problem when selecting the precursor ion in tandem MS experiments. Often, this fact reduces selectivity and sensitivity of

analysis. With the reduced fragmented spectrum given by the APGC source, the precursor ion selection no longer requires a compromise between selectivity and sensitivity allowing more convenient tandem MS experiments. Preliminary results shown in this paper are promising, but further research is required to have a better knowledge on the possibilities of this source in different fields, e.g. environmental MS or food safety.

ACKNOWLEDGMENTS

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of GC-TOF MS (GCT) and to the MS Technologies Centre (Waters Corporation, Manchester, UK) for using the GC-APGC-Xevo QTof. The authors acknowledge the financial support of Generalitat Valenciana, as research group of excellence PROMETEO/2009/054. The authors also acknowledge the Agencia de Salud Pública (Barcelona) for providing the samples and Inés Cervera for sample treatment. T. Portolés is very pleased to University Jaume I for her post-doctoral grant.

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CAPÍTULO 4

Uso combinado de GC-MS y LC-MS con analizadores de triple cuadrupolo y tiempo de vuelo en estudios ambientales y toxicológicos

4.1 INTRODUCCIÓN

La GC es una técnica muy adecuada para la determinación de compuestos apolares y volátiles, mientras que LC está más indicada para compuestos polares/iónicos y no volátiles, complementándose ambas mutuamente.

Como se ha mostrado anteriormente, los analizadores TOF MS son muy útiles con fines de *screening*, confirmación y elucidación de microcontaminantes orgánicos, aportando una gran versatilidad al análisis cuando se combina con GC y LC. Aunque su potencial para fines de cuantificación ha mejorado en los nuevos modelos (generalmente presentaban un bajo rango dinámico de linealidad), los métodos *target*, desarrollados y validados para unos cuantos compuestos seleccionados (por ejemplo, usando GC-MS/MS ó LC-MS/MS con equipos de triple cuadrupolo) parecen ser mas apropiados para ese fin. Considerando el carácter complementario de GC-MS y LC-MS, el uso combinado de estas dos técnicas con analizadores de TOF y triple cuadrupolo en ambos casos constituye, hoy en día, una de las aproximaciones más poderosas en el campo ambiental. Con esta configuración se puede abarcar el mayor número posible de contaminantes orgánicos, con muy diferentes propiedades fisico-químicas, y alcanzar así el alto nivel de multirresidualidad requerido en determinados tipos de análisis. Además, se cubriría tanto el análisis cualitativo, al poder realizar barridos

prácticamente universales, como el cuantitativo, al utilizar MS/MS con equipos de triple cuadrupolo.

En el presente capítulo se explora la capacidad analítica que aporta la combinación de las técnicas GC-MS y LC-MS con analizadores de triple cuadrupolo y tiempo de vuelo para un *screening* amplio de contaminantes orgánicos en dos campos de aplicación, el medioambiental y toxicológico, abordando los aspectos cualitativos y cuantitativos.

En el primer apartado se combinan métodos GC-MS/MS y LC-MS/MS con triple cuadrupolo con fines cuantitativos para investigar la presencia de una amplia variedad de contaminantes orgánicos de diferentes características físico-químicas en aguas de lixiviado de residuos sólidos urbanos. Los métodos aplicados habían sido previamente optimizados y validados en nuestro laboratorio, en términos cuantitativos. Concretamente, el método por GC-(QqQ)MS/MS correspondió al desarrollado en el capítulo 2 de la presente tesis, el cual se aplica de nuevo en este capítulo para la determinación de alrededor de 60 compuestos de diferentes familias químicas, como PAHs, octil/nonil fenoles, PCBs, OCs, insecticidas, herbicidas, y PBDEs. Con respecto al método por UHPLC–(QqQ)MS/MS, el desarrollo del mismo (objetivo ajeno a la presente tesis) se había llevado a cabo previamente en nuestro laboratorio, dando lugar a un método rápido, sensible y selectivo para la determinación de 37 plaguicidas (1). El método está basado en una etapa de preconcentración por SPE off-line con cartuchos HLB previa al análisis por UHPLC-(QqQ)MS/MS. Ambos métodos se aplicaron al análisis de 41 muestras de lixiviados (20 aguas brutas de lixiviado, antes de depurar, y 21 ya depuradas) recogidas entre Marzo de 2007 y Febrero de 2009. Con la finalidad de investigar la presencia de otros contaminantes que no estuvieran en la listas *target* de los métodos mencionados, y ampliar así el nivel de multirresidualidad del *screening*, se analizaron las muestras por GC–TOF MS y LC–QTOF MS, procesándose los dados siguiendo una metodología *non-target*. Los resultados de este trabajo se detallan en el **artículo científico 10**.

En el segundo apartado se explota el potencial que presenta el uso combinado de GC-TOF MS y UHPLC-QTOF MS para investigar de la causa de un episodio de mortandad masiva de abejas en entornos apícolas de la Comunidad Valenciana. El tratamiento de muestra aplicado fue común para las dos técnicas y se basó en una partición con disolventes seguida de una extracción líquido-líquido y preconcentración.

Ante el desconocimiento sobre la posible causa de mortandad, se realizó un *screening non-target* por GC-TOF MS y UHPLC-QTOF MS, aprovechando la elevada información generada en los análisis, al disponer de espectros de iones completos medidos con elevada exactitud de masa. Esta metodología se aplicó en un segundo episodio de mortandad de abejas, en el que no sólo se analizaron las muestras de abejas recibidas, sino también muestras de hojas y flores de nectarina cercanas al área de las abejas y sospechosas de ser responsables del envenenamiento de las mismas. Finalmente, se aprovecharon las ventajas de las técnicas empleadas para investigar los posibles metabolitos de los principales compuestos detectados en las abejas. Los resultados de este trabajo se detallan en profundidad en el **artículo científico 11**.

Es interesante resaltar las diferencias existentes entre GC-TOF MS y LC-QTOF MS cuando se realiza un análisis *non-target*. Tal y como se ha ido mostrando a lo largo de la tesis, el procesamiento de datos en la aproximación *non-target* es una de las claves del éxito. Se puede llevar a cabo con la ayuda de softwares diseñados para la detección de componentes mediante algoritmos CODA (*COmponent Detection Algorithm*), capaces de extraer los componentes de la muestra que se hayan cromatografiado e ionizado, aunque a veces no sean visibles en el cromatograma TIC, porque se encuentran enmascarados por el ruido de fondo de la matriz, o incluso por sangrado de columna en GC o del gradiente en LC. Estos softwares asignan finalmente un espectro de masas deconvolucionado, que se emplea para la identificación del componente. Para la identificación fiable del compuesto responsable del pico cromatográfico, es necesario comparar el espectro deconvolucionado con una librería de espectros. En el caso de GC-(EI)TOF MS, existe la ventaja de disponer de librerías comerciales de espectros de masas obtenidos en la fuente de ionización electrónica. Sin embargo, no se encuentran registrados en masa exacta, por lo que se pierde una información valiosa durante el proceso de comparación de espectros. Por otro lado, en LC-(ESI)TOF MS no existen librerías comerciales de espectros, principalmente por la falta de homogeneidad entre las interfases de los diferentes fabricantes. Por ello se necesitan librerías teóricas o empíricas realizadas por el propio usuario.

Con el ánimo de reducir el número de posibles candidatos para los componentes encontrados, se emplea el valor añadido de la masa exacta. La evaluación de los errores de masa observados para los diferentes candidatos propuestos permite eliminar algunos de ellos, y la disponibilidad de un espectro deconvolucionado con varios iones

fragmento facilitará la asignación de la identidad del componente. En el caso de GC-EI-TOF, con librerías de más de 150.000 espectros se puede confirmar la identidad del compuesto detectado al disponer de su espectro de iones completo medido con elevada exactitud de masa, evaluando a través de los errores de masa si los fragmentos del espectro son compatibles con fragmentos del compuesto identificado. Esta etapa se realiza a través del software, pero es muy importante revisar que las composiciones elementales asignadas a cada ion fragmento son coherentes con la estructura química del compuesto. En el caso de LC-(ESI)TOF MS, los iones fragmento en el espectro son mucho menos abundantes, sino inexistentes por tratarse de una fuente de ionización muy suave. Cuando, para la fórmula molecular más plausible, pueden existir varias estructuras químicas posibles, se pueden utilizar otros parámetros para descartar algunas no viables. Por ejemplo, es interesante adquirir un espectro de iones producto del componente desconocido y evaluar si la fragmentación observada puede justificarse a partir de la estructura propuesta. Esta aproximación se puede llevar a cabo mediante QTOF, aislando el ión precursor y estudiando los iones producto producidos en el analizador TOF en masa exacta, lo que permitirá descartar con mayor facilidad las estructuras no viables.

Una posibilidad que se abre con los instrumentos QTOF actuales es la de obtener los espectros MS a alta (HE) y a baja energía (LE) de colisión de manera "simultánea" (lo que se conoce como MS^E) ya que el equipo trabaja siempre con gas de colisión en la celda. Esto permite minimizar la fragmentación (LE) o favorecerla (HE) modificando la energía aplicada en la celda de colisión, con lo que, en una única inyección, se puede obtener la información del ión precursor y iones fragmento, acelerando el proceso de descarte anteriormente comentado. Finalmente, para la confirmación inequívoca del componente desconocido, es siempre deseable disponer del producto de referencia para comparar el tiempo de tiempo de retención y los espectros MS. Este modo de trabajo de MS^E sería aplicable, en principio, tanto a equipos de GC como de LC siempre que vayan acoplados a un analizador QTOF MS.

En los trabajos que se presentan a continuación se utiliza una librería teórica para LC-QTOF MS confeccionada en nuestro grupo de investigación, que contiene aproximadamente 500 compuestos (plaguicidas, productos de transformación, antibioticos y fármacos). Esta librería está continuamente en proceso de ampliación y en el momento de redacción de esta tesis consta de 1200 compuestos (drogas,

colorantes, fenoles, preservantes, etc, además de los mencionados anteriormente). Para aproximadamente 200 compuestos, se dispone también de tiempo de retención y de iones fragmento ya que se van inyectando los mismos en el equipo a medida que se van obteniendo los productos de referencia, definiendo unas condiciones fijas de trabajo: tipo de columna cromatográfica, fase móvil, gradiente, etc. para que el espectro de masas y los tiempos de retención sean posteriormente comparables.

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4.2 ESTRATEGIAS ANALÍTICAS BASADAS EN EL USO DE LC Y GC ACOPLADO A ESPECTROMETRÍA DE MASAS CON ANALIZADOR DE TRIPLE CUADRUPOLO Y TIEMPO DE VUELO PARA LA INVESTIGACIÓN DE CONTAMINANTES ORGÁNICOS EN AGUAS RESIDUALES.

En este apartado se estudia el uso combinado de LC y GC acopladas a MS con triple cuadrupolo y tiempo de vuelo para la investigación de contaminantes orgánicos en aguas de lixiviado de residuos sólidos urbanos, antes y después del proceso de depuración. El gran potencial analítico de estas técnicas permite abarcar la determinación de un elevado número de contaminantes orgánicos con un amplio rango de polaridades, y así poder evaluar la eficiencia del proceso de depuración aplicado a las aguas brutas por ósmosis inversa.

En una primera etapa se llevó a cabo un análisis *target* con fines cuantitativos aplicando métodos desarrollados y validados previamente para un cierto número de contaminantes prioritarios. En total, aplicando las técnicas, GC-(QqQ)MS/MS y UHPLC-(QqQ)MS/MS se incluyen aproximadamente unos 100 analitos.

En concreto, el método multiresidual GC-(QqQ)MS/MS permite la determinación de unos 60 compuestos no volátiles o semi-volátiles pertenecientes a diferentes familias químicas, tales como insecticidas organoclorados y organofosforados, herbicidas, PCBs, PAHs, PBDEs y octil/nonil fenoles. La mayoría de los contaminantes seleccionados son considerados relevantes según la política de aguas de la Unión Europea, estando incluidos en el Anexo X de la Directiva 2000/60/EC. Este método ha sido explicado con detalle en el capítulo 2 de la presente tesis.

Por otra parte, cabe señalar muy brevemente, ya que no forma parte del objetivo de la presente tesis, que el procedimiento multiresidual complementario mediante LC se basa en la técnica UHPLC-(QqQ)MS/MS (1). La cromatografía líquida de ultra resolución (UHPLC) constituye una poderosa e innovadora técnica cromatográfica que introduce una elevada resolución y sensibilidad así como menores tiempos de análisis. UHPLC se presenta actualmente como una herramienta ideal para el análisis multi-residual de contaminantes orgánicos en muestras medioambientales. Sin embargo, para acoplar la técnica UHPLC con espectrometría de masas es necesario trabajar con analizadores de triple cuadrupolo de rápida adquisición (en nuestro caso, TQD TM), los cuales permiten reducir el valor de *dwell time* y aumentar el número de transiciones

SRM adquiridas sin pérdida de sensibilidad. El método aplicado presenta la ventaja de adquirir tres transiciones para cada analito, una de cuantificación y dos de confirmación. El tratamiento de muestra aplicado incluye una concentración *off-line* basado en SPE con cartuchos OASIS HLB (200 mg). En el caso de las aguas brutas, previamente a la etapa de extracción, la muestra se diluye 50 veces con agua HPLC debido a su alta carga orgánica. El método aplicado permite la determinación de 37 contaminantes orgánicos y ha sido validado en términos de exactitud y precisión, obteniéndose resultados satisfactorios para la mayoría de los compuestos estudiados, con LOQs de 0.025 µg/L.

La metodología descrita, basada en GC-(QqQ)MS/MS y UHPLC-(QqQ)MS/MS, se ha aplicado al análisis de 41 muestras de aguas de la planta de compostaje de RECIPLASA (21 depuradas y 20 aguas de lixiviado brutas antes de someterse al proceso de depuración) con el fin de determinar aproximadamente 100 contaminantes orgánicos (tabla 1 del **artículo científico 10**).

Los positivos detectados se cuantificaron únicamente cuando la concentración era superior al LOQ objetivo, que fue el valor de concentración más bajo para el cual el método fue validado (0.025 µg/L, excepto para heptacloro epóxidos, α- y β-endosulfan, PBDEs, naftaleno y simazina, para los que fue de 0.25 µg/L). Todos los positivos se han confirmado comprobando que el valor de Q/q se encontraba dentro de la tolerancia establecida en la Decisión de la Comisión Europea (2002/657/EC) (2), lo cual ha permitido asegurar una completa fiabilidad en la identificación del contaminante.

En cada secuencia de muestras, se incluyeron muestras controles de calidad (QC) preparadas a partir de un agua depurada y agua bruta de lixiviado fortificadas a un nivel de concentración conocido. En general, los valores de recuperación obtenidos para los QCs fueron satisfactorios (70-120%).

Destacan por su mayor frecuencia de detección los plaguicidas, especialmente herbicidas (fenilureas, triazinas, uracilos y carbamatos), fungicidas (benzimidazole, conazole y anilida) e insecticidas (carbamatos y organofosforados). Entre los compuestos que no son plaguicidas, los más detectados fueron los octil/nonil fenoles y los PAHs.

En las aguas depuradas, los niveles de concentración raramente han superado el valor de 0.1 µg/L, el cual suele tomarse como referencia en aguas por tratarse del

valor máximo permitido para la mayoría de contaminantes orgánicos, incluidos plaguicidas, en aguas de abastecimiento urbano. En el caso concreto de 4-t-octilfenol, clorfenvinfos, clorpirifos, diuron y simazina, positivos encontrados en las aguas depuradas y que aparecen como sustancias prioritarias en las normas de calidad ambiental (NCA) en aguas superficiales (Propuesta Directiva 2006/0129), ninguno de ellos ha superado el nivel de concentración máxima admisible establecido para dichos contaminantes (0.1, 0.3, 0.1, 1.8 y 4 µg/L, respectivamente).

Los resultados obtenidos para las aguas depuradas contrastan con los de las aguas de lixiviado brutas, en las que el porcentaje de detecciones y los niveles de concentración encontrados han sido notablemente superiores, como era de esperar. Así, en muchos casos se ha superado el nivel de 0.1 µg/L. De nuevo, los plaguicidas han sido los compuestos más frecuentemente detectados. Destacan especialmente los herbicidas diuron, simazina, terbacilo y terbutrina, que se han detectado en todas las muestras analizadas, alcanzándose en algunos casos niveles superiores a 10 µg/L. También destacan algunos insecticidas como carbaril, carbofuran, dimetoato y pirimicarb, los cuales también han sido detectados en todas las muestras analizadas. En cuanto a los fungicidas más frecuentes, imazalil y triadimenol se han detectado en todas las muestras en concentraciones inferiores a 2.3 µg/L. Otros contaminantes, como 4-t-octilfenol, se han detectado en las todas las muestras analizadas con un nivel máximo de concentración de 5.6 µg/L.

De los resultados obtenidos, se deduce que el sistema de depuración empleado, basado en ósmosis inversa, es eficaz y consigue rebajar considerablemente los niveles de concentración de los contaminantes orgánicos encontrados en las aguas de lixiviado brutas.

En una segunda etapa, se han analizado las mismas muestras por GC-TOF MS y UHPLC-QTOF MS con el objeto de detectar más contaminantes que los incluidos inicialmente en el método *target*. El análisis por GC-TOF MS se realizó tanto desde una aproximación *post-target* (detectando diazinon, diclorvos, difenilamina y fention) como *non-target* (se ha detectado la presencia de compuestos como el BHT, cafeína, ibuprofeno, nicotina, etc). El análisis *non-target* por UHPLC-QTOF MS ha permitido detectar compuestos como por ejemplo el atenolol, cocaína, paracetamol, eritromicina, entre otros.

La aplicación de métodos basados en el uso de TOF MS utilizando tanto GC como LC, permite ir ampliando las listas de compuestos *target* en función de los resultados obtenidos en modo *non-target*. Es decir, aquellos compuestos que han sido detectados gracias a las aproximaciones *non-target*, se pueden ir incluyendo paulatinamente en los métodos multiresiduales basados en LC-(QqQ)MS/MS o GC-(QqQ)MS/MS actualmente usados en nuestro laboratorio, con el fin de proceder a su control periódico y a su cuantificación.

4.2.1. Bibliografía

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4.2.2 Artículo científico 10

Anal. Bioanal. Chem. 397, 2763-2776, 2010

ANALYTICAL STRATEGY BASE ON THE USE OF LC AND GC COUPLED TO MASS SPECTROMETRY WITH TRIPLE QUADRUPOLO AND TIME-OF-FLIGHT ANALYZERS FOR INVESTIGATING THE PRESENCE OF ORGANIC CONTAMINANTS IN WASTEWATER

Elena Pitarch, Tania Portolés, José M. Marín, María Ibáñez, Fernando Albarrán, Félix Hernández

Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain,

ABSTRACT

The presence of a wide variety of organic pollutants with different physicochemical characteristics has been investigated in wastewater samples from a municipal solid-waste-treatment plant in Castellón, Spain. An advanced analytical strategy was applied—combined used of two powerful and complementary techniques, GC and LC, both hyphenated with tandem mass spectrometry with triple-quadrupole analyzers. The GC–MS–MS method was based on sample extraction using C18 SPE cartridges and enabled the determination of approximately 60 compounds From different chemical families, for example PAHs, octyl/nonylphenols, PCBs, organochlorine compounds, insecticides, herbicides, and PBDEs. Most of the compounds selected are included as priority contaminants in the European Union (EU) Water Directive. The UHPLC–MS–MS method, which provided high chromatographic resolution and sensitivity and short analysis time, used sample extraction with Oasis HLB SPE cartridges and enabled the determination of 37 (more polar) pesticides. The methodology developed was applied to the analysis of 41 water samples (20 untreated raw leachates and 21 treated samples) collected between March 2007 and February 2009. Amounts of the contaminants investigated rarely exceeded 0.5 μg L−1 in the treated (reverse osmosis) water samples analyzed. As expected, in untreated leachates the number of compounds detected and the concentrations found were notably higher

than in treated waters. The most commonly detected pollutants were herbicides (simazine, terbuthylazine, terbutryn, terbumeton, terbacil, and diuron), fungicides (thiabendazole and carbendazim), and 4-t-octylphenol. The results obtained proved that use of reverse osmosis for water treatment was efficient and notably reduced the amounts of organic contaminants found in raw leachate samples. In order to investigate the presence of other non-target contaminants, water samples were also analyzed by using GC–TOF MS and LC–QTOF MS. Several organic pollutants that did not form a part of the previous list of target contaminants were identified in the samples, because of the high sensitivity of TOF MS in full spectrum acquisition mode and the valuable accurate-mass information provided by these instruments. The insecticide diazinon, the fungicide diphenylamide, the UV filter benzophenone, N-butylbenzenesulfonamide (N-BBSA), the insect repellent diethyltoluamide, caffeine, and the pharmaceuticals erythromycin, benzenesulfonanilide, ibuprofen, atenolol, and paracetamol were some of the compounds identified in the water samples analyzed.

KEYWORDS

Organic pollutants .Wastewater .UHPLC . GC .Tandem MS . TOF MS

INTRODUCTION

Nowadays, many organic contaminants can be present in environmental water, normally at μg L−1 levels or below [1]. One of the routes for the contaminants to enter into the aquatic environment is from municipal solid-waste landfill leachates. These leachates frequently contain a variety of hazardous chemicals which may cause severe biological effects in the aquatic environment, because many of them are highly toxic or even carcinogenic [2, 3]. Therefore, efficient treatment of landfill leachates is required and monitoring of organic pollutants is essential to ensure the quality of treated water. When possible, the treatment process should be performed in municipal solid-waste (MSW) treatment plants, generally by the application of membrane technology, which is free from any chemical addition and uses relatively low energy. Membrane filtration, for example micro and ultra filtration or nano filtration and

reverse osmosis could be a choice for treatment of landfill leachates, depending of the type of particles or salts to be removed.

Selection of the analytical methodology to be applied to water-quality control is of outstanding relevance to obtaining realistic results, especially in the analysis of treated water that is discharged into the aquatic environment. The development of sensitive and multi-class methods for determination of organic contaminants in wastewater has become a major issue, because of the presence of many different compounds in this type of sample and strict European Union legal requirements for water quality [4–6]. General reviews relating to water analysis and emerging environmental contaminants [7–10] have been published over the last two years, reporting different analytical methods and new developments in this field.

Because of the complexity of the wastewater matrices, their high organic matter content, the low analyte levels typically found, and the large variety of organic contaminants with quite different physicochemical characteristics, the complementary use of gas chromatography (GC) and liquid chromatography (LC), both coupled to mass spectrometry (MS), is required to obtain a realistic and more complete overview of the organic pollution present in these waters. GC–MS has been the major adopted analytical technique to perform multi-residue analysis of volatile and semi-volatile organic pollutants [11]. Nowadays, enrichment by solid-phase extraction (SPE) using relatively low sample volumes followed by GC–MS or, even better, GC coupled to tandem mass spectrometry (MS–MS) is the preferred approach for GC-amenable micropollutants. Ion trap (IT) and triple quadrupole (QqQ) analyzers offer the possibility of adequate precursor and product-ion selection, which enables improvement of sensitivity (reducing the chemical noise in the chromatograms) and selectivity. The use of two stages of mass analysis in MS–MS systems based on QqQ enables work in selected reaction monitoring (SRM) mode, one of the most selective and sensitive approaches at present for quantification and confirmation, especially in trace water analysis. Our own research group has recently reported the determination of more than 50 priority organic pollutants in water by GC–MS–MS with QqQ [12].

For more polar, less or non-GC-amenable contaminants, LC–MS–MS is surely the most appropriate analytical technique [13–15], leading to satisfactory results for both quantification and confirmation. Recently, ultra-high-pressure liquid chromatography

(UHPLC) has been developed as an innovative and powerful separation technique based on the use of stationary phases of particle size (<2 μm) smaller than in conventional HPLC. UHPLC coupled to MS–MS has been shown as an excellent analytical tool for multi-class analysis of water for compounds such as pharmaceuticals and drugs [16–19], toxins [20], or pesticides [21, 22], because of its improved selectivity and sensitivity. With modern QqQ analyzers even more than two SRM transitions can be acquired for a safe identification without loss of sensitivity.

Despite the improved sensitivity when using UHPLC–MS–MS, the application of a pre-concentration step (e.g. based on SPE) is typically required in multi-class methods in which a large number of contaminants are determined [16–22]. Despite the excellent performance of LC–MS–MS and GC–MS–MS methods, qualitative information that supports the recognition and structural elucidation of compounds other than target is still needed to obtain more information on actual water sample composition. Time-of flight mass spectrometry (TOF MS) is an excellent technique for this purpose. TOF MS provides the selectivity and sensitivity required for an efficient and wide-scope screening, because it combines high full spectral sensitivity with high mass resolution, enabling any LC-ionizable (LC–TOF MS) or GC-amenable (GC–TOF MS) substances in the sample to be accurately mass measured. TOF MS gives a notable amount of chemical information in a single analysis that enables searching for a large number of compounds after MS acquisition. Our own research group has recently reported several applications of both GC–TOF MS and LC–TOF MS for investigation of organic contaminants in water samples [23–27].

TOF MS is also a powerful technique for investigation of non-target compounds, making feasible the identification of unknown compounds without any previous information or analyte selection. On the basis of these improved characteristics, GC has been combined with high resolution TOF MS (GC–HRTOF MS) for non-target screening of GC-amenable organic (micro) pollutants in water [25, 26, 28, 29]. With regard to LC, very few applications using UHPLC–(Q)TOF MS have been reported in non-target field analysis [27].

The objective of the work discussed in this paper was to investigate the presence of a large number of organic pollutants in treated and raw untreated leachates from a MSW treatment plant. Information on the quality of leachates after

the treatment process is required to estimate the feasibility of discharging them into the aquatic environment. For this purpose, an analytical strategy consisting in combined use of GC–MS–MS and UHPLC–MS–MS, both with triple quadrupole MS, has been applied in order to detect and quantify 94 target contaminants. Although most of the analytes selected are regarded as priority pollutants in water, their determination does not result in an unequivocally realistic overview of sample quality, because only a limited number of contaminants are determined. For this reason, all water samples were also analyzed by GC–TOF MS and LC–QTOF MS in order to widen the search to other non-target contaminants, giving, in this way, useful information that could be used to improve future monitoring programs.

EXPERIMENTAL

Reagents and chemicals

Reference standards of organic contaminants (Table 1) were purchased from Dr Ehrenstorfer (Augsburg, Germany), Wellington Laboratories (Guelph, Ontario, Canada), Fluka (Buchs, Switzerland), Riedel de Haën (Seelze, Germany), or Sigma (St Louis, MO, USA).

Isotopically labeled surrogates used for GC–MS–MS were *p,p´*-DDE-d_8, lindane-d_6, benzo(a)anthracene-d_{12}, terbuthylazine-d_5 (all Dr Ehrenstorfer), and hexachlorobenzene (HCB)-$^{13}C_6$ (Cambridge Isotope Labs, Andover, MA, USA). Isotopically labeled surrogates used for UHPLC–MS–MS were dimethoate-d_6, 2-methyl-4-chlorophenoxy acetic acid (MCPA)-d_3, carbofuran-d_3, diuron-d_6, terbuthylazine-d_5, imazalil-d_5, and thiabendazole-d_6 (all Dr Ehrenstorfer).

To prepare calibration curves, working mixed solutions of organic contaminants and isotopically labeled compounds were prepared in hexane or acetonitrile–water (10:90, v/v) for GC–MS–MS or UHPLC–MS–MS, respectively.

Acetone (residue analysis), acetonitrile (HPLC grade), ethyl acetate, dichloromethane, and hexane (ultra-trace quality) were purchased from Scharlab (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralized water in a Milli-QGradient A10 (Millipore, Bedford, MA. USA). Formic acid (HCOOH, content >98%) and ammonium acetate (NH_4Ac, reagent grade) were supplied by Scharlab.

Table 1 List of target compounds included in the analyses

Compound	Family	Method	Compound	Family	Method
4-*n*-Nonylphenol	ONP	1	Heptachlor	INS OC	1
4-*n*-Octylphenol	ONP	1	Heptachlor epoxide A	Heptachlor TP	1
4-*t*-Octylphenol	ONP	1	Heptachlor epoxide B	Heptachlor TP	1
Acenaphthene	PAH	1	Imazalil	FG Conazole	2
Acenaphthylene	PAH	1	Imidacloprid	INS Nitroguanidine	2
Acetamiprid	INS Pyridylmethylamine	2	Indeno(1,2,3-*cd*)Pyrene	PAH	1
Alachlor	HB Chloroacetanilide	1, 2	Isodrin	INS OC	1
Aldrin	INS OC	1	Isoproturon	HB Phenylurea	2
Anthracene	PAH	1	Lindane	INS OC	1
Atrazine	HB Triazine	1, 2	Malathion	INS OP	2
Azinphos-methyl	INS OP	2	MCPA	HB Phenoxyacetic	2
Azoxystrobin	FG Strobilurin	2	Metalaxyl	FG Anilide	2
Benzo(*a*)anthracene	PAH	1	Methidation	INS OP	2
Benzo(*a*)pyrene	PAH	1	Methiocarb	INS Carbamate	2
Benzo(*b*)fluoranthene	PAH	1	Methomyl	INS Carbamate	2
Benzo(*g*,*h*,*i*)perylene	PAH	1	Methoxychlor	INS OC	1
Benzo(*k*)fluoranthene	PAH	1	Metolachlor	HB Chloroacetanilide	1, 2
BDE 100	PBDE	1	Mirex	INS OC	1
BDE 138	PBDE	1	Naphthalene	PAH	1
BDE 153	PBDE	1	*p*,*p*′-DDD	DDT TP	1
BDE 154	PBDE	1	*p*,*p*′-DDE	DDT TP	1
BDE 28	PBDE	1	*p*,*p*′-DDT	INS OC	1
BDE 47	PBDE	1	PCB 101	PCB	1
BDE 66	PBDE	1	PCB 118	PCB	1
BDE 71	PBDE	1	PCB 138	PCB	1
BDE 85	PBDE	1	PCB 153	PCB	1
BDE 99	PBDE	1	PCB 180	PCB	1
Bentazone	HB Carbamate	2	PCB 28	PCB	1

Table 1 List of target compounds included in the analyses

Compound	Family	Method	Compound	Family	Method
Bromacil	HB Uracil	2	PCB 52	PCB	1
Buprofezin	INS Phenylthiadiazinone	2	Pentachlorobenzene	Chlorobenzene	1
Carbaryl	INS Carbamate	2	Phenanthrene	PAH	1
Carbendazim	FG Benzimidazole	2	Pirimicarb	INS Carbamate	2
Carbofuran	INS Carbamate	2	Pirimiphos-methyl	INS OP	2
Chlorfenvinphos	INS OP	1	Propanil	HB Anilide	2
Chlorpyrifos	INS OP	1	Pyrene	PAH	1
Chrysene	PAH	1	Pyridaphenthion	INS OP	2
Cyprodinil	FG Anilinopyrimidine	2	Simazine	HB Triazine	1, 2
Dibenzo(*a*,*h*)anthracene	PAH	1	Terbacil	HB Uracil	2
Dieldrin	INS OC	1	Terbumeton	HB Triazine	2
Dimethoate	INS OP	2	Terbuthylazine	HB Triazine	1, 2
Diuron	HB Phenylurea	2	Terbutryn	HB Triazine	2
Endosulfan ether	Endosulfan TP	1	Thiabendazole	FG Benzimidazole	2
Endosulfan sulfate	Endosulfan TP	1	Thiobencarb	HB Carbamate	2
Fenarimol	FG Pyrimidine	2	Triadimenol	FG Conazole	2
Fluoranthene	PAH	1	Trifluralin	HB Dinitroaniline	1
Fluorene	PAH	1	α-Endosulfan	INS OC	1
HCB	INS OC	1	ß-Endosulfan	INS OC	1

Method 1, GC–MS–MS; method 2, UHPLC–MS–MS

FG, fungicide; HB, herbicide; INS, insecticide; OC, organochlorine; ONP, octyl nonyl phenols; OP, organophosphorus; PAH, polycyclic aromatic hydrocarbons; PBDE, polybrominated diphenyl ether; TP, transformation product

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Cartridges used for solid-phase extraction were 500 mg Bond Elut C18 (Varian, Harbor City, CA, USA) and 200 mg Oasis HLB (Waters, Milford, MA, USA).

Sampling

Treated (21 samples) and raw leachate (20 samples) water samples were collected monthly, during the period between March 2007 and February 2009, from Reciplasa, a municipal solid waste (MSW) treatment plant sited in Castellón province (Spain). Treated water had been submitted to a reversed osmosis process.

Raw leachate samples were diluted 50-fold with HPLC water before analysis, because of their high organic matter content. All samples were stored in the dark at a temperature below −18 °C. Before analysis, water samples were centrifuged at 3500 rpm for 10 min if suspended particulate matter was present.

LC–MS instrumentation

UHPLC–MS–MS

UHPLC analysis was carried out using an Acquity UPLC system (Waters, Milford, MS, USA), equipped with binary solvent pumping. The chromatographic separation was achieved using an Acquity UPLC HSS T3 column, 1.8 μm, 100 mm×2.1 mm I.D (Waters) at a flow rate of 0.3 mL min^{-1}. The mobile phase was a water–methanol gradient (both 0.1 mmol L^{-1} NH_4Ac). The LC system was interfaced with a TQD (quadrupole T-wave quadrupole) mass spectrometer with an orthogonal electrospray ionization source Z-spray (Waters). For operation in MS–MS mode, the collision gas was argon 99.995% (Carburos Metálicos, Valencia, Spain) with a pressure of 2×10^{-3}mbar in the T-wave cell. Further details of the experimental setups can be found elsewhere [22].

UHPLC–QTOF MS

An ultra performance Acquity liquid chromatography (UPLC) system (Waters) was interfaced with a QTOF mass spectrometer (QTOF Premier, Waters) using an orthogonal Z-spray electrospray interface. LC separation was performed using an Acquity UPLC HSS T3 column, 1.8 μm, 100 mm×2.1 mm I.D at a flow rate of 300 μL

min^{-1}. The mobile phase was a water–methanol gradient (both 0.1 mmol L^{-1} NH_4Ac) in which the methanol percentage was changed linearly as follows: 0 min, 5%; 7 min, 90%; 8 min, 90%; 8.1 min, 5%. The injection volume was 20 µL. TOF-MS resolution was ~10,000 FWHM (V-mode) at *m/z* 556. The MCP detector potential was set to 1750 V in positive-ionization mode. A cone voltage of 25 V and a capillary voltage of 3 kV were used. The interface temperature was set to 350 °C and the source temperature to 120 °C. A scan time of 0.05 s was chosen. The automated attenuated function (dynamic range enhancement, DRE) was selected to correct possible mass peak saturations, making it feasible to achieve quantification and accurate mass measurements over a wide concentration range. Calibration experiments from 50 to 1000 *m/z* were performed monthly using the mixture of NaOH 0.05mol L^{-1}– HCOOH 10% (50:50). A 2 mg L^{-1} standard solution of leucine enkephalin was introduced via the lock-spray needle (cone voltage, 90 V) at a flow rate of 30 µL min^{-1}.

GC–MS instrumentation

GC–MS–MS

A GC system (Agilent 6890 N, Palo Alto, USA) equipped with an autosampler (Agilent 7683) was coupled to a triple quadrupole (QqQ) mass spectrometer, Quattro Micro GC (Waters), operating in electron-ionization (EI) mode. The GC separation was performed using a fused silica HP-5MS capillary column with a length of 30 m×0.25 mm i.d. and a film thickness of 0.25 µm (J&W Scientific, Folson, CA, USA). Splitless injections of 1-µL samples were carried out. The system was operated in MS–MS (SRM) mode using argon 99.995% (Carburos Metálicos) as collision gas at a pressure of 2.8×10^{-3}mbar in the collision cell. More detailed information can be found elsewhere [12].

GC–TOF MS

An Agilent 6890 N GC system equipped with an Agilent 7683 autosampler was coupled to a time-of-flight mass spectrometer, GCT (Waters), operating in EI mode. The GC separation was performed using the same conditions as for the above GC–MS–MS system. The interface and source temperatures were both set to 250°C and a solvent

delay of 3 min was selected. TOF MS was operated at 1 spectrum s^{-1} acquiring the mass range *m/z* 50–650 and using a multi-channel plate voltage of 2850 V. TOF MS resolution was approximately 8500 (FWHM) at *m/z* 612 and heptacosa was used for daily mass calibration and lock mass (*m/z* ion monitored was 218.9856). The application manager TargetLynx, a module of the MassLynx software, was used to process the qualitative and quantitative data obtained from standards and samples for target compounds. The application manager ChromaLynx, also a module of the MassLynx software, was used to investigate the presence of non-target compounds in samples. Library searching was performed using the commercial NIST library.

Analytical procedure

The UHPLC–MS–MS procedure was based on previous work in our laboratory on the determination of multi-class pesticides in environmental and wastewater samples [22]. Briefly, 100 mL water sample acidified with HCOOH and containing the surrogate internal standards (ISs) was passed through a previously conditioned Oasis HLB cartridge. After elution with 5 mL acetone, the extract was evaporated and reconstructed with 1 mL acetonitrile–water (10:90, v/v) and 20 µL of the final extract was injected for UHPLC– MS–MS analysis. Three SRM transitions were acquired for each compound.

The GC–MS–MS procedure was based on our previous work on determination of priority organic contaminants in water [12]. Several of the target compounds are relevant to the water policy of the European Union, and, in fact, are included in Annex X of Directive 2000/60/EC [5]. Briefly, 100 mL water sample containing the surrogate ISs was passed through a previously conditioned C_{18} cartridge. After elution with 5 mL ethyl acetate–dichloromethane (50:50), the extract was evaporated and redissolved in 1 mL hexane and 1 µL of the final extract was injected for GC–MS–MS analysis. Two SRM transitions were acquired for each compound. All methods applied were previously validated.

RESULTS AND DISCUSSION

GC–MS–MS and LC–MS–MS target analysis

The study presented here was a part of a project which required the determination of approximately 100 organic pollutants (Table 1) in treated and raw leachate water samples collected from a MSW treatment plant sited in Castellón province. The main objective was to investigate the quality of the leachates after treatment with a reverse osmosis process in order to assess the feasibility of dumping them into the aquatic environment. Moreover, analysis of both types of water (treated and untreated) enabled evaluation of the efficiency of the reverse-osmosis process. On the basis of the polarity of the target analytes and our previous experience [13], a modern and efficient analytical strategy, consisting in the combined use of two complementary techniques, GC–MS–MS and UHPLC–MS–MS, was applied. In this way, we were able to widen the scope of the method, covering approximately 100 target analytes.

This study was carried out between March 2007 and February 2009, and a total of 41 water samples (21 treated and 20 raw leachate) were analyzed. Both methods, GC–MS–MS and UHPLC–MS–MS, were applied for analysis of all the samples collected. The acquisition of, at least, two transitions per compound—one for quantification (Q) and one (or two) additional for confirmation (q_i)—enabled simultaneous quantification and reliable identification of positive findings. Thus, all findings were confirmed by the compliance of both retention time and Q/q_i ratio when compared with a reference standard. Maximum Q/q_i deviations accepted were based on the European Commission Decision [30].

Data obtained from analysis of the samples (Fig. 1) showed that pesticides were by far the most commonly detected compounds in both treated and raw leachate samples, specially herbicides (phenylurea, triazine, uracil, and carbamate), fungicides (benzimidazole, conazole, and anilide) and insecticides (carbamate, organophosphorus (OP), and phenylthiadiazinone). Other contaminants also frequently found were octyl nonyl phenols (ONP) and polycyclic aromatic hydrocarbons (PAH), detected in almost all raw water, and in 86 and 57%, respectively, of treated water samples. Among pesticides, phenylurea herbicides exceeded the concentration of 0.1 μg L^{-1} in more

than 50% of treated water samples analyzed, followed by triazine herbicides (approx. 40%) and benzimidazole fungicides (approx. 30%).

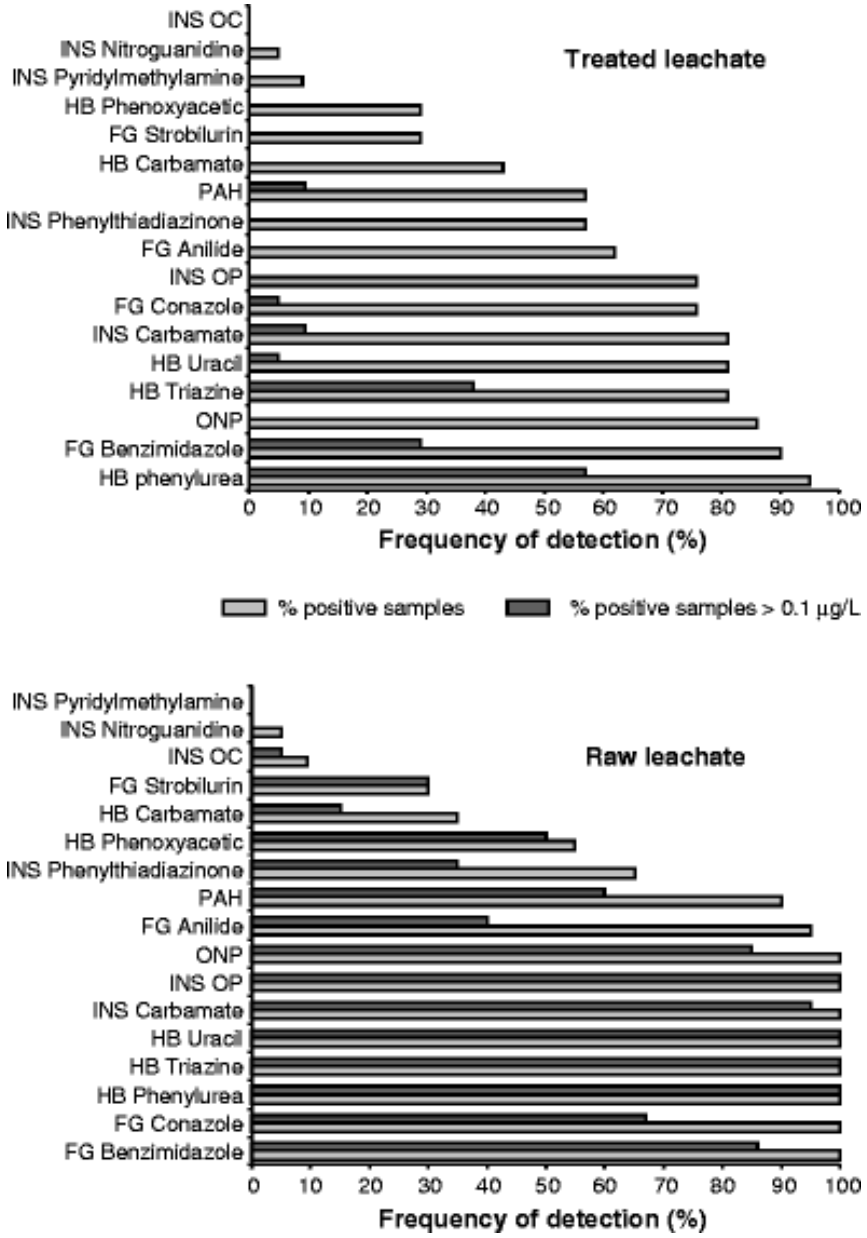

Fig. 1 Frequency of detection (%) of different families of organic contaminants in treated and raw leachate samples collected from the MSW treatment plant between March 2007 and February 2009. INS, insecticide; FG, fungicide; HB, herbicide; OC, organochlorine; ONP, octyl nonyl phenols; OP, organophosphorus; PAH, polycyclic aromatic hydrocarbons

Table 2 shows detection frequencies for the specific organic pollutants detected. It can be seen that most of the positive findings in raw leachate exceeded the 0.1 μg L^{-1} level. However, in treated water only ten contaminants exceeded this value on one or more occasions (six herbicides: terbumeton, terbutryn, terbuthylazine, terbacil, simazine, and diuron; two fungicides: thiabendazole and imazalil; one insecticide: carbaryl; one PAH: naphthalene). Among these, the phenylurea herbicide diuron was present at concentrations higher than 0.1 μg L^{-1} in more than 50% of the treated water samples analyzed (57% of samples), because of its widespread use in Castellón province.

Summarizing the results obtained for treated water, among 349 positive findings in the two-years of monitoring, only 34 were at concentrations higher than 0.1 μg L^{-1}, although, rarely, they exceeded 0.5 μg L^{-1}. The only exceptions were carbaryl and diuron, with maximum concentrations of 1.5 μg L^{-1} (sample of January 2008) and 0.61 μg L^{-1} (sample of February 2008), respectively. For 4-t-octylphenol, chlorphenvinphos, chlorpyrifos, diuron, and simazine, priority substances in Environmental Quality Standards (EQS) for water [6], all were detected on occasion in treated water but never exceeding the maximum permissible concentration (0.1, 0.3, 0.1, 1.8, and 4 μg L^{-1}, respectively).

As expected, detection percentages and concentrations were notably higher for raw leachates than for treated samples. Thus, among 477 positive findings in raw leachate, 373 exceeded 0.1 μg L^{-1}. Several compounds were detected in all the samples analyzed. Within the pesticides, the herbicides diuron, simazine, terbacil, and terbutryn were detected in all 20 water samples analyzed, reaching concentrations as high as 21 μg L^{-1} (terbacil, August 2008). Four insecticides (carbaryl, carbofuran, dimethoate, and pirimicarb) were also found in all untreated samples. The highest concentration was reported for dimethoate in the sample of October 2008 (82 μg L^{-1}). Of the fungicides, imazalil and triadimenol were also detected in all the samples, reaching maximum concentrations of 2.3 μg L^{-1} in both cases. Another compound, 4-t-octylphenol, used as precursor in the manufacture of non-ionic surfactants, was also found in all the samples analyzed, with a maximum concentration of 5.6 μg L^{-1}.

Table 2 Results obtained from UHPLC–MS–MS and GC–MS–MS target analysis of water samples from the MSW treatment plant between March 2007 and February 2009 (total number of samples: 21 treated and 20 untreated)

Compound	Positive samples (%)		Number of samples >0.1 μg L^{-1} (%)		Maximum level (μg L^{-1})	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
Acetamiprid	0	9.5	0	0	nd	<0.025
Atrazine	5	5	0	0	<0.025	<0.025
Azinphos-methyl	15	0	10	0	4.3	nd
Azoxystrobin	30	29	30	0	0.41	<0.025
Benzo(*a*)pyrene	0	5	0	0	nd	<0.025
Bromacil	75	43	50	0	14	0.03
Buprofezin	65	57	35	0	1.0	<0.025
Carbaryl	100	48	90	14	40	1.5
Carbendazim	95	81	75	0	41	0.08
Carbofuran	100	71	95	0	43	0.10
Chlorfenvinphos	75	57	75	0	3.6	0.082
Chlorpyrifos	55	38	50	0	7.5	<0.025
Dieldrin	5	0	5	0	1.3	nd
Dimethoate	100	48	85	0	82	0.10
Diuron	100	95	100	57	19	0.61
Fluorene	5	0	0	0	<0.025	nd
Imazalil	100	48	65	5	2.3	0.31
Imidacloprid	5	5	0	0	<0.025	<0.025
Isoproturon	5	5	0	0	<0.025	<0.025
Lindane	5	0	0	0	<0.025	nd
Malathion	75	24	70	0	64	0.04
MCPA	55	29	50	0	10	0.03
Metalaxyl	95	62	40	0	4.4	<0.025
Methidation	40	5	15	0	13	<0.025
Methiocarb	55	9.5	30	0	1.6	0.030
Naphthalene	55	48	55	14	15	0.31
4-*t*-Octylphenol	100	86	85	0	5.6	0.044
Phenanthrene	85	57	60	0	1.3	<0.025
Pirimicarb	100	71	50	0	13	<0.025
Pirimiphos-methyl	15	0	5	0	0.13	nd
Pyrene	60	43	50	0	0.42	<0.025
Simazine	100	81	65	9.5	17	0.23
Terbacil	100	81	100	5	21	0.14
Terbumeton	85	67	75	5	29	0.16
Terbuthylazine	95	81	95	14	40	0.48
Terbutryn	100	71	95	9.5	14	0.20
Thiabendazole	95	90	85	29	14	0.37
Thiobencarb	35	43	15	0	1.4	<0.025
Triadimenol	100	71	60	0	2.3	0.06

nd, not detected

As illustrative examples, Figs. 2 and 3 show UHPLC–MS–MS and GC–MS–MS chromatograms for treated and raw leachate water samples collected in March 2008.

From all the results obtained, it seems that the treatment process applied (reverse osmosis) in the MSW treatment plant was rather efficient, because it notably reduced the concentrations of organic contaminants found in raw leachate and fulfil analytical characteristics required in the field of residue analysis.

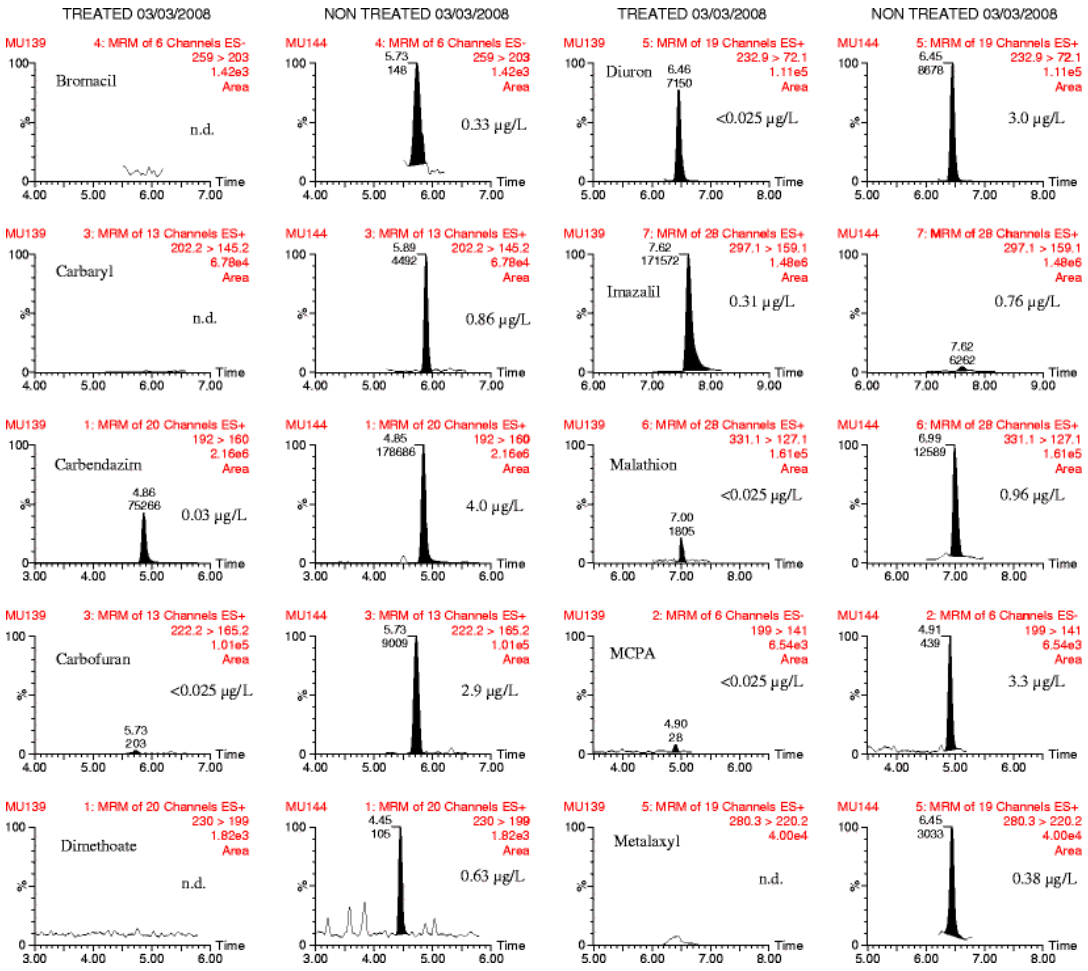

Fig. 2 UHPLC–MS–MS chromatograms obtained from treated and raw leachate samples, both collected on 3 rd March 2008. Only the quantification transition (Q) is shown for every analyte (n.d., not detected)

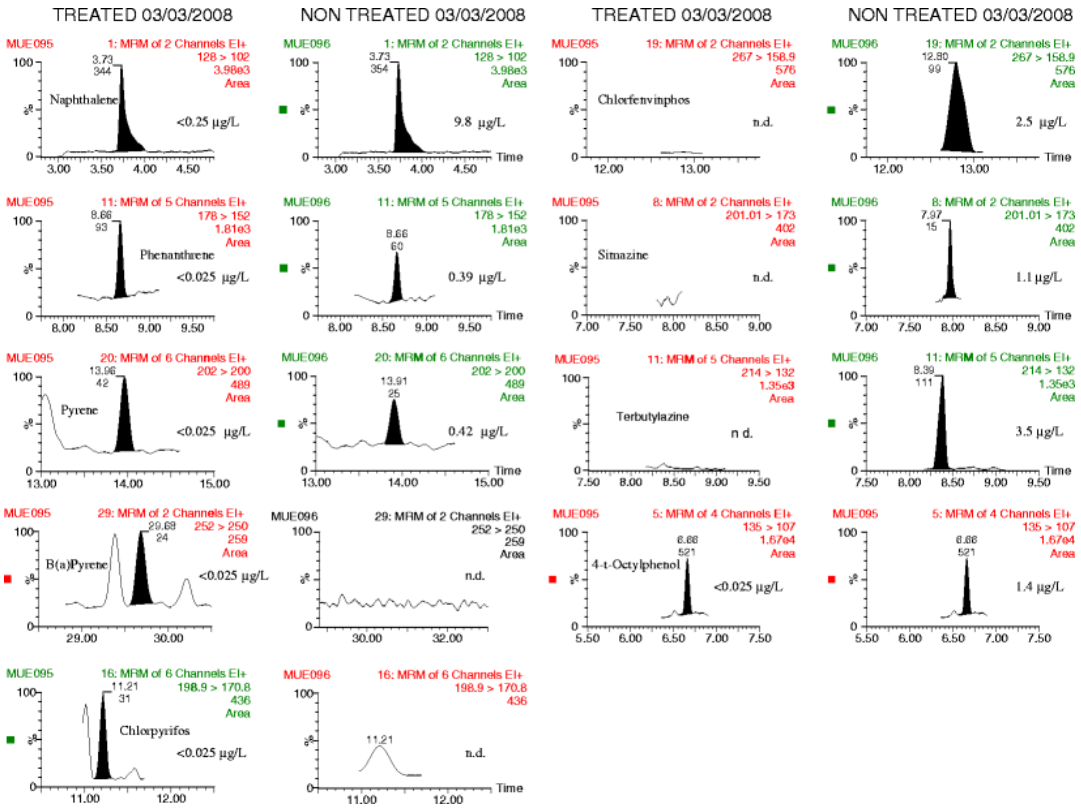

Fig. 3 GC–MS–MS chromatograms obtained from treated and raw leachate samples, both collected on 3 rd March 2008. Only the quantification transition (Q) is shown for every analyte (n.d., not detected)

Analysis of water samples by TOF MS

As illustrated in the previous section, the combined use of GC–MS–MS and LC–MS–MS with triple-quadrupole mass analyzers was a satisfactory approach for quantitative determination of approximately 100 selected contaminants in treated and raw leachate water samples. However, the list of target analytes was limited to a number of contaminants that, although relevant environmentally, are not the only ones present in the samples. So, to obtain more realistic information about the extent of pollution of these samples, investigation of other non-selected contaminants would be necessary. TOF MS was chosen for this purpose because of its great potential for wide-scope screening, as stated in the introduction. Consequently, all water samples were also analyzed by GC–TOF MS and by LC–QTOF MS in order to investigate the presence of other contaminants not included in the list of target analytes. Sample treatment was the same as used for GC–MS–MS (GC–TOF MS analysis) and for UHPLC–MS–MS (UHPLC–QTOF MS analysis). The objective was to identify other pollutants present in the samples that could be added to the list of target analytes in future monitoring programs.

Analysis by GC–TOF MS

Use of GC–TOF MS enabled us to investigate other selected compounds, because of the full-spectrum acquisition at satisfactory sensitivity. In addition, elucidation of several unknown compounds (non-target analytes) was tested. The methodological approach previously developed for screening and confirmation of organic micropollutants in water [25, 26] was applied in this project for searching for target and non-target contaminants in wastewater samples.

Investigation of other selected compounds was carried out in a post-target way, as searching for the compound was performed after MS acquisition [23]. Up to five narrowwindow extracted ion chromatograms (nw-XIC), with a mass window of 0.02 Da, at selected m/z ions were obtained for every compound. The application manager TargetLynx was used to automatically process data and to confirm the identity of compounds detected in samples. Analyte confirmation was performed by comparing the experimental Q/q intensity ratios in samples with the theoretical values, calculated from standards in solvent. The presence of at least two ions measured at their accurate

masses and compliance of their Q/q ratio within specified tolerances [30] was required for a reliable confirmation. In this work, approximately 150 compounds (Electronic Supplementary Material Table S1), including many target analytes investigated by GC–MS–MS QqQ, were investigated in treated and untreated water samples. Calibration curves were included in every sequence of analysis; so semi-quantitative estimation of positive findings could be performed. Table 3 shows four pesticides that were detected in the samples analyzed. These compounds were not included in the target list of either the GC–MS–MS or LC–MS–MS method. Three were OP insecticides (diazinon, dichlorvos, and fenthion) and were detected in several raw leachates, reaching concentrations as high as 79 μg L^{-1} (fenthion, sample of October 2008). The fungicide diphenylamide was detected in one raw sample only (June 2007), although at high concentration (152 μg L^{-1}). In treated water, only diazinon was detected (6 out of 21 samples analyzed), always at concentrations higher than 0.1 μg L^{-1}.

Investigation of non-target compounds in the samples was carried out by applying the ChromaLynx Application Manager. This software automatically detected peaks with a response above a user-defined value, displayed their deconvoluted mass spectra to be searched in the library, and produced at hit list with positive matches (library match >700 was used as criterion). The formulas from the library hit were submitted to the elemental composition calculator and the five most intense ions were scored by exact mass measurement for the confirmation/rejection of the finding [25]. Using this approach, several contaminants were discovered. These compounds were not included in the target list of QqQ-based methods nor in the list of posttarget GC–TOF MS. Table 3 shows the non-target compounds detected by use of this approach. Some of these compounds had been already detected by our group in environmental and biological samples by use of GC–TOF MS [26, 31]. N-Butylbenzenesulfonamide (N-BBSA) used in polyamide and copolyamide plastics and in the manufacture of sulfonyl carbamate herbicides was the compound more frequently detected (100% treated and 90% untreated water). Diethyltoluamide, an insect repellent, was found in all raw water samples and in eight treated water samples. Benzophenone, a UV filter used primarily as photoinitiator and fragrance enhancer, and also used in the manufacture of insecticides, agricultural chemicals, and pharmaceuticals, was identified in approximately 50% of both treated and untreated water. Other compounds frequently

detected in untreated water were caffeine and the pharmaceuticals ibuprofen and benzenesulfonanilide.

Table 3 Results obtained from GC–TOF MS analysis of water samples from the MSW treatment plant between March 2007 and February 2009 (total number of samples: 21 treated and 20 untreated)

Compound	Positive samples (%)		Maximum level (μg L^{-1})	
	Treated	Untreated	Treated	Untreated
Post-target approach				
Diazinon	28	55	7.7	38
Dichlorvos	0	5	nd	11
Diphenylamine	0	5	nd	152
Fenthion	0	5	nd	79
Non-target approach				
Benzenesulfonanilide	5	70		
Benzoguanamine	0	5		
Benzophenone	38	55		
BHT	10	5		
BHT-CHO	5	10		
n-Butylbenzenesulfonamide	100	90		
Caffeine	5	35		
Cotinine	5	0		
3,4-Dichloroaniline	5	10		
Diethyltoluamide	38	100		
8-Hydroxyquinoline	5	0		
Ibuprofen	10	30		
Ibuprofen, trimethylsilyl ether	5	0		
Methylparaben	5	20		
Nicotine	0	5		
Triacetin	0	5		
3,4,5-Trichlorobenzenamine	0	5		

nd, not detected

As an illustrative example, Fig. 4 shows a positive finding of ibuprofen in untreated water using the GC–TOF MS nontarget approach. Accurate mass confirmation automatically performed by the software for four representative ions led to the confirmation of the identity of ibuprofen with mass errors below 1 mDa for three of the ions.

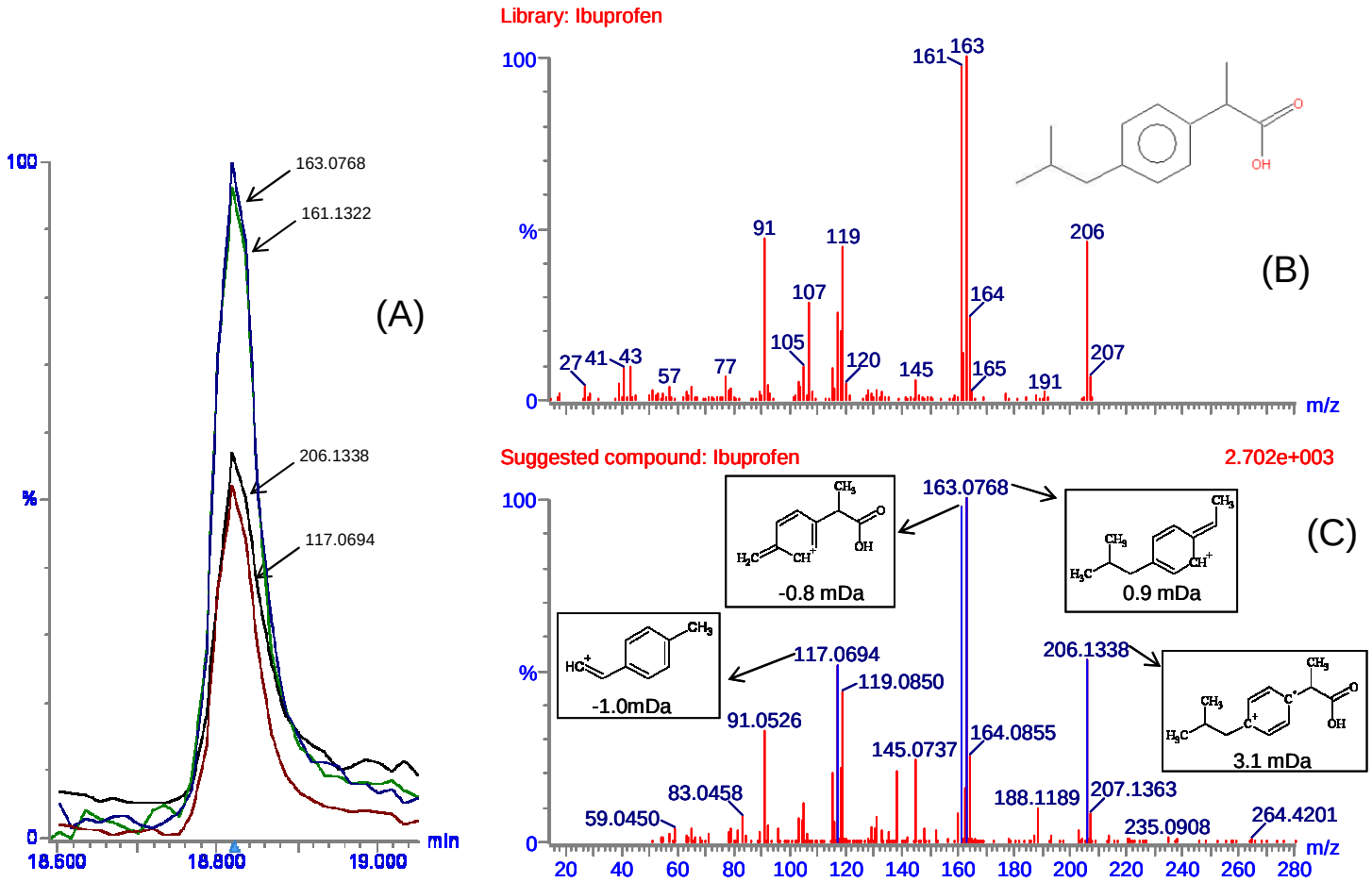

Fig. 4 Identification of non-target ibuprofen by GC-TOF MS in a raw leachate sample collected on 28th August 2008. (a) Extracted-ion chromatogram for four m/z ions. (b) Commercial library mass spectrum of ibuprofen at nominal mass. (c) Deconvoluted accurate mass spectrum of ibuprofen in the sample (mass errors shown in mDa)

Analysis by UHPLC-QTOF MS

The analysis of samples by UHPLC–QTOF MS was carried out in a post-target way searching for approximately 500 compounds that were included in a home-made database. The list contained 377 pesticides and 40 transformation products (TP), and 47 antibiotics, 20 pharmaceuticals, and other emerging contaminants reported to have been detected in the aquatic environment, for example cocaine or caffeine [27]. For investigation of these compounds, ChromaLynx XS software was used.

Briefly, this application manager automatically processes data, and obtains nw-XICs (mass window 0.02 Da) for selected *m/z* ions, usually those corresponding to protonated or deprotonated molecules, based on a selected list of accurate masses and retention times, if available. This software also enables visualization of the complete spectrum of positive findings at accurate masses, which can be compared with a library, if available. This facilitates a rapid and simple review by cataloguing pollutants on colors, as a function of mass errors. In our case, a theoretical home-made library was built without the need to inject reference standards. It showed the theoretical spectrum with information on molecular ion mass (typically $(M + H)^+$ in ESI positive) and the theoretical isotopic pattern.

Following this methodology, most of analytes detected by triple-quadrupole MS were also confirmed by TOF MS. In addition, other contaminants not included in the target list of the QqQ methods were identified. Table 4 shows compounds not investigated by QqQ that were discovered in several samples analyzed. Antibiotics, for example erythromycin and clarythromycin, were detected by UHPLC–QTOF MS in approximately 5% of treated water samples and approximately 50% of raw leachates. The analgesic paracetamol was found in more than 70% of samples analyzed. Atenolol, a beta-antagonist used primarily in cardiovascular diseases for treatment of hypertension, was detected in 85%of samples. Metamizole, an anti-inflammatory drug commonly used as powerful analgesic and antipyretic, was found in 75% of raw samples and in 50% of treated water. Caffeine and the insecticide diazinon, which had already been identified in some samples by use of GC–TOF MS, were also found in approximately 40% of raw leachate and a few treated water samples. Paraxanthine, a caffeine metabolite, was detected in both raw and treated water (approx. 30% samples). Cocaine was detected in one sample only out of the 20 raw leachates

analyzed. However, benzoylecgonine, one of the main metabolites of cocaine, was detected in 95% of raw leachate and 60% of treated water samples. Finally, the pesticide TPs deethylterbumeton, 2-hydroxyterbuthylazine, and deethyl-2-hydroxyterbuthylazine, were also found in several samples.

Table 4 Results obtained from UHPLC–QTOF MS analysis of water samples from MSW treatment plant between March 2007 and February 2009 (total number of samples: 21 treated and 20 untreated)

Compound	**% positive samples**	
	Treated	**Untreated**
Post-target approach		
Atenolol	85	85
Benzoylecgonine[a]	60	95
Caffeine[a]	40	43
Clarythromycin	5	45
Cocaine[a]	0	5
Diazinon[a]	5	42
Erythromycin	5	65
Metamizole[a]	50	75
Paracetamol	70	84
Paraxanthine	25	35
Deethyl-terbumeton	10	15
Deethyl-2-hydroxyterbuthylazine	35	55
2-Hydroxyterbuthylazine	50	70

[a]Compounds that were also discovered by performing the non-target approach

As an example, Fig. 5 shows a positive finding of diazinon in raw leachate using the UHPLC–QTOF MS post-target approach. The nw-XIC at *m/z* corresponding to the exact mass of diazinon [M + H]+ is shown with its accurate mass spectrum and the theoretical spectrum. Accurate mass confirmation automatically performed for the [M + H]+ ion showed a mass error of 0.7 mDa.

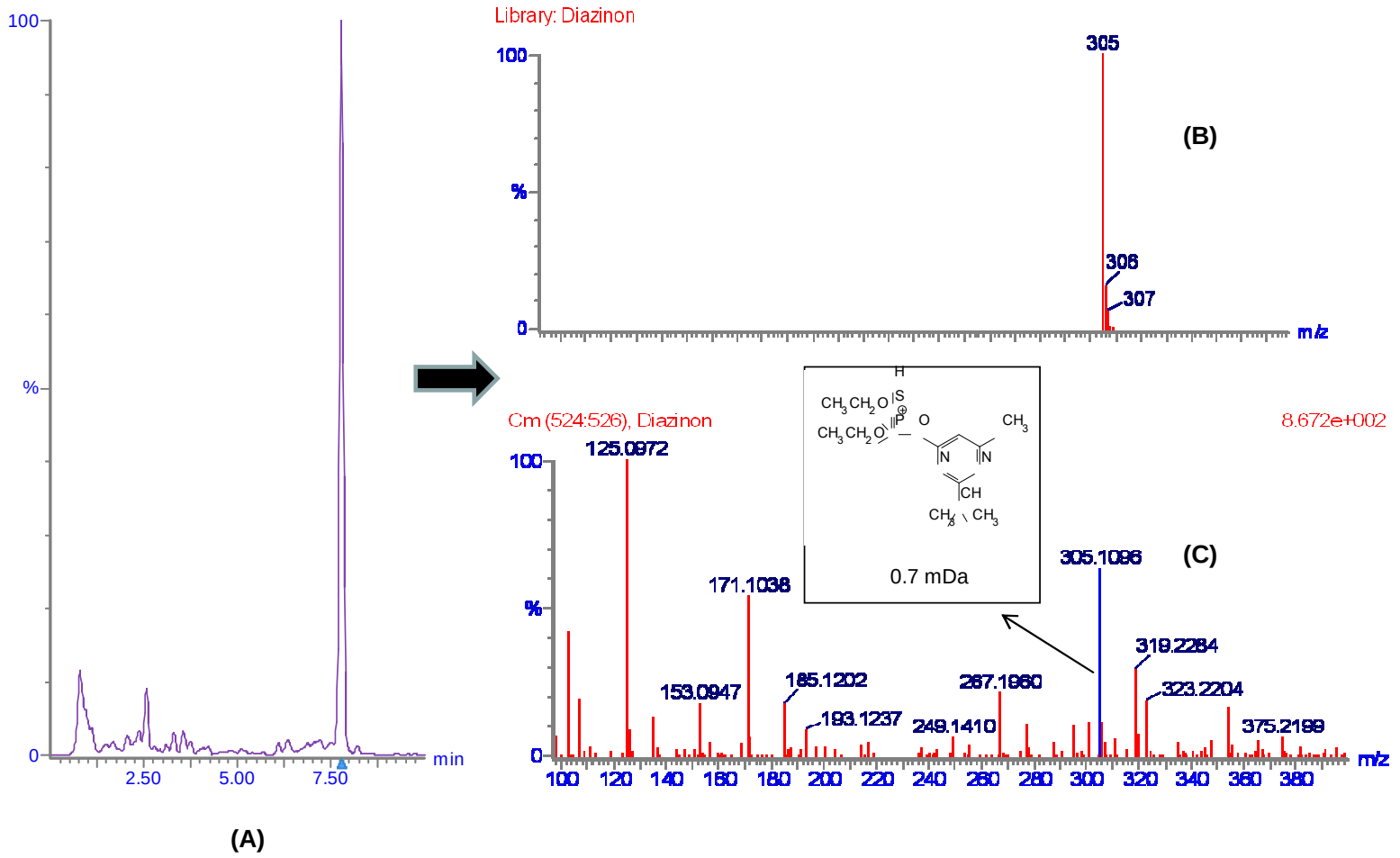

Fig. 5 Identification of diazinon by UHPLC-QTOF MS in a raw leachate sample collected on 31st October 2008. (a) Extracted-ion chromatogram for [M + H]+ ion, m/z 305.1089. (b) Theoretical library mass spectrum of diazinon at nominal mass. (c) Accurate mass spectrum of diazinon in the sample

In addition, the availability of a QTOF instrument made it feasible to perform MS–MS experiments to go further in the identification process of the compounds detected, because of the useful information given by product-ion spectra at accurate mass. Full-acquisition accurate mass data were also processed in a non-target way [27], trying to elucidate "unknown" components detected in samples. The difference from the GC–TOF MS approach was the use of only two ions (softer ionization in ESI in comparison with EI) and the use of the theoretical home-made library previously described, because of the non-availability of extensive and reproducible LC–MS commercial libraries. After processing MS data in the non-target approach, only five contaminants were discovered: paracetamol, cocaine, benzoylecgonine, caffeine, and diazinon. All of these had been previously detected in the post-target screening, and corresponded to those analytes with sensitive response in TOF MS. The other compounds (Table 4) could not be detected by use of this non-target approach, because of either their low sensitivity and/or their low concentration in the samples. It is important to remark that the LC–TOF MS screening of organic contaminants using a non-target approach may not be fully satisfactory at the moment, because the success of this approach becomes notably worse at low analyte responses, being therefore less efficient than the posttarget analysis. The non-availability of wide commercial reproducible libraries, as in GC with EI, is an important limitation. In fact, using non-target analysis only five compounds were elucidated, as stated above.

As an example of the non-target UHPLC–QTOF MS approach, Fig. 6 shows a positive finding of paracetamol in untreated leachate. Accurate mass confirmation automatically performed for two representative ions led to the confirmation of the identity of paracetamol in this sample with mass errors of 0 and −0.7 mDa.

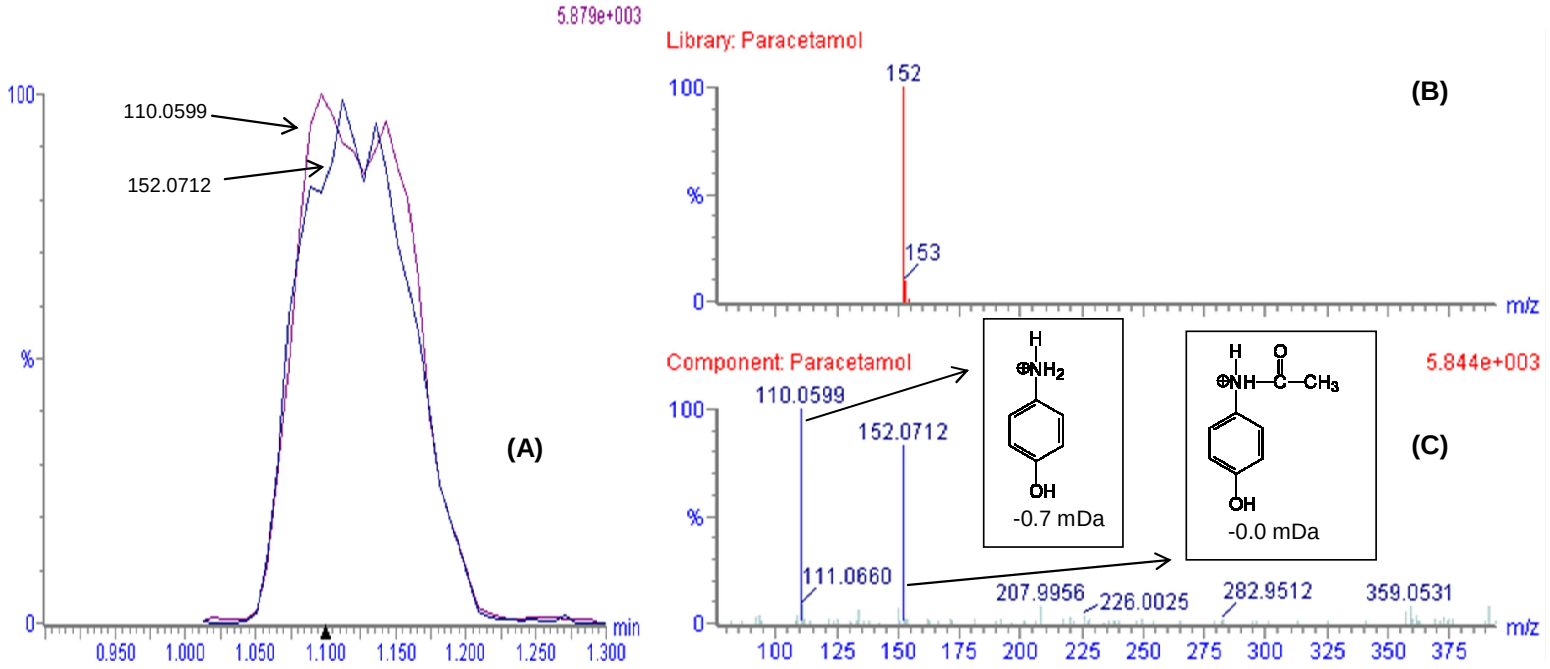

Fig. 6 Identification of non-target paracetamol by UHPLC–QTOF MS in a raw leachate sample collected on 10th October 2008. (a) Extracted-ion chromatogram for two m/z ions. (b) Theoretical library mass spectrum of paracetamol at nominal mass. (c) Deconvoluted accurate mass spectrum of paracetamol in the sample (mass errors shown in mDa)

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CONCLUSIONS

Investigation of organic pollutants of wide polarity ranges in water requires the use of two complementary techniques: GC–MS, for determination of non-polar/semi volatile analytes, and LC–MS, for more polar analytes. In this paper, an analytical strategy consisting on the combined use of GC–MS–MS and UHPLC–MS–MS, both with triple-quadrupole analyzers, has been applied in order to investigate the presence of approximately 100 organic contaminants in treated and raw leachate samples from a municipal solid-waste-treatment plant.

Pesticides were the most commonly detected compounds in both types of sample, especially herbicides (phenylurea, triazine, uracil, and carbamate), fungicides (benzimidazole, conazole, and anilide) and insecticides (carbamate, organophosphorus, and phenylthiadiazinone). Other contaminants widely detected were 4-t-octylphenol and several PAHs, for example naphthalene, phenanthrene, and pyrene.

As expected, frequency of detection and pollutant concentrations in raw leachates were notably higher than in treated samples. Most of the positive findings in untreated samples were at concentrations above 0.1 μg L^{-1}. However, in treated water very few compounds were detected at levels higher than 0.1 μg L^{-1}, and rarely exceeded 0.5 μg L^{-1}. From the results obtained it seems that the reverse-osmosis treatment applied in the MSW treatment plant was rather efficient, because it notably reduced the concentrations of organic contaminants found in raw leachates.

MS–MS techniques using triple-quadrupole analyzers have great potential in environmental analysis because of their high sensitivity and selectivity. However, tandem MS methods are developed on purpose for a limited list of target contaminants (approximately 100 organic contaminants in this work) so, other relevant pollutants that might be present in the samples would be ignored in these analyses. For this reason, all the samples were also analyzed by GC–TOF MS and UHPLC–QTOF MS in order to investigate the presence of many other contaminants, either in a post-target way (searching for selected pollutants after MS acquisition data) or in a non-target way (searching for unknowns without any previous selection nor information on the compounds to be investigated). This was feasible because of to the full MS spectra acquisition by TOF analyzers, which offered the possibility of searching for a large number of contaminants with the help of the accurate mass information of the

molecules and of the fragment ions. This enabled discovery of several compounds not included in the initial target list of organic contaminants. Other pesticides (diazinon, dichlorvos, diphenylamine, and fenthion) and some TPs (deethylterbumeton, 2-hydroxyterbuthylazine, and deethyl-2-hydroxyterbuthylazine), pharmaceuticals (erythromycin, clarythromycin, atenolol, metamizole, benzenesulfonanilide, ibuprofen, and paracetamol), drugs of abuse (cocaine and its metabolite benzoylecgonine), the UV filter benzophenone, N-BBSA, the insect repellent diethyltoluamide, or caffeine and its metabolite paraxanthine, are examples of compounds identified in additional analyses performed by TOF instruments. These discovered analytes could be included in the target quantitative methods applied in future monitoring programs. This paper shows the potential of TOF MS for screening purposes, because this analyzer in combination with GC and LC is able to detect and identify a huge number of pollutants, making it an excellent analytical tool for wide-scope environmental screening.

ACKNOWLEDGMENT

The authors acknowledge financial support from the Ministerio Español de Ciencia e Innovación (Project CTQ2009-12347), and also from Generalitat Valenciana as research group of excellence PROMETEO/2009/054. The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of UHPLC–MS (TQD), GC–TOF MS (GCT), and UHPLC–QTOF MS (QTOF Premier). This work has been developed under financial support of RECIPLASA as a part of the project "Investigación de contaminantes orgánicos en las aguas de lixiviado de residuos urbanos de la planta de compostaje de Onda".

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SUPPLEMENTARY MATERIAL

Table S1: List of compounds investigated by GC-TOF MS in a post-target way

4-n-Nonylphenol	Cyfluthrin	Hexythiazox	PCB 180
4-n-Octylphenol	Cyfluthrin_1	Imazalil	PCB 189
4-t-Octylphenol	Cyfluthrin_2	Indeno(1,2,3,cd)pyrene	PCB 28
Acenaphthene	Cyfluthrin_3	Iprodione	PCB 52
Acenaphthylene	Cyfluthrin_4	Isodrin	PCB 77
Alachlor	Cypermethrin_1	Isofenfos	PCB 81
Aldrin	Cypermethrin_2	lambda-Cyhalothrin	Penconazole
Anthracene	Cypermethrin_3	Lindane	Pentachlorobenze
Atrazine	Cypermethrin_4	Malathion	Permetrhin_1
Atrazine desethyl	Cyprodinil	Metalaxyl	Phenanthrene
Atrazine desisopropyl	Deltamethrin	Metamidophos	Phosmet
Azinphos methyl	Diazinon	Methacrifos	Pirimicarb
BDE 100	Dibenzo(a,h)anthracene	Methidathion	Pirimiphos ethyl
BDE 138	Dichlofluanide	Methiocarb	Pirimiphos methyl
BDE 153	Diclorvos	Methiocarb sulfone	Procymidone
BDE 154	Dieldrin	Metolachlor	Profenofos
BDE 183	Diflufenican	Metoxychlor	Propyzamide
BDE 28	Dimethoate	Mevinfos	Pyrazofos
BDE 47	Diphenylamine	Mirex	Pyrene
BDE 66	Endosulfan ether	Molinate	Quinalfos
BDE 71	Endosulfan sulfate	Naphthalene	Simazine
BDE 85	Ethion	Omethoate	tau-Fluvalinate_1
BDE 99	Etrimfos	Oxadixyl	tau-Fluvalinate_2
Benzo(a)anthracene	Fenarimol	*p,p'*-DDD	Tebuconazole
Benzo(a)pyrene	Fenchlorfos	*p,p'*-DDE	Tecnazen
Benzo(b)fluoranthene	Fenitrothion	*p,p'*-DDT	Terbacil
Benzo(g,h,l)perylene	Fenoxycarb	Parathion ethyl	Terbumeton
Benzo(k)fluoranthene	Fenthion	Parathion methyl	Terbumetona desethyl
Bifentrin	Fenvalerate	PCB 101	Terbuthylazine
Bupimirate	Fluoranthene	PCB 105	Terbuthylazine desethyl
Buprofezin	Fluorene	PCB 114	Terbutryn
Carbaryl	Fonofos	PCB 118	Tetradifon
Carbaryl	Forate	PCB 123	Thiabendazole
Chlorfenvinphos	Fosalone	PCB 126	Trifluraline
Chlorpropham	Fosfamidon	PCB 138	α-Endosulfan
Chlorpyrifos	Heptachlor	PCB 153	ß-Endosulfan
Chlozolinate	Heptachlor epoxide A	PCB 156	
Chlropyriphos methyl	Heptachlor epoxide B	PCB 157	
Chrysene	Heptenofos	PCB 167	
Coumafos	Hexachlorobenzene	PCB 169	

4.3 USO COMBINADO DE GC-TOF MS Y UHPLC-QTOF MS EN LA INVESTIGACIÓN DE UN EPISODIO DE MORTANDAD MASIVA DE ABEJAS

Las abejas (*Apis mellifera*) son insectos cuya persistencia y calidad de vida dependen notablemente de las condiciones toxicológicas del medioambiente que los rodea. Están sometidas a un elevado riesgo de intoxicación por plaguicidas, sobretodo durante la polinización, ya que se exponen directamente al contacto con estos compuestos aplicados para el control de plagas y malas hierbas en la agricultura. El cuerpo de la abeja está cubierto con una vellosidad plumosa que aumenta la superficie de área de captura de partículas, donde éstas se bioacumulan. Por ello, la abeja es un excelente indicador biológico, ya que indica el estado del medio ambiente donde vive, al visitar prácticamente todos los sectores ambientales: suelo, vegetación, aire y agua (1). Los productos consumidos pueden permanecer en su tejido corporal. Por ello, los compuestos encontrados en sus cuerpos son un reflejo fiel de los plaguicidas que se aplican en las zonas agrícolas que rodean los colmenares de dichas abejas. Dentro de los plaguicidas, los que más daños les provocan son los insecticidas, que representan un riesgo importante de envenenamiento, conllevando algunas veces intensas pérdidas que pueden confundirse con los efectos de enfermedades infecciosas de las abejas adultas y de las larvas. El papel de las abejas en el proceso de polinización es crucial, ya que de entre todos los insectos que participan en la misma, la abeja es con mucho la más eficaz. Por ello, provocan gran preocupación los episodios de mortandad de abejas y son muchos los trabajos dedicados al desarrollo de métodos eficaces para la determinación de contaminantes, sobretodo plaguicidas, en abejas y en productos relacionados con ellas, como polen, cera, néctar, miel, etc (2-13)

En el trabajo que se presenta a continuación (**artículo científico 11**) se expone la aplicación de las técnicas GC-TOF MS y UHPLC-QTOF MS en la investigación de las posibles causas de episodios de mortandad de abejas en entornos avícolas de la Comunidad Valenciana.

Ante el inicial desconocimiento sobre los contaminantes que habrían podido causar la mortandad de las abejas, se aplicó un análisis genérico por GC-TOF MS y UHPLC-QTOF MS, y se trataron los datos aplicando una metodología *non-target,* tal como se ha descrito en apartados anteriores. Esta poderosa combinación nos permitió abarcar un amplio abanico de compuestos, sin ninguna selección previa de analitos ni

restricción en las búsquedas de posibles compuestos causantes de la mortandad. En el primer episodio de mortandad estudiado, las muestras de abejas analizadas resultaron positivas a coumafos, un acaricida que se utiliza en el control del ácaro *Varroa destructor* en los panales de miel.

Debido a las elevadas concentraciones de coumafos encontradas en las abejas, se planteó el estudio de sus posibles metabolitos. El software Metabolynx, disponible en los equipos utilizados, está diseñado para este fin, ya que compara el archivo de masas de una muestra conteniendo el analito con el de una muestra control, subrayando las diferencias entre ellos, que son entonces atribuídas a los metabolitos originados. Sin embargo, la aplicación de este software está más enfocada a datos de MS originados por LC, con fuentes de ionización suaves como ESI que promueven la formación del ión molecular. Por ello, en los análisis por UHPLC-QTOF MS, se hizo uso del software Metabolynx para la detección de metabolitos del coumafos en las muestras de abejas, metodología que ha proporcionado resultados satisfactorios en nuestro grupo de investigaciones en otras aplicaciones (14). Dado que esta aplicación está originariamente diseñada para el estudio del metabolismo de fármacos (es decir, búsqueda de metabolitos más o menos esperados) fue necesario un estudio previo con el fin de optimizar todos los parámetros, de manera que el software fuera útil para nuestros objetivos en el campo toxicológico. Siguiendo esta metodología se detectó la presencia de CMHC, coumaphos-OH, coumaphos oxon-OH y potasan en la muestra de abejas que contenía una concentración mayor del acaricida coumafos. Los compuestos detectados pudieron ser confirmados mediante la evaluación de los errores de masa de los principales iones característicos de los espectros de ESI para cada compuesto, todos ellos menores a 3.6 mDa. Todos estos datos se muestran en la Tabla 3 del **artículo científico 11**.

La investigación de metabolitos por GC-TOF MS se llevó a cabo siguiendo una metodología *post-target*. Para ello se buscaron metabolitos del coumafos descritos en la literatura y se seleccionaron aquellos que eran susceptibles de ser analizados por GC, como fue el caso de 7-chloro-7-hydroxy-4-methyl-chromen-2-one (CMHC), potasan, coumafos-oxon y 4-methylumbelliferone (ver figura 3 del artículo científico 11). Los datos de masas necesarios para crear el método de procesamiento de datos fueron

obtenidos de los espectros teóricos disponibles en la librería comercial NIST para todos los compuestos excepto para el potasan, cuyo espectro no estaba disponible en la NIST. En este caso particular, se utilizó únicamente el ion molecular para detectar la presencia de este metabolito que, afortunadamente resultó ser un pico relevante en el espectro del mismo, y pudo ser detectado en las muestras igualmente. Así pues, se detectó la presencia de CMHC y potasan en la muestra de abeja que contenía una concentración mayor de coumafos. Los compuestos detectados pudieron ser confirmados mediante la evaluación de los errores de masa de los principales iones característicos de los espectros de EI para cada compuesto, todos ellos menores a 4.1 mDa. Todos estos datos se muestran en la Tabla 2 del **artículo científico 11**.

La misma metodología se aplicó a un segundo caso de mortandad de abejas remitido a nuestro laboratorio. En este caso dispusimos de tres muestras de abejas diferentes y de una muestra adicional de hojas y flores de nectarina. El interés era estudiar si los plaguicidas que habían sido aplicados sobre estos árboles frutales, habían podido causar la muerte de las abejas, cuyas colmenas se encontraban relativamente cerca de la zona agrícola. Los compuestos detectados en las abejas fueron de nuevo coumafos, además de fenitrotion, clorfenvinfos y metiocarb. En cambio, en las muestras de hojas y flores de nectarina se detectó piriproxifen y tiametoxam. No se detectaron restos de estos dos compuestos en las abejas.

Así pues, podemos concluir que en este caso, el uso combinado de GC-TOF y LC-QTOF permitió descubrir insecticidas y un buen número de sus principales metabolitos que podrían haber sido, con alta probabilidad, los agentes causantes de la mortandad. Cabe destacar que los análisis realizados no estuvieron dirigidos hacia ningún compuesto en particular, sino que se aplicó una metodología *non-target*, que fue capaz de detectar, identificar y confirmar de modo absolutamente fiable la presencia de varios plaguicidas de alta toxicidad para las abejas.

4.3.1. Bibliografía

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4.3.2 Artículo científico 11

J. Agric. Food Chem. 57, 4079-4090, 2009

COMBINED USE OF GC-TOF MS AND UHPLC-(Q)TOF MS TO INVESTIGATE THE PRESENCE OF NONTARGET POLLUTANTS AND THEIR METABOLITES IN A CASE OF HONEYBEE POISONING

Tania Portolés, María Ibáñez, J. Vicente Sancho, F. José López, Félix Hernández

Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain,

ABSTRACT

The combined use of gas chromatography (GC) and ultrahigh-pressure liquid chromatography (UHPLC), both coupled to time-of-flight mass spectrometry (TOF MS), has been explored in this work for the investigation of several cases of honeybee poisoning. The procedure applied involves a previous extraction with acetone followed by liquid−liquid extraction with dichloromethane. Both techniques, GC-TOF MS and UHPLC-(Q)TOF MS, have been applied to discover the presence of compounds that might be responsible of honeybee deaths. The application of a nontarget methodological approach to a first case of poisoning allowed the detection of the insecticide coumaphos at high concentration levels in the samples. The presence of possible metabolites of this organophosphorus insecticide was investigated by using both techniques. UHPLC-(Q)TOF MS showed its higher applicability in this case, as most of the metabolites were more polar than the parent compound. Four metabolites were identified by UHPLC-(Q)TOF MS, whereas only two of them were found by GC-TOF MS. The developed methodology was applied to other subsequent poisoning cases in which insecticides such as coumaphos, thiamethoxam, pyriproxyfen, and chlorfenvinphos were identified by both techniques, whereas GC-TOF MS also allowed the detection of fenitrothion and methiocarb. In all positive cases, the confirmation of the presence of the compound detected was feasible by means of accurate mass measurements of up to five ions together with their ion ratio evaluation.

KEYWORDS

Gas chromatography; ultrahigh-pressure liquid chromatography; time-of-flight; pesticides; honeybee poisoning; metabolites

INTRODUCTION

The honeybee (Apis mellifera) is an important insect worldwide. Its pollinating activity is crucial for the production of high-quality commercial seeds and fruits (1). However, the extensive use of pesticides in agricultural activities is resulting in more and more frequent honeybee poisoning. Within the pesticides that have caused more incidents of honeybee poisoning, insecticides are the main group of concern (2). Toxic compounds are retained and bioaccumulated in honeybee bodies, being therefore good bioindicators of the type of pesticides applied in the area surrounding their hives (3).

In recent years, massive honeybee death has been an issue of increasing concern in several European countries. Although pesticides and agricultural management may play an important role in these losses, it is also recognized that several other factors might be involved, including colony management, diseases, or global climate change. The submission of samples of dead bees is therefore necessary for this forensic investigation. This work involves both field and laboratory assessment and analytical research to look for pesticide residues (4). For this purpose advanced analytical instrumentation is needed.

Determination of pesticides in honeybees has been traditionally carried out using gas chromatography (GC) with electron capture detection (ECD) or nitrogen−phosphorus detection (NPD) (2, 5). In the past decade, a tendency toward the use of more polar pesticides was observed due to their lower persistence and human hazard. For the analysis of these semipolar and polar pesticides and/or metabolites, liquid chromatography (LC) has been traditionally the technique of choice, in combination with UV or diode array detection.

In the past few years, conventional detectors have been replaced by mass spectrometry (MS) analyzers due to their inherent higher selectivity, good sensitivity, and useful information for an approppiate confirmation. Target analysis has been traditionally carried out by gas chromatography−mass spectrometry (GC-MS) or liquid

chromatography−mass spectrometry (LC-MS), typically using quadrupole instruments, ion traps (IT) or, more recently, triple-quadrupole analyzers in environmental, food, and biological samples (6-13). Qualitative information that supports the recognition and structural elucidation of compounds other than the target is still needed to obtain more information on sample composition. To obtain an unbiased data set, full-spectrum acquisition techniques are required. The time-of-flight mass spectrometer (TOF MS) seems to be more appropriate for qualitative purposes, as it provides the selectivity and sensitivity required for an efficient and wide-scope screening. TOF MS combines high full-spectral sensitivity with high mass resolution, allowing any LC ionizable component in the sample (in the case of LC-TOF MS) or GC-amenable (in the case of GC-TOF MS) to be accurately mass-measured. Elemental compositions can be proposed with this technique with low mass errors (typically below 5 ppm, according to the manufacturer's specifications). TOF MS can provide a notable amount of chemical information in a single experiment, so this technique is very attractive for searching for a high number of compounds in a "post-target way", that is, compounds are selected and searched after MS acquisition (14-17).

In a nontarget analysis, the objective is that all compounds eluting from the analytical chromatographic column can be detected and identified without any kind of selection (with the obvious limitations derived from the chromatographic and ionization processes). Here, the analyst is searching for unknown compounds actually, as no previous information about the analytes is taken into account.

On the basis of these improved characteristics, GC has been combined with high-resolution TOF-MS (GC-HR-TOFMS) for nontarget screening of GC-amenable organic (micro) pollutants in water (16, 18, 19), anthropogenic contaminants in biological matrices (20), or flavor research (21).

With regard to LC, very few applications using ultrahigh-pressure LC (UHPLC)-(Q)TOF MS have been reported in the nontarget field. This technique has been successfully applied for a nontarget screening of organic pollutants in water (22) and in the metabolite-profiling field (23). Some applications have been recently reported in other fields, such as impurity profiling of pharmaceutical drug substances (24), metabonomics (25), or food safety (26).

With regard to honeybee analysis, the complexity of the sample matrix together with the presence of wax residues adhered to honeybee bodies may lead to important chromatographic interferences (3). Several analytical procedures for the determination of target pesticides in bees have been published in the past few years. Most of these methods involve an extraction with organic solvent followed by a cleanup step (3, 27, 28). Alternative procedures are based on solid-phase extraction (SPE) (29), solid-phase microextraction (SPME) (5), or matrix solid-phase dispersion (MSPD) (2, 5, 30), among others.

The aim of this work is to investigate the presence of toxic compounds in several honeybee poisoning episodes by the combined use of GC-TOF MS and UHPLC-QTOF MS. The application of a nontarget approach has allowed the detection and safe confirmation of several parent pesticides in samples. Then, the presence of their main metabolites has been investigated by both techniques.

EXPERIMENTAL PROCEDURES

Chemicals and Solvents

Reference materials (thiamethoxam, pyriproxyfen, promecarb, fenitrothion, chlorfenvinphos, methiocarb, coumaphos) with purities of 97−99.7% were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) for standard preparation. Stock solutions (around 500 mg/L) were prepared by dissolving reference standards in acetone and stored in a freezer at −20 °C. Working solutions were prepared by diluting stock solutions with acetone for sample fortification, with ethyl acetate for GC injection, and with methanol/water (10:90) for LC injection.Acetone (pesticide residue analysis), GC-ultra trace analysis grade dichloromethane (DCM), ethyl acetate (ultratrace quality), HPLC-grade methanol, reagent-grade formic acid (HCOOH, content 98−100%), sodium hydroxide, and ammonium acetate (NH_4OAc, >98%) were purchased from Scharlab (Barcelona, Spain). HPLC-grade water was obtained by distilled water passed through a Milli-Q water purification system (Millipore, Bedford, MA). Celite was purchased from Merck (Darmstadt, Germany). Anhydrous sodium sulfate of pesticide residue quality (Baker, Deventer, The Netherlands) was dried for 18 h at 300 °C before use. Sodium chloride of analytical grade from Scharlab was used after purification by heating at 300

°C overnight.Leucine enkephalin and heptacose, used as LC and GC lock masses, respectively, were purchased from Sigma (St. Louis, MO).

Samples

Five honeybee samples (samples 1−5) from different sites of the Valencia area (Spain) suspected to be intoxicated by insecticide applications, together with one sample of nectarine flowers and leaves (sample 6) (possibly related to the sample 3 honeybee intoxication) were received at our laboratory to investigate the reason for the massive intoxications. Additionally, one sample of a supposedly blank honeybee was also provided. After reception of the samples at the laboratory, they were immediately frozen at −18 °C. Analyses were performed after 1 week.

Instrumentation

GC-TOF MS

An Agilent 6890N GC system (Palo Alto, CA) equipped with an Agilent 7683 autosampler was coupled to a time-of-flight mass spectrometer, GCT (Waters Corp., Milford, MA), operating in electron ionization (EI) mode (70 eV). The GC separation was performed using a fused silica HP-5MS capillary column with a length of 30 m × 0.25 mm i.d. and a film thickness of 0.25 μm (J&W Scientific, Folsom, CA). The oven temperature was programmed as follows: 90°C (1 min); 5°C/min to 260°C; 40°C/min to 300°C (2 min). Splitless injections of 1 μL of sample were carried out. Helium was used as carrier gas at 1 mL/min. The interface and source temperatures were both set to 250 °C, and a solvent delay of 3 min was selected. The time-of-flight mass spectrometer was operated at 1 spectrum/s acquiring the mass range *m*/*z* 50−650 and using a multichannel plate (MCP) voltage of 2700V. TOF-MS resolution was about 8500 (fwhm) at *m*/*z* 612.

Heptacosa, used for the daily mass calibration as well as lock mass, was injected via syringe in the reference reservoir at 30°C for this purpose. The *m*/*z* ion monitored was 218.9856.

UHPLC-QTOF MS

An ultraperformance liquid chromatography (UPLC) Acquity system (Waters) was interfaced to a QTOF mass spectrometer (QTOF Premier, Waters) using an orthogonal Z-spray-electrospray interface. The LC separation was performed using an Acquity UPLC BEH C18 1.7 μm particle size analytical column 2.1 × 50 mm (Waters) at a flow rate of 300 μL/min. The mobile phase used was a time-programmed gradient using H2O and MeOH, both 0.1 mM ammonium acetate. The percentage of organic modifier changed linearly from 5 to 90% in 5 min. The injection volume was 10 μL. Desolvation gas as well as nebulizing gas was nitrogen, obtained from a nitrogen generator. The desolvation gas flow was set at 800 L/h. TOF-MS resolution was 10000 fwhm (V-mode) and 17500 fwhm (W-mode) at *m/z* 556. MS data were acquired over an *m/z* range of 50−1000 Da. The MCP detector potential was set to 1750 V in both positive and negative ionization modes. Capillary voltages of 3.5 and 3.0 kV were used in positive and negative ionization modes, respectively. A cone voltage of 20 V was selected. The interface temperature was set to 400 °C and the source temperature to 120 °C. A scan time of 0.1 s was chosen. An auto MS profile was performed. In this work, the automated attenuated function (dynamic range enhancement, DRE) was selected to correct possible mass peak saturations, allowing the exact mass measurement accuracy to be maintained within a wide concentration range. A collision energy ramp from 10 to 30 eV was used to perform MS/MS acquisitions.

Leucine enkephalin (approximately 2 mg/L, in 50:50 methanol/water) was introduced via the lock spray needle at a flow rate of 30 μL/min. The *m/z* ions monitored were 556.2771 and 554.2615 in positive and negative ionization modes, respectively. A cone voltage between 70 and 80 V was selected to obtain adequate signal intensity (around 400 counts/s) for this compound.

Calibration experiments are performed monthly using the built-in single-syringe pump, directly connected to the interface. Calibration from *m/z* 50 to 1000 was conducted in both ionization modes, with a mixture of NaOH 0.05M/HCOOH 10% (50:50) plus imazalil (*m/z* 297.0561) at 500 μg/L.

Analytical Procedure

The analytical procedure applied to the samples was based on that of ref 3. Briefly, 1.5 g of honeybees (fresh weight) was homogenized with 15 g of anhydrous sodium sulfate and 0.5 g of Celite and extracted with 50 mL of acetone in a high-speed blender during 2 min (Ultraturrax T25, Janke and Kunkel, Germany). After filtration by gravity, a 25 mL aliquot was diluted with 50 mL of 2% aqueous NaCl (w/v) and extracted twice with 25 mL of dichloromethane. Organic extracts were preconcentrated in a turbo evaporator under a nitrogen stream at 40 °C until 5 mL. Then, 2 mL aliquots were evaporated to dryness under a gentle nitrogen stream at 40 °C. The final residue was dissolved in 1 mL of ethyl acetate (GC-MS analysis) and in 1 mL of methanol for (LC-MS analysis). In the case of LC-MS, the extract was 10-fold diluted with water before injection in the system to decrease the percentage of organic content.

RESULTS AND DISCUSSION

A First Case of Honeybee Poisoning

Two dead honeybee samples (samples 1 and 2), suspected to be poisoned by insecticide treatment, were received at our laboratory in January 2008. Additionally, one sample of alive honeybees collected from a surrounding area was also provided to be used as a blank sample. As there was no suspicious specific contaminants thought to be responsible for the honeybee poisoning, we applied a nontarget methodology to identify the potential compounds that might be present in the samples. This indicates that we did not work on a list of target compounds. For this purpose, as no information on selected analytes was introduced, specialized deconvolution software was required to detect the components in the sample. In this case ChromaLynx Application Manager was employed (see refs 16 and 22 for more information).

GC-TOF MS Nontarget Screening

Accurate mass GC-TOF MS data were submitted to an automatic nontarget screening by applying the previously mentioned software. This software automatically detected all peaks with a response over user-defined parameters, displayed their

deconvoluted mass spectra to be searched in the library, and produced a hit list with positive matches (library match >700 was used as criterium). To perform accurate mass confirmation/rejection of the library findings, the formula from the library hit was submitted to an elemental composition calculator and up to the five most intense ions were scored by exact mass measurement. Following the described methodology, a large list of compounds was identified. Within this large list, a positive finding of the insecticide coumaphos was detected in both samples (Table 1). Two nominal mass libraries were used for this search, the NIST library and a homemade library, which includes around 1000 compounds, many of them pesticides.

Table 1. Compounds Detected in the Honeybee/Nectarine Samples by GC-TOF MS and UHPLC-(Q)TOF MS

sample	sample type	pesticides identified by GC-TOF MS	pesticides identified by UHPLC-(Q)TOF MS
1	honeybees	coumaphos	coumaphos
2	honeybees	coumaphos	coumaphos
3	honeybees	coumaphos	coumaphos
4	honeybees	fenitrothion, coumaphos	coumaphos
5	honeybees	fenitrothion, chlorfenvinphos, methiocarb	chlorfenvinphos
6	nectarine flowers and leaves	pyriproxyfen, thiamethoxam	pyriproxyfen, thiamethoxam

Table 2 shows the accurate mass confirmation of coumaphos in the honeybee samples 1 and 2, for which the mass errors obtained for five representative ions were lower than 2.2 mDa in all cases. Additionally, when the ion intensity ratios of the positive finding in samples were compared with those from a reference standard, all deviations were within tolerances proposed in European Commission Decision 2002/657/EC (31). Finally, retention times for the reference standards and peak sample were also compared, presenting a deviation of <0.5%. As an example, Figure 1 shows the positive finding of coumaphos in honeybee sample 1 when using the deconvolution process. Accurate mass confirmation automatically performed by the software for five representative ions led to the confirmation of the identity of coumaphos with mass errors normally lower than 2 mDa for every ion.

Table 2. GC-TOF MS Confirmation of Coumaphos and Metabolites in Honeybee Samples 1 and 2[a]

compd	mol formula	mol mass	elemental composition	theor *m/z*	deviation (mDa) sample 1	deviation (mDa) sample 2
1 (coumaphos)	$C_{14}H_{16}ClO_5PS$	362.0145	$C_{14}H_{16}ClO_5PS$	362.0145	−0.2	−0.2
			$C_{14}H_{16}{}^{37}ClO_5PS$	364.0119	−2.0	0.5
			$C_{12}H_{12}O_5SClP$	333.9832	0.3	−1.3
			$C_{10}H_8ClO_5PS$	305.9519	1.2	1.6
			$C_{10}H_7O_2SCl$	225.9855	−2.2	0.2
2 (CMHC)	$C_{10}H_7ClO_3$	210.0084	$C_{10}H_7ClO_3$	210.0084	0.3	nd
			$C_{10}H_7{}^{37}ClO_3$	212.0057	−0.7	
			$C_9H_7ClO_2$	182.0135	1.1	
			C_8H_7ClO	154.0185	−2.2	
			$C_9H_7O_2$	147.0446	2.0	
5 (coumaphos-oxon)	$C_{14}H_{16}ClO_6P$	346.0373	$C_{14}H_{16}ClO_6P$	346.0373	nd	nd
			$C_{12}H_{12}ClO_6P$	318.0060		
			$C_{10}H_8ClO_6P$	289.9747		
			$C_{10}H_7ClO_3$	210.0084		
			$C_9H_7ClO_2$	182.0135		
6 (potasan)	$C_{14}H_{17}O_5P$	328.0543	$C_{10}H_8O_2S$	192.0245	0.0	nd
			$C_{14}H_{17}O_5PS$	328.0543	−4.1	
			$C_{12}H_{13}O_5PS$	300.0221	0.0	
			$C_{10}H_9O_5PS$	271.9908	−1.3	
			$C_{10}H_8O_3$	176.0473	0.0	
7 (4-methylumbelliferone)	$C_{10}H_8O_3$	176.0473	$C_{10}H_8O_3$	176.0473	nd	nd
			$C_9H_8O_2$	148.0524		
			$C_7H_4O_2$	120.0211		

[a] Mass fragments and mass errors for the proposed compounds. nd, not detected.

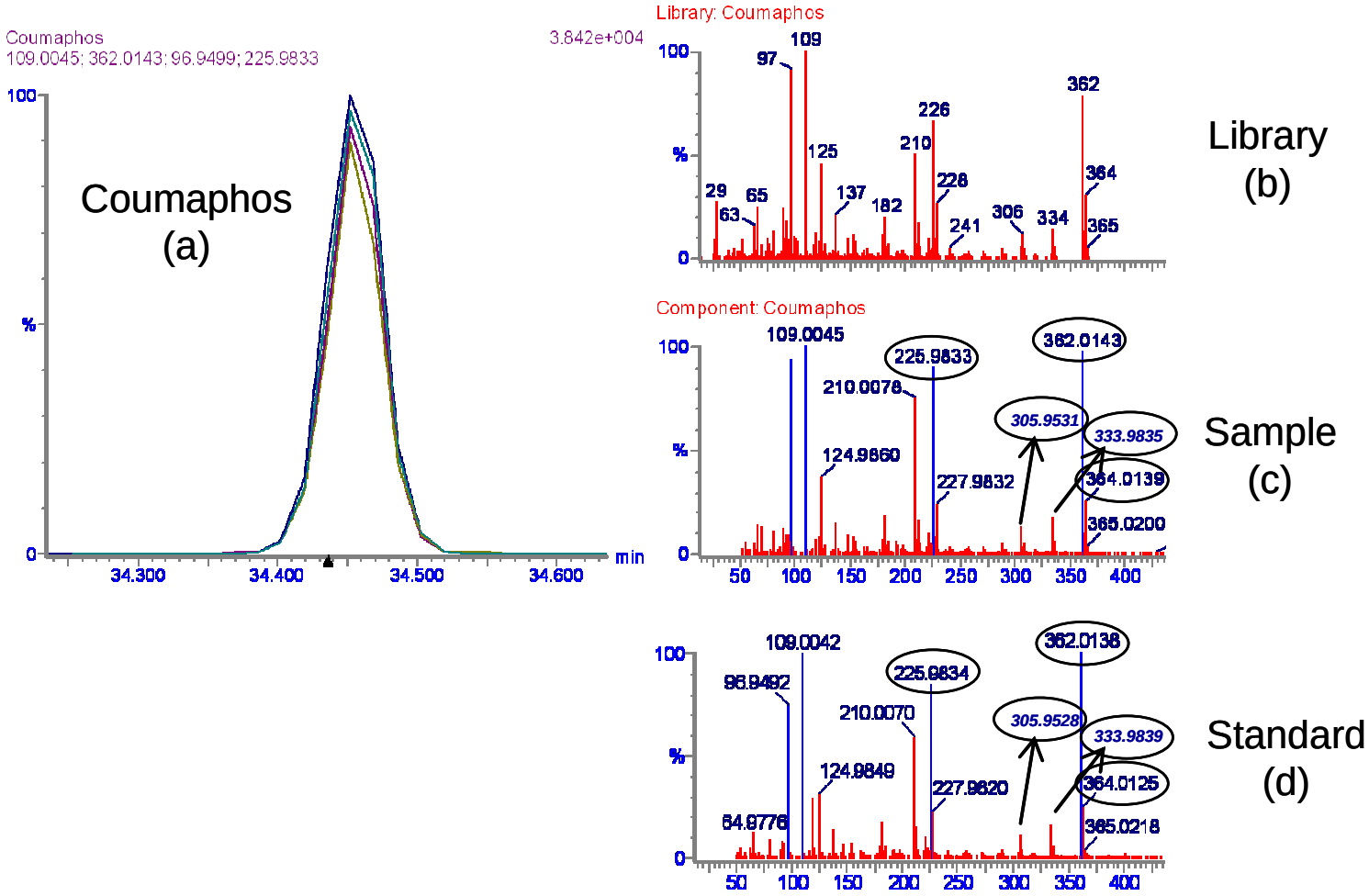

Figure 1. Confirmation of coumaphos in honeybee sample 1 by GC-TOF MS. (a) Extracted ion chromatograms for four ions used for deconvolution. (b) Library mass spectrum at nominal masses. (c) Deconvoluted accurate mass spectrum for the compound detected. (d) Accurate mass spectrum of a coumaphos reference standard in solvent.

UHPLC-TOF MS Nontarget Screening

Accurate mass UHPLC-TOF MS data were also processed in a nontarget way by applying the ChromaLynx Application Manager. The only difference with respect to the GC-MS approach was the use of only the two most intense ions (softer ionization in ESI in comparison to EI) and the use of a theoretical homemade library containing around 500 contaminants, including 377 pesticides and 40 transformation products, but also 47 antibiotics, 20 pharmaceuticals, and other emerging contaminants frequently detected in the environment, such as cocaine or caffeine (22). This library was built without the need of injected standards, and it shows the theoretical spectrum with information on molecular ion mass and the expected isotopic pattern. The insecticide coumaphos was also found in both samples, by applying UHPLC-TOF MS (see Table 1).The availability of a QTOF instrument made it feasible to perform MS/MS experiments for both standards and samples and to go further in the confirmation of the identity of the compound detected. As an example, Figure 2 and Table 3 illustrate the ultimate confirmation achieved in honeybee sample 1 suspected to be positive for coumaphos. Deviations in the measured masses of all product ions were lower than 2.3 mDa. Additionally, when the relative abundances in the suspected positive sample were compared with those of a reference standard, all deviations were within the limits proposed by European Decision 2002/657/EC (31). Finally, retention times for the reference standards and peak sample were also compared, presenting a deviation of <2.5%. Therefore, this sample was confirmed by QTOF to be positive for coumaphos in a highly reliable way.

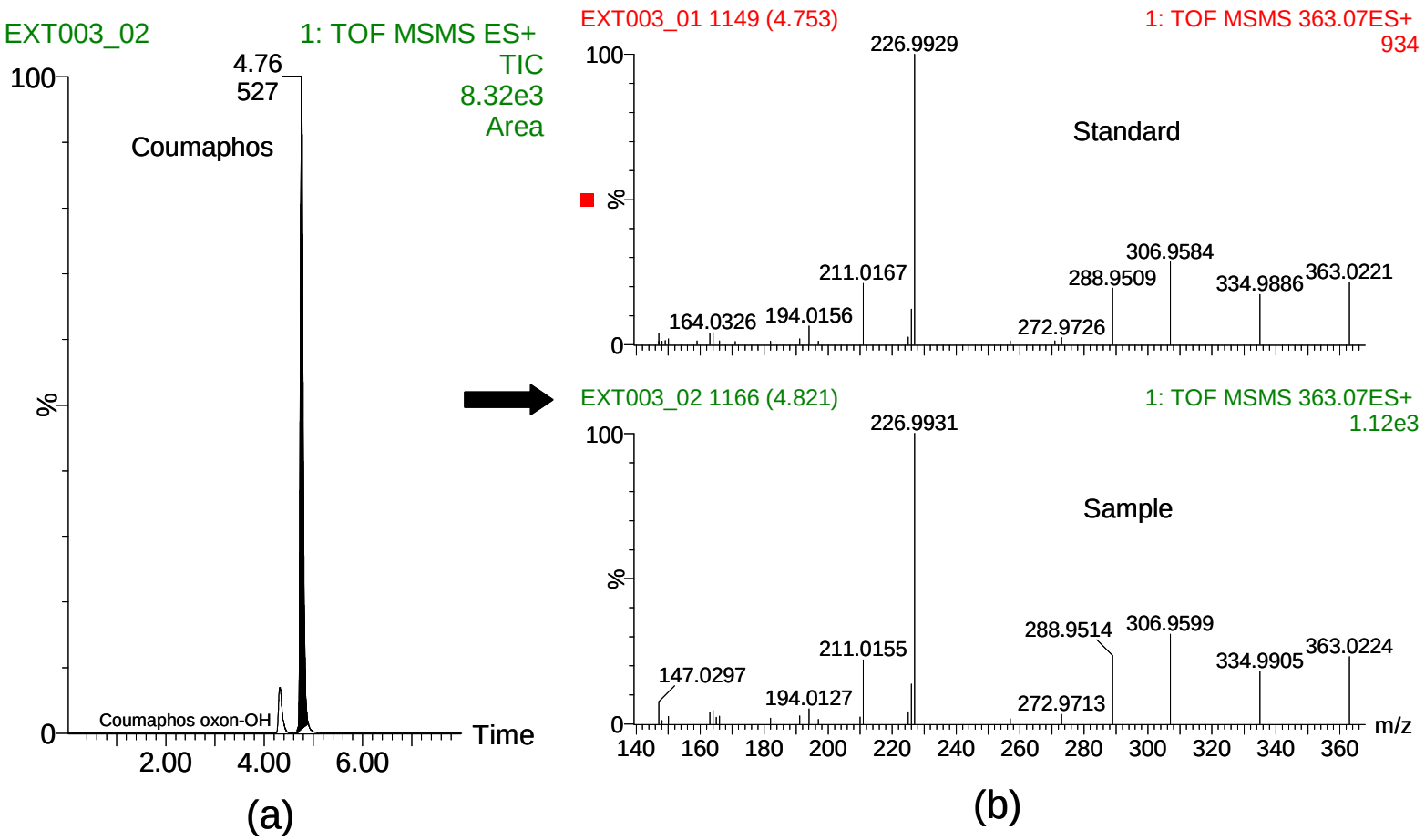

Figure 2. Confirmation of coumaphos in honeybee sample 1 by UHPLC-QTOF MS: (a) UHPLC-QTOF MS/MS chromatogram from the sample; (b) product ion spectrum (precursor ion m/z 363) from the sample and from the reference standard.

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Table 3. UHPLC-(ESI)-QTOF MS Confirmation of Coumaphos and Metabolites in Honeybee Samples 1 and 2[a]

			deviation (mDa)				deviation (mDa)	
compd	**elemental composition precursor ion $[M + H]^+$**	**theor mass precursor ion $[M + H]^+$**	**sample 1**	**sample 2**	**elemental composition product ion**	**theor mass product ion**	**sample 1**	**sample 2**
1 (coumaphos)	$C_{14}H_{17}O_5PSCl$	363.0223	0.1	−0.3	$C_{12}H_{13}O_5PSCl$	334.9910	−0.5	0.8
					$C_{10}H_9O_5PSCl$	306.9597	−0.2	−0.3
					$C_{10}H_7O_4PSCl$	288.9491	−2.3	0.9
					$C_{10}H_8O_2SCl$	226.9934	−0.3	−0.2
					$C_{10}H_8O_3Cl$	211.0162	0.7	1.0
					$C_{10}H_7O_2Cl$	194.0135	0.8	0
2 (CMHC)	$C_{10}H_8O_3Cl$	211.0162	0.8	1.5	$C_9H_8O_2$	148.0524	0.9	
					C_9H_7O	131.0497	0	
					C_8H_7O	119.0497	−1.2	
					C_8H_7	103.0548	0.5	
					C_7H_7	91.0548	−0.1	
3 (coumaphos-OH)	$C_{14}H_{17}O_6PSCl$	379.0172	1.9	0.8	$C_{12}H_{13}O_6PSCl$	350.9862	0.8	
					$C_{10}H_9O_6PSCl$	322.9557	1.0	
					$C_{10}H_7O_5PSCl$	304.9457	0.6	
					$C_{10}H_8O_3SCl$	242.9892	−0.8	
					$C_9H_8O_5P$	227.0096	1.5	
4 (coumaphos oxon-OH)	$C_{14}H_{17}O_7PCl$	363.0400	−1.5	0.9	$C_{12}H_{13}O_7PCl$	335.0087	0.2	
					$C_{10}H_9O_7PCl$	306.9774	1.5	
					$C_{10}H_7O_6PCl$	288.9669	1.9	
					$C_{10}H_8O_4Cl$	227.0111	0.5	
					$C_{10}H_6O_3Cl$	209.0005	0.8	
					$C_9H_6O_2Cl$	181.0051	0.5	
6 (potasan)	$C_{14}H_{18}O_5PS$	329.0613	3.6	nd	$C_{12}H_{14}O_5PS$	301.0300	1.7	
					$C_{14}H_{16}O_5S$	296.0718	1.8	
					$C_{10}H_{10}O_5PS$	272.9987	3.6	
					$C_{10}H_8O_4PS$	254.9881	2.1	

Metabolite Investigation

The high levels of coumaphos found in both samples encouraged us to investigate the presence of its metabolites by both GC-TOF MS and UHPLC-(Q)TOF MS.

GC-TOF MS Studies

As no specialized software for metabolism studies was available for GC-TOF MS, potential metabolites of coumaphos were investigated in a post-target way, that is, by searching for specific compounds after MS data acquisition, based on information available in the scientific literature.

Several metabolites of coumaphos in human urine, soils, and animals have been described (32, 33) as the dechlorination product (potasan, compound 6), the metabolite resulting after hydrolysis of the ester moiety (3-chloro-7-hydroxy-4-methyl-chromen-2-one, CMHC, compound 2), the hydrolysis plus dechlorination product (4-methylumbelliferone, compound 7), and the sulfur atom oxidation product (coumaphos oxon, compound 5) (see Figure 3). To perform an investigation of the presence of these metabolites in honeybee samples, EI spectra of each described analyte were searched for in the NIST library. Up to five *m/z* ions (molecular ion, if available, and fragment ions) were chosen from available nominal library spectrum. A possible elemental composition of those selected *m/z* ions was deduced, and their exact mass calculated and introduced in a target processing method (see Table 2). Experimental GC-TOF MS data were then submitted to the developed post-target processing method, and the presence of the selected ions (nw-XIC of 0.02 Da) in the sample extract was tested. Analyte confirmation was performed by comparison of the experimental intensity ratios in samples with the theoretical ones, calculated from the library spectrum. Additionally, mass accuracy for the most characteristic ions was evaluated.

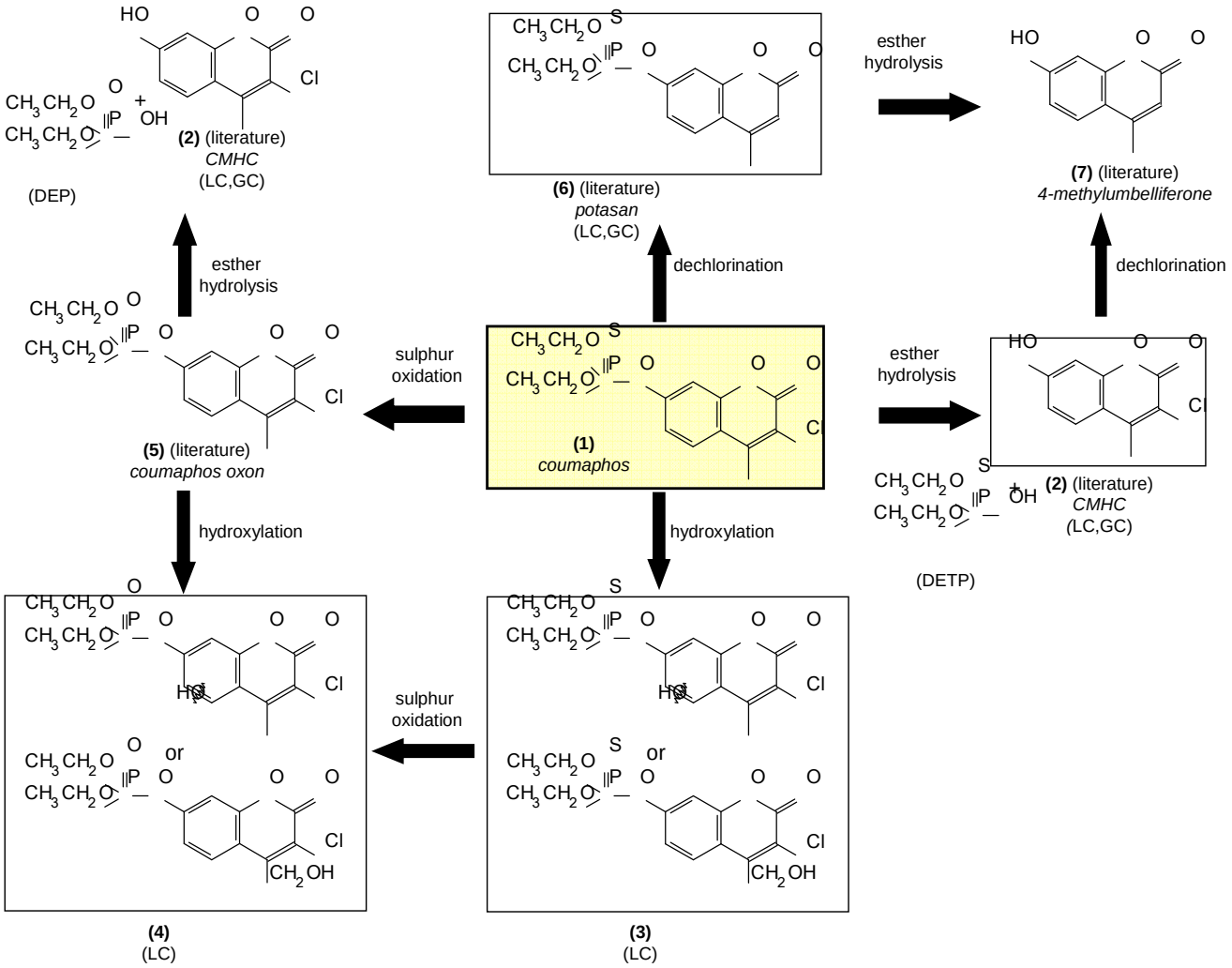

Figure 3. Coumaphos metabolites identified in honeybees. Proposed degradation pathway. "LC, GC" indicates that the metabolite was identified in the honeybee sample in the present work.

This approach was applied for the investigation of CMHC, 4-methylumbelliferone, and coumaphos oxon, as their EI spectra were available in the NIST library. The CMHC metabolite was found in sample 1, which showed a chromatographic peak for the five preselected ions at the same retention time with the ion intensity ratios within specified tolerance. Besides, mass errors for these ions were always below 2 mDa (see Table 2). However, no signal was observed for the other two described analytes. Investigation of metabolite potasan (compound 6) was more difficult as no previous information about its EI spectrum was available in the library. Although no data about the abundance of the molecular ion in potasan EI spectrum were known, a nw-XIC at its theoretical exact *m/z* (328.0543) was performed. As a notable chromatographic peak appeared at 12.65 min, a background-subtracted combined spectrum for this peak was performed. Accurate masses from this spectrum were submitted to an elemental composition calculation program to obtain elemental compositions, which were compared to the theoretical ones. The resulting elemental compositions fit well with possible fragments of potasan, with low mass errors, as Figure 4 shows, leading to the conclusion that the compound detected in honeybee sample 1 was the metabolite potasan.

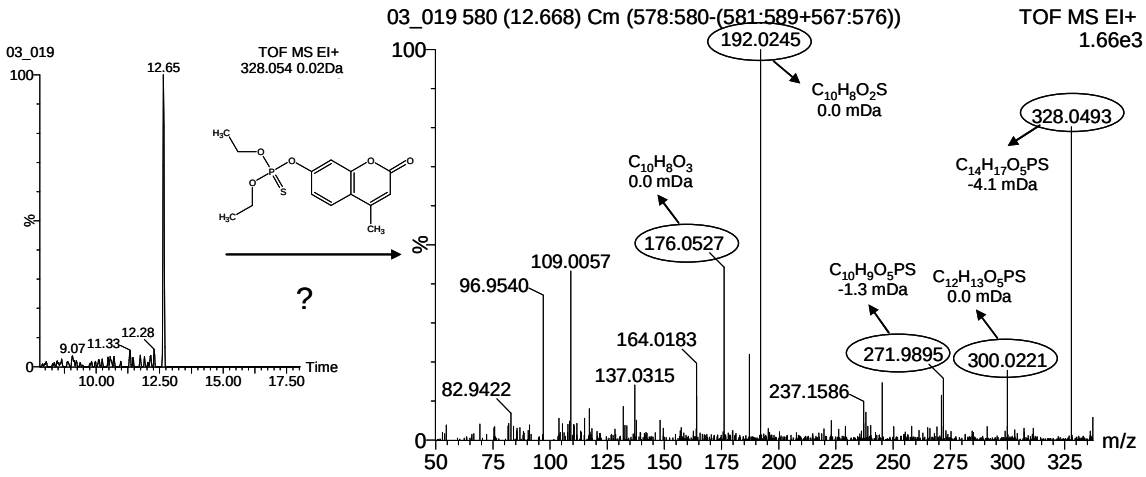

Figure 4. Positive finding of the coumaphos metabolite potasan in honeybee sample 1.

UHPLC-(Q)TOF MS Studies

Regarding LC-MS, data were processed using MetaboLynx software (Micromass v 4.1), which has been proved to be useful in previous pesticide degradation/metabolism studies (34). Two LC-MS data files (one corresponding to the sample and the other one to a blank sample) are compared, and the differences resulting from the presence of new compounds, which could be in principle attributed to transformation processes in the sample, are highlighted. Following the approach applied in previous works for compounds detected by MetaboLynx, the accurate mass of protonated/deprotonated molecules was determined on the basis of averaged spectra obtained in the TOF MS survey scan. On the basis of their accurate mass, possible elemental compositions of the peaks of interest were calculated using the elemental composition calculator with a maximum deviation of 2 mDa from the measured mass. Maximum and minimum parameter settings for all compounds were restricted as a function of the structure of coumaphos: C, 0−14; H, 0−18; O, 0−10; P, 0−1; and S, 0−1. The appropriate number of Cl was determined from the observed isotopic pattern and added if required. The possibility of performing MS/MS experiments helped us to elucidate the structure of several metabolites thanks to the information given by the product ion spectrum with the exact mass of the fragments.

According to the metabolites detected in honeybee samples 1 and 2 by UHPLC, four important processes were found to occur in the metabolism of coumaphos in honeybees, as can be seen in Figure 3: hydrolysis of the ester moiety, hydroxylation, oxidation of the sulfur atom, and dechlorination. A combination of these processes was also observed.

The hydrolysis of the ester moiety originated CMHC (compound 2). However, the metabolite diethyl thiophosphate (DETP) was not found probably due to the low sensitivity for alkylphosphates in negative electrospray interfaces and the need to add an ion-pairing reagent to obtain a good chromatographic separation (35). As shown in Figure 3, hydroxylation was observed in the aromatic or in methyl group (compound 3), as explained in more detail in following paragraphs. A combination of oxidation of the sulfur atom on the PS functional group plus hydroxylation was also observed (compound 4). Finally, our data suggested a loss of the chlorine atom (potasan, compound 6).

In this paper, the potential of TOF MS was useful to distinguish between compounds 1 and 4. Both have the same nominal mass (*m/z* 363), and therefore they would be indistinguishable by quadrupole instruments. However, their accurate masses (*m/z* 363.0219 compound 1, *m/z* 363.0385 compound 4) showed a difference of 16.6 mDa, which was sufficient for an appropriate identification. After the application of elemental composition calculator with the selected parameters (maximum deviation = 5 mDa), only one hit appeared for each compound. Thus, it was easy to assign their correct elemental composition.

The results obtained in LC-(Q)TOF experiments are summarized in Table 3. This table illustrates the identification of the parent compound and four metabolites detected. As can be seen, most of deviations were ≤1 mDa, with the highest values observed for metabolite 6 (potasan). With all of these data, with a minimum of four ions for each compound, one can be confident about the elemental composition given for each analyte.

Despite the capability of TOF analyzers to distinguish between isobaric compounds (mass differences of <1 Da), its usefulness is limited when dealing with isomers, as they present the same molecular formula and, consequently, the same mass. However, hybrid QTOF instruments give the possibility of performing tandem MS acquisitions obtaining product ion spectra with accurate mass, which in some cases can help to differentiate between isomeric analytes in a more confident way than when using nominal mass instruments.

We performed MS/MS experiments with the QTOF to investigate the chemical structure of compound 3. Comparing the elemental composition of coumpahos ([M + H]+ $C_{14}H_{17}O_5PSCl$) with the calculated composition for compound 3 ([M + H]+ $C_{14}H_{17}O_6PSCl$, *m/z* 379.0172), one can predict this compound is a monohydroxylated product of coumaphos. However, there was no information on where the hydroxylation occurred: in the aromatic methyl group, in the ethyl group of the thiophosphoric esther, or in the aromatic group. To elucidate this metabolite, MS/MS experiments on the precursor ion $C_{14}H_{16}O_6PS^{35}Cl$ (*m/z* 379) were carried out. In addition, MS/MS experiments on the isotopic peak ($C_{14}H_{16}O_6PS^{37}Cl$, *m/z* 381) were also performed to learn the product ions that maintained the chlorine atom. These experiments were useful and allowed some candidates to be discarded, as in several cases two plausible

elemental compositions (one with a chlorine atom and the other without) were feasible. In a similar way, MS/MS experiments for all metabolites were carried out.

Product ion spectrum of compound 3 ([M + H]+ $C_{14}H_{17}O_6PSCl$, *m/z* 379.0191) (see Figure 5) showed fragment ions at *m/z* 350.9870 (ΔmDa = 0.8, with regard to the theoretical exact mass), 322.9567 (ΔmDa = 1.0), and 304.9463 (ΔmDa = 0.6), which resulted from losses of one ethyl group, two ethyl groups, and two ethyl groups plus water from the precursor ion *m/z* 379.0191, respectively, showing that the hydroxylation could not occur in the ethyl radicals. Performing MS/MS experiments of both precursor ions (corresponding to ^{35}Cl and ^{37}Cl) led to useful information. Thus, the fragment ion *m/z* 242.9884 was initially assigned to $C_9H_8O_4PS$, which would have resulted from a hydroxylation in the aromatic ring. However, after performing MS/MS experiments (precursor ion *m/z* 381, ^{37}Cl), we observed that this fragment maintained the chlorine atom, being therefore assigned to $C_{10}H_8O_3SCl$ instead of $C_9H_8O_4PS$. Then two possibilities (hydroxylation in the methyl group and hydroxylation in the aromatic ring) were still feasible. Something similar occurred with compound 4 ($C_{14}H_{17}O_6PCl$). Data obtained in the MS/MS spectra were not sufficient to discover if the hydroxylation had occurred in the aromatic ring or in the methyl group.

In a similar way, MS/MS experiments were carried out for all metabolites. Although between 8 and 12 product ions were justified for each compound, only the most abundant ones are shown in Table 3.

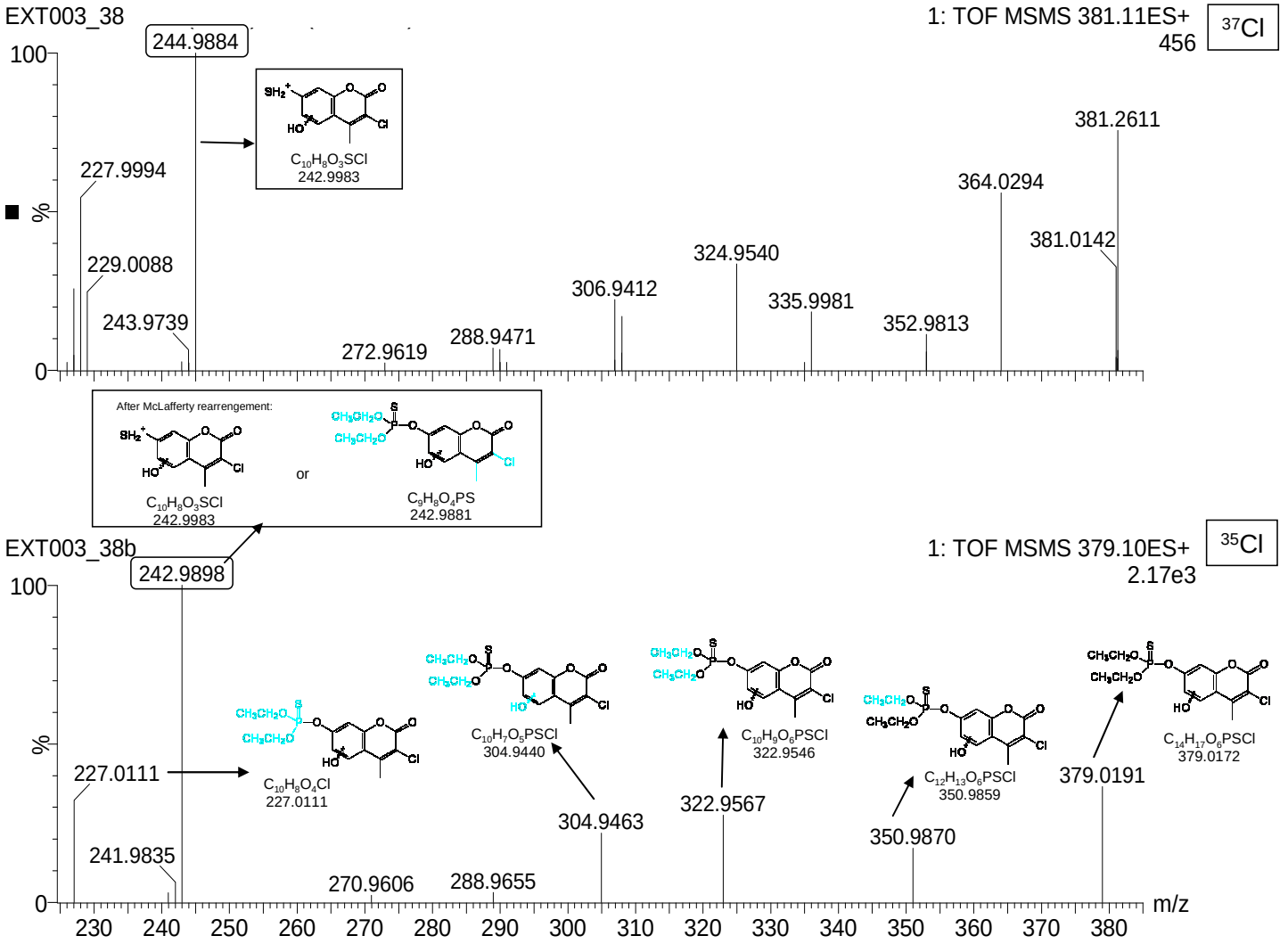

Figure 5. Product ion spectra of the coumaphos precursor ions (a) m/z 379 (35Cl) and (b) m/z 381 (37Cl) from sample 1.

Other Cases of Poisoning

Three additional honeybee samples (samples 3−5) also suspected to be intoxicated by insecticide applications, together with one sample of nectarine flowers and leaves (sample 6) (supposedly responsible for the sample 3 honeybee intoxication), were received at our laboratory a few months after the first poisoning case. These samples were investigated following the above-mentioned methodology.Regarding GC-TOF MS analysis, positive findings of coumaphos, fenitrothion, chlorfenvinphos, and methiocarb were found in the honeybee samples. In the nectarine flower and leaf sample, pyriproxyfen and thiamethoxam were found (Table 1). As an illustrative example, Table 4 shows the confirmation of pesticides detected in honeybee sample 5 and in the nectarine sample (sample 6). Mass errors for every ion were typically below 2 mDa, except for a few low-abundant ions. Additionally, when the ion intensities of findings in samples were compared with the theoretical ones from reference standards, all deviations were within maximum tolerances (31). Figure 6 shows the positive findings of methiocarb and fenitrothion in honeybee sample 5 when using the deconvolution process

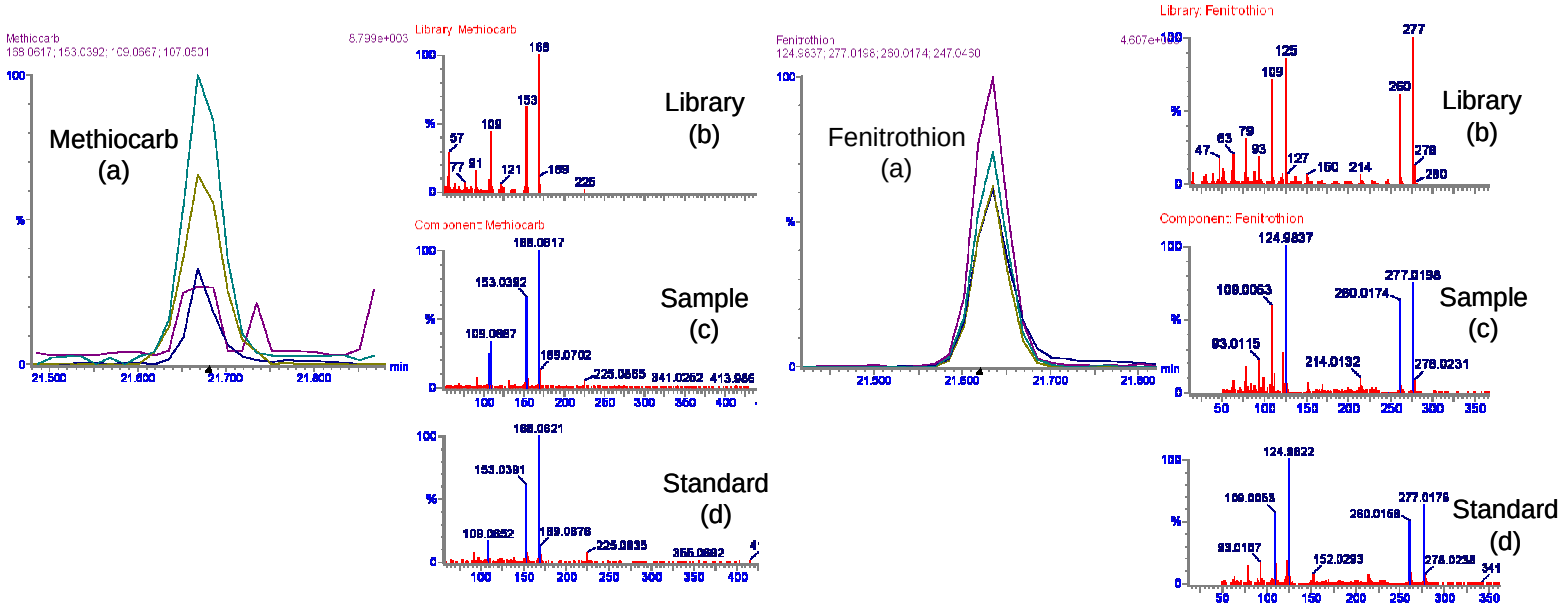

Figure 6. GC-TOF MS confirmation of positive findings of methiocarb and fenitrothion in honeybee sample 5: (a) extracted ion chromatograms for four ions used for deconvolution; (b) library mass spectrum at nominal masses; (c) deconvoluted accurate mass spectrum for compounds detected in the sample; (d) accurate mass spectrum of a reference standard in solvent.

With regard to UHPLC-TOF MS analysis, among the three samples of honeybee, previous positive GC-TOF MS findings of coumaphos and chlorfenvinphos were confirmed. In the nectarine sample, the presence of pyriproxyfen and thiamethoxam was also confirmed (see Table 1). As an example, Figure 7 and Table 4 show the safe confirmation achieved in the nectarine sample suspected to be positive for thiamethoxam and pyriproxyfen. Deviations in the measured masses of all product ions were lower than 2 mDa, except for one product ion of thiamethoxam. Additionally, all relative ion abundances observed in the positive sample were within the maximum values allowed (31). In sample 5, no MS/MS experiments were possible a priori for chlorfenvinphos due to the low level found. To confirm the presence of this compound, MS/MS experiments were carried out but with the raw extract in 100% methanol to avoid the 10-fold dilution. Regarding fenitrothion and methiocarb, these compounds were not detected by LC-MS.

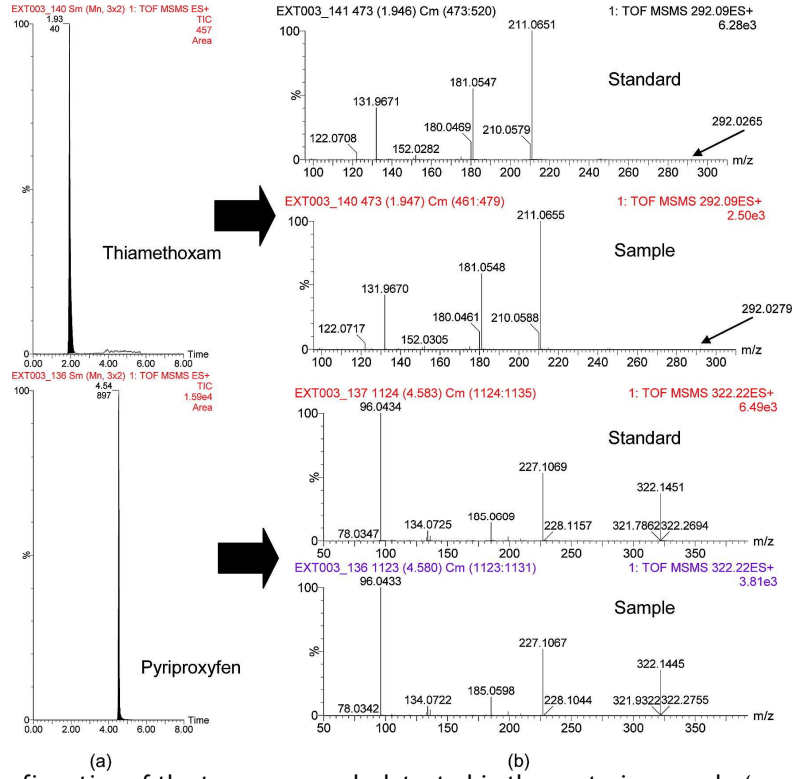

Figure 7. Confirmation of the two compounds detected in the nectarine sample (sample 6) by UHPLC-QTOF MS: (a) UHPLC-QTOF MS chromatograms from the sample; (b) product ion spectrum of the precursor ion (*m/z* 292 for thiamethoxam and *m/z* 322 for pyriproxyfen) from the sample and from the standard.

Table 4. Confirmation of Pesticides Identified in Honeybee Sample 5 and Nectarine Sample (Sample 6)[a]

GC-TOF MS

	molecular peak		ion 1		ion 2		ion 3		ion 4		ion 5	
compd	mol formula	mol mass	elemental composition	theor *m/z* (error in mDa)	elemental composition	theor *m/z* (error in mDa)	elemental composition	theor *m/z* (error in mDa)	elemental composition	theor *m/z* (error in mDa)	elemental composition	theor *m/z* (error in mDa)
Fenitrothion (sample 5)	$C_9H_{12}NO_5PS$	277.0174	$C_2H_6O_2PS$	124.9826 (1.1)	$C_2H_6O_3P$	109.0055 (0.8)	$C_9H_{12}NO_5PS$	277.0174 (2.4)	$C_9H_{11}NO_4PS$	260.0146 (2.8)	CH_4O_2P	78.9949 (0.9)
Methiocarb (sample 5)	$C_{11}H_{15}NO_2S$	225.0824	$C_9H_{12}OS$	168.0609 (0.8)	C_8H_9OS	153.0374 (1.8)	C_7H_9O	109.0653 (1.4)	$C_{11}H_{15}NO_2S$	225.0824 (4.1)		
Chlorfenvinphos (sample 5)	$C_{12}H_{14}Cl_3O_4P$	357.9695	$C_8H_6Cl_2O_4P$	266.9381 (0.4)	$C_8H_6{}^{35}Cl^{37}ClO_4P$	268.9353 (−0.2)	$C_{12}H_{14}Cl_2O_4P$	323.0007 (−0.7)	$C_{10}H_{10}Cl_2O_4P$	294.9694 (−1.4)	$C_{12}H_{14}{}^{35}Cl^{37}ClO_4P$	324.9980 (−1.7)
thiamethoxam (sample 6)	$C_8H_{10}ClN_5O_3S$	291.0193	$C_8H_{10}N_3O_2S$	212.0494 (−1.2)	C_4H_3NSCl	131.9675 (0.3)	$C_7H_8N_3OS$	182.0388 (−1.0)	$C_8H_{10}N_3O_2SCl$	247.0182 (−0.1)	$C_8H_{10}N_3O_2S^{37}Cl$	249.0153 (1.1)
Pyriproxyfen (sample 6)	$C_{20}H_{19}NO_3$	321.1365	$C_8H_{10}NO$	136.0762 (1.0)	$C_{12}H_{10}O_2$	186.0681 (−0.3)	$C_{15}H_{14}O_2$	226.0994 (0.9)				

UHPLC-QTOF MS

	parent ion		product ion 1		product ion 2		product ion 3		product ion 4		product ion 5	
compd	mol formula	theor *m/z* (error mDa)	elemental composition	theor *m/z* (error in mDa)	elemental composition	theor *m/z* (error in mDa)	elemental composition	theor *m/z* (error in mDa)	elemental composition	theor *m/z* (error in mDa)	elemental composition	theor *m/z* (error in mDa)
Chlorfenvinphos (sample 5)	$C_{12}H_{16}O_4PCl_3$	358.9774 (−1.5)	$C_8H_4Cl_3$	204.9379 (1.0)	$C_4H_{12}O_4P$	155.0473 (1.0)	$C_2H_8O_4P$	127.0160 (−0.4)	PO_4H_4	98.9847 (−0.8)	$C_8H_4Cl_2$	169.9690 (−1.8)
Thiametoxam (sample 6)	$C_8H_{11}N_5O_3SCl$	292.0271 (0.8)	$C_8H_{11}N_4OS$	211.0654 (0.1)	$C_7H_9N_4S$	181.0548 (0)	C_4H_3NSCl	131.9661 (−0.9)	$C_6H_8N_3$	122.0718 (−0.1)	$C_6H_6N_3S$	152.0282 (2.3)
Pyriproxyfen (sample 6)	$C_{20}H_{20}NO_3$	322.1444 (0.1)	C_5H_6NO	96.0449 (−1.6)	$C_{15}H_{15}O_2$	227.1072 (−0.5)	$C_{20}H_{20}NO_3$	322.1444 (0.1)	$C_{12}H_9O_2$	185.0603 (−0.5)	$C_9H_{10}O$	134.0732 (−1.0)

[a] Mass fragments and mass errors for the compounds obtained by GC-TOF MS and UHPLC-ESI-QTOF MS.

Validation of the Confirmation Process

To validate the applicability of the procedure used –confirmation of nontarget detected compounds– six honeybee blank samples were spiked at two concentration levels: 1 µg/g (n = 3) and 10 µg/g (n = 3). The "blank" sample was previously analyzed, and no presence of the analytes was found. The spiked samples were extracted and analyzed as previously described and injected in GC-TOF MS and UHPLC-QTOF MS. Six insecticides were studied by GC-TOF MS and four by UHPLC-QTOF MS (see Table 1).

The presence of up to five ions measured at their accurate mass (nw-XIC of 0.02 Da) was evaluated for the six replicates at the two levels tested. Additionally, their intensity ratios were compared to the average ratios calculated from reference standards in solvent: six injections of a 1.5 mg/L standard in GC-TOF MS with RSD < 20% and two injections of standards at four concentration levels (0.15, 0.3, 1.5, and 3.0 mg/L) in UHPLC-QTOF with RSD < 16%. Although European Comission Decision 2002/657/EC (31) requires the attainment of at least one ion ratio deviation, in this study up to four ratio deviations were measured. In all cases, experimental ion ratios in spiked samples, at the two concentration levels, were in agreement with those obtained for reference standards in solvent. All deviations were within the specified tolerances accepted by European guidelines. Data obtained showed that the correct identification and confirmation of analytes could be successfully performed at the concentration range assayed.

In summary, the combination of GC-TOF MS and UHPLC-(Q)TOF MS has been shown as an advanced tool for the screening and confirmation of nontarget analytes in honeybee samples. Without previous selection of the analytes to be searched, the methodology employed (based on a peak deconvolution process followed by a library search and accurate mass scoring) allowed the discovery of the presence of some pesticides, such as pyriproxyfen, chlorfenvinphos, or coumaphos, among others. In addition, the potential of these techniques has been proved by the fact that several pesticide metabolites were also discovered in poisoned honeybee samples. The availability of commercial libraries with more than 150,000 EI spectra normally makes easier the identification of nontarget analytes when using GC-TOF MS instruments

without injecting reference standards. Many detected compounds are normally found in the library, and the accurate mass measurements generated by TOF MS help the confirmation of the identified analyte. The possibility of performing a safe identification and confirmation in a unique analysis is an advantage when using EI spectra, as the number of fragment ions available is normally enough for confirmation purposes. However, the elucidation of a compound that is not present in a library (as normally occurs for most metabolites) is more difficult, as no security in the presence of the molecular ion in the spectrum exists. However, the presence of the molecular ion in the LC-ESI-TOF MS spectrum is one of the main advantages of this technique, which facilitates the obtaining of the elemental composition of an "unknown" compound, both organic pollutants or their metabolites. Furthermore, the possibility of performing MS/MS experiments in QTOF instruments helps to elucidate and/or confirm the structure of the compound detected, as the product ion spectra with the exact mass of fragments is obtained, information that is very useful in the elucidation process.

In this work, making use of a nontarget approach, the insecticides fenitrothion, chlorfenvinphos, coumaphos, and methiocarb were found in the honeybee samples suspected to be poisoned by insecticide applications. Moreover, thiamethoxam and pyriproxyfen were identified in nectarine flowers and leaves, which were supposedly responsible for a honeybee intoxication case. Additionally, up to four metabolites of coumaphos were detected in one honeybee sample that contained high levels of parent coumaphos. To our knowledge, two of these metabolites had not been previously described in the available scientific literature.

ACKNOWLEDGMENT

We are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of UHPLC-QTOF MS (QTOF Premier) and GC-TOF MS (GCT) equipment. We thank our colleague Óscar J. Pozo for useful comments and Fernando Calatayud and Enrique Simó from Agrupación de Defensa Sanitaria Apícola (apiADS) de la Comunitat Valenciana for providing honeybee samples.

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CAPÍTULO 5

CONCLUSIONES

La **conclusión general** que se extrae de la presente tesis es que, en la actualidad, las técnicas avanzadas basadas en el acoplamiento GC-MS, con modernos analizadores de triple cuadrupolo y tiempo de vuelo, constituyen una de las herramientas analíticas más poderosas para fines de *screening* y cuantificación de contaminantes orgánicos poco polares y/o (semi)volátiles. Por un lado, GC-MS/MS con triple cuadrupolo es una técnica muy adecuada para la determinación cuantitativa y confirmación de la identidad de un número amplio de contaminantes orgánicos en muestras biológicas y ambientales. Por otro lado, la técnica GC-TOF MS abre un nuevo escenario para el desarrollo de métodos de *screening* de amplio rango, permitiendo abordar la problemática desde el punto de vista *target* como *non-target*. Ambas técnicas son complementarias para el análisis cuantitativo y cualitativo en los campos de aplicación abordados. La combinación de estas dos técnicas con el análisis por LC también con equipos de triple cuadrupolo y QTOF ofrece un enorme potencial en los laboratorios de Salud Pública al poder enfrentar la investigación de contaminantes orgánicos con una visión "universal".

Conclusiones específicas:

1. El acoplamiento GC-MS/MS con analizador de triple cuadrupolo se ha mostrado como una herramienta analítica muy valiosa para el análisis multirresidual de una gran diversidad de contaminantes orgánicos en aguas a niveles de concentración de µg/L y sub-µg/L. La principal limitación es que el número de transiciones adquiridas simultáneamente es restringido para obtener una buena definición del pico cromatográfico. En este sentido, la optimización cromatográfica es de gran importancia para minimizar el número de compuestos que coeluyen en un determinado tiempo. Con una sencilla etapa de extracción en fase sólida, la elevada sensibilidad y selectividad de GC-(QqQ)MS/MS han permitido determinar los niveles de concentración objetivo por debajo de 0.1 µg/L, con un alto grado de fidelidad para un amplio número de microcontaminantes orgánicos en aguas.
2. El modo de ionización química negativa se ha mostrado como técnica alternativa a la ionización electrónica para ciertos contaminantes prioritarios en aguas

(principalmente compuestos organoclorados), por su mayor sensibilidad y selectividad.

3. El acoplamiento GC-MS/MS con analizador de triple cuadrupolo se ha mostrado como una herramienta analítica muy poderosa para la determinación rápida y selectiva de compuestos xenoestrógenos en muestras de tejido adiposo humano. La purificación de los extractos por HPLC en fase normal con columnas de silica gel, ha permitido minimizar la cantidad de matriz introducida en el sistema GC (principalmente lípidos) facilitando la correcta identificación y cuantificación de los analitos.

4. Desde el punto de vista de la confirmación de la identidad de los compuestos detectados la adquisición de varias transiciones MS/MS mediante triple cuadrupolo y el cálculo de las relaciones iónicas permiten cumplir sobradamente los requisitos de la Decisión Europea 2002/657/CE en cuanto a número de puntos de identificación requeridos (IPs). En general, la adquisición de dos transiciones SRM junto con la medida de su relación de abundancia suele ser suficiente para garantizar la confirmación del analito con equipos de triple cuadrupolo. La confirmación se considera como definitiva si ambas transiciones son suficientemente específicas y la relación iónica está en concordancia con la del patrón.

5. La técnica GC-TOF MS ha resultado ideal para el *screening* y confirmación de contaminantes orgánicos en distintos tipos de muestras, tanto en modo *target* como *non-target*. El procesamiento de datos, que permita gestionar la gran cantidad de información espectral generada por GC-TOF MS, ha resultado crucial en el desarrollo de métodos de *screening target*. Aunque la disponibilidad de patrones es siempre preferible para este fin, se han mostrado alternativas eficientes en el caso de no disponer de patrón de referencia, con los que también se han obtenido resultados satisfactorios.

6. La aplicación de la metodología de trabajo desarrollada para el *screening* en combinación con un tratamiento de muestras basado en microextracción en fase sólida (SPME) ha permitido la rápida detección y confirmación de un buen número de contaminantes en las aguas analizadas.

7. La validación cualitativa de la metodología de *screening* desarrollada en esta Tesis, usando muestras de agua de distintos orígenes y características fortificadas con un amplio grupo de contaminantes seleccionados, ha permitido establecer el nivel más bajo de concentración para el cual un compuesto presente en las aguas podrá ser identificado y confirmado fielmente en todos los tipos de aguas analizadas.

8. GC-TOF MS ha resultado muy adecuada para investigar la presencia de contaminantes de origen antropogénico en muestras de tejido adiposo humano. La comparación de resultados con los obtenidos por GC-(QqQ)MS/MS ha mostrado la mayor idoneidad y sensibilidad del triple cuadrupolo para el análisis cuantitativo *target* de un número limitado de analitos. Sin embargo, la técnica GC-TOF MS se ha mostrado como una técnica más versátil que permite la identificación de más compuestos en las muestras, incluidos algunos inesperados o no buscados, debido a la adquisición del espectro de iones completo medido en masa exacta.

9. El uso de metodologías *target* en combinación con *non-target* en el análisis por GC-TOF MS ofrece una visión más completa y realista sobre la contaminación existente. La aplicación de únicamente metodología *non-target* no resulta completamente satisfactoria por el momento debido a la dificultad detectar componentes a bajo nivel de concentración con esta metodología. Ambas metodologías, *target* y *non-target* son complementarias para el *screening* de contaminantes orgánicos en los distintos campos abordados en esta Tesis.

10. GC-TOF MS ha resultado, gracias a su elevada exactitud de masa y a la información espectral generada, una técnica adecuada para la elucidación de compuestos cuyo espectro de ionización electrónica no se encuentra en librerías comerciales. El uso combinado de las fuentes de ionización electrónica e ionización química para este fin ha resultado ser de gran utilidad debido a la información aportada por la fuente de CI sobre la masa molecular, así como la aportada por los espectros de EI en relación a información estructural del compuesto desconocido, a través de la presencia de sus iones fragmento

11. La nueva fuente de ionización química a presión atmosférica (APCI) en combinación con GC-(Q)TOF MS presenta un gran potencial para el *screening* de residuos de plaguicidas en muestras de alimentos. Los trabajos preliminares

realizados con un prototipo de fuente permiten albergar expectativas muy prometedoras en este y otros campos del *screening*.

12. La confirmación por GC-QTOF mediante un barrido de iones producto de los plaguicidas detectados midiendo la masa exacta de los fragmentos y calculando posteriormente las relaciones iónicas puede considerarse prácticamente como inequívoca, dadas sus excelentes prestaciones en el campo cualitativo, siendo una de las herramienta analíticas más poderosas en este aspecto. Para un mejor aprovechamiento de las capacidades del QTOF se requiere el uso de fuentes de ionización suaves, como CI o la recientemente desarrollada APCI.

13. La combinación de GC-MS y LC-MS con analizadores de triple cuadrupolo y QTOF ha resultado muy eficiente para el *screening* de un elevado número de contaminantes en aguas residuales, muy superior al considerado en las aproximaciones analíticas convencionales. Con ambos tipos de analizadores se cubren de modo excelente tanto los aspectos cualitativos como cuantitativos del *screening*.

14. El uso combinado de GC-TOF MS y UHPLC-(Q)TOF MS ha permitido la investigación en modo *non-target* de contaminantes y metabolitos en casos reales de envenenamiento de abejas, aportando una de las mejores soluciones analíticas en investigaciones relacionadas con el campo de la toxicología.

CONCLUSIONS

The **general conclusion** of this Thesis is that, hyphenation GC-MS, using modern analyzers of triple quadrupole and time of flight, is at present one of the most powerful analytical tools for *screening* and quantification of organic contaminants with low polarity and/or (semi)volatiles. On one hand, GC-MS/MS with triple quadrupole is highly appropriate for the quantitative determination and confirmation of numerous organic contaminants in biological and environmental samples. On the other hand, GC-TOF MS opens a new scenario for wide-scope *screening*, allowing the investigation of compounds present in samples from the *target* and *non-target* point of views. Both techniques are complementary for the quantitative and qualitative analysis as illustrated in this Thesis in applied fields. The combination of GC-MS and LC-MS analysis, both with triple quadrupole and (Q)TOF analyzers, gives an extraordinary strong potential to the Public Health or toxicology laboratories, as this modern configuration allows to face the investigation of organic contaminants in many types of samples from an "universal" point of view.

Specific conclusions:

1. GC-MS/MS with triple quadrupole has been shown as a valuable analytical tool for the multiresidual analysis of organic contaminants in water a µg/L and sub-µg/L concentration levels. As the number of transitions simultaneously acquired is limited, in order to obtain a good chromatographic peak definition, the chromatographic optimization is an important step to minimize the number of coeluting compounds in a time window. With a simple step of solid phase extraction, the elevated sensitivity and selectivity of GC-(QqQ)MS/MS has allowed quantification and confirmation below 0.1 µg/L, with a high degree of confidence, for a wide number of organic microcontaminants in water.
2. The negative chemical ionization mode has been shown as an alternative technique to electron ionization for several priority organic contaminants in water (mainly organochlorine compounds) due to its better sensitivity and selectivity.

3. GC-MS/MS with triple quadrupole analyzer has been shown as a powerful technique for the rapid and selective determination of xenoestrogen compounds in human breast adipose tissue samples. Purification of the extracts by normal phase HPLC with silica columns, has allowed to minimize the quantity of matrix introduced into the GC system (mainly lipids) facilitating the correct identification and quantification of the analytes.

4. As regards confirmation, the acquisition of several MS/MS transitions with triple quadrupole and the estimation of ion ratios allow the accomplishment of European Commision Decision 2002/657/EC requirement regarding the number of identification points (IPs). In general, the acquisition of two SRM transitions together with the attainment of the ion ratio is enough to assure the confirmation of the identity of the analyte when using triple quadrupole instruments. The confirmation is considered as definitive if both transitions are specific enough and its ion ratio is in accordance with that obtained from a reference standard.

5. GC-TOF MS can be seen as an ideal technique for the *screening* and confirmation of organic contaminants in different kinds of samples, in both *target* and *non-target* ways. Data processing, to manage with the huge amount of MS information generated by GC-TOF MS, is a crucial step in the *target screening* method development. Although the availability of reference standards in *target screening* is always welcome, several alternatives have been shown when the reference standard is unavailable, with satisfactory results.

6. The application of developed *screening* methodology in combination with a sample treatment based on solid phase microextraction (SPME) has allowed the rapid detection and confirmation of a wide number of contaminants in the analyzed water samples.

7. The qualitative validation of the *screening* methodology developed in this Thesis, using water samples of different origin and characteristics spiked with a wide number of selected contaminants, has allowed to establish the lowest concentration level at which a given compound can be identified and confirmed in all the water samples analyzed.

8. GC-TOF MS has been shown as an adequate technique to investigate the presence of anthropogenic organic contaminants in human adipose tissue samples. Comparison of the results with those obtained by GC-(QqQ)MS/MS has demonstrated the better performance of triple quadrupole for quantitative *target* analysis of a limited number of analytes. However, GC-TOF MS has been shown as a versatile technique that allows the identification of many other GC-amenable compounds in the samples, including those non selected and unexpected, due to the useful information provided by the full spectrum acquisition spectrum measured at accurate mass.

9. *Non-target screening*, where samples and analytes are treated as unknowns, using component detection algorithm and deconvolution software, may not be completely satisfactory at the moment to investigate the presence of organic contaminants in samples, as the success of this approach gets notably worse at low concentrations. Both, *target* analysis, focused on priority contaminants, and *non-target* analysis, are complementary and both are required to obtain the maximum information on sample composition.

10. The high mass accuracy and full spectrum acquisition capability of GC-TOF MS has make this technique highly adequate for the elucidation of compounds for which their electron ionization spectrum is not available in commercial libraries. The combined use of electron ionization and chemical ionization sources has great potential for this purpose due to the valuable information about molecular mass given by CI, together with the structural information given by fragment ions of the EI spectrum.

11. The new atmospheric pressure chemical ionization source (APCI) in combination with GC-(Q)TOF MS opens a new scenario in the *screening* of pesticide residues in food. Preliminary results using this source prototype exhibit promising features for the use of this approach in many other applied fields.

12. The acquisition of product ions at accurate mass together with the evaluation of the ion ratios can be considered as unequivocal confirmation by GC-(Q)TOF MS. The excellent characteristics of GC-(Q)TOF for qualitative analysis makes this technique one of the most powerful for this purpose. The use of soft

ionization techniques, as CI or the recently developed APCI, is required to fully exploit the QTOF capabilities I combination to GC analysis.

13. The complementary use of GC-MS and LC-MS, both with triple quadrupole and (Q)TOF analyzers, is possibly the most powerful approach for screening, quantification and confirmation of a great number of contaminants in water, superior to that normally considered in other analytical approaches. Using these analyzers, both the qualitative and the quantitative aspects of the *screening* are covered in an excellent way.

14. The combined used of GC-TOF MS and UHPLC-(Q)TOF MS has allowed the investigation of contaminants and metabolites in real cases of honeybee poisoning, giving one of the best analytical solutions to investigate many cases related to the toxicological field.

SUGERENCIAS PARA POSTERIORES TRABAJOS

En la presente Tesis Doctoral se ha puesto de manifiesto el gran potencial del acoplamiento GC-MS/MS con analizadores de triple cuadrupolo y TOF para la determinación de contaminantes orgánicos, lo que se ha evidenciado en muestras de origen ambiental y biológico. A partir de estos resultados y de las conclusiones obtenidas en esta Memoria, se pueden sugerir algunas líneas para la ampliación de los trabajos realizados. A continuación, se muestran dos líneas de investigación futura, que pueden resultar de interés:

- Profundizar en el uso de la nueva fuente de ionización química a presión atmosférica en combinación con las técnicas GC-MS/MS con triple cuadrupolo y QTOF con fines de *screening*. Aplicación a campos de interés aplicado, como son el medioambiental, seguridad alimentaria o el control del dopaje en el deporte.
- Avanzar en la investigación de las capacidades identificativas de GC-TOF MS con fuentes de ionización electrónica e ionización química para la elucidación de compuestos desconocidos. Estudio de las aportaciones de la nueva fuente APCI en combinación con QTOF al respecto.

ARTÍCULOS CIENTÍFICOS QUE COMPONEN LA TESIS DOCTORAL

Artículo científico 1

Gas chromatography/high-resolution time-of-flight mass spectrometry: an advanced analytical tool to investigate the presence of organic compounds in environmental, food safety and toxicology fields

T. Portolés, E. Pitarch, F.J. López, F. Hernández

Trends in Analytical Chemistry (submitted)

Artículo científico 2

Determination of priority organic micro pollutants in water by gas chromatography coupled to triple quadrupole mass spectrometry

E. Pitarch, C. Medina, T. Portolés, F.J. López, F. Hernández.

Anal. Chim. Acta, 583, 246-258, **2007**

Artículo científico 3

Potential of gas chromatography coupled to triple quadrupole mass spectrometry for quantification and confirmation of organohalogen xenoestrogen compounds in human breast tissues

F. Hernández, T. Portolés, E. Pitarch, F.J. López, J. Beltrán.

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Methodical approach for the use of GC-TOF MS for screening and confirmation of organic pollutants in environmental water

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J. Mass Spectrom., 42, 1175-1185, **2007**

Artículo científico 5

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F. Hernández, T. Portolés, E. Pitarch, F.J. López

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Artículo científico 9

Potential of atmospheric pressure chemical ionization source in GC-QTOF MF for pesticide residue analysis

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E. Pitarch, T. Portolés, J. Marín, M. Ibáñez, F. Albarrán, F. Hernández.

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OTROS ARTÍCULOS RELACIONADOS

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Combined use of GC-TOF MS and UPLC-QTOF MS for investigative analysis of honeybee poisoning

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Waters Application Note