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POTENCIAL DE DIFERENTES ANALIZADORES Y FUENTES DE IONIZACIÓN EN CROMATOGRAFÍA DE GASES-ESPECTROMETRÍA DE MASAS EN EL CAMPO DE LA SEGURIDAD ALIMENTARIA Y MEDIOAMBIENTAL

Tesis Doctoral

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Los Dres. **Joaquim Beltran Arandes** y **Elena Pitarch Arquimbau**, Profesores Titulares de Química Analítica de la Universitat Jaume I de Castellón,

CERTIFICAN: que la Tesis Doctoral "Potencial de diferentes analizadores y fuentes de ionización en cromatografía de gases-espectrometría de masas en el campo de la seguridad alimentaria y medioambiental" ha sido desarrollada bajo su dirección en el Instituto Universitario de Plaguicidas y Aguas, Departamento de Química Física y Analítica de la Universitat Jaume I de Castellón, por **Laura Cherta Cucala**.

Lo que certificamos para los efectos oportunos en Castellón de la Plana, a 22 de septiembre de 2014.

Fdo. Dr. Joaquim Beltran Arandes Fdo. Dra. Elena Pitach Arquimbau

Este trabajo responde al compromiso adquirido con la Universitat Jaume I por la concesión de una beca predoctoral para la formación de personal investigador, desde el 1 de mayo de 2010.

Laura Cherta Cucala ha sido beneficiaria de una beca concedida por la Universitat Jaume I para la realización de una estancia en el *Dipartimento di Scienze del Farmaco e dei Prodotti per la Salute, Università degli Studi di Messina* (Italia), desde el 4 de marzo al 7 de junio de 2013. El trabajo realizado llevó por título "Desarrollo de procedimientos de determinación de volátiles y semivolátiles mediante cromatografía de gases bidimensional acoplada a espectrometría de masas", bajo la supervisión del Prof. Luigi Mondello, y permitió ampliar los conocimientos de la doctoranda en el uso de la técnica de cromatografía de gases bidimensional (GCxGC), tanto en los principios teóricos como en los aspectos prácticos.

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Esta Tesis ha sido realizada y será defendida de acuerdo con los requisitos exigidos para la obtención del Título de Doctorado Internacional.

Previamente a la defensa de la Tesis Doctoral, este trabajo ha sido evaluado por dos censores extranjeros independientes relacionados con el área de investigación: Dr. Adrian Covaci (Toxicological Center, University of Antwerp, Belgium) y Dra. Marja Lamoree (Institute for Environmental Studies, VU University, Amsterdam, The Netherlands).

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Resumen

En la presente Tesis Doctoral se han estudiado las posibilidades de la cromatografía de gases (GC) acoplada a espectrometría de masas (MS) con distintos analizadores y fuentes de ionización para la determinación de contaminantes en muestras alimentarias y medioambientales. La singularidad de los cuatro instrumentos diferentes empleados reside en los analizadores de masas que los componen: cuadrupolo simple (Q), tiempo de vuelo (TOF), triple cuadrupolo (QqQ) e híbrido cuadrupolo tiempo de vuelo (QTOF). En los dos primeros sistemas, el modo de ionización aplicado ha sido el más frecuentemente utilizado en GC, la ionización electrónica (EI), mientras que en los dos últimos se ha hecho uso de la ionización a presión atmosférica (APCI), cuyo acoplamiento con la cromatografía de gases es relativamente reciente. El potencial de estas técnicas se ha investigado tanto para el análisis *target* como para *non-target*.

Las metodologías desarrolladas en modo *target* han incluido diferentes compuestos como pesticidas, bifenilos policlorados (PCBs), dioxinas, difenil éteres polibromados (PBDEs), alquilfenoles e hidrocarburos aromáticos policíclicos (PAHs). La presencia de estos contaminantes en el medio ambiente, ya sea por producción inintencionada o por una mala práctica agrícola o industrial, está estrechamente relacionada con la contaminación alimentaria, siendo especialmente preocupante el carácter persistente y tóxico de muchos de ellos. La detección y cuantificación a los bajos niveles de concentración normalmente esperados en alimentos y muestras medioambientales requiere de técnicas analíticas que sean lo suficientemente sensibles y selectivas. Las aplicaciones cuantitativas en este tipo de muestras, como las descritas en los capítulos 2 y 3, suelen involucrar analizadores de masas como el cuadrupolo simple y, especialmente, el QqQ.

Concretamente, el capítulo 2 se centra en el estudio de las capacidades y limitaciones que ofrece el cuadrupolo simple en su acoplamiento a la cromatografía de gases rápida (*fast* GC) para la determinación de contaminantes en aguas, zumos,

frutas y verduras. Las diferentes metodologías desarrolladas han sido validadas considerando los parámetros analíticos definidos en las correspondientes directivas. Así mismo, se han evaluado y comparado las técnicas de extracción SPE, SPME y QuEChERS, tratando de establecer un modelo de tratamiento de muestra rápido y efectivo compatible con la modalidad *fast* GC para conseguir un alto rendimiento por muestra analizada.

En el capítulo 3 se introduce el uso de la nueva fuente APCI acoplada a GC con un QqQ como analizador con el fin de estudiar su potencial en el campo de los pesticidas y las dioxinas. En primer lugar, se han valorado las ventajas aportadas con esta suave ionización en términos de sensibilidad y selectividad, comparándolas con el comportamiento típico observado en la fuente tradicional de EI. La presencia del pico molecular en la mayoría de los espectros de masas estudiados por APCI marca una gran diferencia con respecto a la alta fragmentación que ocurre habitualmente por EI. Así, y aprovechando la capacidad del QqQ de operar en tándem MS (MS/MS), se han desarrollado y validado métodos en modo *Selected Ion Monitoring* (SRM) seleccionando el ión molecular o quasi-molecular como ión precursor.

Por otro lado, con el fin de valorar el nuevo sistema GC-(APCI)QqQ MS/MS como alternativa a las técnicas de alta resolución en el campo de las dioxinas, se ha analizado material de referencia certificado cuyos resultados se han comparado con los obtenidos en trabajos anteriores empleando espectrometría de masas de alta resolución (HRMS).

En cuanto al análisis *non-target*, las metodologías de *screening* o amplio barrido desarrolladas en la presente Tesis, concretamente en el capítulo 4, se han aplicado para investigar la presencia de compuestos desconocidos derivados del procesado de alimentos. La migración de componentes desde el material de envasado (comúnmente plástico) hasta el alimento con el que está en contacto supone una fuente de contaminación potencial. La amplia variedad de sustancias migrantes, muchas de ellas no reguladas en las directivas, requiere el desarrollo de métodos *non-target* capaces de identificar el mayor número posible de contaminantes presentes en

los alimentos (ante la complejidad de las muestras alimentarias, los ensayos de migración y posteriores análisis no se llevan a cabo directamente con alimentos sino con simulantes de los mismos). Los analizadores empleados en esta parte de la Tesis, TOF y QTOF, resultan ideales para estos fines cualitativos.

El *screening* de compuestos capaces de migrar se ha desarrollado, por un lado, mediante la combinación de las técnicas GC-(EI)TOF MS y GC-(APCI)QTOF MS. Con el uso del GC-(EI)TOF MS la búsqueda de desconocidos se ha centrado en una primera lista de candidatos tentativos de acuerdo al porcentaje de concordancia con las librerías de espectros comerciales y a la medida de masa exacta. Con el GC-(APCI)QTOF MS se ha evaluado la información relativa al ión molecular y a los correspondientes fragmentos, con el fin de reducir la lista de candidatos anterior y aproximarse con mayor certeza a la identidad final.

Excepcionalmente, no siendo el objetivo de esta Tesis sino parte de un proyecto de investigación más amplio, se ha aplicado la cromatografía de líquidos (LC) acoplada a MS de manera complementaria al análisis *non-target* por GC-MS para ampliar el rango de compuestos a investigar en este tipo de muestras derivadas del envasado alimentario. En concreto, se ha empleado un equipo de LC de alta resolución (UHPLC) acoplado al analizador híbrido QTOF, realizándose el *screening* en modo *post-target* a partir de una base de datos que contiene alrededor de 700 compuestos.

Summary

In this Doctoral Thesis, the possibilities of gas chromatography (GC) coupled to mass spectrometry (MS) with different analyzers and ionization sources have been studied for the determination of pollutants in food and environmental samples. The singularity of the four different instruments used lies in their mass analyzers: single quadrupole (Q), time-of-flight (TOF), triple quadrupole (QqQ) and hybrid quadrupole time-of-flight (TOF). Electron ionization (EI) (the most frequently ionization mode used in GC) has been applied in the first two systems, while atmospheric pressure chemical ionization (APCI) (whose coupling with GC is relatively new) has been used in the last ones. The potential of these techniques in target and non-target analysis has been investigated.

The developed methodologies in target mode have included different pollutants as pesticides, polychlorinated biphenyls (PCBs), dioxins, polybrominated diphenyl ethers (PBDEs), alkylphenols and polycyclic aromatic hydrocarbons (PAHs). The presence of these pollutants in the environment, due to an intentional production or a bad agricultural or industrial practice, is closely related with the food pollution, and the persistent and toxic character of many of them is especially worrying. The detection and quantification of the low concentration levels usually expected in food and environmental samples require analytical techniques sensitive and selective enough. Quantitative applications in this kind of samples, as the ones described in chapter 2 and 3, usually involve mass analyzers as the single quadrupole and, especially, the QqQ.

Concretely, chapter 2 is focused on the study of the capabilities and limitations of the use of the single quadrupole as analyzer in fast GC for the determination of pollutants in water samples, juices, fruits and vegetables. The different developed methodologies have been validated considering the analytical parameters described in the corresponding directives. Moreover, the extraction techniques SPE, SPME and

QuEChERS have been evaluated in order to establish a rapid and effective sample treatment which fits well with *fast* GC and allows increasing sample throughput.

The use of the new APCI source coupled to GC with a QqQ analyzer is presented in chapter 3, in which its potential is studied in pesticides and dioxins fields. First of all, advantages derived from this soft ionization have been evaluated in terms of sensitivity and selectivity in comparison with the typical behavior observed in the traditional EI source. The presence of the molecular ion in the APCI mass spectra of most of the studied compounds shows a big difference in comparison with the high fragmentation typically observed in EI. Thus, taking profit of the QqQ system to operate under tandem MS (MS/MS), Selected Ion Monitoring (SRM) methods have been developed using the molecular or quasi-molecular ion as precursor ion.

On the other hand, in order to evaluate the new GC-(APCI)QqQ MS/MS system as alternative to high resolution techniques usually applied for dioxins determination, certified reference material has been analyzed and results have been compared with those ones obtained in previous works by using high resolution mass spectrometry (HRMS).

Regarding non-target analysis, screening methodologies developed in this Thesis, specifically in chapter 4, have been applied to investigate the presence of unknown compounds coming from food processing. The migration of components from the packaging material (commonly plastic) to the food in contact is considered a source of potential pollution. The wide variety of migrating substances, most of them non-regulated in the directives, requires the development of non-target methods able to identify as many compounds as possible in the food samples (due to the complexity of food samples, migration assays and further analysis are usually performed using food simulants). The analyzers employed in this part of the Thesis, TOF and QTOF, are appropriate for these qualitative purposes.

The screening of compounds able to migrate has been performed by the complementary use of GC-(EI)TOF MS and GC-(APCI)QTOF MS. The search of unknowns from the GC-(EI)TOF MS data has been focused on a first list of tentative candidates according to the matching percentage with the commercial spectral libraries and the accurate mass measurement. Information relative to the molecular ion and the corresponding fragments coming from the GC-(APCI)QTOF MS data has been evaluated in order to reduce the previous list of candidates and to get an approach to the final identity with more certainty.

Exceptionally, not being the objective of this Thesis but a part of a wider research project, liquid chromatography (LC) coupled to MS has been applied in a complementary way to the non-target analysis carried out by GC-MS in order to widen the scope of contaminants coming from food packaging. Ultrahigh-pressure liquid chromatography (UHPLC) coupled to an hybrid analyzer QTOF has been used and a post-target screening has been performed using a database of around 700 compounds.

ÍNDICE GENERAL

ÍNDICE DE ACRÓNIMOS

ACN	Acetonitrile
AOAC	Association of Analytical Communities
APCI	Atmospheric pressure chemical ionization
ASE	Accelerated solvent extraction
CE	Collision energy
CEN	European Committee for Standardization
CI	Chemical ionization
CID	Collision induced dissociation
EI	Electron ionization
ESI	Electrospray ionization
EPA	United States Environmental Protection Agency
GC	Gas chromatography
GPC	Gel permeation chromatography
HRMS	High resolution mass spectrometry
I.D.	Internal diameter
IT	Ion trap
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
$M^{+\bullet}$	Molecular ion
MAC	Maximum allowable concentration

MAE	Microwave assisted extraction
MRL	Maximum residue level
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
NIAS	Non-intentionally added substances
nw-XIC	Narrow window-extracted ion chromatogram
PAHs	Polycyclic aromatic hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzodioxins
PCDFs	Polychlorinated dibenzofurans
POP	Persistent organic pollutant
PSA	Primary-secondary amine
PTV	Programmable temperature vaporization
Q	Quadrupole
QqQ	Triple quadrupole
Q/q	Ion ratio
QuEChERS	Quick, easy, cheap, effective, rugged and safe
QTOF	Hybrid quadrupole time-of-flight
RSD	Relative standard deviation
SFE	Supercritical fluid extraction
SML	Specific migration limit
SIM	Selected ion monitoring

S/N	Signal to noise ratio
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SRM	Selected reaction monitoring
TEF	Toxic equivalency factor
TEQ	Toxic equivalent
TOF	Time-of-flight
UHPLC	Ultra-high-pressure liquid chromatography
WHO	World Health Organization

Objetivos

El objetivo principal de la presente Tesis Doctoral es la investigación del potencial del acoplamiento cromatografía de gases-espectrometría de masas, tanto en modo simple (GC-MS) como en tándem (GC-MS/MS), con diferentes analizadores (cuadrupolo simple (Q), triple cuadrupolo (QqQ), tiempo de vuelo (TOF) y cuadrupolo tiempo de vuelo (QTOF)) y fuentes de ionización (ionización electrónica (EI) e ionización a presión atmosférica (APCI)), en el campo de la seguridad alimentaria y medioambiental. Con ello se pretende contribuir al desarrollo de nuevas metodologías, o mejorar las existentes, para la detección y cuantificación de contaminantes orgánicos a baja concentración en alimentos y medio ambiente.

Los trabajos presentados en esta Tesis se basan en los siguientes objetivos específicos:

1. Evaluación de las capacidades y limitaciones del cuadrupolo simple empleado como analizador en cromatografía de gases rápida (*fast* GC) acoplada a MS para el análisis de aguas y alimentos. Optimización de las condiciones GC-MS para la determinación de un elevado número de contaminantes seleccionados en el menor tiempo posible.

2. Desarrollo de metodología analítica basada en *fast* GC-MS para la determinación de pesticidas, bifenilos policlorados (PCBs), difenil éteres polibromados (PBDEs), alquilfenoles e hidrocarburos aromáticos policíclicos (PAHs) en aguas, aplicando la extracción en fase sólida (SPE) como técnica de extracción y preconcentración.

3. Desarrollo de metodología analítica basada en *fast* GC-MS para la determinación de pesticidas en frutas, vegetales y zumos. Evaluación de las técnicas QuEChERS, SPE y microextracción en fase sólida (SPME) como tratamiento de muestra.

4. Estudio comparativo de la ionización EI y APCI para la evaluación del potencial del nuevo sistema GC-(APCI)QqQ MS/MS en el campo de los pesticidas.

5. Desarrollo de metodología analítica basada en GC-(APCI)QqQ MS/MS para la determinación de pesticidas en frutas y vegetales, prestando especial atención a la selección del ión molecular o quasi-molecular como ión precursor en el modo *Selected Ion Monitoring* (SRM). Mejora de los métodos clásicos basados en GC-(EI) MS/MS en cuanto a sensibilidad y selectividad.

6. Validación de las metodologías desarrolladas en términos de linealidad, exactitud, precisión, límites de detección (LOD) y cuantificación (LOQ), así como de la capacidad de confirmación para asegurar la calidad de los resultados analíticos.

7. Estudio del potencial del sistema GC-(APCI)QqQ MS/MS para la determinación de dioxinas en muestras de origen ambiental y alimentario con el fin de proponer una alternativa a las técnicas de alta resolución consideradas oficiales en este campo.

8. Evaluación de la aplicación conjunta de GC-(EI)TOF MS y GC-(APCI)QTOF MS para la investigación en modo *non-target* de contaminantes capaces de migrar desde el material de envasado hasta el alimento.

9. Desarrollo de una estrategia de trabajo para el procesamiento de datos generados por GC-(EI)TOF MS y GC-(APCI)QTOF MS mediante softwares específicos.

10. Aplicación complementaria de la cromatografía de líquidos de alta resolución (UHPLC) acoplada a QTOF MS para ampliar el rango de detección de contaminantes derivados del procesado de alimentos.


Objectives

The main objective of this Doctoral Thesis is the investigation of the potential of gas chromatography coupled to mass spectrometry, both in single (GC-MS) and tandem modes (GC-MS/MS), by using different mass analyzers (single quadrupole (Q), triple quadrupole (QqQ), time-of-flight (TOF) and quadrupole time-of-flight (QTOF)) and ionization sources (electron ionization (EI) and atmospheric pressure chemical ionization (APCI)) in food safety and environmental control fields. In this way, it is expected to contribute to the development of new methodologies, or to improve the current ones, for the detection and quantification of organic contaminants at low levels in food and environmental samples.

Detailed work described in this Thesis is based on the following specific objectives:

1. Evaluation of capabilities and limitations of the single quadrupole used as analyzer in fast GC-MS for water and food analysis. Optimization of GC-MS conditions for the determination of a large number of selected contaminants in the shortest possible time.

2. Development of analytical methodology based on fast GC-MS for the determination of pesticides, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), alkylphenols and polycyclic aromatic hydrocarbons (PAHs) in water samples by applying solid-phase extraction (SPE) as sample treatment and pre-concentration step.

3. Development of analytical methodology based on fast GC-MS for the determination of pesticides in fruits, vegetables and fruit juices. Evaluation of QuEChERS, SPE and solid-phase microextraction (SPME) as sample treatment.

4. Comparative study of the ionization using EI and APCI for the evaluation of the potential of the new system GC-(APCI)QqQ MS/MS in the pesticides field.

5. Development of analytical methodology based on GC-(APCI)QqQ MS/MS for the determination of pesticides in fruits and vegetables considering the selection of the molecular or quasi-molecular ion as precursor ion under Selected Ion Monitoring (SRM) mode. Improvement of classical methodologies based on GC-(EI) MS/MS regarding sensitivity and selectivity.

6. Validation of the developed methodologies in terms of linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ), as well as confirmation capability to ensure the quality of the analytical results.

7. Study of the potential of GC-(APCI)QqQ MS/MS for the determination of dioxins in food and environmental samples in order to propose an alternative to the high resolution techniques considered official in this field.

8. Evaluation of the complementary use of GC-(EI)TOF MS and GC-(APCI)QTOF MS for the non-target investigation of pollutants able to migrate from the packaging material to food.

9. Development of a work strategy for the data processing generated by GC-(EI)TOF MS and GC-(APCI)QTOF MS through specific softwares.

10. Application of ultra-high pressure liquid chromatography (UHPLC) coupled to QTOF MS in a complementary way in order to widen the detection range of pollutants derived from food packaging.

Capítulo I

Introducción general

I.1. Problemática de los contaminantes orgánicos en alimentos y medio ambiente

Desde la década de 1960 hasta la actualidad, la preocupación pública ante el uso desmedido de compuestos químicos de síntesis se ha ido acrecentando ante la revelación de los efectos negativos de estos compuestos que, en sus ámbitos de uso, han sido beneficiosos para la humanidad. El uso de muchas de estas sustancias tuvo su auge tras el descubrimiento de la eficacia del pesticida sintético organoclorado DDT en la década de 1940.

Los pesticidas se definen como sustancias químicas empleadas para matar, repeler o controlar cualquier tipo de plaga, tanto las que afectan al crecimiento y conservación de productos agrícolas como los vectores de enfermedades humanas o de animales. Su uso se extiende, además, hacia prácticas relacionadas con la higiene pública y las aplicaciones domésticas (insecticidas domésticos y sprays repelentes de insectos). Sin embargo, a pesar de los innegables beneficios que aportan a la agricultura y a nuestra vida cotidiana, su naturaleza no excluye a los seres vivos ni al medio ambiente de sufrir sus efectos adversos (Mrema *et al*., 2013). Tras 20 años de elevado consumo, mayoritariamente en el ámbito agrícola, la revelación de la toxicidad del DDT hacia muchos otros organismos no objeto de su aplicación empezó a generar alertas ambientales y de seguridad alimentaria (Casida & Quistad, 1998; Koh, 1996). En 1962, Rachel Carson publicó el libro titulado “Silent Spring”, que vino a ser el germen de inicio en la concienciación de la problemática asociada al amplio uso de estos compuestos orgánicos, hoy y desde entonces considerados como contaminantes (muchos de ellos persistentes), y haciendo patente la problemática de la contaminación del medio ambiente y la necesidad de su protección.

En la década de 1970, otros compuestos químicos de síntesis, los bifenilos policlorados (PCBs), que constituyen una familia de 209 congéneres, fueron ampliamente utilizados en diversas aplicaciones industriales y popularmente empleados como aislantes para equipos eléctricos. Tras descubrirse sus efectos

nocivos sobre la salud, su fabricación y uso se prohibió en la década de 1980. Sin embargo, su presencia en el medio ambiente aún resulta alarmante debido a su producción inintencionada como resultado de procesos industriales o de incineración de residuos urbanos e industriales (DeCaprio *et al*., 2005).

Cabe destacar que algunos PCBs son análogos en cuanto a estructura y toxicidad a las dioxinas, unos de los compuestos con mayor potencial tóxico conocido. Las dioxinas comprenden dos grupos de compuestos organoclorados, las policlorodibenzodioxinas (PCDD) y los policlorodibenzofuranos (PCDF). Ambos se liberan al medio ambiente como subproductos de procesos industriales o naturales, resultando muy persistentes y dañinos para los seres vivos (Birnbaum *et al.*, 2003).

Otros compuestos estructuralmente similares a los PCBs cuyo uso empezó a extenderse en la década de 1970 son los difenil éteres polibromados (PBDEs), los cuales también constituyen un grupo de 209 congéneres. Éstos son frecuentemente utilizados como retardantes de llama en una amplia gama de productos cotidianos como plásticos, textiles, electrónica, muebles, automóviles y otros materiales. Aunque su naturaleza contaminante no es homóloga a la de los PCBs, la exposición a algunos de los congéneres resulta especialmente perniciosa para la salud como resultado de su liberación al entorno y su persistencia (Domingo, 2004).

Los alquilfenoles son otro grupo de contaminantes domésticos persistentes que se emplean en la elaboración de agentes tensioactivos (detergentes), dispersantes, emulsionantes y humectantes y como plastificantes. Así mismo, pueden originarse como productos de degradación de algunos pesticidas, detergentes y productos para el cuidado personal, resultando dañinos (Rudel & Perovich, 2009).

Por otro lado, los hidrocarburos aromáticos policíclicos (PAHs) constituyen un grupo de contaminantes particular por su producción continua e inadvertida, ya que se originan de forma natural a partir de combustiones incompletas de materia orgánica a altas temperaturas y están presentes en el petróleo, carbón y depósitos de alquitrán. Los PAHs están constituidos por dos o más anillos aromáticos unidos, sin sustituyentes ni heteroátomos, tratándose también de compuestos difícilmente

degradables. Los mayores porcentajes de su generación residen en procesos industriales y domésticos, como en la combustión de petróleo y madera, en motores de combustión y en la incineración de desechos, aunque también pueden liberarse de forma natural en incendios forestales (Srogi, 2007).

La presencia en el medio ambiente de estos contaminantes, muchos de ellos persistentes, conlleva, a su vez, la problemática de la posible bioacumulación y transferencia de dichos compuestos a los alimentos consumidos por los seres humanos. Así, los aspectos relacionados con la seguridad alimentaria toman especial relevancia en las últimas décadas.

Además de los contaminantes y residuos presentes en los alimentos, en los últimos años ha emergido la necesidad de controlar otra fuente de contaminación en el campo de los productos envasados: la migración de sustancias peligrosas desde el envase hasta el alimento. Los materiales empleados en el envasado, comúnmente plásticos, no son inertes y el contacto directo con el alimento puede propiciar la migración de contaminantes hacia el producto, pudiendo alterar su composición e incluso provocar riesgos toxicológicos a los consumidores. Además, la posible existencia de reacciones de polimerización y degradación desconocidas e incontroladas resultan una fuente de incontables impurezas en la composición final del envase que pueden pasar fácilmente desapercibidas en los análisis químicos (Lau & Wong, 2000).

I.2. Legislación

Con el fin de evitar problemas ambientales y de salud pública, diversos organismos como la Agencia de Protección Ambiental (EPA) de Estados Unidos y la Comisión Europea establecen normativas que fomentan un uso sostenible de estos compuestos químicos, así como leyes de regulación en las que se implantan niveles máximos de residuos permitidos en alimentos y en el medio ambiente, algunos de los cuales reciben la consideración de contaminantes prioritarios. Además de una regulación en cuanto a uso y control, existen documentos relativos a los criterios de calidad, y características de validación de métodos y criterios de interpretación de los resultados analíticos para asegurar buenas prácticas en los laboratorios de análisis. A modo de ejemplo, en la **Tabla I.1** se enuncian los actos legislativos de la UE relacionados con los temas tratados en esta Tesis Doctoral.

Tabla I.1. Principales documentos legislativos relativos al control de los niveles máximos de residuos de pesticidas y otras sustancias y al funcionamiento de los métodos analíticos relacionados con la presente Tesis Doctoral.

Documento	Fundamento
Directive 2013/39/EU	• Normas de calidad ambiental en el ámbito de la política de aguas • Concentración máxima admisible (MAC) de contaminantes prioritarios como algunos pesticidas, PCBs, PAHs y alquilfenoles
Regulation (EC) No 396/2005	• Aplicable a alimentos y piensos de origen vegetal y animal • Límites máximos de residuos (MRL) de pesticidas
Commission Regulation (EU) No 589/2014 and No 1259/2011	• Métodos de análisis para el control de contaminantes como dioxinas, furanos y PCBs similares a dioxinas en productos alimenticios • Contenidos máximos expresados en equivalentes tóxicos de la Organización mundial de la salud (WHO-TEQ), utilizando los factores de equivalencia de toxicidad de la misma organización (WHO-TEF)
Commission Regulation (EU) No 10/2011	• Aplicable a materiales y objetos plásticos destinados a entrar en contacto con alimentos • Límites de migración específicos (SML) de monómeros, aditivos y otras sustancias para la producción de polímeros
Guidance document SANCO/12571/2013	• Criterios de validación de métodos y control de calidad analítica • Aplicable a residuos de pesticidas en alimentos
Commission Decision 2002/657/CE	• Funcionamiento de los métodos analíticos e interpretación de los resultados

En el mismo sentido, cabe destacar el Convenio de Estocolmo sobre los Contaminantes Orgánicos Persistentes (POPs), que entró en vigor en 2004 con objeto de regular el tratamiento de estas sustancias potencialmente tóxicas y persistentes (Fiedler *et al.*, 2013). Este convenio se fundamenta en una lista de contaminantes sobre los que es preciso emprender acciones prioritarias, ya sea restringir o prohibir su producción y uso, o reducir y/o eliminar las emisiones inintencionadas de algunos de ellos. En la lista se incluyen algunos pesticidas como el DDT y otros organoclorados, las dioxinas y furanos y también compuestos químicos industriales como los PCBs y algunos BDEs; sobre todos ellos se han reportado numerosos estudios relativos a la contaminación humana y ambiental (El-Shahawi *et al.*, 2010; Jones & de Voogt, 1998; Dirtu & Covaci, 2010). En cuanto a los PAHs, aunque no registrados en el Convenio de Estocolmo, 16 de ellos se consideran contaminantes orgánicos prioritarios según la EPA y son contemplados en la Directiva 2013/39/CE, del mismo modo que ocurre con algunos alquilfenoles.

La disposición de listas predefinidas supone una gran ventaja en el ámbito de la química analítica en salud pública, especialmente en estudios destinados a detectar la presencia de estas sustancias y determinar sus niveles de concentración en diferentes matrices. Los laboratorios de rutina suelen trabajar de acuerdo a las legislaciones vigentes, las cuales resultan de gran ayuda para la selección de los compuestos de interés. Este modo de búsqueda de compuestos previamente seleccionados es lo que en el argot analítico se conoce como análisis *target*.

A pesar de que la aproximación de análisis *target* permite cumplir las reglamentaciones en cuanto a control en el ámbito de salud pública, el número de contaminantes excluidos en este tipo de estudios puede ser muy elevado y de gran relevancia. La determinación de los compuestos desconocidos (*non-target*) entraña mayores dificultades por la falta de información relativa a su presencia, identidad, niveles de concentración y métodos de determinación. En estos casos, el trabajo resulta más laborioso y se requiere instrumentación analítica compleja, en ocasiones diferente a la empleada en modo *target*.

I.3. Determinación analítica

Actualmente, tanto la cromatografía de gases (GC) como la de líquidos (LC) acopladas a la espectrometría de masas (MS) se consideran herramientas poderosas para la determinación de contaminantes orgánicos a niveles de concentración del orden de partes por billón, e incluso partes por trillón, e identificarlos con una alta probabilidad de certeza. Los numerosos estudios reportados sobre la determinación de una amplia variedad de contaminantes mediante estas técnicas consolidan la preferencia de las mismas en el ámbito alimentario y ambiental (Richardson, 2012; Lehotay *et al*., 2008; Botitsi *et al*., 2011; Tang, 2013).

El desarrollo de métodos multiresiduales basados en GC-MS o LC-MS suele perseguir la inclusión del mayor número posible de compuestos a determinar en un mismo método de análisis. Esta exigencia supone un reto analítico dadas las diferentes propiedades físico-químicas de los analitos a determinar, la complejidad de la muestra a analizar y/o los bajos niveles máximos de concentración impuestos por la legislación.

Las técnicas GC-MS abarcan la temática principal de esta Tesis Doctoral, cuyos fundamentos se describen a continuación. Cabe destacar que en el capítulo IV, la cromatografía de líquidos se aplica paralelamente a la cromatografía de gases como técnica complementaria en el análisis *non-target*, con lo que los principios de LC-MS se comentan también en este capítulo aunque más brevemente. Para una descripción más detallada de ambas técnicas pueden consultarse obras específicas (Grob & Barry, 2004; Dass, 2007; Boyd *et al*., 2008; Niessen, 2006).

I.3.1. Cromatografía de gases

La cromatografía de gases es una técnica de separación especialmente adecuada para compuestos volátiles, apolares y termoestables. La mayoría de los POPs contemplados en el Convenio de Estocolmo como los pesticidas

organoclorados, dioxinas y furanos, PCBs y BDEs son compuestos compatibles con esta técnica, al igual que los PAHs y muchas de las sustancias presentes en los materiales de envasado (Xu *et al.*, 2013; Martinez *et al.*, 2004; Félix *et al.*, 2012).

El corazón de la cromatografía de gases reside en la columna cromatográfica, que en la gran mayoría de las aplicaciones es de tipo capilar, aprovechando su alta eficacia de separación. La principal desventaja de este tipo de columnas es la baja capacidad de carga, ya que no suelen tolerar inyecciones de masas elevadas de analitos (o interferentes) sin afectar negativamente a la resolución y a la forma de pico, lo que consecuentemente puede perjudicar a la calidad de la separación. No obstante, los componentes del cromatógrafo de gases -desde el sistema de inyección, pasando por las distintas fases estacionarias disponibles, hasta el sistema de detección- ofrecen diversas posibilidades para adaptarse, en la medida de lo posible, a la naturaleza y concentración de las muestras y compuestos a determinar, compensando así ciertas limitaciones en otros aspectos.

I.3.1.1. Sistemas de inyección

La inyección en GC puede efectuarse básicamente en cuatro modos diferentes, en función de la estabilidad de la muestra, la concentración de los analitos y la polaridad o volatilidad del disolvente en que se encuentra la muestra. En la **Tabla I.2** se resumen los principios básicos de cada uno, así como las principales aplicaciones y ventajas y desventajas.

Tabla I.2. Resumen de las principales características de los modos de inyección en GC.

	Principios	Ventajas/Aplicaciones	Desventajas
Split	División del flujo de la muestra entrante según una relación de división previamente establecida	• Muestras muy complejas o sucias • Analitos muy volátiles	• Posible discriminación de una parte de la muestra • Menor sensibilidad
Splitless	La válvula de *split* se mantiene cerrada durante la inyección, con lo que no existe división de flujo y la totalidad de la muestra se transfiere a la columna	• Mayor sensibilidad • Análisis de trazas	• Limitación en el abanico de disolventes compatibles • Obtención de picos más anchos
On-column	La muestra se introduce directamente en el interior de la columna sin previa vaporización	• Se evitan los problemas de discriminación y posible degradación térmica	• Posibilidad de deterioro de la columna
PTV	Programación de la temperatura de inyección aplicando diferentes modos de operación	• Inyección de grandes volúmenes • Eliminación selectiva del disolvente en frío	• Optimización de muchas variables y complejidad para lograr una adecuada retención de los analitos durante la eliminación del disolvente

Por la menor sensibilidad que comporta frente a la inyección *splitless*, las aplicaciones en modo *split* suelen ser menos habituales en los ámbitos de estudio descritos en esta Tesis. Por su parte, la mayor reproducibilidad, sencillez y sensibilidad de la inyección *splitless* hacen que éste sea el modo de inyección más común. No obstante, el modo de vaporizador con temperatura programada (PTV) ofrece una mayor versatilidad ya que permite trabajar en los modos de *split* y *splitless* convencional y resulta particularmente útil en casos de incompatibilidad con el disolvente, con lo que es también bastante empleado. A diferencia de los modos anteriores, la inyección *on-column* permite la introducción directa de la muestra líquida, lo que supone una ventaja frente a la discriminación y posible degradación térmica de los analitos sometidos a las altas temperaturas de vaporización en los otros modos; sin embargo, compuestos poco volátiles pueden acumularse en la cabeza de la columna, favoreciendo un deterioro rápido de la misma, lo que la convierte en una opción menos extendida y limitada a muestras muy limpias.

El uso de los modos splitless y PTV se evalúa en el capítulo II de esta Tesis.

I.3.1.2. Sistemas de detección

Existen diversos tipos de detectores convencionales que generan las señales correspondientes en respuesta a la llegada de los compuestos previamente separados en la columna cromatográfica; entre ellos se pueden encontrar detectores de conductividad térmica, de ionización, electroquímicos y espectroscópicos. No obstante, en la gran mayoría de aplicaciones actuales relacionadas con el análisis medioambiental y alimentario, el sistema de detección más adecuado/utilizado suele ser el de espectrometría de masas.

A finales de la década de 1950 se diseñó el primer acoplamiento GC-MS, pero no fue hasta la llegada de la cromatografía de gases capilar, unos años más tarde, cuando se extendió su comercialización. Desde entonces, las técnicas y metodologías basadas en GC-MS han ido evolucionando de la mano de continuos progresos instrumentales. Actualmente, el acoplamiento GC-MS combina un elevado poder de resolución (derivado de la GC capilar) con una alta selectividad, sensibilidad y capacidad de identificación (derivado del uso de MS), con lo que numerosas aplicaciones se basan en esta técnica tanto para la identificación cualitativa de compuestos desconocidos como para la determinación cuantitativa. En el siguiente apartado se describen de forma general algunos aspectos básicos de la espectrometría de masas (en cuanto a su acoplamiento con la cromatografía).

I.3.2. Espectrometría de masas

El funcionamiento de un espectrómetro de masas se basa en la generación de iones en fase gas y su posterior separación en función de su relación masa/carga (m/z) y detección. Los espectros de masas resultantes proporcionan información muy valiosa para la identificación de los compuestos detectados y para la cuantificación en base a la respuesta para una m/z determinada. A continuación, se comentan algunas características básicas de las fuentes de ionización y de los analizadores de masa, que

son los responsables de la separación de los iones generados en la fuente y verdadero corazón de los sistemas de MS.

I.3.2.1. Fuentes de ionización

Las fuentes de ionización más comunes para GC son las que operan en condiciones de vacío, como la ionización electrónica (EI) y la ionización química (CI), aunque en los últimos años, la instrumentación analítica ha progresado y ha permitido compatibilizar la técnica GC con fuentes de ionización química a presión atmosférica (APCI). En la **Tabla I.3** se muestran las principales características de cada una.

Tabla I.3. Resumen de las principales características de las fuentes de ionización para GC.

	Principios	Ventajas	Desventajas
EI	Ionización por bombardeo con un haz de electrones de alta energía (normalmente a 70 eV)	• Universal, robusta y muy reproducible • Genera espectros clásicos compatibles con la búsqueda en librerías de espectros comerciales	• Excesiva fragmentación en muchos casos • Posible ausencia del ión molecular
CI	Ionización por reacción con los iones de un gas reactivo en donde la transferencia de energía generalmente no supera los 5 eV	• Ionización más suave que EI y mayor abundancia del ión molecular • Mejor sensibilidad y selectividad para familias específicas de compuestos	• No universal • Poco reproducible y estable • No existen librerías espectrales
APCI	Ionización por reacción con los iones de un gas reactivo. Dos mecanismos posibles: 1) transferencia de carga desde el gas reactivo, que suele ser N_2 2) protonación con trazas de vapor de agua presentes en la fuente	• Ionización suave y universal • El primer mecanismo fomenta la presencia abundante del ión molecular • El segundo mecanismo puede ser exaltado con el uso de modificadores para favorecer la generación de la molécula protonada	• No existen librerías espectrales • Poco consolidada ya que el acoplamiento con GC es relativamente nuevo • Falta de *background* teórico/práctico

La EI ha venido considerándose la fuente de ionización por excelencia en GC-MS por su universalidad, robustez y reproducibilidad, empleándose comúnmente en métodos multiresiduo. Además, la disponibilidad de librerías de espectros comerciales resulta de gran utilidad y facilita el proceso de identificación. Por el

contrario, la CI goza de menos popularidad ya que no es efectiva por igual para todos los compuestos, pero ofrece una mayor sensibilidad y selectividad especialmente en modo negativo para determinadas familias de compuestos con elevada electronegatividad.

Por otro lado, a diferencia de la suave ionización que tiene lugar en la fuente de CI, la alta energía que se emplea en la EI suele favorecer la ausencia del ión molecular ($M^{+\bullet}$), hecho que se considera el punto débil de esta fuente de ionización, ya que ello puede afectar negativamente a la sensibilidad, selectividad y capacidad de identificación.

Por su parte, la fuente de APCI combina la universalidad de la EI con la suave fragmentación de la CI, favoreciendo la presencia del $M^{+\bullet}$ en una amplia variedad de compuestos. Así, la aparición de esta nueva fuente compatible con GC se revela como una potencial alternativa en aplicaciones medioambientales y de seguridad alimentaria, como se verá en el capítulo III.

I.3.2.2. Analizadores

El analizador de masas es la esencia del espectrómetro de masas y responsable de la mayoría de las características analíticas del sistema MS. Existen varios tipos de analizadores que presentan diferentes características y posibilidades en cuanto a la sensibilidad, el máximo rango de masas capaz de separar y el poder de resolución. Los analizadores más populares empleados en la determinación de contaminantes orgánicos mediante GC son el cuadrupolo simple (Q), la trampa de iones (IT), el analizador de tiempo de vuelo (TOF), el triple cuadrupolo (QqQ) y el híbrido cuadrupolo tiempo de vuelo (QTOF), aunque este último ha venido siendo acoplado mayoritariamente a la cromatografía de líquidos. Seguidamente se resumen las principales características de cada uno de ellos.

CUADRUPOLO SIMPLE (Q)

El filtro de masas cuadrupolar o cuadrupolo simple está formado por cuatro barras cilíndricas de metal dispuestas paralelamente dos a dos. Los potenciales de corriente continua y radiofrecuencia que se aplican entre las barras opuestas determinan las relaciones m/z de los iones que son capaces de describir trayectorias estables a través de ellas hasta alcanzar el detector.

Así, con la variación del potencial es posible realizar un barrido completo de iones en un determinado rango de m/z, lo que se conoce como *full scan*, o seleccionar iones con una m/z específica, trabajando en modo *Selected Ion Monitoring* (SIM). En el primer caso se obtiene el espectro de masas completo, lo que proporciona información cualitativa aunque con baja sensibilidad. En cambio, la selección de iones que tiene lugar en el modo SIM aumenta la sensibilidad y selectividad de la adquisición, con lo que este modo suele aplicarse para análisis cuantitativos tipo *target* con gran robustez y reproducibilidad.

TRAMPA DE IONES (IT)

En este analizador, la separación de los iones se produce en el mismo espacio pero a distintos tiempos (separación en el tiempo). Consiste en dos electrodos hiperbólicos enfrentados y un electrodo anular situado entre ellos que forman una cavidad en donde la acción de potenciales de corriente continua y radiofrecuencia aplicados permite almacenar a los iones en órbitas/trayectorias estables. El valor del potencial va aumentando y produciendo, secuencialmente de menor a mayor, la desestabilización de las trayectorias con una determinada m/z, que abandonan la trampa y llegan al detector.

De este modo, es posible obtener espectros en *full scan* con una alta sensibilidad, pudiéndose además trabajar en modo de espectrometría de masas en tándem (MS/MS). En este caso se selecciona un ión precursor para que quede confinado dentro de la trampa y posteriormente se fragmenta, realizando un barrido de los iones producto, lo que aporta mayor información estructural.

TIEMPO DE VUELO (TOF)

El analizador TOF se basa en un tubo de vuelo al que todos los iones llegan inicialmente con la misma energía cinética y se separan en función del tiempo que tardan en atravesarlo, de modo que los de menor m/z (mayor velocidad) llegan antes al detector.

La ventaja relevante de estos analizadores es la capacidad de medición de la masa de los iones detectados con exactitud, gracias a la elevada resolución que alcanzan, lo que sumado a la excelente sensibilidad en modo *full scan* los hace ideales para análisis *non-target*.

Así, considerando las propiedades de cada uno de estos tres analizadores, la elección dependerá de la aplicación particular considerada y de sus requisitos. En la **Tabla I.4** se muestra una comparación de los principales analizadores utilizados en GC en función de los parámetros más relevantes.

Tabla I.4. Comparación de los parámetros más relevantes de los analizadores empleados en GC.

	Resolución	Rango de masas (m/z)	Velocidad de escaneo	Modo de trabajo	Aplicaciones
Q	1 uma	50-4000	4000-10000 uma/s	Scan SIM	Cualitativa y cuantitativa
IT	1 uma	6000	13000 uma/s	Scan MS/MS	Cualitativa y cuantitativa
TOF	> 10000	Ilimitado	1000000 uma/s	Scan, masa exacta	Cualitativa

Como se observa, la baja resolución del cuadrupolo y de la trampa limita su uso en fines cualitativos, aunque la capacidad de la trampa para operar en modo MS/MS resulta útil para la elucidación estructural (Thurman & Ferrer, 2003). El rango de masas de ambos analizadores es más limitado que en el caso del TOF pero suele ser

suficiente en los análisis convencionales, especialmente cuando se considera el acoplamiento GC-MS.

Por su parte, la velocidad de escaneo constituye un factor a tener en cuenta especialmente en métodos *target* que incluyen un número de analitos considerable. Como se comentará en el capítulo II, el elevado número de iones adquiridos a una velocidad limitada puede afectar significativamente a la sensibilidad y al número de puntos por pico cromatográfico obtenidos y, por tanto, a la efectividad de la cuantificación.

Aunque también puede emplearse en análisis cuantitativos, la trampa no presenta una respuesta lineal a la concentración tan adecuada como el cuadrupolo. Además, hay que tener en cuenta la alta probabilidad de producirse interacciones entre los iones durante el tiempo de residencia en la trampa, lo que puede llegar a degradar la calidad del espectro, especialmente en matrices muy complejas. El intervalo lineal del TOF también es limitado, aunque en los últimos años se ha mejorado para adecuar su uso en aplicaciones cuantitativas (Cervera *et al.*, 2012).

En definitiva, el TOF se considera el analizador preferente para análisis *non-target* (Portolés *et al.*, 2011), mientras que el cuadrupolo es uno de los más empleados en la determinación cuantitativa de contaminantes orgánicos (*target*) en una amplia variedad de matrices (Cunha & Fernandes, 2011). No obstante, analizando las citas reportadas en los últimos años se observa una evolución hacia el empleo de MS/MS por su mayor selectividad y sensibilidad. El uso de combinaciones de dos o más analizadores aumenta el potencial de las técnicas aplicadas, como en el caso de los analizadores QTOF y QqQ. Algunos estudios relevantes sobre el uso de estos analizadores avalan su capacidad para la búsqueda de desconocidos y cuantificación de contaminantes orgánicos, tanto en GC como en LC (Hernández *et al.*, 2005a; Botitsi *et al.*, 2011). Seguidamente se muestran algunas de sus principales características.

TRIPLE CUADRUPOLO (QqQ)

Esta combinación de analizadores está formada por tres cuadrupolos en serie (Q1, q2 y Q3), aunque realmente el q2 actúa únicamente como celda de colisión, siendo habitualmente un sistema hexapolar u octapolar donde se produce la fragmentación de los iones por disociación inducida por colisión con las moléculas de un gas inerte (argón o nitrógeno). Existen diferentes modos de adquisición dependiendo de la finalidad del análisis, tanto en MS como en MS/MS, que permiten trabajar con velocidades de escaneo muy altas en comparación con las que ofrece el cuadrupolo simple. A pesar de que el MS no es el típico modo de trabajo en estos sistemas, es posible adquirir en *full scan* o trabajar en modo SIM, como si de un Q simple se tratara, generalmente estableciendo las condiciones de trabajo para Q1 y q2 en modo de transmisión. Por su parte, el uso de un QqQ permite realizar barrido de iones producto (*product ion scan*) y pérdidas neutras (*neutral loss scan*), búsqueda de iones precursores (*precursor ion scan*) y monitorización de una transición concreta (*Selected Reaction Monitoring*, SRM).

En el modo SRM, el más aplicado en análisis *target*, el Q1 se encarga de seleccionar el ión precursor, que se fragmenta en q2 aplicando una determinada energía de colisión (CE) y, finalmente, el Q3 selecciona un ión producto de entre todos los generados. De esta manera se monitorizan transiciones específicas que mejoran la sensibilidad y selectividad del método gracias a la discriminación de interferentes y reducción del ruido de fondo (mayor relación señal/ruido (S/N)).

Con ello, el QqQ se afianza como preferente en análisis *target* cuantitativos, especialmente para el análisis de trazas donde se requieren límites de detección muy bajos (Pitarch *et al*., 2007; Camino-Sánchez, 2011; Hernández *et al*., 2013).

CUADROPOLO TIEMPO DE VUELO (QTOF)

El acoplamiento del analizador híbrido QTOF se consigue a través de una celda de colisión del mismo modo que con el QqQ. En este caso también es posible trabajar en modo *full scan*, aunque la utilidad más práctica reside en el modo *product ion scan*, de manera que los iones precursores seleccionados en el cuadrupolo son

fragmentados y, posteriormente, el TOF realiza un barrido de los iones producto con una elevada resolución, exactitud de masa y sensibilidad.

De este modo, la información estructural de los iones producto a través de experimentos MS/MS supone una herramienta complementaria muy útil para el análisis de desconocidos así como para la elucidación estructural (Hopfgartner *et al.*, 1999; Zhang *et al.*, 2012).

Cabe destacar que el desarrollo de las nuevas fuentes de APCI para GC ha aportado ventajas adicionales en este campo gracias a la favorable presencia del ión molecular en el espectro de masas. Por un lado, el acoplamiento de esta fuente con el analizador QqQ (Portolés *et al.*, 2012) supone un aumento de la sensibilidad y selectividad derivado de la suave o nula fragmentación del ión molecular, como se verá en el capítulo III. Por otro lado, estos nuevos instrumentos disponibles comercialmente basados en GC-APCI han incluido al QTOF como analizador de masas (empleado en el capítulo IV), con lo que la presencia del ión molecular contribuye a una mayor fiabilidad en la identificación de desconocidos en análisis *non-target* (Portolés *et al.*, 2010)

I.3.3. *Fast* GC-MS

Cada vez es mayor el interés en el desarrollo de métodos multiresiduales capaces de determinar un elevado número de compuestos en un mismo análisis debido, principalmente, a la necesidad de satisfacer las demandas actuales de análisis rápidos y económicos. En este sentido, la cromatografía de gases rápida (*fast* GC) resulta una opción adecuada puesto que permite acortar el tiempo de separación ofreciendo un mayor rendimiento en el análisis con una instrumentación no demasiado sofisticada.

Basándose en la premisa de que una separación más rápida implica un menor tiempo de residencia de los analitos en la columna y, por tanto, una reducción de la anchura de banda, van Deursen *et al.* (2000) proponen una clasificación de *fast* GC

según la anchura del pico cromatográfico obtenido y que se muestra en la **Tabla I.5**. Como puede observarse, el rango de tiempo de los análisis puede variar desde minutos a sub-segundos, aunque la primera modalidad es la más habitual.

Tabla I.5. Clasificación de *fast* GC en base a la anchura de pico cromatográfico (van Deursen *et al.*, 2000).

Clasificación	Rango de tiempo	Anchura de pico a media altura
Fast	minutos	1-3 s
Very fast	segundos	30-200 ms
Ultra-fast	sub-segundos	5-30 ms

En cualquier caso, para trabajar en cualquier modalidad de *fast* es preciso que los instrumentos dispongan de elementos específicos como reguladores de presión internos de amplio rango, sistemas de inyección adecuados para evitar el ensanchamiento de los picos, sistemas de calentamiento rápido y detectores con velocidad de escaneo aceptable (debe ser suficientemente rápida como para representar un número suficiente de puntos por pico sin que la sensibilidad y el proceso de cuantificación se vean afectados) (Klee & Blumberg, 2002). En este sentido, el TOF es un analizador especialmente útil en *fast* GC (van Deursen *et al.*, 2000), aunque el uso del simple y triple cuadrupolo puede resultar suficiente en algunas aplicaciones (Kirchner *et al.*, 2005a; Dallüge *et al.*, 2002).

La bibliografía existente sobre *fast* GC reporta diversas estrategias (**Tabla I.6**) que permiten conseguir separaciones más rápidas con la modificación de ciertos parámetros (Korytár *et al.*, 2002; Matisová & Dömötörová, 2003; Mastovská & Lehotay, 2003). Existen además diversos estudios en este campo aplicados a la determinación de diferentes tipos de contaminantes como los comentados al principio de este capítulo (Cochran, 2002; Nikonova & Gorshkov, 2012; Tollbäck *et al.*, 2003), aunque la gran mayoría de aplicaciones sobre *fast* GC se centra en la determinación de pesticidas (Dömötörová & Matisová, 2008; Matisová & Hrouzková, 2012; Kirchner *et al.*, 2005b).

Tabla I.6. Resumen de las rutas y estrategias posibles para acelerar los análisis por GC.

Rutas	Estrategias
Minimizar resolución hasta un valor suficiente	Reducir longitud columna
	Reducir espesor de fase
	Aumentar temperatura inicial/final del horno
	Aumentar rampas de temperatura
	Usar programas de presión/flujo
	Aumentar velocidad lineal del gas portador
Maximizar selectividad	Usar fase estacionaria más selectiva
	Trabajar con GG x GC
	Usar detección selectiva
	Aplicar *backflush*
Reducir tiempo de análisis a resolución constante	Reducir diámetro interno de la columna
	Usar hidrógeno como gas portador
	Aplicar condiciones *vacuum-outlet*
	Aplicar condiciones flujo turbulento

De entre éstas, las opciones más empleadas implican modificaciones en los parámetros de la columna cromatográfica y el uso de programas de temperatura y detectores rápidos. También son comunes las técnicas de cromatografía de gases bidimensional y la de baja presión, pero el requerimiento de instrumentación más compleja o específica limita sus aplicaciones, especialmente en análisis rutinarios.

La simple reducción de la longitud de la columna conlleva una disminución del tiempo cromatográfico pero también contribuye a la pérdida de resolución (Tranchida *et al*., 2008). En este caso, si el número de compuestos a determinar es elevado pueden producirse importantes coeluciones que sólo un analizador con elevada resolución de masas podría solucionar. La reducción del diámetro interno (I.D.) de la columna es otra alternativa frecuente que consigue restablecer la resolución a valores aceptables con un drástico descenso del tiempo de análisis (Dömötörová *et al*., 2006), obteniéndose picos más estrechos y, por consiguiente, un aumento de la sensibilidad (mayor S/N). La principal desventaja de esta estrategia es la consecuente menor capacidad de muestra, lo que se traduce en una cierta pérdida

de sensibilidad, pero que puede compensarse con sistemas de inyección adecuados (Kirchner *et al.*, 2004, Korenková *et al.*, 2003; Matisová *et al.*, 2002). En combinación con cualquier aproximación de las anteriores, el uso de programas de temperatura rápidos es una estrategia muy común y efectiva para acelerar los análisis (van Deursen *et al.*, 1999), aunque, en cualquier caso, la elección final de la estrategia más apropiada vendrá determinada por los requerimientos específicos de cada análisis.

I.3.4. LC-MS

Como se ha comentado anteriormente, la determinación analítica de los contaminantes orgánicos también comprende el uso de la cromatografía de líquidos, especialmente para aquellos con carácter polar, baja volatilidad y termolábiles. La aplicación de GC y LC como técnicas complementarias es necesaria para abarcar un amplio abanico de contaminantes de diferente naturaleza (como es el caso de la aplicación *non-target* desarrollada en el capítulo IV).

El acoplamiento de la cromatografía de líquidos con la espectrometría de masas empezó a investigarse a principios de 1970, tratando de solventar los problemas de incompatibilidad de las fases líquidas procedentes del LC con el alto vacío requerido en el MS. Así, antes de que la muestra líquida alcance el MS, las interfases deben eliminar el disolvente y, posteriormente, vaporizar la muestra e ionizarla. Estos sistemas han tardado décadas en perfeccionarse, siendo la ionización por electrospray (ESI) y la APCI (ambas basadas en la ionización a presión atmosférica) las interfases más comunes hoy en día para la determinación de compuestos de polaridad media-alta.

La particularidad de la ESI reside en la generación de un aerosol de pequeñas gotas altamente cargadas cuya dispersión va provocando la evaporación del disolvente. A diferencia de la APCI, la ionización por electrospray permite la formación de iones múltiplemente cargados, resultando más adecuada para la ionización de analitos muy polares o incluso iónicos y de un amplio rango de masas, a

la vez que aporta mayor sensibilidad. La **Figura I.1** muestra la relación de las diferentes interfases en función de la polaridad y peso molecular de los analitos, siendo la ESI la interfase que permite el mayor rango de aplicación (Hernández *et al.*, 2005b).

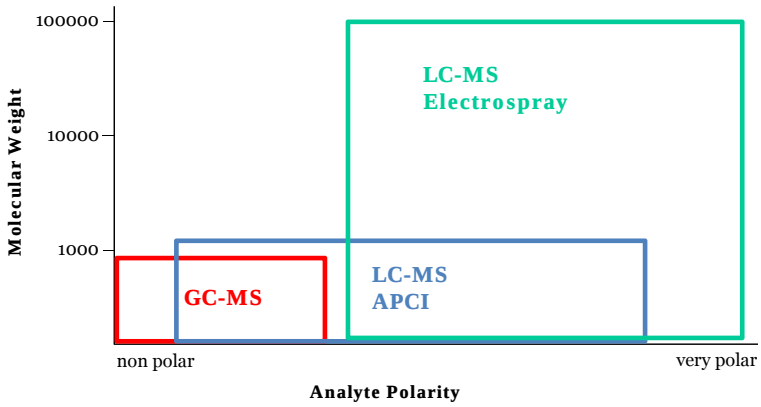

Figura I.1. Rango de aplicación de las diferentes interfases empleadas en GC-MS y LC-MS en función de la polaridad de los compuestos y su masa molecular.

En cuanto a los analizadores, siguiendo la misma tendencia que en GC-MS, la alta sensibilidad y selectividad del triple cuadrupolo y la exactitud de masa y la posibilidad de trabajar en MS/MS del QTOF convierten a estos analizadores en herramientas poderosas en LC-MS para fines cuantitativos y cualitativos, respectivamente (Hernández *et al.*, 2008; Masiá *et al.*, 2013).

1.3.5. Tratamiento de muestra

En general, el análisis de muestras mediante GC requiere de una etapa previa de tratamiento de muestra que, habitualmente, implica una extracción de los analitos de la matriz en un disolvente orgánico compatible con GC. A su vez, resulta necesario eliminar/reducir aquellos componentes de la matriz que pueden interferir en el análisis, disminuyendo de este modo el llamado efecto matriz. Con el fin de obtener extractos lo más limpios posible, pueden aplicarse etapas adicionales de purificación

o *clean-up*, con las que se puede reducir este efecto. Por otro lado, los bajos límites de detección exigidos por las legislaciones existentes implican, en algunos casos, incluir etapas de pre-concentración en el tratamiento de muestra que mejoren la sensibilidad del método global. En el caso de LC, las muestras suelen requerir un menor tratamiento, pudiéndose recurrir, en ocasiones, a la inyección directa de muestras acuosas, simplificando la metodología analítica.

Actualmente existe una gran variedad de técnicas para el tratamiento de muestras medioambientales y alimentarias, cuya elección dependerá de los analitos a determinar y de la naturaleza y complejidad de las muestras (Dean, 2009). Entre las más empleadas en aguas y alimentos se encuentran las siguientes: extracción Soxhlet, extracción con fluidos supercríticos (SFE), extracción asistida por microondas (MAE), extracción acelerada con disolventes (ASE), extracción líquido-líquido (LLE), QuEChERS (*Quick, Easy, Cheap, Effective, Rugged and Safe*), microextración en fase sólida (SPME), extracción en fase sólida (SPE) y cromatografía de permeación en gel (GPC). En la **Tabla I.7** se resumen los fundamentos, así como algunas ventajas y desventajas de cada técnica.

Tabla I.7. Resumen de las principales características de los tratamientos de muestra más habituales.

	Principios	Ventajas/Aplicaciones	Desventajas
Soxhlet	Destilación en la que un disolvente extrae a los analitos de la muestra en un proceso cíclico continuo	• Recuperaciones elevadas • Muestras sólidas	• Alto consumo de tiempo y disolvente
SFE, MAE y ASE	Uso de disolventes de alta difusividad, elevando la temperatura y controlando la presión	• Extracciones más rápidas • Muestras sólidas	• Elevado coste del equipamiento
LLE	Transferencia de los analitos desde una muestra líquida a otra inmiscible o parcialmente miscible con ésta	• Versatilidad (la eficacia depende de la afinidad del analito por los disolventes, de la relación de volúmenes de cada fase y del número de extracciones sucesivas)	• Uso elevado de disolvente y riesgo de exposición a disolventes tóxicos • Posible formación de emulsiones • Difícil automatización (elevado consumo de tiempo)
QuEChERS	Extracción con acetonitrilo (ACN) (disolvente más habitual) y *clean-up* basado en una extracción en fase sólida dispersiva con una amina primaria-secundaria (PSA) como sorbente y $MgSO_4$ anhidro para eliminar trazas de agua	• Sencillo y rápido • Extracción de pesticidas en sólidos con alto contenido en agua • Varias versiones disponibles en función de la naturaleza de los pesticidas	• ACN no compatible con la inyección *splitless* por su gran volumen de expansión en el liner y baja volatilidad (inyección directa requiere PTV) • Inyección *splitless* requiere cambio de disolvente (riesgo de pérdida de analitos o contaminación de las muestras) • No pre-concentración
SPME	Absorción de los analitos en una fibra de sílice fundida cubierta con una fase polimérica y posterior desorción térmica en el inyector del GC	• Extracción y pre-concentración en una sola etapa • Dos modos posibles en función de la naturaleza de los analitos: *head-space* e inmersión directa • No requiere uso de disolvente • Muestras sólidas y líquidas	• Extracción de las muestras una a una (bajo rendimiento en cuanto a número de muestras extraídas por día) • Aplicación a muestras complejas limitada debido a la posible absorción de interferentes de la matriz en la fibra
SPE	Retención de los analitos en un sorbente y posterior elución con un disolvente orgánico adecuado	• Permite pre-concentración • Versatilidad (amplia variedad de sorbentes y disolventes de elución disponibles) • Rápida y con poco consumo de disolvente • Modo *on-line* facilita la automatización y aumenta la reproducibilidad y velocidad de extracción • Muestras líquidas	• Número de muestras a extraer bastante limitado si se realiza en modo manual • Elevado coste del modo *on-line* • Obturación en muestras sin filtrar
GPC	Separación de los analitos por tamaño mediante el paso de la muestra a través de un gel polimérico	• Posible automatización (separaciones más eficaces, mayor reproducibilidad, menor consumo de disolvente) • Muy aplicado como tratamiento de purificación adicional de muestras complejas o con elevado contenido graso	• En muestras muy complejas puede ser insuficiente para eliminar todos los componentes grasos de la matriz y es necesario repetir el tratamiento o aplicar una etapa adicional de SPE

El alto consumo de tiempo y disolvente de los tratamientos de muestra basados en Soxhlet y extracción líquido-líquido, así como el elevado coste del equipamiento necesario para SFE, MAE y ASE, suponen unas desventajas importantes a tener en cuenta. Al igual que cada vez se demandan métodos GC-MS más rápidos y económicos, el interés en aplicar métodos de extracción simples, rápidos y con el menor consumo posible de disolvente también va en aumento. En este sentido, la aplicación de QuEChERS, SPME y SPE goza de gran popularidad en la actualidad.

Desde que fue diseñado en 2003 para la extracción de pesticidas en muestras sólidos (Anastassiades *et al.*, 2003a), la creciente popularidad del QuEChERS ha derivado en múltiples modificaciones que lo convierten en un procedimiento muy flexible, adaptable a matrices y pesticidas de diversa naturaleza, así como a las características instrumentales y a las preferencias de los analistas (Lehotay *et al.*, 2010; Andraščíková & Hrouzková, 2013). Entre ellas, existen dos modificaciones consideradas oficiales que agregan tampones durante la extracción con el objetivo de reducir los efectos negativos del método original sobre los pesticidas pH-dependientes: el método 15662 del Comité Europeo de Normalización (CEN) (Payá *et al.*, 2007), que emplea tampón de citrato (pH 5-5.5) y el método oficial 2007.01 de la Asociación de Comunidades Analíticas (AOAC) (Lehotay *et al.*, 2005), que usa acetato sódico como tampón (pH 4.8). Además, este último incluye el uso de otros sorbentes como C_{18} o carbón negro grafitizado para mejorar el *clean-up* de muestras grasas y pigmentadas, respectivamente.

El uso de acetonitrilo (ACN) como disolvente habitual de extracción representa el principal inconveniente con respecto a la determinación analítica por GC debido a su incompatibilidad con la inyección *splitless*. La inyección directa al cromatógrafo requiere de un inyector PTV, aunque otra alternativa muy común es el cambio de disolvente a tolueno, compatible con el modo *splitless*, lo que a su vez permite la pre-concentración del extracto en caso de ser necesario. Las numerosas citas reportadas demuestran que ambas opciones son muy comunes y ofrecen buenos resultados (Lehotay, 2011; Du *et al.*, 2011; Norli *et al.*, 2011), aunque con diferente complejidad en cuanto al tratamiento de muestra.

Para el tratamiento de muestras sólidas y líquidas también es comúnmente aplicada la SPME, desarrollada por Pawliszyn en la década de 1990 (Belardi & Pawliszyn, 1989). La modalidad *head-space* resulta útil para el tratamiento de muestras complejas, ya que la fibra se expone al espacio de cabeza existente sobre la muestra de manera que sólo los analitos más volátiles se retienen en la misma (Schurek *et al*., 2008). En la modalidad de inmersión directa, la fibra se sumerge en la muestra permitiendo la absorción de un mayor número de compuestos sin discriminación por volatilidad, aunque ello conlleva también una mayor absorción de los componentes de la matriz (Beltran *et al*., 2003). En este caso una opción sencilla es la dilución de las muestras para reducir la complejidad de la matriz, aunque con la consiguiente disminución de la sensibilidad.

La versatilidad que ofrece la SPE, vigente desde mediados de la década de 1970 y firmemente consolidada para análisis de aguas, la convierte en una técnica apropiada para una gran variedad de analitos. La amplia diversidad de sorbentes permite adaptarse a las diferentes características físico-químicas de los analitos, incluso aplicarse en métodos multiresiduales con alta eficacia (Bizkarguenaga *et al*., 2012). En este caso los cartuchos con relleno C_{18} y los Oasis HLB (*Hydrophilic-Lipophilic Balance*) son los más empleados.

La SPE también se utiliza como tratamiento de purificación adicional, requerida en el análisis de muestras muy complejas o con elevado contenido graso, al igual que la GPC, que representa otra alternativa muy aplicada en estos casos.

A pesar de la efectividad del tratamiento de muestra para reducir el efecto matriz, éste aún puede ser notorio en algunos tipos de muestras. En la determinación por GC, este efecto puede originarse por retención de la matriz en los sitios activos del liner, provocando una exaltación de la señal en relación con la de un patrón en solvente, o puede ocurrir en la fuente de ionización, obteniendo generalmente una supresión de la señal. Contrarrestar este efecto es importante para evitar errores en la cuantificación y falsos positivos que se pueden reportar por la presencia de

interferentes coextraídos de la matriz. En la **Tabla I.8** se comentan las principales características de las opciones más empleadas para reducir el efecto matriz.

Tabla I.8. Estrategias habituales para reducir el efecto matriz en los análisis de muestras por GC.

	Principios	Ventajas	Desventajas
Patrones internos	Similitud con los analitos de interés	• Similitud en el comportamiento GC-MS de analitos y patrones	• Elevado coste • Disponibilidad comercial
Protectores de analitos	Interacción con los sitios activos del liner	• Reducción de la interacción de los analitos con el liner	• Optimización compleja
Calibrado en matriz	Preparación del calibrado con matriz blanco	• Efecto matriz actúa por igual en las muestras y en el calibrado	• Disponibilidad de matrices blanco representativas
Adiciones estándar	Adición a la muestra de cantidades conocidas del analito a determinar	• No necesidad de matriz blanco representativa	• Elevado número de inyecciones por muestra • Previsión del nivel de concentración
Dilución de las muestras	Dilución de las muestras con un disolvente adecuado	• Disminución de los interferentes	• Dilución no selectiva

En el caso de patrones internos, el uso del mismo compuesto objeto de estudio marcado isotópicamente (^{13}C, ^{2}H) es la opción más adecuada, pero la poca disponibilidad comercial o el elevado coste promueven el uso de patrones lo más semejantes posibles a los analitos en cuanto a estructura y tiempo de retención. Del mismo modo ocurre con los protectores de analitos (Anastassiades *et al*., 2003b), siendo una opción menos habitual por la dificultad de encontrar protectores adecuados para todos los compuestos incluidos en métodos multiresiduo.

Por otro lado, el calibrado en matriz ofrece la posibilidad de asemejar muestras y calibrado, aunque la falta de matrices blanco representativas para algunos tipos de muestras condiciona el uso de esta metodología. En este caso el método de adiciones estándar resulta útil, aunque también tedioso por el elevado número de inyecciones por muestra que requiere. Otra opción factible es la dilución de las muestras (y

consecuente dilución de los interferentes) siempre y cuando la sensibilidad del método lo permita, ya que los límites de detección también pueden verse afectados.

El desarrollo de los diferentes métodos analíticos que componen esta Tesis Doctoral se ha basado en el estudio y optimización de parámetros relacionados tanto con la etapa de determinación mediante GC-MS como con la del tratamiento de muestra, con el fin de mejorar las metodologías existentes para la determinación de los contaminantes descritos en el primer apartado.

Estudio de las posibilidades de *fast* GC-MS (analizador cuadrupolo simple) para la determinación de pesticidas y otros contaminantes en aguas y alimentos

II.1. Introducción

En los últimos años el factor tiempo se ha convertido en un parámetro relevante a considerar en el desarrollo de métodos analíticos. Aunque las bases teóricas de la cromatografía de gases rápida ya se establecieron en la década de 1960, las aplicaciones fueron implementadas lentamente, quedándose bastante limitadas. No es hasta finales de 1990 cuando se reaviva el interés por conseguir separaciones más rápidas que ofrezcan ventajas adicionales a los métodos existentes. La posibilidad de incluir un elevado número de compuestos para su determinación simultánea en un método con un tiempo reducido aporta un aumento del rendimiento en un laboratorio de rutina, por lo que las novedades en este campo son bien acogidas.

Un análisis convencional por GC-MS suele durar entre 20 y 60 minutos, dependiendo del tipo de muestra y del número de analitos a determinar. Las condiciones empleadas en *fast* GC son tales que permiten una buena resolución cromatográfica, lo que habitualmente unido a la selectividad proporcionada por un espectrómetro de masas hace que se desarrollen métodos adecuados para el análisis multiresidual. De este modo, métodos multiresiduo pueden ejecutarse en menos de 10 minutos, con la obtención de picos cromatográficos de entre 1 a 3 segundos.

Remontándose a la teoría básica de la cromatografía, algunos autores realizan estudios en base a las ecuaciones que rigen el comportamiento de los picos cromatográficos, deduciendo las rutas generales hacia la cromatografía rápida (Cramers & Leclercq, 1999). De entre ellas, descritas anteriormente en el capítulo I, la estrategia más directa es el uso de columnas con un diámetro interno reducido (Lieshout *et al.*, 1998), que permiten una mayor velocidad lineal óptima. En las aplicaciones *fast* GC-MS descritas en este capítulo se emplean columnas con un I.D. de 0.1 mm, consiguiéndose análisis rápidos con picos

estrechos y con buena relación S/N frente a los obtenidos en GC convencional con el uso de columnas de 0.25 mm. El inconveniente que lleva asociado esta opción es una menor capacidad de carga de muestra que a largo plazo puede conllevar el deterioro de la columna, traduciéndose en picos más anchos (*band broadening*), con colas o incluso "picos fantasma" (Dömötörová *et al.*, 2006). Con el fin de conservar la eficacia de la columna y que estos efectos descritos no se agraven, se requieren tanto sistemas de inyección como de control de temperatura de la columna suficientemente rápidos. El uso de programas de temperatura rápidos favorece la reducción de la anchura de pico gracias a un menor efecto de la difusión longitudinal en la columna. A diferencia de las rampas de 10-20 ºC/min que se suelen aplicar tradicionalmente, rampas del orden de 50-100 ºC/min (como las aplicadas en los métodos desarrollados en este capítulo) permiten la obtención de picos cromatográficos de entre 1 y 3 segundos de amplitud, reduciendo considerablemente el tiempo de análisis (McNair & Reed, 2000). Ello requiere a su vez sistemas de detección capaces de monitorizar con suficiente rapidez un número adecuado de puntos para estos picos tan estrechos.

En este sentido, el TOF resulta el analizador ideal para el acoplamiento con la cromatografía rápida. No obstante, analizadores cuya velocidad de escaneo no es tan alta como la del TOF pueden resultar adecuados para trabajar en modo *fast* GC en determinadas aplicaciones (Kirchner *et al.*, 2005). Además, actualmente existen sistemas de MS con cuadrupolos simples diseñados para trabajar en modo *fast* que alcanzan velocidades de escaneo más elevadas que las habituales en este tipo de analizador. En el presente capítulo, se ha trabajado con un sistema GC-MS Shimadzu QP2010 Plus que cuenta con un cuadrupolo simple capaz de alcanzar velocidades de escaneo de hasta 10000 uma/s, permitiendo trabajar con bajos tiempos de escaneo. En este sistema, el tiempo de escaneo o *scan time* viene determinado por el tiempo empleado en la monitorización de todos los iones comprendidos en un determinado rango de tiempo, ya sea en *full scan* o en modo SIM. Considerando que el analizador debe proporcionar un número suficiente de puntos por pico para todos los compuestos incluidos en un método *target*, a menor tiempo de escaneo, mayor

capacidad para monitorizar adecuadamente un mayor número de compuestos, aunque ello también puede suponer una disminución de la sensibilidad. Se trata, pues, de encontrar un compromiso entre sensibilidad y número de puntos por pico.

Aprovechando las ventajas que ofrece la cromatografía rápida, en este capítulo se han desarrollado métodos *fast* GC-MS para la determinación de contaminantes en aguas y alimentos. La consecución de estas metodologías, descritas en los tres artículos que comprenden el capítulo, se asienta sobre las bases de un primer trabajo no publicado cuyo objetivo era la determinación de 117 compuestos en un corto tiempo de análisis. Durante la optimización del método, diversos parámetros GC-MS no pudieron ser ajustados adecuadamente para conseguir buenos resultados cuantitativos, haciéndose patentes las limitaciones del cuadrupolo simple para este tipo de aplicaciones. Aunque la validación del método no pudo llevarse a cabo, el proceso de desarrollo permitió evaluar las posibilidades del cuadrupolo simple como analizador en *fast* GC-MS.

Los compuestos estudiados incluyen diferentes POPs como pesticidas, PCBs, PBDEs y alquilfenoles, así como los 16 PAHs regulados por la EPA. La selección de los mismos se hizo en base a su frecuente presencia en muestras medioambientales, así como a su potencial tóxico. Los bajos límites de estos contaminantes impuestos por las legislaciones (MRL y MAC) requieren de métodos analíticos suficientemente sensibles para una detección y cuantificación fiables de niveles mínimos de 10 ng/L en aguas (*Directive 2013/39/EU*) y de 10 μg/kg en alimentos (*Regulation (EC) No 396/2005*).

Todos estos compuestos constituyen un grupo de contaminantes especialmente preocupantes en el medio ambiente, de modo que una de las metodologías desarrolladas se ha aplicado a su determinación en el análisis de aguas (Artículo científico 1), utilizando la SPE como tratamiento de muestra. En este trabajo se han optimizado cuidadosamente las condiciones cromatográficas para separar un total de 66 compuestos en un tiempo corto y, al mismo tiempo, se han evaluado las capacidades y limitaciones del cuadrupolo simple como analizador acoplado a *fast*

GC. La contribución más destacable es la aplicación de *fast* GC a la determinación de un mayor número de compuestos que los que normalmente se incluyen en este tipo de métodos.

Los trabajos posteriores de este capítulo se han centrado en la determinación de pesticidas en el ámbito alimentario. La presencia de estos contaminantes en muestras de alimentos es bien conocida, tal y como queda recogido en obras específicas (Tadeo, 2008; Barceló & Hennion, 1997).

Las matrices estudiadas en el Artículo científico 2 incluyen frutas y verduras, cuyo tratamiento se ha basado en el método QuEChERS. La disponibilidad de un inyector PTV (además del *splitless*) en este sistema GC-MS Shimadzu QP2010 Plus permite la inyección directa de los extractos de acetonitrilo obtenidos por QuEChERS en modo *solvent venting*, en el que se produce la eliminación selectiva del disolvente tras la inyección en frío con la válvula de *split* abierta. Así, es posible el desarrollo de métodos que combinen la sencillez y rapidez en el tratamiento de muestra con la rápida separación cromatográfica que ofrece el *fast* GC. En cuanto al uso del cuadrupolo simple en muestras complejas, se ha prestado especial atención a los factores que pueden afectar a la confirmación de los analitos detectados en las muestras.

El último trabajo comprendido en este capítulo (Artículo científico 3) pretende ampliar el campo de aplicación al análisis de zumos de frutas. Este tipo de muestra es compatible con diferentes tratamientos de extracción como SPE, SPME y QuEChERS, y dado que existen pocas referencias de aplicaciones por *fast* GC en la bibliografía, en este trabajo se persigue el desarrollo de una metodología cuyo tratamiento de muestra case con las capacidades de la cromatografía rápida para el análisis de zumos. La comparación de las tres extracciones se realiza principalmente en función de la exactitud, precisión, límites de detección (LOD) y cuantificación (LOQ), así como del tiempo de extracción. El objetivo perseguido es maximizar el rendimiento en cuanto a número de muestras a analizar por día, sin descuidar la sensibilidad final del método GC-MS para poder alcanzar los

límites establecidos por la legislación. No obstante, por tratarse de alimentos procesados, los MRLs en los zumos no siempre se encuentran bien definidos. Las concentraciones de residuos de pesticidas encontradas en zumos suelen ser menores que las detectadas en las frutas de las que proceden, debido a procesos de degradación durante su tratamiento o eliminación tras el lavado (Picó & Kozmutza, 2007). Así, los límites de residuos considerados en los zumos corresponden a los MRLs de la fruta de partida, teniendo en cuenta los factores de concentración o dilución aplicados durante el procesado (en el caso de estar disponibles).

II.2. Artículo científico 1

Multiclass determination of 66 organic micropollutants in environmental water samples by fast gas chromatography–mass spectrometry

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Multiclass determination of 66 organic micropollutants in environmental water samples by fast gas chromatography–mass spectrometry

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Abstract

A multiresidue method has been developed for quantification and identification of 66 multiclass priority organic pollutants in water by fast gas chromatography (GC) coupled to mass spectrometry (MS). Capabilities and limitations of single quadrupole mass spectrometer as detector in fast GC were studied evaluating the chromatographic responses in terms of sensitivity and chromatographic peak shapes, as they were influenced by scan time. The number of monitored ions in a selected ion monitoring (SIM) group strongly conditioned the scan time and subsequently the number of data points per peak. A compromise between peak shape and scan time was adopted in order to reach the proper conditions for quantitative analysis. An average of 10–15 points per peak was attained for most compounds, involving scan times between 0.1 and 0.22 s. The method was validated for mineral, surface, and groundwater. A solid-phase extraction pre-concentration step using C_{18} cartridges was applied. Four isotopically labeled standards were added to the samples before extraction and used as surrogates to ensure a reliable quantification. Analyses were performed by GC–MS in electron ionization mode, monitoring the three most abundant and/or specific ions for each compound and using the intensity ratios as a confirmatory parameter. With a chromatographic run of less than 10 min, SIM mode provided excellent sensitivity and identification capability due to the monitoring of three ions and the evaluation of their intensity ratio. Limits of detection below

10 ng/L were reached for most of the 66 compounds in the three matrices studied. Accuracy and precision of the method were evaluated by means of recovery experiments at two fortification levels (10 and 100 ng/L), obtaining recoveries between 70% and 120% in most cases and relative standard deviations below 20%. The possibilities of a simultaneous SIM scan method have also been explored for non-target qualitative analysis. The developed method has been applied to the analysis of surface water samples collected from the Mediterranean region of Spain.

Keywords

Pesticides; Organic pollutants; Fast gas chromatography; Mass spectrometry; Water analysis.

INTRODUCTION

The presence of organic pollutants in environmental water is related to the wide use of many synthetic products mainly in the agricultural and industrial practices, but urban wastewater can also be an important source of pollution in the aquatic environment. Although there can be hundreds of potential contaminants, only a few have been defined as priority contaminants in the framework of the Water Directive 2008/105/CE [1], and maximum allowable concentrations have been established for them in order to perform a strict control on their concentration levels. Most concentration levels regulated are over 10 ng/L; therefore, the development of highly sensitive analytical methods that ensure the reliable quantification and confirmation of the compounds in samples at the nanograms per liter level is required.

Gas chromatography coupled to mass spectrometry (GC–MS) has been widely applied for determination of semivolatile and volatile organic pollutants with satisfactory sensitivity and selectivity [2]. Single quadrupole has been commonly used [3–5], although this MS analyzer does not always ensure the sensitivity and

selectivity required for most analyte/matrix combinations. This fact has led to an increased use of ion trap detector and triple quadrupole analyzers, which allow working in tandem mass spectrometry mode (MS–MS) [6, 7]. The use of MS–MS techniques dramatically minimizes matrix interferences and chemical noise in the chromatograms, notably improving the selectivity and sensitivity [6, 7]. However, gas chromatographic runs are still long in most multiresidue multiclass analysis, even when using capillary GC instruments. Nowadays, the interest in reducing analysis time has increased, and methods able to determine as many compounds as possible in a single analysis in a short time are encouraged. The use of fast GC reveals itself as a good approach to reduce analysis time in routine analysis due to the similar or even higher separation efficiency than conventional capillary GC, the higher sensitivity and simultaneous reduction of operating cost of a GC analysis. Nevertheless, despite the benefits, the technique has not been implemented yet as a common routine analysis in analytical laboratories. Some reviews have been published in the last decade [8–11] illustrating the advantages, limitations, and practical applications, looking for the best way to speed up the GC separations. Different routes towards a faster separation have been described in the literature, but the option to be selected greatly depends on the combination of sample and analytes (number and type), on the analysis purposes, and on the application under study [12–15].

As a first approach, shortening the column length [12, 13], but maintaining the internal diameter, reduces chromatographic times but also contributes to loss in resolution. This option is only adequate for the determination of a few compounds since coelutions can become an important drawback. In this way, using faster temperature programming is sometimes better than using shorter columns [14]. Moreover, increasing the carrier gas velocity, as well as modifying pressure or flow conditions, is another option to reduce analysis time [13].

Alternatively, the use of narrow-bore columns (I.D., <0.15 mm) [15] combined with any of the previously indicated approaches restores the resolution to adequate values, allowing the determination of a larger number of compounds, but still maintaining short chromatographic runs. Several authors have also reported another

way to reduce analysis time by applying two-dimensional GC [16]. This is a powerful technique for analysis of complex matrices, but it requires more complex instrumentation.

For an effective application of fast GC, the detector has to contribute with adequate characteristics, specially related to scan speed, selectivity, and also sensitivity. The use of selective MS detection allows to speed up separations and ensures reliable quantitative and qualitative determinations. An adequate scan speed (high sampling frequency) is required in order to provide sufficient number of data points across the peak. Thus, one of the best choices is time-of-flight analyzer [17], which fits well with fast GC since it provides data acquisition rates faster than, for example, ion trap or quadrupole. Quadrupole analyzers that are typically applied in conventional GC have also been coupled to fast GC on narrow-bore capillary columns with satisfactory results. Kirchner et al. [18] studied the possibilities and limitations of single quadrupole in fast GC by the measurement of 27 n-alkanes and pesticides in a run time of less than 10 min, evaluating at the same time the limitations of the acquisition in selected ion monitoring (SIM) mode related to the quality of the spectra obtained. These authors indicate that only 11 ions could be acquired as a maximum in a SIM group looking for quantitative purposes. Discussion is only related to standard solutions, and the application to real samples is missing. Mondello et al. [19] applied fast GC–MS with satisfactory results using single quadrupole for the determination of 25 allergens in fragrances in full scan mode. They reported the use of a QP2010 Shimadzu, allowing a scan speed of 1000 amu/s, in scan mode, which is adequate when working with high concentration samples (>100 mg/L). The ultratrace analysis of 25 pesticides in non-fatty food matrices has been also performed combining fast GC–MS with negative chemical ionization, with a total analysis time of 11.45 min. The results confirm that quadrupole acquisition rates are fast enough for a proper reconstruction of the chromatographic peaks and are sensitive enough when combined with NCI mode [20].

The main objective of this paper is to study the capabilities of fast GC coupled to MS with single quadrupole analyzer, using narrow-bore capillary column, in the

field of environmental (water) analysis. The number of compounds typically included in fast GC has been notably increased, developing and validating a method for the determination of 66 organic pollutants, belonging to different chemical classes, in water samples. During optimization, special effort has been made to find a compromise between efficient chromatographic separation and short run time, still maintaining satisfactory sensitivity.

EXPERIMENTAL

Reagents

Organic pollutants investigated in this work, which included several chemical classes, are listed in **Table 1**. All pesticide standards (organochlorine (OC) and organophosphorus (OP) insecticides and herbicides) and octyl/nonyl phenols were purchased from Dr. Ehrenstorfer (Augsburg, Germany). PCB Mix 3 from Dr. Ehrenstorfer (100 μg/mL in cyclohexane) was used for single quantification of PCB congeners 28, 52, 101, 118, 138, 153, and 180. PAH Mix 9 from Dr. Ehrenstorfer (10 μg/mL in cyclohexane) provided 16 polycyclic aromatic hydrocarbons regulated by the US Environmental Protection Agency. Standards of brominated diphenyl ethers (BDEs) were purchased from Chiron (Stiklestadveien, Trondheim, Norway).

Stock standard solutions (nominal concentration of 500 μg/mL) were prepared by dissolving reference standards in acetone and stored in a freezer at −20 °C. Working standard mixtures were prepared by volume dilution of stock solutions in acetone, for sample fortification, and in hexane, for GC injection.

Table 1. List of compounds studied and GC–MS parameters used.

Peak number	t_R (min)	Time window (min) (SIM group)	Compound	Monitored ions in SIM		
				Target ion	Reference ions	Scan time (s)
1	2.900	2.3-3.2	*Naphtalene* [a]	128	102, 127	0.10
2	3.484	3.2-3.7	*Acenaphtylene* [a]	152	151, 153	0.10
3	3.554		*Acenaphthene* [a]	153	152, 154	
4	3.626		*Pentachlorobenzene* [b]	250	248, 252	
5	3.742	3.7-3.95	*4-t-Octylphenol* [c]	107	108, 206	0.12
6	3.770		*Fluorene* [a]	166	165, 167	
7	3.821		Chlorpropham [d]	127	171, 213	
8	3.833		*Trifluralin* [c]	264	290, 306	
9	4.035	3.95-4.24	alfa-HCH [c]	181	183, 219	0.22
10	4.043		*Simazine* [d]	201	173, 186	
11	4.060		*Atrazine* [d]	200	202, 215	
12	4.081		*4-n-Octylphenol* [c]	107	108, 206	
13	4.087		Hexachlorobenzene-$^{13}C_6$ *	292		
14	4.089		*Hexachlorobenzene* [b]	284	282, 286	
15	4.125		Terbuthylazine-D_5 *	219		
16	4.135		Terbuthylazine [d]	214	173, 229	
17	4.155		beta-HCH [c]	181	183, 219	
18	4.161		Propyzamide [d]	173	175, 255	
19	4.202		Lindane [c]	181	183, 219	
20	4.289	4.24-4.44	*Phenantrene* [a]	178	176, 179	0.10
21	4.318		*Anthracene* [a]	178	176, 179	
22	4.362		*4-n-Nonylphenol* [c]	107	108, 220	
23	4.452	4.44-4.65	Metribuzin [d]	198	144, 199	0.12
24	4.463		Endosulfan ether [c]	241	239, 277	
25	4.499		PCB 28 [c]	256	186, 258	
26	4.545		*Alachlor* [d]	160	146, 188	
27	4.703	4.65-4.97	PCB 52 [c]	220	290, 292	0.13
28	4.800		Metolachlor [d]	162	146, 238	
29	4.805		*Chlorpyrifos* [d]	197	199, 314	
30	4.883		*Aldrin* [c]	263	261, 293	
31	5.043	4.97-5.19	Pendimethalin [d]	252	162, 192	0.10
32	5.085		*Chlorfenvinphos* [d]	267	269, 323	
33	5.103		*Isodrin* [c]	193	195, 263	
34	5.225	5.19-5.42	*Fluoranthene* [a]	202	200, 203	0.10
35	5.337		PCB 101 [c]	326	254, 328	
36	5.448	5.42-5.81	*Pyrene* [a]	202	200, 203	0.13
37	5.455		*Endosulfan I* [c]	241	195, 339	

Table 1 (continued).

Peak number	t_R (min)	Time window (min) (SIM group)	Compound	Monitored ions in SIM		
				Target ion	Reference ions	Scan time (s)
38	5.540		*p,p'*-DDE-D_8 *	254		
39	5.558		*p,p'*-DDE [c]	246	248, 318	
40	5.667		*Dieldrin* [c]	263	79, 277	
41	5.939	5.81-6.03	*Endrin* [c]	263	261, 265	0.16
42	5.875		PCB 118 [c]	326	254, 328	
43	5.906		BDE 28 [c]	246	406, 408	
44	5.928		*p,p'*-DDD [c]	165	176, 199	
45	5.936		*Endosulfan II* [c]	195	241, 339	
46	6.050	6.03-6.36	PCB 153 [c]	360	290, 362	0.10
47	6.219		*p,p'*-DDT [c]	235	165, 237	
48	6.245		Endosulfan sulfate [c]	237	274, 387	
49	6.260		PCB 138 [c]	360	290, 362	
50	6.563	6.36-6.76	Benzo(a)anthracene-D12 *	240		0.10
51	6.581		*Benzo(a)anthracene* [a]	228	226, 229	
52	6.612		*Chrysene* [a]	228	226, 229	
53	6.617		BDE 71 [c]	326	484, 486	
54	6.659		PCB 180 [c]	324	394, 396	
55	6.691		BDE 47 [c]	326	484, 486	
56	6.781	6.76-7	BDE 66 [c]	326	484, 486	0.10
57	7.205	7-7.3	BDE 100 [c]	404	406, 564	0.10
58	7.347	7.3-7.58	BDE 99 [c]	404	406, 566	0.10
59	7.395		*Benzo(b)fluoranthene* [a]	252	126, 250	
60	7.412		*Benzo(k)fluoranthene* [a]	252	126, 250	
61	7.661	7.58-7.8	*Benzo(a)pyrene* [a]	252	126, 250	0.10
62	7.677		BDE 85 [c]	404	406, 566	
63	7.832	7.8-8	BDE 154 [c]	484	482, 486	0.10
64	8.093	8-8.35	BDE 153 [c]	484	482, 486	0.10
65	8.527	8.35-8.64	BDE 138 [c]	484	482, 486	0.10
66	8.704	8.64-8.9	*Dibenzo(a,h)anthracene* [a]	279	139	0.10
67	8.703		*Indeno (1,2,3,cd)pyrene* [a]	124	272	
68	8.979	8.9-9.6	*Benzo(g,h,i)perylene* [a]	276	138, 277	0.10
69	9.140		BDE 183 [c]	562	564, 566	
70	-		BDE 209 [c]	799	400, 487	

* ILIS used in this work.

(a), (b), (c), (d) indicates the internal standard used for quantitative purposes: (a) benzo(a)anthracene-D_{12}, (b) hexachlorobenzene-$^{13}C_6$, (c) *p,p'*-DDE-D_8, (d) terbutylazine-D_5.

Acetone (pesticide residue analysis), ethyl acetate, dichloromethane (DCM), and hexane (ultra-trace quality) were purchased from Scharlab (Barcelona, Spain).

Four isotopically labeled internal standards (ILIS) were used: *p,p′*-DDE-D_8, benzo(*a*)anthracene-D_{12}, 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 was prepared by volume dilution of individual stock solutions with hexane, for calibration preparation, and with acetone, for sample fortification, and stored at 4 °C.

Bond Elut solid-phase extraction (SPE) cartridges (500 mg C_{18}; Varian, Harbor City, CA, USA) were used for SPE.

Sample matrices

Three different water samples were used during the validation study: mineral, surface, and groundwater. Mineral water was purchased directly from a local market in Castellón (Spain), surface water samples were collected from Mijares River (Vila-Real, Castellón), and groundwater samples were collected from an irrigation well (Serra d'Irta, Castellón).

Additionally, ten surface water samples from the Spanish Mediterranean area (Tarragona) were analyzed to investigate the presence of selected organic contaminants and to test the applicability of the method.

GC instrumentation

Measurements were performed on a Shimadzu QP2010 Plus GC system equipped with an autosampler (Shimadzu AOC-5000) and coupled to a single quadrupole mass spectrometer (Shimadzu GCMS-QP2010 Plus). Compounds were separated on a SAPIENS-5MS capillary column (length 20 m × I.D. 0.10 mm × film 0.10 μm) purchased from Teknokroma. Injector was operated in splitless mode, injecting 1 μL at 320 °C; splitless time was 1 min. The oven was programmed as follows: 80 °C (1.2 min), 90 °C/min to 225 °C, 15 °C/min to 270 °C, and 150 °C/min

to 330 °C (3.4 min), resulting in a total chromatographic time of 9.6 min. Helium was used as carrier gas. A pressure pulsed injection was carried out using an initial pressure of 850 kPa (1.25 mL/min) maintained during 1.2 min and then changed to a constant flow of 0.75 mL/min (this corresponds to a linear velocity of 39 cm/s).

Mass spectrometer was operated in the electron ionization mode (70 eV). The source and the interface temperatures were adjusted to 225 °C and 300 °C, respectively. The scan time in scan mode was set initially at 0.1 s; when SIM mode was applied, scan time ranged from 0.1 to 0.22 s. A solvent delay of 1.5 min was used to prevent damage to the filament in the ion source.

Shimadzu software GCMSsolution was used through all the work to process the data automatically.

Analytical procedure

Samples were prepared by adding 1 mL of surrogate standard mixture in acetone (containing the four ILIS) to 250 mL of water. SPE cartridges were conditioned by passing 6 mL methanol, 6 mL ethyl acetate:DCM (50:50), 6 mL methanol, and 6 mL deionized water, avoiding dryness. The water sample was loaded and passed through the cartridge using vacuum. Then, the cartridge was washed with 3 mL deionized water and dried by passing air, using vacuum for at least 30 min to ensure no residual water would be eluted with the final extract. The retained analytes were eluted with 5 mL ethyl acetate:DCM (50:50). The collected extract was evaporated to dryness under a gentle nitrogen stream at 40 °C, redissolved in 0.5 mL of hexane, and injected into the GC system under the experimental conditions indicated before.

Quantification of analytes in samples was carried out from calibration curves prepared with standards in solvent also containing ILIS, using relative responses of each compound to the corresponding internal standard. The selection of the ILIS to be used for each analyte was based on their chromatographic behavior and similarity in chemical structure, and it is shown in **Table 1**.

Validation study

The developed method was validated in mineral, surface, and groundwater. The analytical parameters evaluated were linearity, accuracy, precision, limits of detection and quantification, and confirmation capability of the method for positive samples.

Linearity was studied by means of calibration curves obtained with standard solutions ($n = 3$), at eight concentration levels: 0.5, 1, 5, 10, 50, 100, 200, and 250 μg/L. Linearity was considered satisfactory when regression coefficient was higher than 0.99 and the residuals lower than 30%, and without any clear tendency in their distribution (aleatory distribution of positives and negatives).

Accuracy was estimated from recovery experiments, analyzing six replicates of the water spiked at two levels (10 and 100 ng/L). Precision was expressed as repeatability in terms of relative standard deviation (RSD, percent; $n = 6$) calculated for each fortification level.

Limit of quantification (LOQ), as the analyte concentration that produced a peak signal of ten times the background noise, was estimated from the chromatogram at the lowest fortification level tested with satisfactory recovery (70–120%) and precision (RSD, <20%). Limit of detection (LOD) was estimated in the same way but using a signal-to-noise ratio of 3.

In order to confirm peak identity in samples, the ratio between the quantification ion (target, Q) and the reference ions (q_i) was evaluated and compared with the theoretical value obtained for reference standards. The confirmation criterion is based on the European Commission Decision 2002/657/EC [*21*], which also established the maximum tolerances as a function of relative intensities. Although this Decision applies to the determination of contaminants and residues in food of animal origin, it is also widely applied in environmental pollution measurements due to the lack of guidelines in this field. Coincidence between the retention time in a sample and the corresponding standard was also required to confirm a positive finding (maximum allowed deviation, ±0.5%).

RESULTS AND DISCUSSION

GC–MS optimization

Preliminary experiments for optimization of chromatographic conditions were performed using hexane standard solutions, with the GC–MS operating in full scan mode using a 10-m × 0.1-mm I.D. GC column. Due to its short length, the chromatographic run was shorter than 6 min and showed a great number of coelutions that could not be avoided even by modifying temperature programming or by adjusting MS parameters. Problems with coelutions became even more important when the SIM mode was developed for the 66 selected compounds (and four ILIS), making an adequate quantification impossible. Therefore, a 20-m column with the same internal diameter (0.10 mm) and film thickness (0.10 μm) was considered instead, since increasing the column length should also increase the number of theoretical plates and resolution, although at the cost of higher chromatographic times.

GC parameters that could affect peak resolution and analysis time, such as initial and final temperature, linear velocity of the carrier gas, and oven temperature program, were evaluated and optimized. Early eluting compounds determined the choice of the best value for initial temperature, which was studied between 60 °C and 100 °C (the lower value selected was 60 °C since hexane was used as injection solvent). Final temperature was selected according to the behavior of the last eluting compounds, which required temperatures between 300 °C and 350 °C. The optimum results responded to a compromise between resolution, sensitivity, and peak shape. Linear velocity of the carrier gas was also optimized in order to obtain satisfactory results, testing values between 30 and 50 cm/s. Although linear velocity changes did not produce an important impact on the results, a good compromise between sensitivity and resolution was achieved at 39 cm/s, and this value was used for further experiments.

The oven temperature program was the most complex parameter to be optimized since it notably influenced analysis time and resolution. Programs with a

single temperature ramp (50 °C/min, 100 °C/min, and 120 °C/min) were tested, but they resulted in too many chromatographic coelutions that could not be overcome by adjusting MS parameters. The main goal was to achieve a rapid separation with adequate resolution. In this way, different temperature ramp rates were tested: slower rates were selected for chromatographic zones where many compounds eluted at nearly the same time and higher rates were applied to speed up the analysis time. After several experiments, the best conditions corresponded to 80 °C (1.2 min); then, a high-speed ramp rate of 90 °C/min was applied up to 225 °C to accelerate the elution of the most volatile compounds and facilitate the elution of the rest. Later, due to the large number of compounds that eluted in the next minutes, a low rate of 15 °C/min was used up to 270 °C, allowing good resolution without increasing significantly the analysis time (lower rates did not imply significant chromatographic changes, but time did increase considerably). Finally, a rate of 150 °C/min up to 330 °C was selected to speed up the elution of the less volatile compounds, which did not show coelution problems.

The last GC parameter studied was the use of pressure pulsed injection, which was found to improve the sensitivity for most compounds, as a result of a faster transfer from the injector to the column, thus allowing a very narrow initial chromatographic band. **Figure 1** shows the total ion chromatogram for a standard mixture in hexane obtained in scan mode under the optimum conditions. This chromatogram illustrates that all compounds (except BDE 209) elute in less than 10 min, with good sensitivity for most of them.

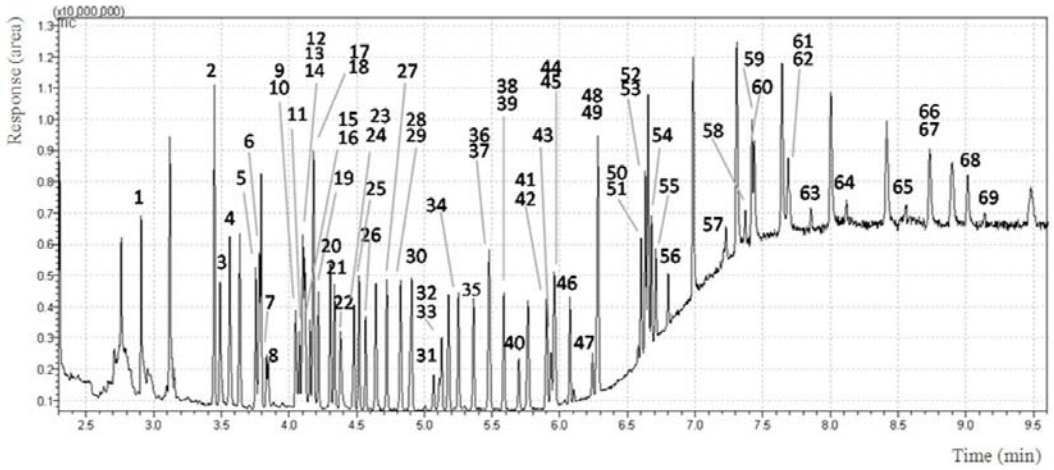

Figure 1. Full scan chromatogram of a 100-ng/mL standard mixture in hexane obtained by fast GC–MS in full scan mode.

MS parameters for scan mode were also optimized in order to obtain good peak shapes but still maintaining satisfactory sensitivity for each compound. The ion source temperature was modified between 175 °C and 225 °C, and the interface temperature between 225 °C and 300 °C to obtain the best performance. The scan time that notably affects the peak shape was tested between 0.1 and 0.3 s. As expected, a scan time of 0.1 s led to the best peak shapes with 10–15 points per peak, corresponding to a scan speed of 3333 amu/s, without a decrease in sensitivity.

Once the GC–MS conditions for scan mode were optimized, the selection of target and reference ions for each compound was carried out. Generally, the most abundant and/or characteristic ions were selected for identification and quantification of the analytes. In cases where coelutions were unavoidable, a careful selection of *m/z* values was necessary in order to use those ions that did not interfere in the quantitative determination of the coeluting analytes. Thus, in spite of the large number of compounds to be determined in a very short chromatographic time, selected analytes could be determined using the mass spectrometer capabilities. **Table 1** shows the quantitative (target) and the reference (confirmative) ions selected for each compound.

The developed scan mode method allowed the determination of all compounds, except BDE 209, in a short time (9.6 min). In order to improve method sensitivity, a SIM method was created automatically from the scan injection selecting appropriate target and reference ions (as indicated before, with three ions per compound), with some manual corrections.

Compounds had to be sorted into groups (time window) of at least 0.2 min (because this is a system restriction limiting the minimum SIM group width). Another aspect to take into account is that 64 is the maximum number of ions that system is capable of acquiring in a SIM group, so this limits the number of compounds to be included. Furthermore, in this acquisition mode, scan time depends on the number of ions included in each group, in such a way that scan time increases when the number of ions acquired increases [*22*, *23*].

The minimum number of data points required to have satisfactory chromatographic peaks for quantitative purposes has been widely discussed in the literature [*24–26*]. A number of eight to ten data points per peak (including the baseline points) are commonly accepted for a satisfactory peak reconstruction and quantification [*18*, *27*]. The convenience of "collecting as many points across the peak as possible to meet quantitative and qualitative needs of the application" has also been reported [*28*].

Considering that the method proposed in this work strongly emphasizes the mass spectrometer aspect, including the large number of compounds that can be determined in a very short analysis time, it is necessary to study the maximum number of ions that can be included in a SIM group without degrading peak shape more than it is acceptable. In this way, several chromatographic methods were prepared including a SIM group with a variable number of ions monitored, from 9 to 52. In order to determine peak quality, a model compound (mevinphos) was selected and its extracted ion chromatogram (*m/z* 127) obtained after each injection (**Figure 2**).

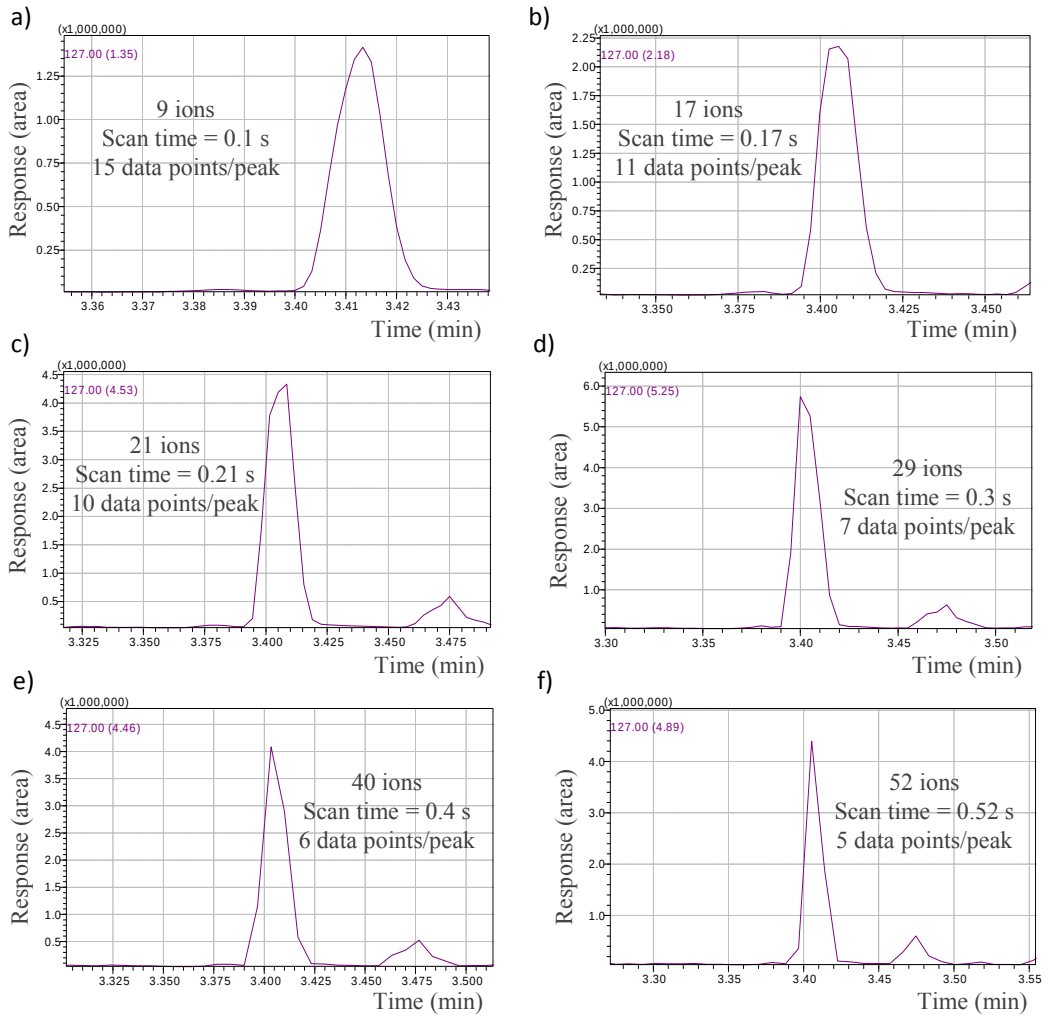

Figure 2. Effect of scan time over peak shape in SIM mode. Peak shape of the pesticide mevinphos when included in a SIM group where 9, 17, 21, 29, 40, or 52 ions were monitored.

As can be seen, when nine ions were monitored in the SIM group (a total of three compounds), it was possible to select a scan time of 0.1 s (giving a total of 15 points per peak). When the number of ions increased, the scan time also increased resulting in fewer data points for mevinphos peak. From the results obtained (as depicted in **Figure 2**), it is concluded that, with our mass spectrometer system, a maximum of 20 ions should be included in a SIM group (corresponding to a scan time of 0.2 s) to obtain satisfactory peak shape.

The number of compounds to be detected in a given (short) time and resolution between peaks determines the number of SIM groups. If the number of compounds in a SIM group exceeds the maximum number of ions recommended before, two approaches in method design can be considered. In the first approach, monitoring only one or two ions per compound should maintain the number of compounds per SIM group without losing satisfactory peak shape. This approach has not been considered in the present work, as adequate confirmatory capabilities of the method have to be achieved, being necessary to record at least three ions per compound as indicated in the Directive 96/23/CE [*21*]. The second approach relies in reducing the SIM group width (time), increasing the total number of SIM groups with less compounds in each one. This is not an easy task, as in many cases, there are no gaps between compounds to establish the cut of the SIM group, making it necessary to sacrifice one or more compounds that elute in the group change zone.

These limitations forced a compromise to be reached between peak shape, number of compounds, and analysis time. In the present work, most SIM groups (of a total of 22 groups) contained less than 15 ions, enabling the attainment of 10–15 points per peak. Only one SIM group included 20 ions (see **Table 1**; time window, 3.95–4.24 min), resulting in seven to ten data points per peak depending on the analyte peak width, which is in the limit of acceptability for satisfactory peak shape. Even in this unfavorable case, the quantification of these compounds was likewise satisfactory, as supported by the validation data.

Once an adequate SIM mode quantitative method was developed for the 66 studied compounds, the possibility of simultaneous scan and SIM mode was tested in order to fully exploit the capabilities of our GC–MS instrument. This option, available in this equipment, seems useful for screening purposes. Working under this mode, selected analytes can be quantified (target analysis in SIM mode), while simultaneous full scan acquisition allows the identification of unknown analytes (non-target analysis in scan mode).

Thus, three SIM scan methods were prepared, monitoring different number of target analytes (SIM) in each method, in all cases including a simultaneous scan

event in the 70–500 *m/z* range. For this purpose, five surface water samples, fortified at 0.1 μg/L with all the 66 target analytes, were analyzed using the three SIM scan methods. The first conclusion is that when performing a scan event simultaneously to SIM detection, a decrease in the number of points per peak occurs; thus, the number of compounds included in the SIM group was decreased from the initially selected 66 down to 33 (all priority pollutants considered in the Directive 2008/105/CE [*1*]) or to 10.

When only ten compounds (30 ions distributed in eight SIM groups) were included in the SIM acquisition, the chromatographic peak shapes were satisfactory, achieving eight to ten points per peak. However, even under these conditions, two analytes could not be satisfactorily quantified (recoveries around 50%), as they were in the limit of acceptability for satisfactory peak shape. As it seems reasonable to develop a method for the determination of the regulated contaminants, the 33 priority contaminants studied in this work (shown in italic, **Table 1**) were included in a second SIM scan method. As expected, a notable reduction in the number of points per peak was observed, and around 20 compounds could not be properly quantified (recoveries around or below 50%) since they presented four to five points per peak.

On the other hand, scan spectra obtained by the three SIM scan methods were compared, without observing notable differences among them. Although scan acquisition was also affected by the increment of the scan time in the simultaneous mode, a non-target screening in the SIM scan method can be performed in parallel to the quantification of the target analytes for which the method was satisfactory. In this way, when performing an automatic search of the scan data, some of the spiked compounds could be identified (by library match) even at the relatively low concentration 0.1 μg/L (50 pg injected). This means that non-target scan (in a SIM scan method) can be easily applied for those compounds chromatographically separated and at relatively high concentrations (above 0.5 μg/L, 250 pg injected).

As satisfactory quantification of target analytes under SIM mode strongly depends on the number of data points per peak, it is concluded that the best results

were obtained from single scan and SIM injections, rendering the acquisition under simultaneous SIM scan mode futile.

SPE procedure

The SPE step applied in this work is based on previous work performed at our laboratory for the determination of organic micropollutants in water [*29*]. Using SPE with the well-known C_{18} cartridges is widely accepted and commonly applied for organic contaminants that are GC-amenable [*29–32*]. This step was used under the experimental conditions applied at our laboratory [*29*] without further optimization.

Ethyl acetate:DCM (50:50) was chosen as the elution solvent (5 mL). Two hundred fifty milliliters of water sample was pre-concentrated to a final extract volume of 0.5 mL. The 500-fold concentration factor allowed reaching the required sensitivity for determination of the selected analytes at the sub-parts per billion levels.

Analytical parameters

Validation of the method was carried out in terms of accuracy, precision, LODs and LOQs, as well as confirmation criteria for compound identity. These parameters were evaluated in three different types of water. All the samples were fortified at two levels, using four ILIS added before the SPE procedure (surrogate standards), to correct possible losses along the overall procedure and/or instrumental deviations. The selection of the internal standards was based on our previous experience on their extraction and chromatographic behavior [*29*]. HCB-$^{13}C_6$ was used as internal standard for pentachlorobenzene and HCB; terbutylazine-D_5 for herbicides and OP insecticides; DDE-D_8 for octyl/nonyl phenols, BDEs, PCBs, and OC pesticides; and benzo(*a*)anthracene-D_{12} for PAHs. The internal standard applied for each individual compound is shown in **Table 1**.

Linearity was evaluated with pure solvent standard solutions also containing the internal standards, so relative responses were used; each concentration level was

injected in triplicate. For the most sensitive compounds, like PCBs, trifluralin, metolachlor, or chlorpyrifos, the concentration range studied was 0.5–250 μg/L. For chlorfenvinphos, endosulfan I and II, endrin, endosulfan sulfate, dibenzo(*a*,*h*)anthracene, and indeno(1,2,3-*cd*)pyrene, it was 10–250 μg/L, and for BDE 183, 50–250 μg/L. The regression coefficients were higher than 0.99 for all compounds (ranging from 0.9913 for endrin to 0.9999 for aldrin) over the whole range tested and the residuals lower than 30%. BDE 209 could not be measured with adequate sensitivity, so this analyte could not be validated at realistic environmental levels since the selected chromatographic conditions were not appropriate for this compound. As discussed in the literature [*33*], shorter columns and higher final temperatures would be recommended for the determination of this BDE.

Precision and accuracy were evaluated by means of recovery experiments ($n = 6$) of samples fortified at 10 and 100 ng/L. **Table 2** shows the results obtained for the three types of water tested. Most compounds presented recoveries between 70% and 120% at both spiking levels in all matrices. Some compounds presented recoveries over 120% at the lowest level in the three matrices (as BDE 28, 71, 47, and 66), but recoveries were in all cases satisfactory at the highest level. Furthermore, some other compounds could not be quantified at the lowest level in none of the matrices due to their low sensitivity. The two more volatile PAHs, naphthalene and acenaphthylene, and also PAHs like dibenzo(*a*,*h*)anthracene and indeno(1,2,3-*cd*)pyrene were poorly recovered in groundwater and surface water, which is in compliance with the literature [*32*], so the method was not fully satisfactory for these compounds. Other remarkable cases were aldrin and isodrin, whose recoveries at the highest level were lower than 60%, probably due to an inappropriate correction from the ILIS used.

RSD lower than 20% were obtained for most compounds and only in some specific cases, like chlorfenvinphos in mineral water or 4-*t*-octylphenol in surface water, slightly higher values were obtained, although still with satisfactory recoveries. Several problematic analytes, whose recoveries were unsatisfactory, also presented

poor precision with high RSD, sometimes nearly 50%, indicating that the method did not properly work for these few compounds.

LOQs were typically in the range 0.2–20 ng/L, with exceptions like chlorpropham (in surface and groundwater) and endosulfan sulfate, beta-HCH, metribuzin, chlorpyrifos, endrin, endosulfan II and *p,p′*-DDT (in groundwater) that presented values ≥40 ng/L. Low LOD values, between 0.1 and 10 ng/L, were reached for the majority of the compounds. In general, the values obtained for both LODs and LOQs were rather similar in the three types of water tested, although, as expected, in some particular cases, they were slightly higher in surface and groundwater due to the higher complexity of the matrix (e.g., chlorpropham, beta-HCH, metribuzin, endrin, or endosulfan II). These LOQ and LOD values are in the same order than those reported in the literature for most of the compounds studied under similar conditions [*20*], and even for analytes determined by conventional GC in MS–MS [*34*], so this data show the extensive quantitative capabilities of single quadrupole in fast GC.

Table 2. Average recovery (percent) and RSD (in parenthesis) obtained after the application of the GC-MS method to mineral, ground and surface water samples (n=6) fortified at two concentration levels.

Compounds	Mineral water				Groundwater				Surface water			
	Fortification levels (ng/L)		LOD (ng/L)	LOQ (ng/L)	Fortification levels (ng/L)		LOD (ng/L)	LOQ (ng/L)	Fortification levels (ng/L)		LOD (ng/L)	LOQ (ng/L)
	10	100			10	100			10	100		
Naphthalene	89 (14)	70 (11)	0.1	0.3	101 (4)	39 (14)	0.2	0.8	52 (30)	14 (0)	0.4	n.e.
Acenaphthylene	94 (2)	13 (28)	1	4	30 (12)	26 (18)	0.1	n.e.	60 (12)	15 (43)	0.1	n.e.
Acenaphthene	101 (18)	78 (18)	0.1	0.4	92 (20)	65 (14)	0.6	3	88 (19)	38 (35)	0.,5	2
Pentachlorobenzene	86 (5)	88 (4)	0.1	0.3	92 (9)	83 (11)	0.1	0.2	89 (6)	76 (20)	0.2	0.6
4-*t*-Octylphenol	124 (2)	113 (10)	5	10	79 (9)	83 (4)	0.6	2	102 (25)	83 (8)	2	7
Fluorene	51 (25)	74 (17)	0.5	2	74 (19)	65 (13)	0.4	2	69 (11)	38 (49)	0.2	0.6
Chlorpropham	-	100 (8)	10	15	-	67 (8)	32	100	-	71 (3)	17	60
Trifluralin	101 (11)	80 (8)	0.7	3	95 (21)	78 (4)	0.6	2	74 (14)	66 (11)	0.4	2
alpha-HCH	78 (8)	131 (5)	2	5	89 (21)	93 (7)	2	6	108 (16)	82 (16)	3	9
Simazine	-	79 (13)	10	15	107 (12)	76 (6)	2	6	120 (5)	64 (6)	2	4
Atrazine	105 (12)	74 (13)	0.8	3	97 (13)	77 (6)	0.1	0.5	95 (8)	67 (8)	0.4	2
4-*n*-Octylphenol	26 (30)	91 (11)	5	15	95 (14)	67 (13)	2	6	99 (10)	74 (9)	3	9
Hexachlorobenzene	69 (10)	109 (3)	0.1	0.2	109 (6)	109 (1)	0.1	0.3	101 (3)	108 (4)	0.1	0.5
Terbuthylazine	104 (13)	72 (14)	0.5	2	85 (18)	74 (9)	0.2	0.8	97 (10)	67 (7)	0.4	2
beta-HCH	-	119 (4)	10	15	-	108 (7)	19	64	-	109 (8)	10	26
Propyzamide	80 (20)	73 (12)	2	6	89 (17)	43 (19)	2	6	117 (5)	64 (4)	2	4
Lindane	113 (14)	117 (7)	3	9	112 (18)	90 (4)	2	6	95 (12)	85 (12)	2	6
Phenanthrene	72 (17)	71 (5)	0.1	0.5	96 (14)	71 (19)	0.1	0.3	66 (20)	68 (22)	0.1	0.3
Anthracene	70 (11)	69 (20)	0.2	0.6	75 (20)	77 (14)	2	5	68 (13)	63 (19)	0.2	0.8
4-*n*-Nonylphenol	102 (14)	76 (8)	1	4	31 (25)	48 (14)	0.3	n.e.	94 (7)	80 (10)	0.6	3
Metribuzin	-	66 (11)	10	15	-	70 (9)	27	90	-	135 (18)	10	n.e.
Endosulfan ether	170 (5)	119 (5)	2	5	156 (9)	90 (5)	9	30	92 (22)	87 (12)	2	6
PCB 28	101 (8)	89 (8)	0.2	0.8	92 (17)	83 (10)	0.2	0.6	87 (6)	74 (15)	0.1	0.5
Alachlor	109 (17)	76 (12)	2	6	93 (18)	75 (10)	2	6	84 (12)	63 (4)	2	6
PCB 52	105 (10)	97 (10)	0.3	0.9	101 (11)	88 (10)	0.3	1	86 (8)	85 (12)	0.3	2
Metolachlor	68 (24)	72 (9)	0.6	2	63 (21)	75 (11)	0.3	1	105 (6)	70 (6)	0.3	2
Chlorpyrifos	-	87 (10)	10	19	-	72 (6)	13	44	-	76 (7)	10	15
Aldrin	95 (6)	53 (7)	0.6	3	87 (11)	56 (7)	0.6	2	79 (8)	48 (5)	0.6	2
Pendimethalin	-	67 (10)	10	15	-	69 (9)	10	22	-	50 (10)	10	n.e.
Chlorfenvinphos	-	110 (24)	10	15	-	100 (8)	10	23	-	99 (12)	10	15
Isodrin	70 (9)	50 (11)	2	6	72 (16)	60 (10)	2	5	71 (9)	41 (6)	2	6
Fluoranthene	106 (11)	82 (19)	0.1	0.3	108 (5)	93 (10)	0.1	0.3	93 (5)	75 (19)	0.1	0.4
PCB 101	103 (8)	83 (7)	0.1	0.5	104 (4)	79 (6)	0.1	0.4	96 (4)	79 (4)	0.1	0.4
Pyrene	99 (13)	86 (17)	0.1	0.4	79 (8)	80 (12)	0.1	0.4	95 (5)	82 (19)	0.1	0.5

Table 2 (continued).

Compounds	Mineral water				Groundwater				Surface water			
	Fortification levels (ng/L)		LOD (ng/L)	LOQ (ng/L)	Fortification levels (ng/L)		LOD (ng/L)	LOQ (ng/L)	Fortification levels (ng/L)		LOD (ng/L)	LOQ (ng/L)
	10	100			10	100			10	100		
Endosulfan I	-	112 (6)	10	15	-	98 (5)	10	28	-	97 (9)	10	31
p,p'-DDE	120 (14)	99 (8)	0.2	0.8	113 (3)	95 (2)	0.2	0.8	107 (3)	98 (3)	0.2	0.8
Dieldrin	-	105 (5)	10	15	-	100 (6)	10	33	-	97 (6)	10	8
Endrin	-	115 (10)	10	29	-	108 (9)	33	100	-	115 (9)	10	21
PCB 118	114 (7)	90 (5)	0.3	0.9	118 (2)	82 (3)	0.3	2	104 (5)	87 (2)	0.2	0.8
BDE 28	154 (7)	96 (4)	2	5	130 (5)	104 (6)	1	3	136 (2)	101 (2)	1	3
p,p'-DDD	104 (14)	94 (7)	2	5	126 (6)	102 (2)	10	16	115 (6)	96 (6)	3	9
Endosulfan II	-	115 (11)	10	15	-	104 (7)	21	70	-	113 (6)	10	20
PCB 153	112 (6)	80 (5)	0.2	0.6	111 (4)	78 (4)	0.1	0.4	116 (4)	120 (10)	0.1	0.5
p,p'-DDT	-	109 (9)	10	15	-	118 (6)	18	59	-	119 (15)	10	16
Endosulfan sulfate	-	129 (26)	17	n.e.	-	121 (2)	52	172	-	120 (7)	10	24
PCB 138	102 (10)	87 (5)	1	3	111 (14)	84 (5)	1	3	112 (7)	86 (8)	1	3
Benzo(*a*)anthracene	124 (6)	96 (3)	0.5	2	112 (3)	99 (2)	0.4	2	104 (2)	95 (2)	0.2	0.7
Chrysene	134 (12)	91 (3)	0.5	2	107 (4)	99 (4)	0.3	1	101 (3)	92 (7)	0.5	2
BDE 71	131 (3)	102 (6)	1	3	143 (12)	103 (6)	0.7	3	149 (5)	105 (2)	0.8	3
PCB 180	139 (8)	106 (7)	1	3	128 (9)	97 (9)	1	3	116 (11)	102 (14)	1	3
BDE 47	157 (7)	109 (7)	0.8	3	154 (19)	108 (6)	2	6	204 (4)	113 (8)	2	6
BDE 66	120 (7)	104 (7)	0.9	3	153 (14)	100 (10)	0.7	3	176 (4)	114 (3)	0.6	3
BDE 100	94 (14)	101 (7)	1	4	136 (17)	98 (6)	2	6	137 (4)	105 (6)	2	6
BDE 99	-	102 (8)	10	15	-	95 (14)	10	15	-	112 (5)	10	14
Benzo(*b*)fluoranthene	171 (2)	92 (2)	2	6	117 (7)	88 (8)	2	6	124 (4)	93 (6)	2	6
Benzo(*k*)fluoranthene	198 (9)	92 (4)	2	6	115 (7)	87 (10)	2	6	104 (8)	89 (5)	2	6
Benzo(*a*)pyrene	107 (13)	79 (8)	2	6	114 (8)	80 (9)	2	6	106 (7)	78 (11)	2	6
BDE 85	-	96 (9)	10	15	-	98 (15)	10	17	-	105 (7)	10	17
BDE 154	88 (18)	85 (12)	2	4	-	118 (13)	10	15	-	94 (10)	10	15
BDE 153	147 (13)	77 (15)	2	5	-	105 (3)	10	15	-	89 (8)	10	15
BDE 138	-	58 (27)	10	n.e.	-	103 (12)	10	17	-	82 (11)	10	18
Dibenzo(*a,h*)anthracene	-	106 (11)	10	15	-	59 (50)	10	n.e.	-	55 (26)	10	n.e.
Indeno(1,2,3-*cd*)pyrene	-	94 (11)	10	30	-	46 (35)	10	n.e.	-	28 (64)	10	n.e.
Benzo(*g,h,i*)perylene	83(17)	103 (8)	5	15	115 (14)	75 (19)	5	15	152 (6)	80 (10)	10	16
BDE 183	-	-	-	n.e.	-	-	-	n.e.	-	-	-	n.e.
BDE 209	-	-	-	n.e.	-	-	-	n.e.	-	-	-	n.e.

Underlined, not acceptable results.

n.e., LOQ not estimated as validation parameters at both fortification levels were not satisfactory.

Detection (LOD) and quantification (LOQ) limits.

Confirmation of the identity of compounds in the sample was based on acquisition of specific ions under SIM mode. The general criterion was the acquisition of three ions (target (Q) and two reference ions (q_i)) leading to the presence of the three corresponding chromatographic peaks, together with the retention time of the reference standard. Ideally, the comparison of Q/q ratios measured in samples with those measured from reference standard shall lie within the maximum permitted tolerances [*21*]. Acquiring three ions means that two Q/q ratios are available. Our experience was that the achievement of the two Q/q ratios was rather exceptional, even when performing experiments with a "clean" matrix, like mineral water. It was especially difficult at the low concentration level tested, where the lower abundance of the ions can alter the expected Q/q ratios. According to the literature [*24*] and to our own data, a low number of data points per peak also makes more difficult to get ion ratios within the permitted tolerances. Therefore, a more realistic criterion was applied for confirmation: three ions monitored were observed in the sample, and at least one ion ratio was fulfilled (including retention time agreement).

Matrix effects were also checked comparing responses of standards prepared in hexane and in sample extracts at different concentration levels. Since no severe matrix effects were observed, quantification of samples was carried out using calibration curves prepared in solvent for all water samples analyzed. **Figure 3** shows illustrative chromatograms for metolachlor, taken as an example.

It is noteworthy the interest of determining 66 compounds in a chromatographic run time as short as 10 min. Most reported methods dealing with fast GC limit the scope to a reduced number of compounds, determining around 30 or less analytes in 10 min [*3*, *18*, *20*, *35*]. In this way, our method has shown that it is possible to determine a higher number of compounds in the same analysis time using a single quadrupole. Taking into account the limitations of this analyzer, mentioned above, it is possible to increase the number of analytes in a fast single run if they are selected to be separated within the appropriate gaps for changing SIM groups.

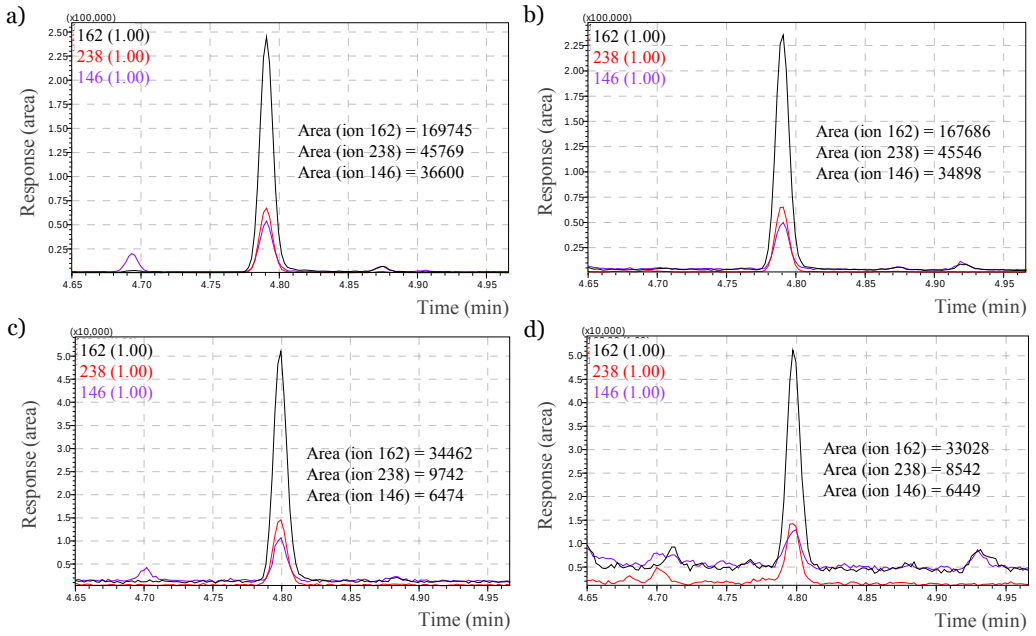

Figure 3. Matrix effect. Comparison of metolachlor responses in (**a**) standard solution in hexane at 50 µg/L and (**b**) surface water matrix-matched standard at 50 µg/L; (**c**) standard solution in hexane at 5 µg/L and (**d**) surface water matrix-matched standard at 5 µg/L.

Application to the analysis of water samples

Ten surface waters collected in the Spanish Mediterranean area (Tarragona province) were analyzed in order to test the applicability of the developed method.

The herbicides terbutylazine, propyzamide, and metolachlor and the PAHs phenanthrene and pyrene were detected in all the ten samples at levels higher than their respective LOQs. Terbutylazine was found at concentration levels between 30 and 70 ng/L, higher than those for propyzamide or metolachlor, which generally were around 10 ng/L, except in one sample that contained 340 ng/L of metolachlor. These herbicides are used on rice crops, which are predominant in the area under study. Phenanthrene and pyrene presented lower levels (in the range of 0.5–3 ng/L), close to the LOQs. Fluoranthene was occasionally detected but at low levels, sometimes below the LOQ. The persistent organic pollutant 4-*n*-nonylphenol (between 5 and

15 ng/L) was also frequently detected. A few samples gave positive findings for the herbicide atrazine (two samples, 10 ng/L) and the OP insecticide chlorpyrifos (one sample, 24 ng/L). Naphthalene and indeno(1,2,3-*cd*)pyrene were found in most samples, but they could not be quantified since this method could not be validated for these analytes in surface water. None of the compounds detected exceeded the maximum allowable concentration established for surface waters [*1*].

Confirmation of a positive finding requires reproducible retention times, the presence of the three monitored ions, and at least one Q/q ratio within the allowed tolerance. As an example of worse case situations, **Figure 4a** shows the ion chromatograms for metolachlor and phenanthrene, where retention time is an important factor for confirmation, as only two of the three ions are clearly detected and only one of the Q/q ratios is accomplished. Other analytes, shown in **Figure 4b**, were satisfactorily identified, as the sensitivity for all m/z ions was good enough due to the higher concentrations found in the samples. The main drawback derives from the fact that, in some particular cases, the selected reference ions showed low sensitivity. This led to the non-compliance of the Q/q ratios, or even to the absence of some of the reference ions. This problem could be surely avoided with more sensitive detectors or applying sample preparation procedures with a higher pre-concentration factor.

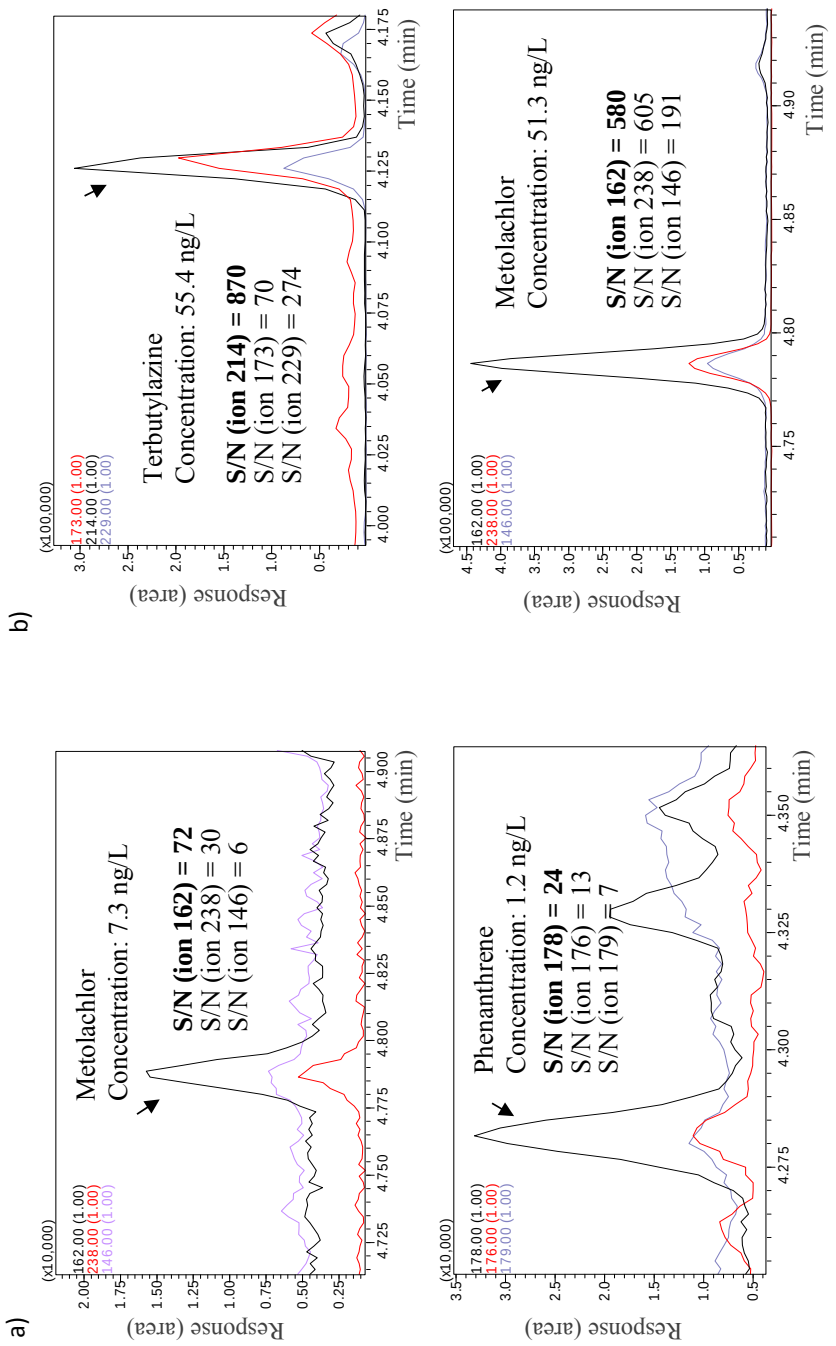

Figure 4. Typical chromatograms obtained after SPE and fast GC–MS applied to surface water samples quantified at (**a**) low concentration levels and (**b**) higher concentration levels. Signal-to-noise ratio (S/N) has been calculated for target (*bold letter*) and reference ions.

CONCLUSIONS

Capabilities and limitations of fast GC–MS for the determination of organic micropollutants in water have been studied in this paper. A multiresidue method has been developed for the rapid determination of around 60 compounds in water samples with a chromatographic run of less than 10 min, based on a compromise between analysis time and resolution. The operating SIM mode provided good sensitivity, making the quantification feasible at levels as low as 0.01 μg/L, which was the lowest concentration level validated. The presence of the three ions monitored for each compound and the compliance of, at least, one intensity ratio was used as confirmatory parameter. The variability of ion intensities observed in replicate injections of standards was obviously higher in sample extracts, especially at low analyte concentrations, making difficult the compliance of the two intensity ratios available in spiked samples. Agreement of retention time between standard and sample was also required for confirmation.

The scan time was the parameter that mostly limited the SIM mode, so it was thoroughly studied. Scan time, which depends on the number of ions in a SIM group, affects the chromatographic analyte peak shape. A value of 0.2 s was established as the maximum scan time that allowed the acquisition of sufficient number of data points per peak to perform the quantitative analysis of the 66 compounds selected.

A simultaneous scan and SIM mode was also optimized in order to test the capabilities of the mass spectrometer, but a loss of sensitivity in comparison with the SIM mode was noticed. Moreover, the extension of the mass range acquired in this mode increased the scan time and, consequently, the number of data points per peak was reduced. In this way, the SIM scan mode would be efficiently applied only for a low number of target analytes, still maintaining short chromatographic run times, but requiring higher analyte concentrations than in SIM mode. The main advantage is the possibility of performing a non-target analysis at high concentration levels simultaneously with target analysis in SIM mode of a limited number of compounds.

Analysis of surface water samples showed the presence of several target pollutants in the samples, and the most frequently found were terbutylazine, propyzamide, metolachlor, phenanthrene, and pyrene.

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II.3. Artículo científico 2

Application of fast gas chromatography-mass spectrometry in combination with the QuEChERS method for the determination of pesticide residues in fuits and vegetables

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Application of fast gas chromatography-mass spectrometry in combination with the QuEChERS method for the determination of pesticide residues in fruits and vegetables

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Abstract

A fast gas chromatography–mass spectrometry method has been developed for multiresidue determination of up to 56 pesticides in fruits and vegetables in a chromatographic run time of <10 min, using a single quadrupole mass spectrometer operating in selected ion monitoring mode. The well-known acetate-buffering version of the QuEChERS method has been used for sample preparation. Programmable temperature vaporizer injection of 3 μL allowed reaching limits of detection between 0.15 and 15 μg/kg for most compounds in the sample matrices tested. The applicability of the method has been evaluated in apple, orange, carrot and tomato. Recoveries at three fortification levels (0.01, 0.1 and 0.5 mg/kg) ranged from 70 to 120 % for most compounds, with relative standard deviations below 20% in all cases. The developed method has been applied to fruit and vegetable samples from different Spanish provinces.

Keywords

Pesticides; Fast gas chromatography-mass spectrometry; Fruits and vegetables; Matrix effects; QuEChERS.

INTRODUCTION

An extensive range of pesticides is widely used nowadays to protect crops from pests. Pesticide residues are strictly controlled by legislation in order to prevent water and food contamination and avoid unnecessary risks for animals and human beings. The European Commission (2005) has set harmonized maximum residue levels (MRLs) so as to regulate pesticides in food and assure food safety. Sensitive analytical techniques are required to verify MRLs accomplishment due to the low concentrations (e.g., at the micrograms per kilogram level) allowed by the legislation.

Many multiresidue gas chromatography–mass spectrometry (GC-MS) methods have been reported in the literature in different food commodities (Sandra et al. 2003; Mezcua et al. 2009), most of them using a single quadrupole mass spectrometer as analyzer. In the last years, tandem mass spectrometry (MS/MS) has emerged as a very interesting approach since it allows minimizing matrix interferences and chemical noise in the chromatograms, improving selectivity and sensitivity. Numerous applications based on GC-MS/MS have been reported for the determination of multiclass pesticides using triple quadrupole (Cervera et al. 2010; Frenich et al. 2005; Medina et al. 2009) or ion trap detector analyzers (González-Rodríguez et al. 2008; Zhang et al. 2008), some of them including around or more than 100 target analytes. In parallel to the interest of increasing the number of compounds in a single chromatographic run, there is a trend to decrease chromatographic run times in multiresidue analysis. At this point, the use of fast GC becomes an attractive approach since it allows an important reduction of analysis time (Dömötörová and Matisová 2008; Kirchner et al. 2005). The relevance of fast GC also comes from the attainment of better instrumental sensitivity, comparable in some cases to that reached with analyzers working under MS/MS mode. Thus, in a previous study (Cherta et al. 2012), low limits of quantification (LOQs) were achieved for pesticides and other organic pollutants in water matrices working with fast GC-MS using single quadrupole. A challenge would be to demonstrate the same capabilities for more complex matrices as fruits and vegetables. It is well known that limitations of single quadrupole are more evident when complex matrices are

analyzed, especially due to the matrix effects (Hercegová et al. 2010). In this way, not only adequate chromatographic resolution is required but also an efficient separation of the analytes from the matrix components. An effective sample treatment is also necessary to facilitate the subsequent GC analysis. Nowadays, the trend is to head towards fast and simple approaches, as the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method, which was first developed by Anastassiades et al. (2003). Since then, it has been successfully implemented for a wide range of commodities in many routine laboratories and has been subjected to several changes to expand the method capabilities and applications.

The original QuEChERS method was designed to allow the extraction of pesticide residues in fruits and vegetables with high percentage of water. It was based on solvent extraction carried out with acetonitrile (MeCN) and subsequent cleanup based on dispersive solid-phase extraction (d-SPE) using a primary–secondary amine (PSA) sorbent and anhydrous $MgSO_4$ to remove water. Later, two remarkable modifications of the original unbuffered method have been reported. These modifications have been adopted as the Association of Analytical Communities (AOAC) Official Method 2007.01 (Lehotay et al. 2005 a), which uses strong acetate buffering (pH 4.8), and the European Committee for Standardization (CEN) Standard Method EN 15662 (Payá et al. 2007), which uses a weaker citrate buffering (pH 5–5.5). Both approaches pursue modifying buffering conditions, since the unbuffered original method had negative effects on some pH-dependent pesticides. The AOAC method also includes the use of sorbents, such as C_{18} or graphitized carbon black (GCB) for fatty and pigmented foods, respectively, in order to improve the cleanup procedure. In the CEN version, disodium hydrogen citrate sesquihydrate and trisodium citrate dihydrate are also used in the extraction step (Payá et al. 2007; Camino-Sánchez et al. 2011). From the results published, it can be concluded that QuEChERS is a very flexible procedure that can be used as a template for adapting the method to analytes under study, matrix composition, analytical instruments, and analyst preferences (Lehotay et al. 2010). The use of MeCN as extraction solvent and its direct injection can be a drawback for splitless injection in GC due to its large expansion volume in the glass-liner, low volatility, or coextracted water presence. The

use of programmable temperature vaporizer (PTV) injection becomes an interesting alternative that has received much attention when dealing with injection of MeCN extracts in GC. Another option is to make a solvent exchange into toluene (Zhou et al. 2011), ethyl acetate (Shi et al. 2010), or cyclohexane (Moreno et al. 2008), which also allows concentrating the final extract, compensating one of the disadvantages of QuEChERS, the absence of an extract pre-concentration step. The use of other solvents, such as ethyl acetate or acetone as extractants, has been tested (Lehotay et al. 2010; Cunha et al. 2007), but MeCN still remains as the priority solvent for the QuEChERS procedure. The combination of MeCN as extractant combined with PTV injection has allowed reaching low limits of determination when combined with fast GC techniques (Hada et al. 2000; Hercegová et al. 2005; Korenková et al. 2003).

As it can be seen in the literature, only a limited number of studies have been reported about the combination of QuEChERS with fast GC for the determination of pesticide residues. Thus, Húšková et al. (2008) reported the determination of 61 pesticides in apple in a total chromatographic run time of 11 min, demonstrating the possibilities of the single quadrupole as analyzer in fast GC. The separation of 18 pesticides in apple was achieved in 10 min working with single quadrupole, and the performance of analyte protectants compared with matrix-matched standards after the application of QuEChERS was studied (Kirchner et al. 2008). In that work, only apple was selected as the matrix under study. In another paper, 20 pesticides were determined in baby food using GC-MS with single quadrupole in 8 min, testing the capabilities of the QuEChERS procedure versus other sample preparation methods (Hercegová et al. 2006). The possibility of working in negative chemical ionization mode was also tested for the determination of 25 pesticides in fruits and vegetables treated by QuEChERS with a chromatographic run time of only 11 min (Húšková et al. 2009). In general terms, several fast GC methods have been developed and validated for pesticides but, in most cases, for quite a limited number of compounds and sample matrices. Moreover, detailed studies of matrix effects when using single quadrupole are not frequent (Hajšlová and Zrostlíková 2003; Poole 2007).

In the present work, QuEChERS (based on the AOAC Official Method 2007.01) has been applied for the extraction of 56 pesticides from 5 fruit and vegetable sample matrices. Subsequent determination has been made by fast GC-MS using single quadrupole as analyzer. The appropriate selection of target and reference ions for each analyte in each type of matrix has allowed the detection and quantification of most compounds (between 45 and 52 depending on the matrix and the concentration level) with satisfactory sensitivity. Single quadrupole provided sufficient fast data acquisition rates, so that an efficient determination of the analytes was achieved in short chromatographic time. Advantages of fast GC-MS and limitations of single quadrupole in the analysis of complex matrices have been also discussed, including the relevant aspect of confirmation of the analytes detected in samples.

EXPERIMENTAL

Reagents

The pesticides investigated in this work are listed in **Table 1**. Reference standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock standard solutions (around 500 µg/mL) were prepared by dissolving reference standards in acetone and stored in a freezer at −20 °C. Working standard mixtures for sample fortification and GC injection were prepared by dilution of stock solutions in acetonitrile.

Acetone, acetonitrile (MeCN), glacial acetic acid (HAc), anhydrous $MgSO_4$ and anhydrous sodium acetate (NaAc) were purchased from Scharlab (Barcelona, Spain). All solvents were for pesticide residue analysis or were high-performance liquid chromatography grade. Two types of 2 mL microcentrifuge tubes for QuEChERS d-SPE containing 50 mg PSA and 150 mg anhydrous $MgSO_4$ or 50 mg PSA, 150 mg anhydrous $MgSO_4$ and 50 mg C_{18} were obtained from Teknokroma (Barcelona, Spain).

Table 1. List of pesticides studied and experimental conditions of the optimized GC-MS method.

Peak number	t_R (min)	Window (min)	Compounds	Monitored ions under SIM mode		
				Target ion	Reference ions	Scan time (s)
1	3.757	3.6-3.8	Dichlorvos [a]	185	109, 187	0.10
2	4.567	3.8-4.63	Chlorpropham [a]	213	127, 154	0.10
3	4.587		Trifluralin [b]	264	290, 306	
4	4.704	4.63-4.85	Phorate [a]	260	121, 231	0.13
5	4.777		alpha-HCH [b]	219	181, 217	
6	4.817		Atrazine [a]	200	202, 215	
7	4.832		Hexachlorobenzene-$^{13}C_6$ *	292		
8	4.832		Hexachlorobenzene [c]	284	282, 286	
9	4.886	4.85-5.05	terbuthylazine-D_5 *	219		0.13
10	4.897		Terbuthylazine [a]	214	173, 229	
11	4.905		beta-HCH [b]	217	181, 219	
12	4.920		Propyzamide [a],[d]	175	173, 255	
13	4.929		Diazinon [a],[d]	152	137, 179	
14	4.948		Lindane [b],[d]	181	183, 219	
15	5.091	5.05-5.25	Pirimicarb [a]	166	138, 238	0.10
16	5.100		Chlorothalonil [c]	266	264, 268	
17	5.215		Metribuzin [a]	198	144, 199	
18	5.274	5.25-5.52	Chlorpyriphos methyl [a]	286	197, 288	0.20
19	5.274		Parathion methyl [a]	263	216, 246	
20	5.312		Alachlor [a]	160	132, 188	
21	5.393		Heptachlor [b]	272	100, 102	
22	5.413		Pirimiphos methyl [a]	290	125, 244	
23	5.438		Fenitrothion [a]	277	109, 260	
24	5.467		Malathion [a]	173	125, 127	
25	5.567	5.52-5.72	Fenthion [a]	245	279, 280	0.15
26	5.572		Metholachlor [a]	162	146, 238	
27	5.583		Chlorpyriphos [a]	314	197, 199	
28	5.588		Parathion ethyl [a]	291	139, 155	
29	5.645		Aldrin [b]	263	101, 261	
30	5.784	5.72-5.99	Cyprodinil [c]	224	210, 225	0.18
31	5.827		Pendimethalin [a]	252	162, 192	
32	5.876		Chlofenvinphos [a]	267	269, 323	
33	5.868		Isodrin [b]	193	195, 263	
34	5.922		Quinalphos [a],[d]	146	156, 157	
35	5.953		Tolylfluanid [c]	238	137, 240	

Table 1 (continued).

Peak number	t_R (min)	Window (min)	Compounds	Monitored ions under SIM mode		
				Target ion	Reference ions	Scan time (s)
36	6.059	5.99-6.28	Methidathion [(a),(d)]	145	93, 125	0.10
37	6.115		trans-Chlordane [(b)]	375	371, 373	
38	6.230		Endosulfan I [(b)]	170	239, 241	
39	6.337	6.28-6.55	*p,p'*-DDE-D_8 *	254		0.10
40	6.355		*p,p'*-DDE [(b)]	246	248, 318	
41	6.419		Buprofezin [(c),(d)]	105	104, 172	
42	6.453		Dieldrin [(b)]	263	265, 277	
43	6.655	6.55-6.88	Endrin [(b)]	263	261, 345	0.15
44	6.723		Endosulfan II [(b)]	195	241, 339	
45	6.732		p,p'-DDD [(b)]	165	176, 199	
46	6.738		Ethion [(a),(d)]	125	153, 384	
47	6.757		Oxadixyl [(c)]	132	120, 146	
48	6.974	6.88-7.2	Propiconazole I [(c)]	173	175, 259	0.10
49	7.020		Propiconazole II [(c)]	173	175, 259	
50	7.020		*p,p'*-DDT [(b),(d)]	165	199, 212	
51	7.032		Endosulfan sulfate [(b)]	272	227, 274	
52	7.306	7.2-7.42	Bifenthrin [(a)]	181	165, 166	0.10
53	7.333		Phosmet [(a),(d)]	160	104, 161	
54	7.356		Methoxychlor [(b),(d)]	227	212, 228	
55	7.498	7.42-7.65	Tetradifon [(c),(d)]	159	227, 229	0.10
56	7.548		Pyriproxyfen [(c)]	136	137, 186	
57	7.730	7.65-7.85	Fenarimol [(c),(d)]	139	219, 251	0.10
58	8.244	7.85-8.9	Cypermethrin [(a),(d)]	163	127, 181	0.10
59	8.554		Fenvalerate [(a),(d)]	125	167, 169	

* ILIS used in this work.

(a), (b), (c) indicates the internal standard used for quantitative purposes: (a) terbutylazine-D_5, (b) *p,p'*-DDE-D_8, (c) hexachlorobenzene-$^{13}C_6$.

(d) Target ion modified in some matrices: propyzamide, 173 in carrot and tomato; diazinon, 179 in tomato; lindane, 219 in carrot and tomato; quinalphos, 156 in carrot; methidathion, 93 in tomato; buprofezin, 104 in orange and carrot and 172 in tomato; ethion, 384 in carrot and 153 in tomato; p,p'-DDT, 199 in apple, orange and tomato; phosmet, 104 in carrot; methoxychlor, 228 in orange; tetradifon, 227 in carrot; fenarimol, 219 in carrot; cypermethrin, 181 in carrot and tomato; fenvalerate, 169 in carrot.

Three isotopically labeled internal standards (ILIS) were used as surrogates: *p*,*p*′-DDE-D_8, terbuthylazine-D_5 (Dr. Ehrenstorfer, Augsburg, Germany) and hexachlorobenzene (HCB)-$^{13}C_6$ (Cambridge Isotope Labs Inc., Andover, MA, USA). A working ILIS mixed solution (of around 1000 ng/mL) was prepared by dilution of individual stock solutions with MeCN and stored at 4 °C.

Sample material

Five types of commodities, selected following the European Control Guidelines SANCO/3131/2007, 31 October 2007, were used in the validation study: orange was selected as a food with high acidity, apple and tomato as high water content commodity, carrot as high protein content commodity and olive as a representative matrix with a high fat content. Blank samples, used to perform the matrix-matched calibration and the validation study, were obtained from organic cultivars (pesticide-free).

Four different varieties from each food commodity were analyzed so as to test the applicability of the method and to investigate the presence of pesticides. Orange varieties were purchased from local markets in the Castellón province: Clementine (a variety of the mandarin orange), from Benicarló and Vila-real; Navelina, from Vila-real; and Navelate, from Almassora. All the four apple varieties, Royal Gala, Golden, Granny, and Fuji, were obtained from local markets in Castellón and Vila-real. Raff tomato variety was from Murcia (Spain); Kumato and Pear Cherry Tomato were purchased from local markets in Castellón; and hanging tomato was from Almería (Spain). Commercial carrots and Baby carrots were also purchased from local markets in Castellón, and the variety Mantesa and Mokum came from the Northern and Southern Spain, respectively.

GC instrumentation

Determinations were performed on a GC system (Shimadzu QP2010 Plus) equipped with an autosampler (Shimadzu AOC-5000) and coupled to a single quadrupole mass spectrometer (GCMS-QP2010 Plus). Compounds were separated on

a SAPIENS-5MS capillary column (length 20 m × I.D. 0.10 mm × film thickness 0.10 μm) from Teknokroma. Injection (3 μL) was performed in PTV mode, programmed as follows: 40 °C (hold time, 0.5 min), maintaining the split valve open; once the valve is closed, heating at a rate of 400 °C/min to 320 °C (hold time, 0.5 min), resulting in a total injection time of 1.70 min.

Initial oven temperature was maintained at 60 °C for 1.70 min and then heated at a rate of 90 °C/min to 225 °C, then 15 °C/min to 270 °C and finally 150 °C/min to 330 °C (2 min), resulting in a total analysis time of 8.93 min. Helium was used as carrier gas at a constant flow of 0.77 mL/min (corresponding to a linear velocity of 39.1 cm/s).

MS was operated in the electron ionization mode (70 eV). The source and the interface (transfer line) temperatures were adjusted to 225 and 300 °C, respectively. The scan time in scan mode was set at 0.1 s; when selected ion monitoring (SIM) mode was applied, scan time ranged from 0.1 to 0.2 s. A solvent delay of 3.3 min was used to prevent damage to the filament of the ion source. Shimadzu software GCMSsolution was used to automatically process the data.

Analytical procedure

Fruit and vegetable samples were firstly homogenized in a food chopper. Then, 15 g of sample was weighted in a 50-mL polypropylene centrifuge tube and 375 μL of surrogate solution mixture in MeCN (containing the three ILIS) was added and mixed on a vortex for 1 min. Extraction was carried out using 15 mL MeCN (with 1 % HAc), shaking by hand for 30 s. Then, 6 g anhydrous $MgSO_4$ and 1.5 g anhydrous NaAc were added and immediately shaken vigorously by hand to prevent formation of $MgSO_4$ agglomerates. Then, the tube was centrifuged at 3000 rpm for 2 min.

For the cleanup step, 1 mL of the upper MeCN extract was poured into a d-SPE tube containing 150 mg $MgSO_4$ and 50 mg PSA (or 150 mg $MgSO_4$, 50 mg PSA and 50 mg C_{18} when oranges and olives were extracted). The tubes were shaken on a

vortex for 30 s and centrifuged at 3000 rpm for 2 min. The final MeCN extract was injected into the GC system under the experimental conditions indicated before.

Matrix-matched standards for each sample matrix were prepared as follows: 500 µL of the MeCN extract obtained from a blank sample were mixed with 50 µL of the pesticide standard solution in MeCN at different concentrations, also containing the three ILIS. Each compound was quantified by using relative responses to the corresponding internal standard, as shown in **Table 1**.

Validation study

Validation study was carried out for apple, orange, carrot, tomato and olive samples in terms of linearity, accuracy, precision, LOQ and limit of detection (LOD). Blank samples were used to prepare spiked samples as follows: 15 g of sample was mixed with 150 µL of the pesticide standard solution in MeCN at 1 or 5 µg/mL in order to obtain spiked samples at 0.01 or 0.05 mg/kg, respectively; spiked samples at 0.1 mg/kg were obtained by mixing 15 g of sample with 1500 µL of the pesticide standard solution in MeCN at 5 µg/mL. In all cases, 375 µL of surrogate solution mixture in MeCN (containing the three ILIS) were also added and then left to stand over during an hour. Confirmation capability of the method for positive samples was also evaluated using ion intensity ratios. The effect of interfering peaks was also carefully studied.

Linearity was studied by injecting matrix-matched calibration standards ($n = 3$) in the range 1–500 ng/mL (corresponding to 0.001–0.5 mg/kg in sample). Linearity was considered satisfactory when the determination coefficient was higher than 0.99 and the residuals lower than 30 % without any clear tendency.

Accuracy was estimated from recovery experiments at three concentration levels (0.01, 0.05 and 0.5 mg/kg) ($n = 6$ each). Precision was expressed as repeatability in terms of relative standard deviation (RSD, in percent) ($n = 6$) at each fortification level.

LOQ was estimated as the analyte concentration that produced a peak signal ten times that of the background noise. It was calculated using the chromatograms at the lowest fortification level tested with satisfactory recovery (70–120%) and precision (RSD < 20%). LOD was estimated in the same way, but for a signal-to-noise ratio of 3.

In order to confirm peak identity in the samples, the ratio between the quantification ion (target, Q) and the reference ions (q_i) was calculated for each compound in the samples and compared with the value obtained from matrix-matched standards. As a start point, maximum tolerances for Q/q ratio deviation based on the European Commission Decision 2002/657/EC (European Commission Decision 2002) were considered, but modified in some cases. Agreement between retention time in the sample and the corresponding standard was also required to confirm a positive finding (maximum deviation ± 0.5%).

RESULTS AND DISCUSSION

GC–MS optimization

Optimization of the chromatographic conditions was first performed by injecting pesticide standard solutions in MeCN with the mass spectrometer operating in full scan mode. GC and MS parameters optimized in our previous paper (Cherta et al. 2012) were used, since several pesticides in common were determined in both cases. Injection mode was the only change to be considered on the chromatographic system, so optimization was focused on the PTV injection mode parameters.

Several injection temperatures were tested (40–60 °C), evaluating the sensitivity and chromatographic peak shape of early eluting compounds (more volatile). A temperature of 40 °C led to the best responses for these pesticides, so it was selected for further experiments. Initial column temperature was then studied between 50 and 80 °C; 60 °C was chosen as the best value that provided better sensitivity and chromatographic peak shape. Final temperature was selected

according to the chromatographic behavior of the last eluting compounds, which required temperatures between 300 and 350 °C; 330 °C was selected, as it was high enough to elute these compounds with satisfactory sensitivity. All these experiments were performed by injecting 1 μL of 100 ng/mL standard solution in MeCN using a glass liner packed with glass wool.

On the other hand, considering the PTV possibilities of injecting larger volumes, sensitivity was evaluated using injection volumes between 1 and 5 μL, paying special attention to solvent vent times. An injection volume of 3 μL, which required a solvent vent time of 0.5 min, was considered as the most satisfactory. Once the MeCN was eliminated, the valve was closed and a heating rate of 400 °C/min was applied until 320 °C (hold time, 0.5 min). The total injection time was 1.70 min. During this time, column temperature was maintained at 60 °C.

MS parameters were optimized previously (Cherta et al. 2012); ion source and interface temperatures were maintained at 225 and 300 °C, respectively, and scan time was set at 0.1 s in scan mode (scan speed of 3333 amu/s), allowing the acquisition of 10 to 15 data points per peak.

In order to perform the simultaneous identification and quantification of the analytes, the three most abundant and/or characteristic ions for each compound were selected as target (typically the most abundant) and reference ions. Considering that a large number of compounds were determined in short chromatographic time, the unavoidable existence of some coelutions made necessary an accurate selection of m/z values in order to use those ions that did not interfere in the quantitative determination of coeluting analytes. **Table 1** shows the quantitative (target) and the reference (confirmative) ions selected for each compound.

The developed scan mode allowed the determination of 56 pesticides in run time as short as 8.93 min, as can be seen in **Fig. 1**, which illustrates the total ion chromatogram for a standard mixture in MeCN at 200 ng/mL. The lowest concentration level that could be detected under scan mode ranged from 10 to 70 ng/mL, depending on the pesticide under study. In this way, an increase in

sensitivity was required for an adequate quantification at low concentration levels, so a SIM method was created from the scan injection selecting the target and reference ions to be acquired. As the increase in the number of ions included in a SIM group also increases the scan time, resulting in the acquisition of less data points per peak, we established for our mass spectrometer a maximum of 20 ions in a SIM group (corresponding to a scan time of 0.2 s) to obtain good peak shape and satisfactory quantification (Cherta et al. 2012; Maštovská and Lehotay 2003).

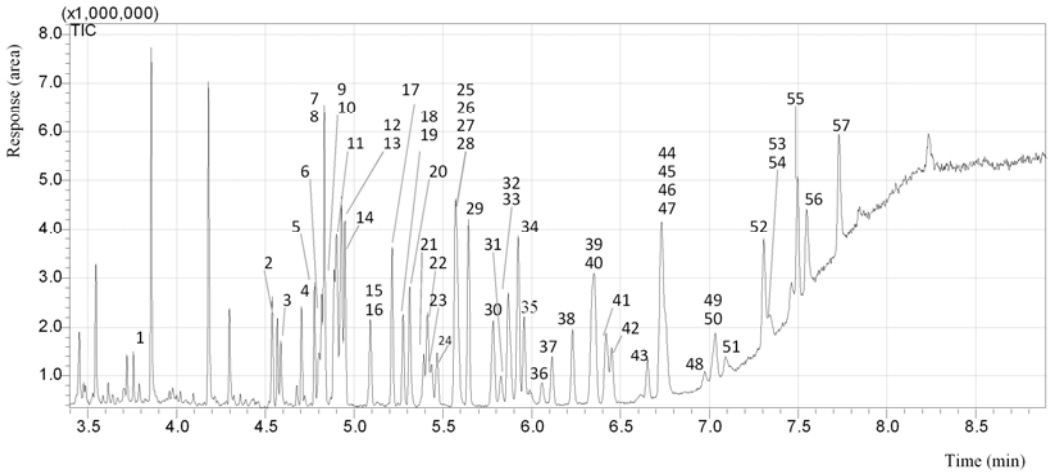

Fig. 1. GC-MS chromatogram of a mixed standard in acetonitrile (200 ng/mL) under the full scan method conditions (cypermethrin and fenvalerate could not be detected at 200 ng/mL, so higher concentration levels were required for their determination under scan mode).

Finally, compounds were sorted into 16 SIM groups with 3 to 20 ions monitored in each one (from 1 to 7 compounds included in the different groups). Scan time varied between 0.1 and 0.2 s, depending on the number of compounds included in each group (**Table 1**). Under these conditions, standard solutions down to 1–5 ng/mL could be easily analyzed and quantified.

QuEChERS procedure

The extraction procedure applied in this work was based on the QuEChERS AOAC Official Method 2007.01, which uses acetate buffering (Lehotay et al. 2005 a, 2010; Koesukwiwat et al. 2011). It was applied without changes, but including the use of C_{18} in the cleanup step for oranges and olives. Application of this sample preparation method allows preparing around ten real samples in <2 h (including matrix-matched calibration standards).

The QuEChERS method does not include solvent evaporation or concentration steps, leading to a ratio of approximately 1 g sample/mL in the final extract. As previously stated, the injection of 3 μL allowed to notably increase sensitivity. Injection volumes above 3 μL led to detector saturation under the selected conditions due to the introduction of larger amounts of sample matrix.

Although relatively clean extracts were obtained following this procedure, some interferences were observed in the chromatograms, depending on the type of matrix analyzed. Apple and orange samples presented less interferences than carrot and tomato. The olive matrix presented the worst chromatographic background, probably due to the presence of large lipid amounts that could not be completely removed even by adding C_{18} in the cleanup step. Moreover, we observed several interfering peaks, even at the characteristic analyte ions, making the quantification of analytes troublesome. On the other hand, fat usually forms an oily layer between the aqueous and MeCN phases, in which some pesticides could be retained, resulting in lower recoveries, as it has been reported in the literature (Lehotay et al. 2005 b). Improvement of the procedure should involve the use of other approaches reported to analyze olive matrices, such as the use of GCB as additional sorbent in the cleanup (Cunha et al. 2007; Lehotay et al. 2005 b; Gilbert-López et al. 2010 a, b), conventional SPE using Florisil as sorbent (Garrido Frenich et al. 2008), or direct sample introduction instead of PTV injection (Cunha et al. 2007), but they have not been tested in this work.

Study of matrix effects

In order to evaluate matrix effects on MS responses, calibration curves prepared in pure solvent (MeCN) and in matrix were compared. Considerable differences were observed in terms of calibration slopes, as illustrated in **Fig. 2**, using diazinon and *trans*-chlordane as representative examples. Higher values for the slope of fitted calibration curves were obtained for standards in solvent as a general tendency, even when using relative responses to ILIS. Thus, the use of matrix-matched calibration was necessary to correct for matrix effects and to achieve satisfactory quantitative applications. Additionally, due to the complexity of the sample, matrix components interfered with the analyte ions monitored. In a few cases, when the ion selected as target was heavily interfered in a specific matrix, the "cleanest" reference ion was then established as target ion in order to perform a correct quantification. Modifications related to the ions selected as target ions are indicated in **Table 1** for each type of matrix. Reference ions were also interfered in some cases, making the confirmation of the analytes problematic. **Fig. 3** shows three representative cases of spectral interferences: **Fig. 3a** illustrates the ideal situation, where none of the ions is interfered, so both quantification and identification/confirmation are adequate; in **Fig. 3b**, one of the reference ions is interfered, thus reducing identity confirmation capabilities; and **Fig. 3c** shows interferences observed for all the analyte ions, even for the target ion, making unfeasible the determination of tetradifon.

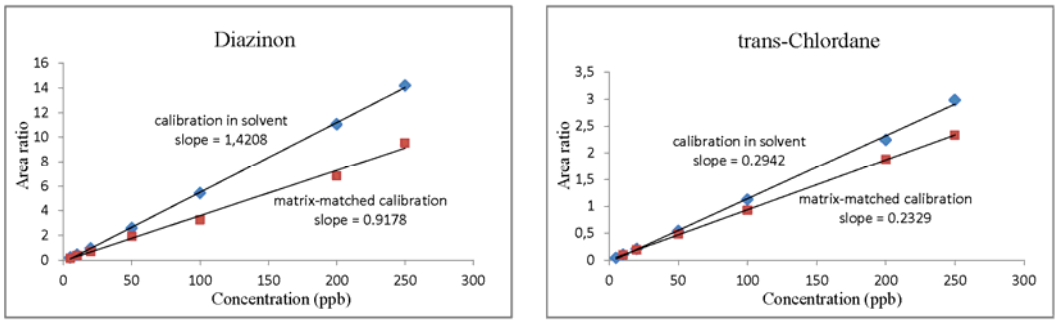

Fig. 2. Comparison of calibration curves obtained in solvent and in matrix for diazinon and *trans*-chlordane pesticides.

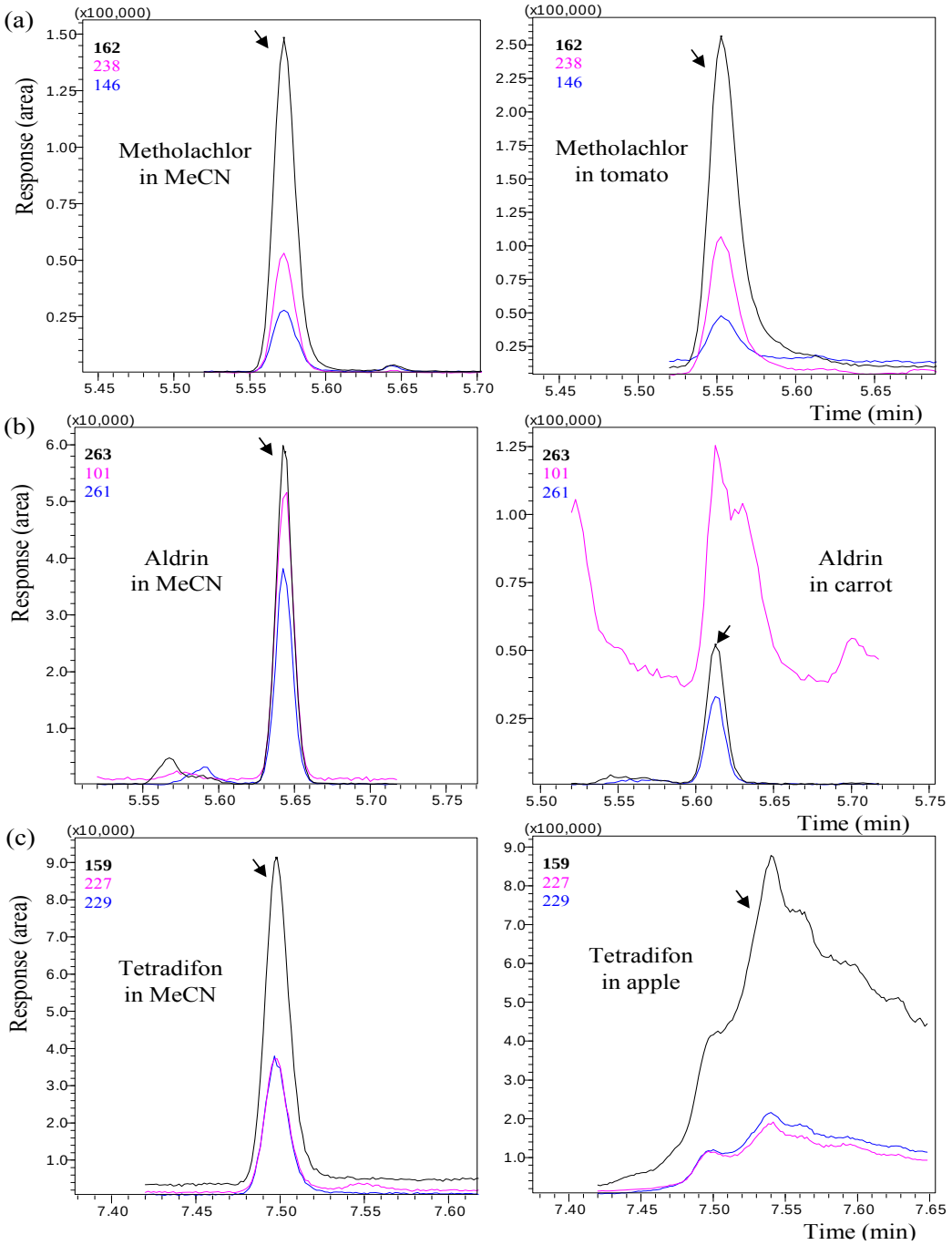

Fig. 3. Comparison of chromatographic responses for three selected pesticides in solvent at 50 ng/mL and in sample extracts spiked at 0.05 mg/kg. Target ion (in bold) and two reference ions are shown. **a)** Metolachlor; **b)** aldrin; **c)** tetradifon.

Therefore, a careful selection of the SIM ions has to be done due to coelutions between sample matrix components and analytes. The use of specific ions not interfered by coeluting components is necessary, although it was found difficult to be applied in some particular analyte/matrix combinations. **Table 2** shows the target and reference ions interfered in each matrix.

Table 2. Interferences observed for target and reference ions in each type of matrix at the 0.01 mg/kg level.

Compounds	Q	q_1	q_2	Apple			Orange			Carrot			Tomato		
				Q	q_1	q_2	Q	q_1	q_2	Q	q_1	q_2	Q	q_1	q_2
Dichlorvos	185	109	187					X			X				
Chlorpropham	213	127	154		X			X			X			X	
Trifluralin	264	290	306												
Phorate	260	121	231												
alpha-HCH	219	181	217		X			X			X			X	
Atrazine	200	202	215												
Hexachlorobenzene	284	282	286												
Terbuthylazine	214	173	229		X										
beta-HCH	217	181	219			X			X		X			X	
Propyzamide	175	173	255							X[a]			X[a]		
Diazinon	152	137	179										X[a]		
Lindane	181	183	219			X			X	X[a]	X		X[a]	X	
Pirimicarb	166	138	238												
Chlorothalonil	266	264	268												
Metribuzin	198	144	199			X					X	X	X	X	X
Chlorpyriphos methyl	286	197	288											X	
Parathion methyl	263	216	246												
Alachlor	160	132	188												
Heptachlor	272	100	102			X		X	X			X			X
Pirimiphos methyl	290	125	244		X			X			X			X	
Fenitrothion	277	109	260		X			X			X			X	
Malathion	173	125	127								X			X	
Fenthion	245	279	280												
Metholachlor	162	146	238												
Chlorpyriphos	314	197	199												
Parathion ethyl	291	139	155		X	X		X	X		X	X	X	X	X
Aldrin	263	101	261		X						X				
Cyprodinil	224	210	225										X	X	X
Pendimethalin	252	162	192					X			X			X	X
Chlofenvinphos	267	269	323												
Isodrin	193	195	263												
Quinalphos	146	156	157							X[a]		X			
Tolylfluanid	238	137	240		X			X			X			X	
Methidathion	145	93	125		X			X			X		X[a]		X
trans-Chlordane	375	371	373												
Endosulfan I	170	239	241												
p,p'-DDE	246	248	318												
Buprofezin	105	104	172				X[a]			X[a]			X[a]		
Dieldrin	263	265	277												

Table 2 (continued).

Compounds	Q	q_1	q_2	Apple			Orange			Carrot			Tomato		
				Q	q_1	q_2	Q	q_1	q_2	Q	q_1	q_2	Q	q_1	q_2
Endrin	263	261	345												
Endosulfan II	195	241	339												
p,p'-DDD	165	176	199												
Ethion	125	153	384							X^a	X		X^a		
Oxadixyl	132	120	146							X	X	X	X	X	X
Propiconazole I	173	175	259												
Propiconazole II	173	175	259												
p,p'-DDT	165	199	212	X^a			X^a						X^a		
Endosulfan sulfate	272	227	274					X						X	
Bifenthrin	181	165	166					X							
Phosmet	160	104	161				X	X	X	X^a		X		X	
Methoxychlor	227	212	228			X	X^a	X							
Tetradifon	159	227	229	X	X	X		X		X^a					
Pyriproxyfen	136	137	186	X	X	X									
Fenarimol	139	219	251							X^a					
Cypermethrin	163	127	181							X^a			X^a		
Fenvalerate	125	167	169							X^a	X			X	

X matrix interference with the ion selected; Xa target ion is interfered and replaced for one reference ion in order to perform quantitative analysis.

Purple, blue and red colors indicate interferences in one, two and three ions, respectively.

Apple was the "cleanest" matrix since most of compounds were not affected by matrix interferences. Moreover, the quantitative ion (*Q*) was not interfered, except for *p,p'*-DDT, methoxychlor, tetradifon and pyriproxyfen, which prevented the validation of the method for these compounds. A similar trend was found for most pesticides in orange, although a higher number of ions, including the *Q* ion in four cases, were interfered. Worse results were obtained in carrot and tomato matrices: half compounds presented interferences in at least one analyte ion; in some cases, all the three ions were interfered, so their validation could not be performed. The most common interferences occurred at low *m/z* values, as 109 for fenitrothion, 102 for heptachlor and 125 for pirimiphos methyl, which were observed in all matrices.

This can be considered as a limitation of single quadrupole MS for the analysis of complex matrix samples. The determination of problematic analytes would require the use of more powerful analyzers like triple quadrupole working under MS/MS or high-resolution MS. Results obtained are similar and, in some cases, better (in terms of sensitivity) than those obtained in our group by GC-TOF MS (Cervera et al. 2012) but with some lack in terms of specificity.

Validation results

The method developed was validated for apple, orange, carrot and tomato samples. Due to the higher complexity of olive samples, validation results were not satisfactory for most of the compounds. As indicated before, three ILIS were used as surrogates in order to correct for possible losses of analytes during the extraction process and/or instrumental deviations. Terbuthylazine-D_5 was used as internal standard for herbicides, organophosphate insecticides, carbamates and pyrethroids; *p*,*p′*-DDE-D_8 was used for organochlorine pesticides and insecticides and trifluralin; and HCB-$^{13}C_6$ was used for fungicides and insecticides, such as buprofezin, pyriproxyfen and tetradifon. The internal standard used for each individual compound is shown in **Table 1**.

Linearity of responses using matrix-matched standards was studied in the range 0.001–0.5 mg/kg. Only those analytes for which sensitivity was higher, like dichlorvos, atrazine, chlorpyrifos methyl, chlorpyrifos, or bifenthrin, could be detected at the lowest calibration level tested, although the type of matrix also conditioned this value. Apple was the matrix that allowed extending the calibration range to the lowest concentration levels. Determination coefficients were better than 0.99 for all compounds and the residuals were lower than 30% in all matrices.

Accuracy and precision were evaluated by means of recovery experiments ($n = 6$) at three concentration levels (0.01, 0.05 and 0.1 mg/kg) for each sample matrix. Results are shown in **Tables 3**, **4**, **5** and **6**. Apple matrix presented the best results in relation to recoveries and number of validated compounds. Four analytes (*p*,*p′*-DDT, methoxychlor, tetradifon and pyriproxyfen) were interfered by the matrix and could not be validated at any concentration level; methoxychlor showed poor sensitivity and could not be detected. The rest of the compounds presented recoveries between 70 and 120% in this matrix, with RSD lower than 20%, and the wide majority were satisfactorily validated at the 0.01 mg/kg level. In orange, carrot and tomato samples, most analytes could be validated at the 0.05 and 0.5 mg/kg levels with satisfactory recoveries and precision. The number of compounds interfered by matrix components was higher in these matrices, as well as those compounds with

poor sensitivity. Thus, chlorothalonil and tolylfluanid could not be validated in any of these matrices. It is known that these compounds are problematic in multiresidue analysis since they easily degrade during sample preparation, GC injection, and/or solution storage (Lehotay et al. 2005 c, 2007; Peruga 2012). No satisfactory results were also obtained for some pyrethroids, like cypermethrin and fenvalerate in carrot and tomato. Pyrethroids can also be problematic pesticides from an analytical point of view according to the literature (Lehotay et al. 2005 c).

LOQs between 2 and 20 μg/kg were obtained for most compounds in apple, orange, carrot, and tomato samples. A few values were around 30 μg/kg and higher LOQs (100 μg/kg) were obtained for some particular analyte/matrix combinations, as shown **in Tables 3**, **4**, **5** and **6**. LODs were typically in the range 0.5–15 μg/kg, which are of the same order to those reported in the recent literature (Nguyen et al. 2009; Steiniger et al. 2010; Qu et al. 2010) and in agreement with regulations requirements (European Commission 2005).

Confirmation of peak identity in the samples was also conditioned by the presence of matrix interferences in some particular cases. A strict criterion based on the acquisition of one target (Q) ion and two reference ions (q_i) and the accomplishment of Q/q ratios in comparison with the reference standard values within acceptable deviations (European Commission Decision 2002) was firstly applied. The agreement in the retention time between sample and standard was also required. However, when matrix interferences coeluted with analytes, the Q/q ratio could not be properly measured. But even without apparent interferences occurring, only in a very few cases the two Q/q ratios were accomplished. This occurred especially at low analyte levels, where the low abundance of the ions can alter the expected Q/q ratios. Thus, a more realistic criterion was applied for confirmation, consisting of agreement in retention time, three ions monitored observed in the sample, and at least one Q/q ratio fulfilled (instead of the two available).

Table 3. Average recovery (%) and R.S.D. (%, in parenthesis) obtained for apple samples (n=6) fortified at three concentration levels. Detection (LOD) and quantification (LOQ) limits.

Compounds	Fortification levels (mg/kg)			LOD (µg/kg)	LOQ (µg/kg)
	0.01	0.05	0.5		
Dichlorvos	88 (12)	75 (10)	107 (5)	0.5	2
Chlorpropham	105 (3)	104 (6)	97 (9)	0.8	3
Trifluralin	99 (7)	80 (9)	96 (8)	0.6	2
Phorate	104 (9)	99 (7)	102 (7)	0.6	2
alpha-HCH	117 (11)	100 (12)	109 (7)	0.6	2
Atrazine	99 (4)	90 (6)	103 (5)	0.6	2
Hexachlorobenzene	99 (1)	108 (2)	95 (2)	0.5	2
Terbuthylazine	97 (2)	88 (12)	103 (5)	0.5	2
beta-HCH	111 (5)	89 (4)	99 (6)	4	14
Propyzamide	110 (5)	100 (6)	86 (5)	4	14
Diazinon	96 (9)	98 (6)	103 (7)	6	19
Lindane	77 (4)	70 (11)	108 (5)	0.6	2
Pirimicarb	82 (10)	96 (9)	103 (5)	0.5	2
Chlorothalonil	-	40 (10)	93 (19)	30	100
Metribuzin	101 (8)	96 (6)	103 (6)	0.5	2
Chlorpyriphos methyl	94 (7)	72 (9)	106 (12)	0.3	1
Parathion methyl	-	72 (7)	107 (13)	5	15
Alachlor	92 (5)	94 (6)	104 (6)	0.5	2
Heptachlor	107 (7)	69 (5)	104 (4)	2	6
Pirimiphos methyl	117 (3)	94 (9)	101 (7)	0.5	2
Fenitrothion	113 (4)	78 (8)	82 (13)	2	6
Malathion	105 (7)	66 (5)	95 (17)	2	6
Fenthion	-	96 (5)	101 (6)	5	15
Metholachlor	95 (8)	98 (7)	105 (6)	0.5	2
Chlorpyriphos	118 (1)	106 (9)	106 (7)	0.5	2
Parathion ethyl	-	86 (7)	98 (6)	5	15
Aldrin	101 (5)	96 (3)	109 (5)	0.6	2
Cyprodinil	92 (8)	99 (8)	87 (7)	0.5	2
Pendimethalin	-	86 (7)	93 (4)	4	12
Chlofenvinphos	-	73 (3)	98 (7)	5	15
Isodrin	92 (15)	96 (2)	103 (3)	2	5
Quinalphos	110 (5)	90 (10)	104 (6)	2	5

Table 3 (continued).

Compounds	Fortification levels (mg/kg)			LOD (µg/kg)	LOQ (µg/kg)
	0.01	0.05	0.5		
Tolylfluanid	-	-	92 (2)	30	100
Methidathion	-	74 (4)	100 (12)	4	12
trans-Chlordane	114 (3)	93 (4)	103 (4)	2	6
Endosulfan I	-	88 (6)	104 (3)	5	15
p,p'-DDE	103 (4)	99 (3)	102 (4)	0.6	2
Buprofezin	-	102 (6)	90 (3)	5	15
Dieldrin	-	92 (4)	110 (4)	7	21
Endrin	-	100 (6)	107 (6)	7	21
Endosulfan II	-	-	103 (3)	30	100
p,p'-DDD	88 (9)	73 (11)	101 (5)	2	6
Ethion	-	109 (8)	102 (6)	8	25
Oxadixyl	-	105 (8)	90 (8)	5	15
Propiconazole I	-	104 (7)	89 (6)	12	38
Propiconazole II	-	101 (5)	93 (5)	14	46
p,p'-DDT	-	-	-	-	-
Endosulfan sulfate	-	40 (23)	108 (15)	30	100
Bifenthrin	95 (6)	99 (16)	102 (6)	0.6	2
Phosmet	107 (6)	78 (12)	103 (15)	2	6
Methoxychlor	-	-	-	-	-
Tetradifon	i.	i.	i.	-	-
Pyriproxyfen	i.	i.	i.	-	-
Fenarimol	87 (7)	102 (10)	107 (5)	0.4	2
Cypermethrin	-	-	106 (7)	30	100
Fenvalerate	-	113 (11)	108 (3)	15	40

Underlined, not acceptable results.

i., analyte not detected due to matrix interfences on the three analyte ions.

Table 4. Average recovery (%) and R.S.D. (%, in parenthesis) obtained for orange samples (n=6) fortified at three concentration levels. Detection (LOD) and quantification (LOQ) limits.

Compounds	Fortification levels (mg/kg)			LOD (µg/kg)	LOQ (µg/kg)
	0.01	0.05	0.5		
Dichlorvos	92 (5)	78 (6)	91 (6)	0.5	2
Chlorpropham	-	102 (8)	97 (7)	5	15
Trifluralin	69 (9)	107 (7)	105 (7)	1	4
Phorate	114 (5)	89 (6)	98 (5)	2	6
alpha-HCH	97 (16)	103 (12)	102 (10)	2	6
Atrazine	100 (11)	87 (6)	101 (4)	2	6
Hexachlorobenzene	76 (5)	110 (4)	97 (4)	0.6	2
Terbuthylazine	95 (3)	92 (10)	98 (9)	0.6	2
beta-HCH	-	94 (9)	104 (7)	8	25
Propyzamide	-	98 (5)	103 (7)	6	20
Diazinon	-	84 (13)	98 (7)	8	25
Lindane	76 (16)	90 (12)	111 (8)	2	6
Pirimicarb	107 (13)	91 (4)	99 (9)	1	3
Chlorothalonil	-	-	-	-	-
Metribuzin	-	91 (11)	92 (8)	5	15
Chlorpyriphos methyl	101 (4)	81 (6)	90 (14)	0.6	2
Parathion methyl	-	89 (6)	89 (11)	5	15
Alachlor	107 (7)	86 (8)	92 (10)	2	6
Heptachlor	-	91 (16)	82 (10)	4	12
Pirimiphos methyl	110 (7)	78 (6)	87 (15)	2	6
Fenitrothion	-	-	82 (15)	5	15
Malathion	-	-	75 (16)	30	100
Fenthion	-	96 (11)	90 (14)	7	21
Metholachlor	118 (5)	78 (4)	99 (10)	1	3
Chlorpyriphos	-	93 (6)	89 (12)	10	30
Parathion ethyl	-	87 (8)	87 (14)	5	15
Aldrin	106 (9)	90 (10)	92 (8)	0.6	2
Cyprodinil	111 (8)	120 (5)	105 (9)	2	6
Pendimethalin	-	85 (12)	79 (15)	8	25
Chlofenvinphos	-	80 (10)	79 (16)	8	25
Isodrin	-	97 (15)	94 (11)	4	12
Quinalphos	-	79 (6)	100 (10)	6	18

Table 4 (continued).

Compounds	Fortification levels (mg/kg)			LOD (µg/kg)	LOQ (µg/kg)
	0.01	0.05	0.5		
Tolylfluanid	-	-	-	-	-
Methidathion	-	-	91 (9)	30	100
trans-Chlordane	-	95 (12)	101 (10)	5	15
Endosulfan I	-	100 (3)	105 (8)	6	20
p,p'-DDE	105 (6)	93 (12)	94 (6)	2	6
Buprofezin	-	118 (13)	113 (6)	5	15
Dieldrin	-	-	110 (10)	30	100
Endrin	-	-	104 (9)	30	100
Endosulfan II	-	-	112 (9)	30	100
p,p'-DDD	-	94 (13)	111 (7)	5	15
Ethion	-	87 (14)	107 (7)	7	25
Oxadixyl	-	114 (8)	109 (14)	9	30
Propiconazole I	-	117 (7)	117 (3)	8	24
Propiconazole II	-	115 (7)	116 (6)	12	38
p,p'-DDT	-	-	-	-	-
Endosulfan sulfate	-	-	-	-	-
Bifenthrin	-	82 (17)	96 (8)	5	15
Phosmet	i.	i.	i.	-	-
Methoxychlor	-	-	-	-	-
Tetradifon	-	90 (8)	109 (12)	10	30
Pyriproxyfen	-	118 (10)	116 (13)	5	15
Fenarimol	110 (5)	97 (11)	101 (8)	1	4
Cypermethrin	-	-	108 (12)	30	100
Fenvalerate	-	-	102 (5)	30	100

i., analyte not detected due to matrix interfences on the three analyte ions.

Table 5. Average recovery (%) and R.S.D. (%, in parenthesis) obtained for carrot samples (n=6) fortified at three concentration levels. Detection (LOD) and quantification (LOQ) limits.

Compounds	Fortification levels (mg/kg)			LOD (µg/kg)	LOQ (µg/kg)
	0.01	0.05	0.5		
Dichlorvos	96 (9)	97 (5)	87 (4)	0.6	2
Chlorpropham	-	106 (4)	86 (5)	4	12
Trifluralin	92 (7)	97 (4)	93 (11)	1	5
Phorate	107 (10)	107 (3)	90 (6)	2	6
alpha-HCH	-	110 (13)	94 (6)	5	15
Atrazine	-	109 (4)	81 (6)	4	12
Hexachlorobenzene	91(3)	92 (3)	91 (5)	0.6	2
Terbuthylazine	96 (6)	108 (2)	89 (5)	3	8
beta-HCH	-	81 (9)	86 (10)	7	20
Propyzamide	118 (4)	101 (5)	90 (6)	3	8
Diazinon	-	95 (2)	85 (7)	6	18
Lindane	-	80 (9)	94 (9)	5	15
Pirimicarb	104 (9)	102 (6)	87 (6)	3	9
Chlorothalonil	-	-	200 (8)	-	-
Metribuzin	-	113 (5)	105 (12)	7	20
Chlorpyriphos methyl	82 (8)	98 (12)	85 (8)	1	5
Parathion methyl	-	-	90 (7)	30	100
Alachlor	103 (18)	109 (8)	89 (6)	2	5
Heptachlor	92 (11)	100 (6)	91 (8)	2	5
Pirimiphos methyl	86 (12)	107 (3)	89 (8)	0.6	2
Fenitrothion	-	119 (5)	81 (7)	9	27
Malathion	-	118 (2)	91 (7)	7	20
Fenthion	-	112 (4)	85 (5)	8	24
Metholachlor	117 (9)	101 (6)	90 (6)	1	5
Chlorpyriphos	-	111 (3)	90 (5)	4	12
Parathion ethyl	-	110 (7)	86 (7)	7	22
Aldrin	93 (9)	87 (3)	95 (7)	2	6
Cyprodinil	-	106 (6)	n. a.	5	15
Pendimethalin	-	88 (7)	90 (7)	4	12
Chlofenvinphos	-	108 (6)	81 (5)	6	18
Isodrin	-	88 (3)	88 (8)	4	12
Quinalphos	-	100 (5)	100 (7)	5	15

Table 5 (continued).

Compounds	Fortification levels (mg/kg)			LOD (µg/kg)	LOQ (µg/kg)
	0.01	0.05	0.5		
Tolylfluanid	-	-	-	-	-
Methidathion	-	-	-	-	-
trans-Chlordane	-	98 (4)	91 (7)	6	18
Endosulfan I	-	85 (7)	88 (6)	10	30
p,p'-DDE	96 (9)	99 (1)	90 (7)	2	6
Buprofezin	-	74 (13)	96 (7)	7	21
Dieldrin	-	-	94 (6)	30	100
Endrin	-	98 (7)	91 (7)	7	25
Endosulfan II	-	-	95 (7)	30	100
p,p'-DDD	-	109 (4)	68 (7)	7	25
Ethion	-	-	95 (7)	30	100
Oxadixyl	i.	i.	i.	-	-
Propiconazole I	-	54 (14)	90 (8)	30	100
Propiconazole II	-	93 (14)	95 (10)	15	45
p,p'-DDT	-	-	92 (6)	30	100
Endosulfan sulfate	-	-	-	-	-
Bifenthrin	94 (16)	97 (7)	90 (4)	2	6
Phosmet	-	-	-	-	-
Methoxychlor	-	-	102 (4)	30	100
Tetradifon	-	-	-	-	-
Pyriproxyfen	-	112 (3)	96 (10)	4	12
Fenarimol	-	-	-	-	-
Cypermethrin	-	-	-	-	-
Fenvalerate	-	-	-	-	-

Underlined, not acceptable results.

i., analyte not detected due to matrix interfences on the three analyte ions.

Table 6. Average recovery (%) and R.S.D. (%, in parenthesis) obtained for tomato samples (n=6) fortified at three concentration levels. Detection (LOD) and quantification (LOQ) limits.

Compounds	Fortification levels (mg/kg)			LOD (µg/kg)	LOQ (µg/kg)
	0.01	0.05	0.5		
Dichlorvos	93 (10)	94 (12)	83 (6)	0.6	2
Chlorpropham	-	91 (6)	91 (6)	6	20
Trifluralin	113 (11)	109 (9)	113 (9)	0.6	2
Phorate	97 (6)	113 (7)	95 (6)	0.6	2
alpha-HCH	104 (9)	104 (8)	105 (11)	0.6	2
Atrazine	-	108 (10)	91 (12)	5	15
Hexachlorobenzene	100 (4)	120 (1)	98 (2)	0.3	1
Terbuthylazine	93 (9)	111 (10)	100 (8)	2	6
beta-HCH	110 (10)	91 (12)	86 (11)	2	6
Propyzamide	-	69 (29)	107 (8)	30	100
Diazinon	87 (11)	117 (12)	94 (6)	0.6	2
Lindane	110 (8)	105 (13)	107 (8)	2	6
Pirimicarb	98 (14)	89 (9)	96 (6)	2	5
Chlorothalonil	-	-	-	-	-
Metribuzin	i.	i.	i.	-	-
Chlorpyriphos methyl	93 (11)	99 (14)	102 (11)	0.6	2
Parathion methyl	-	-	86 (13)	30	100
Alachlor	109 (6)	117 (8)	102 (7)	2	5
Heptachlor	105 (8)	97 (6)	103 (13)	1	3
Pirimiphos methyl	98 (5)	107 (10)	99 (10)	0.6	2
Fenitrothion	-	-	95 (13)	50	150
Malathion	-	112 (3)	86 (10)	3	15
Fenthion	-	-	95 (13)	30	100
Metholachlor	97 (7)	93 (14)	101 (7)	0.6	2
Chlorpyriphos	101 (12)	104 (10)	97 (8)	1	5
Parathion ethyl	i.	i.	i.	-	-
Aldrin	105 (5)	119 (8)	104 (6)	1	3
Cyprodinil	i.	i.	i.	-	-
Pendimethalin	-	96 (11)	104 (9)	5	15
Chlofenvinphos	-	91 (17)	101 (9)	5	15
Isodrin	111 (7)	110 (10)	105 (7)	2	6
Quinalphos	-	105 (16)	109 (10)	5	15

Table 6 (continued).

Compounds	Fortification levels (mg/kg)			LOD (µg/kg)	LOQ (µg/kg)
	0.01	0.05	0.5		
Tolylfluanid	-	-	-	-	-
Methidathion	-	-	-	-	-
trans-Chlordane	116 (6)	106 (7)	105 (8)	0.6	2
Endosulfan I	-	108 (3)	105 (8)	16	48
p,p'-DDE	100 (5)	113 (6)	103 (7)	1	3
Buprofezin	-	118 (7)	99 (4)	5	15
Dieldrin	-	106 (5)	110 (5)	5	15
Endrin	-	115 (2)	110 (8)	7	22
Endosulfan II	-	-	116 (11)	30	100
p,p'-DDD	-	89 (5)	85 (12)	10	32
Ethion	-	125 (7)	101 (15)	30	100
Oxadixyl	i.	i.	i.	-	-
Propiconazole I	-	-	111 (9)	20	60
Propiconazole II	-	-	106 (4)	20	60
p,p'-DDT	-	-	-	-	-
Endosulfan sulfate	-	-	97 (16)	30	100
Bifenthrin	102 (11)	113 (13)	97 (7)	1	3
Phosmet	-	-	-	-	-
Methoxychlor	-	-	81 (22)	30	100
Tetradifon	-	-	90 (11)	40	115
Pyriproxyfen	104 (15)	99 (10)	103 (5)	3	10
Fenarimol	-	-	116 (7)	30	100
Cypermethrin	-	-	-	-	-
Fenvalerate	-	-	-	-	-

Underlined, not acceptable results.

i., analyte not detected due to matrix interfences on the three analyte ions.

As discussed before, several compounds presented heavy interferences in the reference ions, making the confirmation problematic in some cases. As can be seen in **Table 2**, most of matrix interferences affected only one reference ion (excluding of course the non-validated compounds that were interfered in the three ions selected), so the other one was available to be used for confirmation. Parathion ethyl, heptachlor, methoxychlor, lindane, metribuzin, quinalphos, ethion, phosmet, fenvalerate, pendimethalin, methidathion, propyzamide, pirimicarb and fenarimol showed interferences in both reference ions in some of the matrices. At higher pesticide concentrations (around or above 0.05 mg/kg), the number of cases where the two reference ions were interfered was much lower and, although one reference ion was sometimes interfered, the other one commonly accomplished the ion ratio, making confirmation feasible. Thus, when the two reference ions were interfered, confirmation can be doubtful, and this occurred especially at the 0.01 mg/kg level. In these situations, a more selective analyzer as TOF MS or the use of tandem MS would be required for confirmation.

Application to real samples

The developed GC-MS procedure was applied to apple, orange, carrot and tomato samples, analyzing 4 different varieties of each matrix (in total, 16 samples analyzed). The insecticide chlorpyrifos was predominant in apple and orange samples. It was found in the apple Royal Gala and Golden varieties at 0.03 and 0.04 mg/kg (MRL, 0.5 mg/kg), respectively, and in the Navelate (0.05 mg/kg) and Clementine (0.17 mg/kg) orange varieties (MRL, 0.3 mg/kg). This insecticide is commonly applied in Spain for pest control in these crops. The herbicide terbuthylazine was also found in the Clementine orange variety, but at lower concentration (0.006 mg/kg; MRL, 0.1 mg/kg). Bifenthrin was detected in the Navelina orange sample below the LOQ level (MRL, 0.1 mg/kg) and also in the Royal Gala apple (0.05 mg/kg) and in the Fuji apple (0.035 mg/kg) varieties (MRL, 0.3 mg/kg). The herbicides trifluralin and metolachlor and the fungicide HCB were found in the Raff tomato variety at levels around 0.002 mg/kg (MRL, 0.5, 0.05 and 0.01, respectively). All the compounds detected were present at concentrations below

the corresponding MRLs (for illustrative chromatograms of positive samples, see **Fig. 4**).

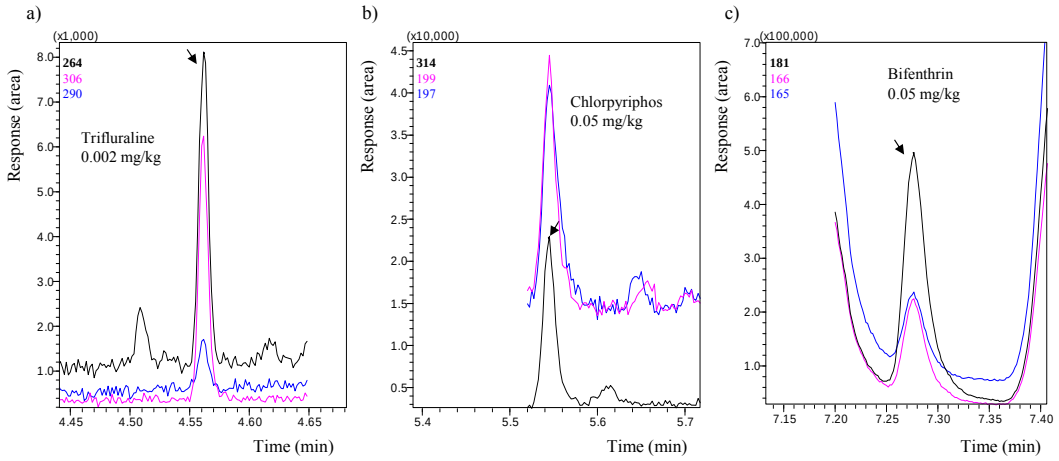

Fig. 4. Chromatograms for several compounds detected in **a)** Raff tomato, **b)** Navelina orange and **c)** Royal Gala apple.

Despite some problems found during validation in the accomplishment of ion ratios, this was not the case in the analysis of the 16 samples presented here, as none of the positive findings presented relevant matrix interferences, as shown in the examples of **Fig. 4**. So, the confirmation criterion based on the presence of three ions and the accomplishment of, at least, one ion ratio could be satisfactorily applied in all of them.

A non-target analysis was also applied in these samples using the same GC-MS conditions under scan mode. Although sensitivity in scan mode does not allow reaching concentration levels as low as in the SIM mode, screening can be satisfactorily performed under this acquisition mode for compounds present at higher concentrations. None of the samples showed positive findings for non-target pesticides, probably due to the low concentrations involved, but the potential of this technique for more concentrated compounds was demonstrated, with some positive findings for pyrene or major fruit components that, in any case, are not covered by the scope and aim of this work.

Additional analysis of the samples performed by GC-TOF MS using a method developed in our group (Cervera et al. 2012) allowed confirming all the positive findings reported by GC-MS, with the exception of trifluralin, HCB and metolachlor in the Raff tomato variety (concentrations around 0.002 mg/kg) that could not be detected by GC-TOF due to its lower sensitivity.

CONCLUSIONS

The potential of GC-MS using single quadrupole for multiresidue determination of pesticides in fruit and vegetable samples has been evaluated in this paper. A fast GC method has been developed for quantitative determination of 56 pesticides with a chromatographic run time of <10 min. Acquisition under SIM mode (three m/z ions) provided satisfactory sensitivity although not enough selectivity for some analyte/matrix combinations, especially at the 0.01 mg/kg level. Quantification was satisfactory since acceptable results for accuracy and precision were obtained for most compounds in apple, orange, carrot, and tomato matrices at the three fortification levels (0.01, 0.1 and 0.5 mg/kg). However, confirmation of positive findings was strongly conditioned by the presence of matrix-interfering peaks that coeluted with some reference ions. As a consequence, the accomplishment of two available Q/q ratios was problematic, especially at low analyte levels. However, the confirmation criterion based on the measurement of three ions and the accomplishment of just one ion ratio was satisfactorily reached in the wide majority of analyte/matrix at all concentration levels tested.

QuEChERS sample treatment became an essential step in order to minimize as much as possible matrix coextractants in the matrices analyzed. An effective extraction was achieved and clean extracts were obtained for apple, orange, carrot and tomato samples, although the presence of interfering peaks could not be completely avoided for a few analytes. Despite using PSA and C_{18} for cleanup purposes in olive samples, their high fat content made this cleanup insufficient, impeding the accomplishment of satisfactory recoveries.

Analysis of samples allowed detecting, identifying and quantifying several pesticides like chlorpyrifos and bifenthrin in apple and orange, terbuthylazine in orange, and trifluralin, metolachlor and HCB in tomato. In all cases, the pesticide concentrations were below the MRLs set by the EU. Sample throughput was notably increased by applying the developed methodology, making the analysis of around 30 samples in 1 day with good sensitivity feasible.

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II.4. Artículo científico 3

Comparison of simple and rapid extraction procedures for the determination of pesticide residues in fruit juices by fast gas chromatography-mass spectrometry

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Comparison of simple and rapid extraction procedures for the determination of pesticide residues in fruit juices by fast gas-chromatography-mass spectrometry

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Abstract

Three sample treatment methods, based on QuEChERS, solid-phase extraction (SPE) and solid-phase microextraction (SPME), were compared and evaluated in order to obtain the best conditions to determine pesticide residues in fruit juice by fast gas chromatography–mass spectrometry (GC-MS) with a single quadrupole. Analysis were performed under selected ion monitoring, acquiring the three most abundant and/or specific ions for each analyte and using their relative intensity ratios as a confirmatory parameter. The three methodologies (QuEChERS, SPE and SPME) were validated taking 15 selected pesticides as model compounds, using commercial apple juice. QuEChERS procedure was based on the AOAC Official Method 2007.01, using acetonitrile (containing 1% acetic acid) as extraction solvent and primary–secondary amine during the dispersive solid-phase extraction. Oasis hydrophilic–lipophilic balance cartridges were used for SPE, and polyacrylate fibers were used for direct immersion SPME procedure. Three isotopically labeled standards were added to the samples before extraction and used as surrogate standards. Validation parameters as recoveries, limits of detection, and limits of quantification (LOQ), as well as matrix effects and sample throughput, were obtained and compared for the three extraction procedures. QuEChERS was considered faster and led to the best quantitative results. In this way, validation was extended to up to 56 pesticides by applying QuEChERS in multi-fruit juice samples, obtaining LOQs ranging from 2 to

20 μg/L for most compounds. Accuracy and precision were evaluated by means of recovery experiments at two concentration levels (10 and 100 μg/L), obtaining recoveries between 70 and 120% in most cases and relative standard deviations below 15%. Finally, the QuEChERS method was applied to the analysis of commercial juices, including mango–apple, pineapple, grapefruit and orange.

Keywords

QuEChERS; SPE; SPME; Pesticides; Fast gas chromatography-mass spectrometry; Juices.

INTRODUCTION

Pesticide residues can remain in food after they are applied to crops, even after being washed, processed and prepared, and may result in adverse consequences to the human health. Their concentrations in processed food are usually lower than those observed in whole fruit due to their degradation through oxidative mechanisms or elimination during food processing, mainly after washing and peeling (Picó and Kozmutza 2007; Burchat et al. 1998; Patyal et al. 2004). The European Commission (2008) has set harmonized maximum residue levels (MRL) based on comprehensive assessment of the properties of the active substance and the residue behavior on treated crops. In most cases, no MRLs are set for processed food as juices. Then, the limit MRL applied for juice is the corresponding MRL for raw agricultural commodity, taking into account the concentration or dilution factor related to the manufacturing process (if available). Regulations and monitoring programs have to be adopted in order to strengthen food safety and control pesticide exposure to unacceptable levels in food. Analytical methodologies must be able to accurately determine the low concentration levels set up by the legislation. This is especially relevant for fruit and vegetable juices to have better knowledge of the pesticide levels actually present in this type of processed samples.

As it is already well-known, chromatographic techniques coupled to mass spectrometry (MS) are the most powerful tools for the identification and quantification of pesticides and other contaminants in food. Gas chromatography (GC) coupled to MS with single quadrupole analyzer operating in selected ion monitoring (SIM) has been widely applied for the multiresidue analysis of GC-amenable pesticides in different vegetable and fruit matrices (Mezcua et al. 2009; Mladenova and Shtereva 2009). The interest on reducing analysis time in multiresidue analysis has increased in the last years, looking for methods designed to determine as many compounds as possible in a short time. The use of fast GC allows rapid separations, satisfying current demands of higher sample throughput with not much sophisticated instrumentation (Dömötörová and Matisová 2008; Kirchner et al. 2005). However, determination of pesticide residues in food typically requires multiple steps: extraction, cleanup and subsequent determination by GC, in some cases after derivatization; thus, faster sample treatment methods are also desirable to reach high sample throughput.

When dealing with liquid samples, like juices, a classical technique for sample preparation is solid-phase extraction (SPE). A wide variety of sorbents and elution solvents can be used depending on the characteristics of the compounds to be extracted. C_{18} and Oasis hydrophilic–lipophilic balance (HLB) cartridges are among the most widely used in multiresidue methods (Marín et al. 2009; Xue et al. 2006; Piedra et al. 2000; Sabik et al. 2000; Picó et al. 2007; Cherta et al. 2012; Pitarch et al. 2007). Albero et al. (2005) developed a multiresidue method for the determination of pesticides using 10 mL juice samples C_{18} cartridge. Pang et al. (2006) used graphitized carbon black SPE cartridges in order to extract pesticides from 15 g of fruit juice. An immunoaffinity-based SPE procedure has been also applied for the determination of triazines in fruit juices (Dallüge et al. 1999).

A fast and simple alternative for sample treatment is the QuEChERS procedure (Anastassiades et al. 2003), which has been widely and successfully applied for the determination of pesticide residues in fruits and vegetables. It has been subjected to several modifications based on authors' preferences, but the AOAC Official Method

2007.01 (Lehotay et al. 2005) and the Standard Method EN 15662 (Payá et al. 2007) are the two official and most known versions. This methodology offers some advantages such as high sample throughput, high recoveries for a wide polarity and volatility range of pesticides and accurate results. QuEChERS combined with fast GC becomes a good choice to speed up multiresidue analysis. Although this procedure has been implemented for a wide range of commodities, especially fruits and vegetables in many routine laboratories with satisfactory results (Cieślik et al. 2011; Dai et al. 2011; Jiang et al. 2009; Kolberg et al. 2011; Park et al. 2011; Cherta et al. 2012a), only few publications have been reported for the analysis of juice samples, especially using GC or fast GC. The original QuEChERS version has been applied for the determination of 118 pesticides in vegetable juice by GC-MS and liquid chromatography–tandem mass spectrometry (Nguyen et al. 2009). Furlani et al. (2011) reported the determination of pesticide residues in sugarcane juice by GC with electron capture detection, also applying the unbuffered original QuEChERS version.

Another interesting approach for juice samples is the use of solid-phase microextraction (SPME), which has been successfully applied in pesticide residue analysis in water, soil, food, and biological samples (Beltran et al. 2000, 2001, 2003; Kataoka et al. 2000; Cervera et al. 2011; Fuster et al. 2005). SPME has gained in popularity since it minimizes sample preparation and also allows performing extraction and pre-concentration in a single step. The most common approach for non-volatile pesticides is the application of SPME by direct immersion (DI-SPME) (Fidalgo-Used et al. 2006; Farajzadeh and Hatami 2004; Natangelo et al. 2002; Simplício and Vilas Boas 1999), but its application to complex matrices is troublesome due to the absorption of interferences onto the fiber. This fact can be overcome if a previous solvent extraction is performed and the subsequent DI-SPME is applied over the separated aqueous extract (Kataoka et al. 2000; Zambonin et al. 2002) or by simply diluting the sample in order to simplify the matrix complexity (Sen et al. 1997). In the case of volatile compounds, the use of SPME in headspace mode (Hernández et al. 2002; López et al. 2001; Schurek et al. 2008; Serrano et al. 2009) allows minimizing the matrix interferences.

The aim of this work has been to critically compare three sample treatment methods based on QuEChERS, SPME and SPE in order to evaluate their applicability for pesticide residue analysis in fruit juice samples. All methods have been validated using apple juice samples. Determination has been performed by fast GC-MS with single quadrupole working under SIM mode.

EXPERIMENTAL

Reagents and materials

Pesticide standards used for this work were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock standard solutions (nominal concentration, 500 μg/mL) were prepared by dissolving reference standards in acetone and were stored in a freezer at −20 °C. Working standard mixtures for sample fortification were prepared by dilution of stock solutions in acetonitrile (for QuEChERS) and in acetone (for SPME and SPE).

Three isotopically labeled internal standards (ILIS) were used as surrogates: *p*,*p*′-DDE-D_8, terbuthylazine-D_5 (Dr. Ehrenstorfer) and hexachlorobenzene (HCB)-$^{13}C_6$ (Cambridge Isotope Labs Inc., Andover, MA, USA). A working mixed solution of labeled standards was prepared by volume dilution of individual stock solutions with acetonitrile (MeCN) and acetone and stored at 4 °C.

Acetone, hexane, MeCN, dichloromethane (DCM), glacial acetic acid (HAc), anhydrous magnesium sulfate ($MgSO_4$), anhydrous sodium acetate (NaAc) and sodium chloride (NaCl) were purchased from Scharlab (Barcelona, Spain). All solvents were for pesticide residue analysis or high-performance liquid chromatography (HPLC) grade. Two types of 2 mL microcentrifuge tubes for dispersive solid-phase extraction (d-SPE; used for the cleanup step) containing 50 mg primary–secondary amine (PSA) and 150 mg anhydrous $MgSO_4$ or 50 mg PSA, 150 mg anhydrous $MgSO_4$ and 50 mg C_{18} were obtained from Teknokroma (Barcelona, Spain).

Oasis HLB cartridges (200 and 60 mg) were purchased from Waters (Milford, MA, USA) and Bond Elut cartridges C_{18} (500 mg) were obtained from Varian (Harbor City, CA, USA). SPME fibers of polydimethylsiloxane (PDMS, 100 μm), polyacrylate (PA, 85 μm), and divinylbenzene/carboxen/PDMS (DVB/CAR/PDMS, 50/30 μm) were purchased from Supelco (Madrid, Spain).

Sample material

Apple and multi-fruit juice used for the validation study were purchased from a local market in Castellón (Spain). Once the optimum method was validated, four different juices were analyzed to investigate the presence of pesticides and test the applicability of the method. Apple–mango, pineapple and grapefruit juices were purchased from a local market in Castellón. Natural orange juice was obtained from fresh oranges collected from local harvesters.

GC instrumentation

Chromatographic measurements were performed on a GC system (Shimadzu QP2010 Plus) equipped with an autosampler (Shimadzu AOC-5000) and coupled to a single quadrupole mass spectrometer (GCMS-QP2010 Plus). Compounds were separated on a SAPIENS-5MS (Teknokroma) capillary column (length 20 m × I.D. 0.10 mm × film 0.10 μm).

For the chromatographic analysis of QuEChERS extracts (in MeCN), injections (3 μL) were performed in programmable temperature vaporization (PTV) mode, which was programmed as follows: 40 °C (hold time, 0.5 min), maintaining the split valve open; once the valve was closed, a rate of 400 °C/min to 320 °C (hold time, 0.5 min) was applied, resulting in an injection total time of 1.70 min. During this time, initial oven temperature was maintained at 60 °C and then heated at a rate of 90 °C/min to 225 °C, then 15 °C/min to 270 °C, and finally 150 °C/min to 330 °C (2 min), resulting in a total analysis time of 8.93 min. Helium was used as carrier gas at a flow of 0.77 mL/min (corresponding to a linear velocity of 39.1 cm/s).

When SPME was performed, the injector was operated in splitless mode at 280 °C and the splitless time was 5 min. During this time, initial column oven temperature was maintained at 50 °C and then programmed as previously indicated. In this case, the total analysis time was 12.14 min.

The injector was also operated in splitless mode (1 μL) when SPE extracts were analyzed, although injection temperature was 320 °C, initial column temperature was 80 °C and splitless time was 1.2 min, so chromatographic run time was 8.01 min.

The mass spectrometer was operated in the electron ionization mode (70 eV). The source and the interface (transfer line) temperatures were adjusted to 225 and 300 °C, respectively. The scan time in SIM mode was set at 0.1 s. In SIM mode, the three most abundant and/or characteristic ions for each analyte were selected as target and reference ions. Solvent delay times of 3.5, 4 and 7 min for SPE, QuEChERS and SPME, respectively, were used to prevent damage to the filament of the ion source. Shimadzu software GCMSsolution was used to automatically process the data.

Analytical procedures

- ***QuEChERS extraction: AOAC Official Method 2007.01*** *(Lehotay et al. 2005)*

15 mL of juice was poured in a 50-mL polypropylene centrifuge tube and 375 μL of surrogate standard solution mixture of 1 mg/L in MeCN was added and mixed on a vortex for 1 min. Extraction was carried out using 15 mL MeCN (with 1% HAc) and shaking by hand during 30 s. Then, 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous NaAc were added and immediately shaken vigorously by hand to prevent the formation of $MgSO_4$ agglomerates. Then, the tube was centrifuged at 3000 rpm during 2 min.

For the cleanup step, 1 mL of the upper MeCN extract was poured into the d-SPE tubes containing 150 mg $MgSO_4$ and 50 mg PSA (in the case of orange juice samples, d-SPE tubes also contained 50 mg C_{18}). The tubes were shaken on a vortex for 30 s and centrifuged at 3000 rpm for 2 min. The final MeCN supernatant extract

was directly injected into the GC system under the experimental conditions indicated before (PTV mode).

Matrix-matched calibration was used for each sample matrix in order to be able to adequately quantify analytes in real samples. In this way, 500 µL of MeCN extract obtained from a blank sample were mixed with 50 µL of the pesticide standard solution in MeCN of adequate concentration, also containing the three ILIS. Each analyte was quantified by using relative responses (areas) to the corresponding internal standard.

- ***SPE extraction***

25 µL of surrogate standard mixture in acetone of 1 mg/L was added to 1 mL of juice sample and passed through the 200-mg (6 mL) Oasis cartridge, previously conditioned by passing 6 mL of methanol, 6 mL of ethyl acetate/DCM, 6 mL of methanol and 6 mL of deionized water. After loading the sample, cartridges were washed with 6 mL of deionized water and dried by passing air, using a vacuum for at least 30 min. The retained analytes were eluted with 5 mL ethyl acetate/DCM (50:50). The collected extract was evaporated, after the addition of 1 mL hexane, under a gentle nitrogen stream at 40 °C until 0.5 mL, adjusted to 1 mL with hexane and injected into the GC system under the experimental conditions indicated before. Quantification of analytes in samples was carried out from calibration curves prepared with standards in solvent, using relative responses of each compound to the corresponding ILIS.

- ***SPME extraction***

Extraction of juice samples was performed by direct immersion of a PA fiber into the sample, under magnetic stirring (600 rpm) for 30 min. Samples were prepared by adding 25 µL of surrogate mixture (200 ng/mL) in acetone to 0.5 mL of juice and subsequent dilution with 1.5 mL of deionized water in a septum-capped 4-mL clear glass vial. Desorption of the fiber was carried out at 280 °C for 5 min in the splitless injector.

Quantification of analytes in samples was carried out using calibration curves prepared by spiking 2 mL of deionized water with 25 μL of pesticide standard solution of adequate concentration and 25 μL of surrogate mixture, both in acetone, and extracting these samples under the SPME procedure previously indicated. Relative responses of each compound to the corresponding internal standard were used.

Validation study

The three extraction methods used were validated using commercial apple juice samples in terms of linearity, accuracy, precision, limits of quantification (LOQ) and limits of detection (LOD). Confirmation capability of the method for positive samples was also evaluated.

Linearity was studied using calibration standards injected by triplicate. It was considered linear when regression coefficient was higher than 0.99 and the residuals lower than 30% without any clear tendency.

Accuracy was estimated from recovery experiments at two concentration levels (10 and 100 μg/L for QUEChERS and SPE; 1 and 10 μg/L for SPME) ($n = 6$). Precision was expressed as repeatability (intraday precision) in terms of relative standard deviation (RSD, in percent) ($n = 6$) at each fortification level.

LOQ was estimated as the analyte concentration that produced a peak signal ten times that of the background noise and it was calculated using the chromatograms at the lowest fortification level tested with satisfactory recovery (70–120%) and precision (RSD <20%). LOD was estimated in the same way, but for a signal-to-noise ratio of 3.

In order to confirm peak identity in samples, the ratio between the quantification ion (target, Q) and the reference ions (q_i) was evaluated and compared with the theoretical value obtained from reference standard solutions. The confirmation criterion was based on the European Commission Decision 2002/657/EC (European Commission Decision 2002), which establishes the

maximum tolerances for Q/q ratio deviation from theoretical values as a function of relative intensities. Coincidence between the retention time in a sample and the corresponding standard was also required to confirm a positive finding (maximum deviation, ± 0.5%).

RESULTS AND DISCUSSION

In a first step, for the optimization and comparison of extraction procedures, 15 selected pesticides (from a total of 56 pesticides studied in this work) were used (**Table 1**). Three sample treatments (QuEChERS, SPE and SPME) were studied in order to evaluate their advantages and disadvantages using apple juice sample as model matrix sample. The QuEChERS procedure was not optimized as it was based on the AOAC Official Method and applied in the same conditions as in our previous work (Cherta et al. 2013). SPME and SPE were subjected to an optimization study.

Table 1. List of compounds studied in method optimization.

Compound	QUECHERS		SPME		SPE		Monitored ions in SIM	
	t_R (min)	Time window (min) (SIM group)	t_R (min)	Time window (min) (SIM group)	t_R (min)	Time window (min) (SIM group)	Target ion	Reference ions
Trifluralin	4.542	4.30-4.70	7.931	7.80 – 8.05	3.772	3.60-3.86	264	290, 306
Atrazine	4.780	4.70-5.00	8.156	8.05 – 8.40	3.994	3.86-4.30	200	202, 215
Hexachlorobenzene-$^{13}C_6$ *	4.780		8.172		4.013		292	
Hexachlorobenzene	4.780		8.173		4.013		284	282, 286
Terbuthylazine-D_5 *	4.843		8.219		4.054		219	
Terbuthylazine	4.853		8.229		4.064		214	173, 229
Chlorpyriphos methyl	5.214	5.00-5.40	8.586	8.40 – 8.80	4.423	4.30-4.60	286	125, 288
Alachlor	5.248		8.618		4.453		160	132, 188
Chlorpyrifos	5.508	5.40-5.65	8.868	8.80 – 9.10	4.703	4.60-4.90	314	197, 199
Aldrin	5.566		8.937		4.771		263	101, 261
Isodrin	5.783	5.65-5.90	9.148	9.10 – 9.35	4.982	4.90-5.10	193	195, 263
Endosulfan I	6.135	5.90-6.19	9.489	9.35 – 9.80	5.323	5.10-5.61	241	170, 239
p,p'-DDE-D_8 *	6.240	6.19-6.45	9.575		5.413		254	
p,p'-DDE	6.256		9.591		5.428		246	248, 318
Dieldrin	6.348		9.693		5.528		263	265, 277
Endrin	6.548	6.45-7.00	9.889	9.80 – 10.20	5.723	5.61-6.10	263	261, 345
Endosulfan II [(a)]	6.626		9.960		5.796		241	243, 339
Bifenthrin	7.227	7.00-7.30	10.544	10.20 – 10.80	6.389	6.10-6.50	181	165, 166

* ILIS used in this work

[(a)] Target ion modified to 243 in QuEChERS extraction.

SPME optimization

In order to establish the optimum conditions for the extraction of the selected pesticides in apple juice samples, several parameters of the SPME procedure (type of fiber, sample dilution, salt and solvent addition and extraction and desorption times) were considered separately. Extraction temperature was set at room temperature and magnetic stirring at 600 rpm.

Firstly, the selection of the fiber was carried out by testing three different fibers: PDMS, PA and DVB/CAR/PDMS, under the same SPME conditions and comparing the chromatographic responses obtained. Each fiber was immersed during 15 min into 0.5 mL of juice spiked at 50 µg/L (diluted with 2.5 mL of water) and desorbed into the GC at 280 °C during 5 min (DVB/CAR/PDMS was desorbed at 270 °C, according to the manufacturer's recommendation). The best results were obtained for the PA fiber, as shown in **Fig. 1**, so this fiber was used for further experiments.

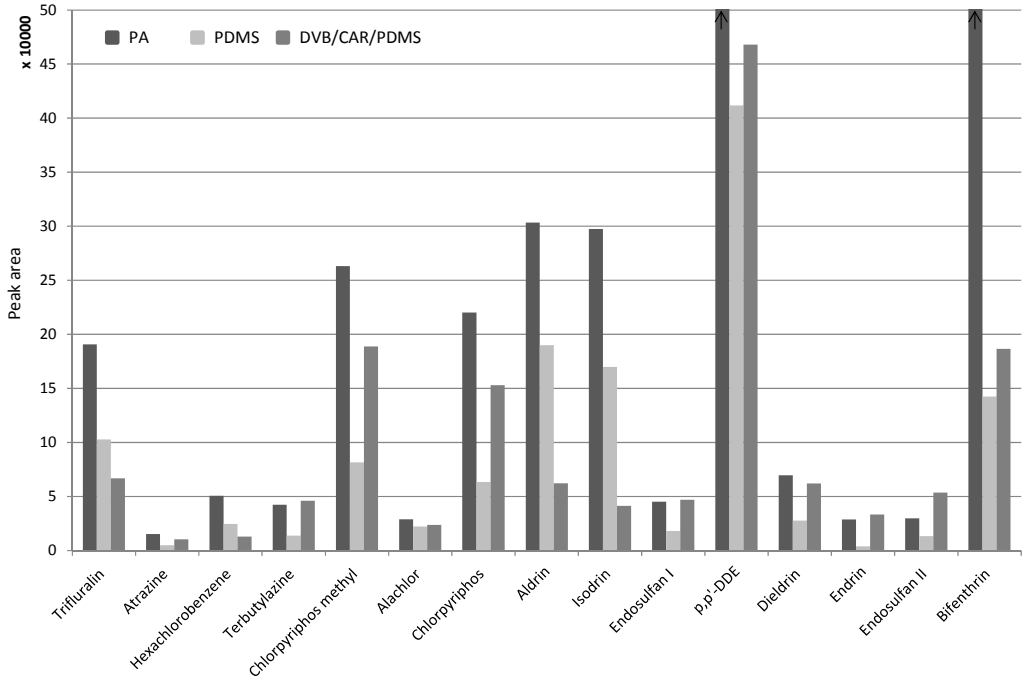

Fig. 1. Effect of SPME fiber type over extraction efficiency for selected pesticides (0.5 mL of 50 µg/L spiked apple juice, diluted with 2.5 mL of water; 15 min extraction).

A statistical optimization procedure based on a full factorial experiment design was applied. It allowed not only determining the optimum values for the selected variables but also detecting interactions between variables or identifying which ones did not affect the response. Optimization was carried out in a two-step scheme: first, a two-level full factorial design was applied to detect significant variables and, then, a surface response design was applied to determine the optimum values for those significant variables.

Three variables (addition of hexane/acetone (1:1), salting-out effect, and sample dilution) were studied at two levels (0 and 400 µL for hexane, 0 and 20% for NaCl, and 1.5 and 3.5 mL for H_2O). A 2^3 factorial design was performed, including 3 central points, so a total number of 11 randomized experiments were done. The statistical software package Statgraphics Centurion XV was used to generate the table of experiments and to evaluate the results obtained. The area of each pesticide was used as response function. The main effects of each variable and all the interactions were studied by means of the resulting Pareto charts. **Fig. 2** illustrates an example of the corresponding Pareto chart obtained for HCB (as all compounds showed the same general trend).

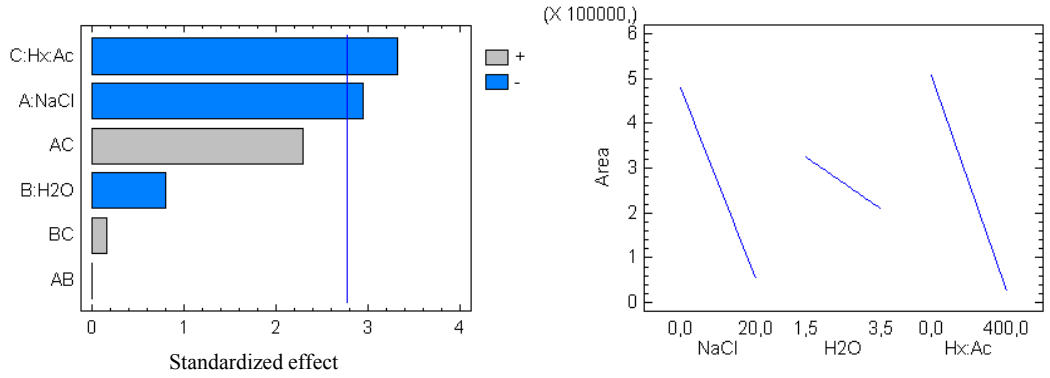

Fig. 2. Pareto chart of standardized effects of 2^3 factorial design for HCB, using the peak area as the function response, and main effects plots.

The length of the horizontal bar in the chart is proportional to the absolute value of the estimated effect; the vertical line defines the 95% confidence level. An effect is considered statistically significant if it exceeds this line. A general behavior was that solvent and salt addition presented a significant and negative effect, so both variables were selected for the next step in the optimization. As sample dilution was not significant but also had a negative effect, the lowest value tested, 1.5 mL, was selected. This minimum value was selected in order to have enough sample volume to cover the stationary phase of the SPME fiber and to have some matrix dilution effect that would improve quantification, as already described in the literature (Beltran et al. 2000).

Then, a 3^2 factorial design, including three central points, was performed in order to study salt and solvent addition at three levels. Values for these variables were set at the same levels as in the first design. This case required 12 randomized experiments and the response function used again was the peak area for each compound. The response surface for HCB (**Fig. 3**) obtained from the results of these experiments shows the negative effect of adding salt and solvent since extraction efficiency decreases proportionally to the addition of NaCl and hexane/acetone.

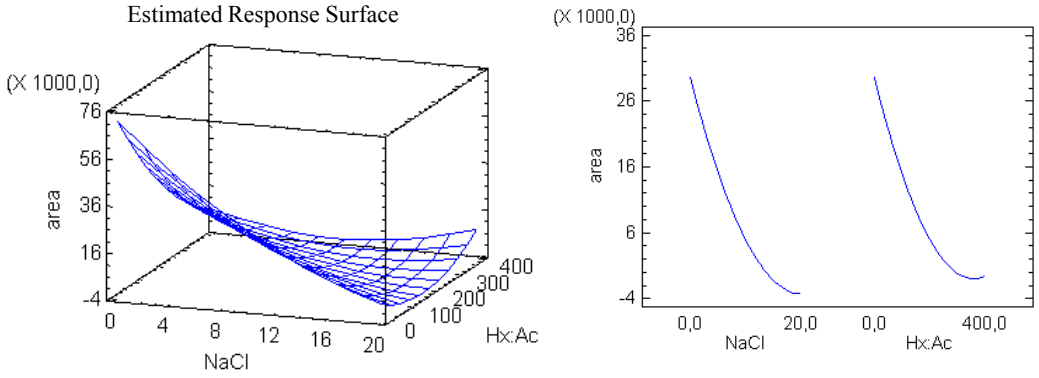

Fig. 3. Response surface obtained from a quadratic model for simultaneous optimization of salt and solvent addition, using the peak area as the function response, and main effects plots for HCB.

Therefore, the optimal conditions were found to be without modifying ionic strength or adding solvent. The effect of NaCl (or other ionic salts) has been widely discussed and different behaviors have been reported. In most cases, the salting out effect tends to increase extraction efficiency (Beltran et al. 1998; Boyd-Boland and Pawliszyn 1995), but this effect also depends on the solubility and polarity of the analytes and sometimes a decrease in sensitivity is noticed when larger amounts of salt are added (Cortés-Aguado et al. 2008; Magdic et al. 1996). Moreover, at higher concentration levels, NaCl crystals can occupy some of the active fiber sites and thus decrease extraction recoveries (Farajzadeh and Hatami 2004).

Optimization of the absorption equilibrium was performed by extracting replicate samples at different times (from 10 to 120 min). Analyte mass absorption, expressed as the peak area, was adjusted to a time-dependent equation given by Ai (1997):

$$n = n_0 (1 - e^{-at})$$

where n and n_0 are the amounts of analyte absorbed at a time t and at the equilibrium, respectively, and a is a parameter that measures how fast the absorption equilibrium can be reached in the SPME process. **Fig. 4** shows the results and the curves obtained after fitting the experimental data to the mentioned equation using the Statgraphics Centurion XV software for three of the studied compounds. Equilibrium time, estimated as the time necessary to extract 95% of n_0, was calculated for all the pesticides, giving values higher than 120 min in all cases. The feasibility of working in non-equilibrium conditions was considered, and thus, using the fitted equations, it was stated that establishing an extraction time of 30 min would lead to an extraction of around 50% with respect to the equilibrium situation for most compounds, and thus, analysis time would be considerably reduced.

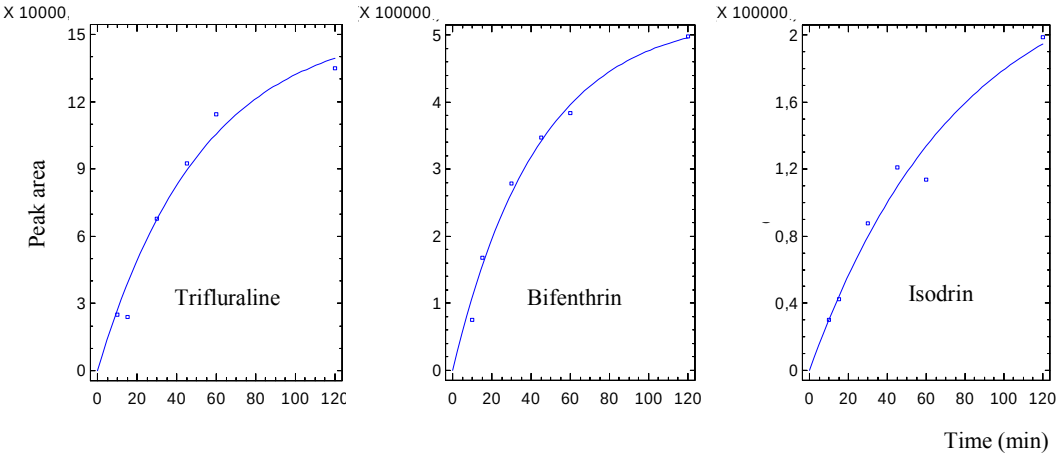

Fig. 4. SPME absorption time profiles for selected pesticides (0.5 mL of 10 µg/L spiked apple juice, diluted with 2.5 mL of water).

Finally, desorption time was studied in the range of 1–9 min under the optimum extraction conditions previously indicated. Peak areas increased with longer desorption times until complete desorption was reached, at about 5 min for most compounds. During this long desorption time, in order to profit from the effect of cold trapping, the oven temperature was maintained isothermal at 50 °C.

SPE optimization

SPE parameters as sorbent type, elution volume, elution solvent, volume of sample and evaporation step were optimized in order to find the optimum conditions for the extraction of the selected pesticides in juice samples.

As a first step, 200 and 60 mg Oasis HLB cartridges and 500 mg Bond Elut cartridges C_{18} were tested using 1 mL of apple juice sample fortified with the 15 selected pesticides. Elution was carried out with 5 mL of ethyl acetate/DCM. More consistent recoveries with lower RSD were obtained when 200 mg Oasis HLB cartridges were used, so this sorbent was selected for further experiments.

Different solvents and mixtures of solvents were also studied to set the best elution conditions. Acetone, ethyl acetate, DCM and a mixture of ethyl acetate and

DCM (50:50) were used to elute analytes retained in the cartridges at different volumes (from 2 to 10 mL). The elution using ethyl acetate/DCM mixture led to higher recoveries (although not still completely satisfactory), and 5 mL was selected since poor recoveries were obtained using lower elution volumes and no significant differences were observed at higher volumes.

The evaporation step was carefully studied by evaluating the effect of evaporation until dryness. Poor recoveries were obtained when the SPE extract was evaporated until dryness and re-dissolved with hexane until 1 mL. Thus, the addition of 1 mL hexane before the evaporation was considered; in this case, the extract was evaporated until 0.5 mL and then adjusted to 1 mL with hexane, avoiding possible losses of analytes during the evaporation process.

Different volumes of sample were tested in order to evaluate the maximum amount of sample to be passed through the cartridge without affecting the retention of the analytes. 1 mL of apple juice fortified at 100 μg/L, 10 mL fortified at 10 μg/L and 100 mL fortified at 1 μg/L were loaded to the cartridge and the signal intensity was evaluated. No chromatographic peaks were observed when using 100 mL, since the cartridge was overloaded with matrix components, impeding the retention of the analytes. The use of 10 mL also had a negative effect on the chromatographic signal since a loss of 75% with respect to the use of 1 mL was observed. Therefore, 1 mL of sample volume was selected, achieving satisfactory sensitivity and maintaining the high speed of sample preparation.

Comparison of analytical characteristics

In order to critically compare the three extraction procedures, accuracy, precision, LOD and LOQ were evaluated using apple juice blank samples spiked with the 15 pesticides selected as model. Three ILIS were used as surrogates in order to correct possible losses of the analytes during the extraction process and/or instrumental deviations. Terbuthylazine-D_5 was used as internal standard for herbicides, organophosphorus (OP) insecticides and pyrethroids; DDE-D_8 was used

for organochlorine pesticides and trifluralin; and HCB-$^{13}C_6$ was used for HCB. The specific internal standard used for each individual compound is indicated in **Table 2**.

Linearity was studied in the range 1–500 μg/L (n = 3) when QuEChERS and SPE were applied. In the case of SPME, the linearity was studied in the range 0.5–50 μg/L, and fitting the experimental data to quadratic curves; this concentration range could not be extended to higher values due to the large signal intensity of most compounds that saturated the detector (although a wider range could be achieved by the dilution of the sample before the SPME). The regression coefficients were higher than 0.99 for all compounds over the whole range tested in the three methodologies and the residuals lower than 30%.

As regards matrix effects, the corresponding study for QuEChERS procedure was performed in our previous work (Cherta et al. 2013), concluding that matrix-matched calibration curves were necessary to compensate for matrix effects in quantitative applications. On the contrary, no severe matrix effects were observed when SPE was applied, so calibration curves prepared in solvent could be used in this case, being this an important advantage. In the SPME procedure, matrix effects were evaluated by comparison of chromatographic responses of spiked water (2 mL) and spiked juice samples (0.5 mL juice and 1.5 mL water), both extracted by SPME. No significant differences or signal enhancements were observed, probably due to the matrix dilution already considered in the development of the procedure. Then, calibration curves prepared in water and juice were analyzed, obtaining similar calibration slopes for most compounds, so calibration prepared in HPLC water could be used instead of matrix-matched calibration curves in the SPME procedure.

Table 2. Average recovery (%) and R.S.D. (in parenthesis) after the application of three extraction techniques in apple juice (n=6) fortified at two concentration levels.

Compounds	QuEChERS				SPE				SPME			
	Fortification levels (µg/L)		LOD (µg/L)	LOQ (µg/L)	Fortification levels (µg/L)		LOD (µg/L)	LOQ (µg/L)	Fortification levels (µg/L)		LOD (µg/L)	LOQ (µg/L)
	10	100			10	100			1	10		
Trifluralin [a]	98 (8)	101 (9)	0.05	0.2	96 (9)	77 (8)	0.2	0.6	98 (15)	80 (7)	0.001	0.003
Atrazine [b]	102(5)	84 (13)	0.7	3	105 (4)	85 (5)	0.5	2	101 (5)	101 (4)	0.05	0.2
Hexachlorobenzene [c]	106 (1)	116 (2)	0.2	0.7	93 (4)	104 (1)	0.1	0.3	92 (19)	67 (2)	0.007	0.03
Terbuthylazine [b]	107 (6)	99 (8)	0.5	2	85 (4)	87 (6)	0.3	0.9	85 (5)	92 (5)	0.005	0.02
Chlorpyriphos methyl [b]	94 (5)	83 (15)	0.1	0.4	147 (7)	114 (5)	5	15	191 (10)	94 (8)	0.05	0.2
Alachlor [b]	104 (12)	94 (12)	0.3	1	88 (5)	71 (5)	0.2	0.6	132 (6)	107 (4)	0.1	0.3
Chlorpyriphos [b]	112 (6)	85 (14)	0.2	0.7	109 (6)	107 (4)	0.5	2	117 (5)	75 (9)	0.003	0.01
Aldrin [a]	102 (4)	109 (6)	0.2	0.7	50 (7)	67 (3)	3	10	94 (9)	104 (12)	0.02	0.06
Isodrin [a]	117 (5)	104 (8)	0.9	3	66 (5)	74 (4)	1	4	99 (7)	111 (9)	0.02	0.06
Endosulfan I [a]	-	103 (2)	6	18	-	82 (5)	9	27	94 (9)	101 (9)	0.04	0.2
p,p'-DDE [a]	100 (3)	103 (2)	0.08	0.3	94 (1)	113 (2)	0.2	0.6	41 (49)	71 (8)	0.008	0.03
Dieldrin [a]	68 (4)	108 (3)	0.9	3	86 (4)	80 (7)	0.7	3.0	103 (5)	101 (8)	0.02	0.06
Endrin [a]	111 (5)	105 (5)	1	4	-	105 (10)	5	15	- *	99 (12)	0.05	0.2
Endosulfan II [a]	-	103 (3)	5	15	112 (3)	111 (5)	1	3	48 (9)	107 (10)	0.05	0.2
Bifenthrin [b]	118 (10)	93 (9)	0.2	0.7	89 (4)	80 (8)	0.3	0.9	109 (18)	112 (6)	0.03	0.1

(a), (b), (c) indicates the internal standard used for each analyte: (a) *p,p'*-DDE-D_8, (b) terbutylazine-D_5, (c) hexachlorobenzene-$^{13}C_6$.

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

*Data not available due to heavy matrix interferences.

Accuracy and precision were evaluated by analyzing juice samples fortified at two concentrations ($n = 6$) of 10 and 100 μg/L for QuEChERS and SPE and 1 and 10 μg/L for SPME. Recoveries and RSD obtained for each analyte were calculated. As shown in **Table 2**, satisfactory recoveries (between 70 and 120%) were obtained for all compounds at both spiking levels for the QuEChERS procedure, as well as adequate RSD values (lower than 15%). Only two analytes, endosulfan I and II, could not be quantified at the lowest level due to poor sensitivity. LOQs ranged from 0.2 to 4 μg/L, except for endosulfan I and II (around 15 μg/L). Similar results were obtained for SPE, with the exceptions of chlorpyriphos methyl and aldrin, whose LOQs were slightly higher due to the inadequate recoveries presented at the lowest level. Endosulfan I and endrin could not be quantified at 10 μg/L, but satisfactory recoveries were obtained at the highest fortification level. The LOQs achieved with both methodologies are in agreement to those previously obtained by other authors for pesticides in fruit juices (Albero et al. 2005). On the other hand, the application of SPME led to an important gain in sensitivity, which can be appreciated in **Fig. 5**, reaching lower LOQs than those obtained in the other extraction methods (even 200 times lower for some compounds). This behavior was also reported in the literature for OP pesticides (Beltran et al. 1998), but in general terms, LODs obtained for pesticide residues ranges from 0.1 to 10 μg/L (Hernández et al. 2002; López et al. 2001; Cortés-Aguado et al. 2008), so an important enhancement of sensitivity is achieved under conditions used in this work. However, five compounds presented inadequate recoveries at the lowest level, although they could be validated at 10 μg/L. LOQs obtained for QuEChERS and SPE could not reach the nanograms per liter level, but were low enough for regulations purposes, considering that MRLs are commonly set at 10 μg/L in food commodities.

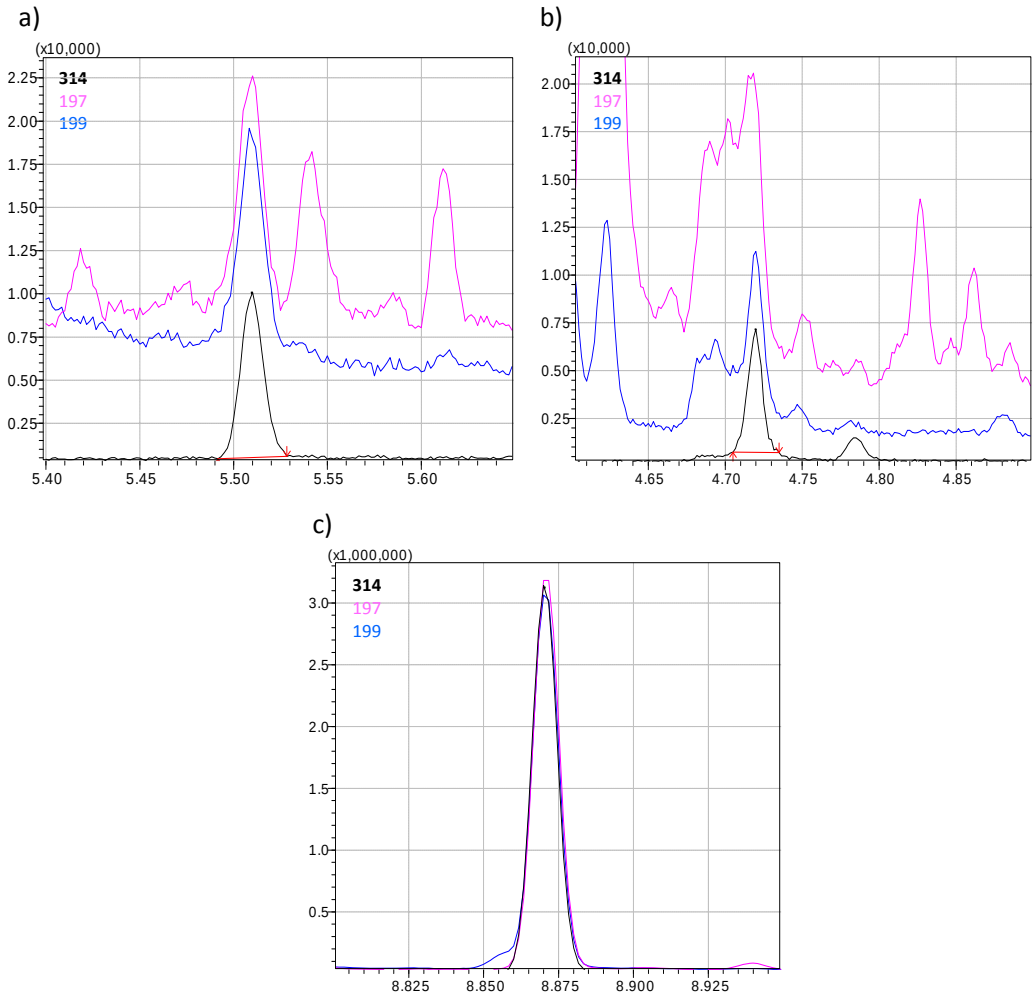

Fig 5. Comparison of chromatographic responses for chlorpyriphos in apple juice extract spiked at 0.01 mg/L after applying **a)** QuEChERS, **b)** SPE, and **c)** SPME. Target ion (*bold*) and two reference ions are shown.

As regards the confirmation of positive samples, the guidelines of the European Commission Decision (2002) establish that, after the acquisition of three ions (target (Q) and two reference ions (q_i)), the comparison of the two Q/q ratios measured in samples with those measured from reference standards shall lie within the maximum permitted tolerances. However, the expected Q/q ratios can be altered mainly due to matrix interferences. This is specially noticed at low concentration levels owing to the

lower abundance of the ions. In this work, we applied a more realistic criterion for the three methodologies: at the retention time of the analyte, three ions (target and reference) have to be observed in the sample and at least one Q/q ratio has to be accomplished.

It is noteworthy that differences on Q/q ratio accomplishment were observed depending on the extraction procedure applied. A higher number of compounds did not get ion ratios within the permitted tolerances after applying the SPE procedure. This seemed to be related to the fact that a higher number of reference ions were interfered by matrix coeluting components when SPE was applied, as illustrated in **Fig. 5** in the case of chlorpyriphos. Better results of Q/q ratio accomplishment were obtained for the QuEChERS procedure, surely due to the cleanup step included in the procedure. Thus, QuEChERS seemed to be a more adequate sample treatment for complex matrices than SPE. In the case of SPME, the higher sensitivity favored the compliance of Q/q ratios similar to QuEChERS, so it can be concluded that SPE is not a good enough extraction method for juice samples.

Extraction time was also evaluated, considering that the analysis method was based on fast GC. SPME involved a longer extraction time since samples were extracted one by one (30 min of extraction for sample), so it reduced dramatically the sample throughput. Decreasing the extraction time to a value similar to that of the chromatographic run (maximizing sample throughput) would lead to lower extraction efficiency (around 20% with respect to the equilibrium situation for most compounds). Moreover, this technique requires an additional desorption time once injected into the GC, so it resulted in longer chromatographic time (10.8 min). QuEChERS is considered as a rapid method and less labor-consuming; around 10 samples can be extracted in approximately 2 h. Moreover, shorter chromatographic time was possible after applying QuEChERS extraction (chromatographic time was around 9 min), taking more benefit from the fast GC.

In summary, the main advantage of SPME was the null solvent consumption and the possibility of reaching very low LOQs. However, the poor reproducibility of SPME specially noticed at low levels complicated the performance of the calibration

curves and the subsequent quantification process. As regards QuEChERS, it was the faster extraction procedure, fitting well with fast GC, and led to more satisfactory quantification results. Therefore, QuEChERS was selected for further validation of a wider list of pesticides, included in **Table 3**.

Table 3. Fast GC-MS conditions for 56 pesticides studied in the QuEChERS extraction applied to multi-fruit juice.

Compound	t_R (min)	Window (min)	Scan time (s)	Monitored ions in SIM	
				Target ion	Reference ions
Dichlorvos	3.735	3.60-3.80	0.10	185	109, 187
Chlorpropham	4.520	3.80-4.61	0.10	127	154, 213
Trifluralin	4.542			264	290, 306
Phorate	4.652	4.61-4.81	0.13	260	121, 231
alpha-HCH	4.723			219	181, 217
Atrazine	4.780			200	202, 215
Hexachlorobenzene-$^{13}C_6$ *	4.780			292	
Hexachlorobenzene	4.780			284	282, 286
Terbuthylazine-D_5 *	4.843			219	
Terbuthylazine	4.853	4.81-5.01	0.13	214	173, 229
beta-HCH	4.880			181	217, 219
Propyzamide	4.882			173	175, 255
Diazinon	4.885			137	152, 179
Lindane	4.890			181	183, 219
Pirimicarb	5.038	5.01-5.27	0.18	166	138, 238
Chlorothalonil	5.127			266	264, 268
Metribuzin	5.172			144	198, 199
Chlorpyriphos methyl	5.214			286	197, 288
Parathion methyl	5.235			263	216, 246
Alachlor	5.248			160	132, 188
Heptachlor	5.311	5.27-5.47	0.11	272	100, 102
Pirimiphos methyl	5.332			290	125, 244
Fenitrothion	5.388			109	260, 277
Malathion	5.400	5.47-5.67	0.15	127	125, 173
Fenthion	5.502			245	279, 280
Metholachlor	5.505			162	146, 238
Chlorpyriphos	5.508			314	197, 199
Parathion ethyl	5.566			291	139, 155
Aldrin	5.566	5.67-5.91	0.18	263	101, 261
Cyprodinil	5.822			224	210, 225
Pendimethalin	5.736			252	162, 192
Clofenvinphos	5.780			267	269, 323
Isodrin	5.783			193	195, 263

Table 3 (continued).

Compound	t_R (min)	Window (min)	Scan time (s)	Monitored ions in SIM	
				Target ion	Reference ions
Quinalphos	5.838			146	156, 157
Tolylfluanid	5.843	5.91-6.20	0.10	137	238, 240
Methidathion	5.985			145	93, 125
trans-Chlordane	6.011			375	371, 373
Endosulfan I	6.135			170	239, 241
p,p'-DDE-D_8 *	6.240			254	
p,p'-DDE	6.256	6.20-6.50	0.10	246	248, 318
Buprofezin	6.309			105	104, 172
Dieldrin	6.348			263	265, 277
Endrin	6.548	6.50-6.88	0.15	263	261, 345
Endosulfan II	6.626			195	241, 339
p,p'-DDD	6.631			165	176, 199
Ethion	6.633			125	153, 384
Oxadixyl	6.713			132	120, 146
Propiconazole I	6.915	6.88-7.18	0.10	173	175, 259
Propiconazole II	6.640			173	175, 259
p,p'-DDT	6.650			165	199, 212
Endosulfan sulfate	6.952			272	227, 274
Bifenthrin	7.227	7.18-7.42	0.10	181	165, 166
Phosmet	7.337			160	104, 161
Methoxychlor	7.338			227	212, 228
Tetradifon	7.470	7.42 -7.65	0.10	159	227, 229
Pyriproxyfen	7.518			136	137, 186
Fenarimol	7.705	7.65-7.85	0.10	139	219, 251
Cypermethrin	8.168	7.85-8.90	0.10	163	127, 181
Fenvalerate	8.470			125	167, 169

* ILIS used in this work

Validation for QuEChERS procedure for 56 pesticides

A complete validation of QuEChERS was performed for 56 pesticides in multi-fruit juice. The three ILIS were again used as surrogates. The specific internal standard used for each compound is indicated in **Table 4**.

Table 4. Average recovery (in percent) and RSD (in parenthesis) for multi-fruit juice after QuEChERS extraction and fast GC-MS analysis.

Compounds	Fortification levels (µg/L)		LOD (µg/L)	LOQ (µg/L)
	10	100		
Dichlorvos [a]	96 (10)	86 (11)	0.6	2
Chlorpropham [a]	103 (12)	90 (10)	0.6	2
Trifluralin [b]	95 (13)	105 (7)	0.3	0.9
Phorate [a]	99 (8)	87 (9)	2	6
alpha-HCH [b]	99 (7)	104 (5)	2	6
Atrazine [a]	-	83 (12)	6	18
Hexachlorobenzene [c]	92 (5)	113 (3)	0.3	0.9
Terbuthylazine [a]	109 (6)	95 (7)	2	6
beta-HCH [b]	88 (8)	87 (7)	2	6
Propyzamide [a]	-	100 (5)	7	21
Diazinon [a]	112 (6)	86 (8)	3	10
Lindane [b]	91 (9)	83 (8)	2	6
Pirimicarb [a]	104 (8)	82 (7)	2	6
Chlorothalonil [c]	-	-	-	-
Metribuzin [a]	-	86 (14)	12	36
Chlorpyriphos methyl [a]	91 (9)	81 (12)	0.3	0.9
Parathion methyl [a]	-	-	-	-
Alachlor [a]	96 (10)	88 (15)	1	3
Heptachlor [b]	68 (14)	98 (9)	2	6
Pirimiphos methyl [a]	120 (4)	111 (8)	2	6
Fenitrothion [a]	-	-	-	-
Malathion [a]	-	80 (11)	4	12
Fenthion [a]	-	109 (8)	10	30
Metholachlor [a]	105 (3)	88 (13)	0.7	2
Chlorpyriphos [a]	119 (7)	104 (7)	0.9	3
Parathion ethyl [a]	-	-	-	-
Aldrin [b]	110 (6)	85 (6)	2	6
Cyprodinil [c]	-	-	-	-
Pendimethalin [a]	-	95 (6)	8	24
Chlofenvinphos [a]	-	105 (10)	9	27
Isodrin [b]	76 (15)	78 (10)	2	6
Quinalphos [a]	-	92 (9)	6	18
Tolylfluanid [c]	-	-	-	-
Methidathion [a]	i.	i.	-	-
trans-Chlordane [b]	78 (12)	89 (6)	0.6	2
Endosulfan I [b]	-	89 (9)	8	24
p,p'-DDE [b]	87 (11)	82 (4)	0.6	2
Buprofezin [c]	-	120 (11)	15	45

Table 4 (continued).

Compounds	Fortification levels (μg/L)		LOD (μg/L)	LOQ (μg/L)
	10	100		
Dieldrin [(b)]	-	85 (10)	4	12
Endrin [(b)]	-	93 (9)	5	15
Endosulfan II [(b)]	-	107 (9)	10	30
p,p'-DDD [(b)]	119 (15)	78 (4)	2	6
Ethion [(a)]	-	88 (7)	5	15
Oxadixyl [(c)]	-	-	-	-
Propiconazole I [(c)]	-	120 (2)	20	60
Propiconazole II [(c)]	-	117 (8)	20	60
p,p'-DDT [(b)]	-	-	-	-
Endosulfan sulfate [(b)]	-	108 (12)	10	30
Bifenthrin [(a)]	83 (8)	72 (8)	1	3
Phosmet [(a)]	i.	i.	-	-
Methoxychlor [(b)]	-	-	-	-
Tetradifon [(c)]	-	126 (5)	-	-
Pyriproxyfen [(c)]	-	127 (5)	-	-
Fenarimol [(c)]	-	129 (7)	-	-
Cypermethrin [(a)]	-	-	-	-
Fenvalerate [(a)]	-	-	-	-

(a), (b), (c) indicates the internal standard used for each analyte: (a) terbutylazine-D_5, (b) *p,p'*-DDE-D_8, (c) hexachlorobenzene-$^{13}C_6$.

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

i., analyte not detected due to matrix interferences on the three analyte ions.

Linearity using matrix-matched standards was studied in the range 5–500 μg/L ($n = 3$). Residuals were lower than 30% and correlation coefficients by linear curves were higher than 0.99.

Accuracy and precision were evaluated by analyzing juice samples fortified at two levels (0.01 and 0.1 mg/L, $n = 6$). Results of recoveries and RSD are shown in **Table 4**. Half of the compounds could not be validated at the lowest level due to insufficient sensitivity and/or matrix interferences, in agreement with previous works (Cherta et al. 2013), but most of them presented satisfactory recoveries (between 70 and 120%) at 0.1 mg/L, as well as adequate RSD values (lower than 15 %). LOQs

ranged from 2 to 25 μg/L in most cases; exceptions were metribuzin, fenthion, buprofezin, endosulfan II, propiconazole and endosulfan sulfate, with LOQs between 30 and 60 μg/L. Results are in accordance with recent literature (Nguyen et al. 2009; Furlani et al. 2011).

In order to test the applicability of the GC-MS method developed for the 56 studied pesticides, it was applied to real commercially obtained samples. Representative samples of four matrices were selected and analyzed, including mango–apple, pineapple, grapefruit and natural orange juices. Multi-fruit juice was used to perform calibration curves. As it corresponds to healthy commercial juices, no positive findings were detected in any of the samples.

CONCLUSIONS

Three different sample treatments based on QuEChERS, SPE and SPME have been applied and evaluated for the determination of pesticides in juice samples by fast GC-MS. A comparative study in terms of validation results, extraction efficacy and extraction times has been carried out for 15 representative pesticides in order to establish the best extraction conditions.

Most compounds presented a similar behavior in terms of recoveries and RSD. However, SPME resulted in the most sensitive approach allowing to reach better LOQs (up to 200 times lower) in comparison with QuEChERS and SPE. On the other hand, more matrix interferences were observed after injecting the SPE extracts, leading to poorer Q/q ratio accomplishment that made identification of compounds in samples more problematic. Thus, SPE without additional cleanup seemed less adequate for complex matrices. Better results were obtained for the other two methodologies due to the cleanup step included in QuEChERS and the higher sensitivity achieved with SPME. As regards extraction times, SPME was the most time-consuming procedure and involved the longest chromatographic run time since an additional 5 min of desorption step in the injector was necessary. On the contrary,

QuEChERS led to the highest sample throughput, making feasible the analysis of around 30 samples in one day.

QuEChERS was considered the most appropriate sample treatment for juice samples, although SPME allowed reaching lower quantification limits. The QuEChERS procedure in combination with fast GC-MS was extended to the residue determination of 56 pesticides in multi-fruit samples, with acceptable results for the wide majority of compounds. Analysis of fruit juice samples of apple–mango, pineapple, grapefruit and orange revealed that any of the pesticides investigated were present at levels above the LOD, all well below the MRLs.

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II.5. Discusión de los resultados obtenidos

En los tres trabajos presentados en este capítulo se han desarrollado diferentes metodologías basadas en *fast* GC utilizando el Shimadzu QP2010 Plus con el fin de mejorar las ya existentes en este campo en base al número de compuestos a determinar y el tiempo de análisis. Previamente, se llevó a cabo un estudio para valorar las posibilidades de la cromatografía rápida en este sistema. Para ello, se investigó el desarrollo de un método para la determinación de un número elevado de compuestos en el menor tiempo posible. Se seleccionaron 117 pesticidas pertenecientes a diferentes familias físico-químicas y se trabajó con una mezcla de los correspondientes patrones de referencia en hexano.

En primer lugar se experimentó con una columna de 10 m x 0.1 mm I.D., cuya longitud reducida con respecto a las columnas de 30 m (empleadas comúnmente en GC convencional) supuso un notable descenso del tiempo de análisis, aunque también una considerable pérdida de resolución cromatográfica. Sumado a ello, la aplicación de rampas de temperatura tan altas como 100 ºC/min se tradujo en cromatogramas de menos de 7 minutos, con un elevado número de coeluciones, como se observa en el cromatograma en *full scan* (o *Total Ion Chromatogram* (TIC)) de la **Figura II.1**.

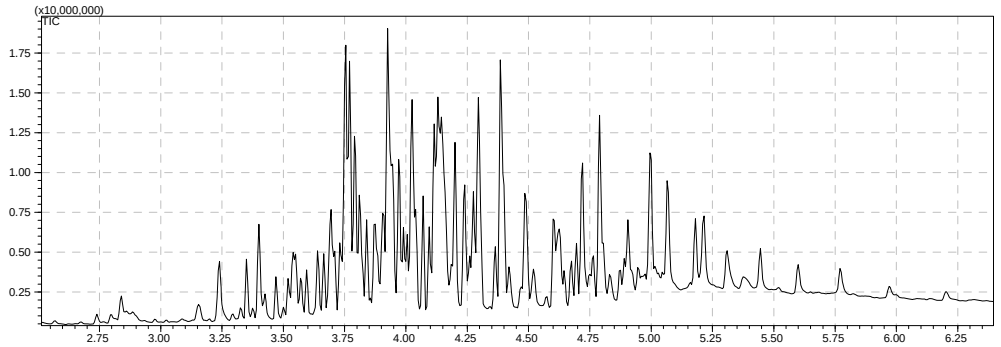

Figura II.1. TIC de una mezcla de patrones de pesticidas (117 compuestos) en hexano a una concentración de 100 ng/mL obtenido con una columna de 10 m x 0.1 mm I.D.

Aunque a simple vista parece complicada la identificación de los analitos, las coeluciones cromatográficas no suponen un problema siempre que el MS sea lo suficientemente selectivo como para solventarlas. En la **Figura II.2 a** se ilustra un ejemplo en el que el TIC no permite distinguir a priori tres pesticidas (fentión, metolaclor y clorpirifos) que eluyen a tiempos de retención muy próximos (entre 4.9 y 4.96 min); por el contrario, la extracción de las señales correspondientes a sus iones específicos (fentión: 278, metolaclor: 162 y clorpirifos: 197) permite la determinación de cada analito sin interferencias de los coeluyentes (**Figura II.2 b**).

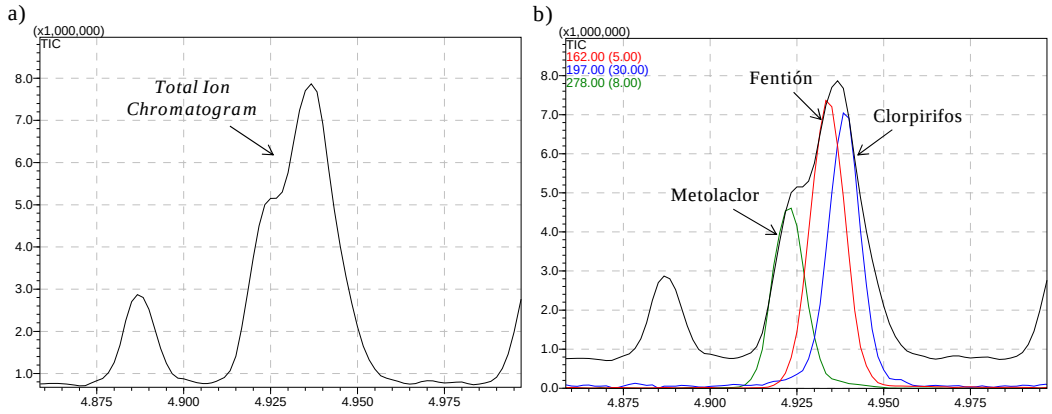

Figura II.2. a) Pico cromatográfico en *full scan* correspondiente a tres pesticidas coeluyendo; b) picos cromatográficos correspondientes a los iones específicos de cada uno de los pesticidas coeluyentes.

El siguiente paso en el diseño del método pasa por la preparación de un método SIM para mejorar la sensibilidad, basado en la adquisición selectiva de los iones más abundantes y/o característicos de cada analito. Especial atención se ha de prestar a las coeluciones, especialmente en estas condiciones de *fast* GC, con el fin de evitar la adquisición de iones comunes en analitos con tiempos de retención próximos. Como se observa en la **Figura II.1**, el primer analito eluye a un tiempo de 2.8 min y el último a 5.4 min, siendo el tiempo de separación de los 117 pesticidas menor de 3 minutos, lo que requiere una optimización compleja. Además, un número tan elevado de coeluciones puede generar una disminución de la sensibilidad ya que, en

ocasiones, los iones específicos que se deben utilizar en ciertos casos no siempre son los más abundantes o sensibles.

No obstante, el principal problema encontrado en el desarrollo del método SIM fue debido a limitaciones instrumentales. En el GC-MS Shimadzu QP2010 Plus, la duración mínima de un grupo o ventana SIM es de 0.2 minutos, con la adquisición de un máximo de 64 iones por ventana. Dado el rango del tiempo de elución de los 117 pesticidas seleccionados, una ventana de 0.2 minutos puede resultar demasiado grande ya que incluye a demasiados compuestos. En estas condiciones y considerando que en un método cuantitativo se requiere la adquisición de al menos 3 iones por compuesto (uno para cuantificar y otros dos de referencia), el número de iones superó en ocasiones el máximo de 64 que el sistema es capaz de adquirir en una ventana. A ello se le sumó la dificultad de encontrar espacios vacíos en el cromatograma que permitieran separar los grupos SIM sin cortar ningún pico cromatográfico, ya que el software no permite el solapamiento de ventanas.

Con ello, a pesar de que las coeluciones cromatográficas pueden resolverse con éxito gracias al monitoreo selectivo de iones, el potencial de este sistema en modo SIM no resulta suficiente como para separar un número elevado de analitos en un tiempo de análisis tan corto.

La situación planteada nos enfrentó a dos medidas alternativas: la eliminación de algunos compuestos de la lista del método o el aumento del rango de tiempo de la separación cromatográfica para poder generar ventanas SIM adecuadas.

Siguiendo la segunda alternativa descrita y con el fin de mejorar la resolución en experiencias posteriores, la columna de 10 m se sustituyó por una de 20 m, manteniendo el mismo I.D. de 0.1 mm. Así mismo, la rampa de temperatura se optimizó tratando de reducir el número de coeluciones y conseguir los huecos apropiados para cambiar los grupos SIM y ajustar el número de iones incluidos en los mismos a la capacidad máxima delimitada por el sistema. Las condiciones GC-MS

(modo SIM) indicadas en la **Tabla II.1** condujeron a una primera propuesta de método para la determinación de los 117 pesticidas en unos 10 minutos.

Tabla II.1. Condiciones *fast* GC-MS del primer método propuesto con la columna de 20 m x 0.1 mm.

GC	
Columna	SAPIENS-5MS: 20 m x 0.1 mm x 0.1 µm
Gas portador	He, 0.75 mL/min (39 cm/s)
Inyector	*Pulsed Splitless* (1.25 mL/min durante 1.2 min), 320 ºC
Programa de temperatura	80 ºC (1.2 min); 90 ªC/min hasta 225 ºC; 15 ºC/min hasta 300 ºC; 70 ºC/min hasta 330 ºC (2 min)
MS	
Temperatura fuente	225 ºC
Temperatura interfase	300 ºC
Adquisición	Modo SIM, 3 iones/compuesto

La lista de los pesticidas estudiados en estas condiciones se muestra en la **Tabla II.2**, donde se indica el tiempo de retención y los iones adquiridos para cada analito; también puede observarse la distribución de los compuestos en los diferentes grupos SIM, así como el tiempo de escaneo empleado en la monitorización de todos los iones que comprenden un grupo.

Tabla II.2. Condiciones *fast* GC-MS para los 117 pesticidas estudiados.

t_R (min)	Time window (min)	Scan time (s)	Compound	Monitored ions in SIM		
				Target ion (m/z)	Reference ion 1 (m/z)	Reference ion 2 (m/z)
3.015	2.53-3.52	0.1	Methamidophos	94	95	141
3.059			Dichlorvos	109	185	187
3.418	3.10-3.52	0.1	Mevinphos	127	109	192
3.446			Acephate	136	94	142
3.483			3,4-Dichloroaniline	161	126	163
3.573	3.52-3.99	0.26	Methacrifos	125	180	208
3.713			Pentachlorobenzene	250	248	252
3.759			Heptenophos	124	109	126
3.812			Omethoate	110	109	156
3.881			Tecnazene	203	215	259
3.89			Diphenylamine	169	167	170
3.892			Ethoprophos	158	200	242
3.911			Chlorpropham	127	154	213
3.929			Trifluralin	306	264	290
4.056	3.99-4.47	0.46	Phorate	121	231	260
4.135			HCH-alfa	181	183	217
4.138			Simazine	201	173	186
4.153			Dimethoate	125	143	229
4.158			Atrazine	200	202	215
4.192			Hexachlorobenzene	284	282	286
4.237			Terbutylazine	214	173	229
4.248			Lindane	181	183	217
4.264			Propyzamide	173	145	175
4.27			Terbufos	231	103	288
4.271			Diazinon	137	152	179
4.295			Tefluthrin	177	197	199
4.306			HCH-beta	181	183	217
4.311			Pyrimethanil	198	184	199
4.324			Fonofos	137	110	246
4.331			Quintozene	237	214	249
4.361			Disulfoton	88	97	186
4.367			Etrimfos	292	168	181
4.416			HCH-delta	181	183	217
4.433			Pirimicarb	166	138	238
4.44			Chlorothalonil	266	264	268
4.522	4.47-4.96	0.54	Phosphamidon	127	138	267
4.56			Metribuzin	198	144	199
4.578			Endosulfan ether	241	170	277
4.6			Vinclozolin	212	178	198
4.623			Parathion methyl	263	139	246
4.627			Chlorpyriphos methyl	286	288	290
4.662			Alachlor	188	202	237

Tabla II.2 (continuación).

t_R (min)	Time window (min)	Scan time (s)	Compound	Monitored ions in SIM		
				Target ion (m/z)	Reference ion 1 (m/z)	Reference ion 2 (m/z)
4.662			Carbaryl	201	115	144
4.665			Tolclofos methyl	265	250	267
4.678			Metalaxyl	192	121	220
4.723			Fenchlorphos	285	125	287
4.754			Pirimiphos methyl	290	233	305
4.757			Heptachlor	272	237	337
4.774			Methiocarb	168	153	225
4.783			Fenitrothion	277	125	260
4.804			Malathion	125	127	173
4.877			Dichlofluanid	123	167	224
4.912			Fenthion	278	153	169
4.923			Metholachlor	162	238	240
4.928			Chlorpyriphos	197	199	314
4.932			Parathion ethyl	291	137	139
4.99	4.96-5.56	0.57	Dicofol deg	139	251	253
5.012			Aldrin	263	261	293
5.023			Pirimiphos ethyl	333	304	318
5.12			Cyprodinil	224	210	225
5.165			Chlozolinate	259	188	331
5.172			Pendimethalin	252	162	281
5.192			Penconazole	248	159	250
5.214			Isofenphos	213	121	185
5.214			Chlofenvinphos	267	269	323
5.233			Tolylfluanid	137	181	238
5.236			Isodrin	193	195	197
5.237			Captan	149	117	264
5.265			Quinalphos	146	118	157
5.281			Triflumizole	278	179	206
5.321			Procymidone	283	255	285
5.358			Folpet	260	130	295
5.404			Methidathion	145	85	125
5.446			Tetrachlorvinphos	329	109	331
5.475			trans-Chlordane	371	373	375
5.578	5.56-6.20	0.42	Imazalil	215	173	217
5.58			Chlorfenson	175	111	302
5.595			Endosulfan I	195	170	241
5.642			Profenofos	337	139	206
5.696			p,p'-DDE	246	248	318
5.715			Myclobutanil	179	181	206
5.728			Bupirimate	273	193	316
5.748			Buprofezin	105	172	175
5.812			Dieldrin	263	265	279

Tabla II.2 (continuación).

t_R (min)	Time window (min)	Scan time (s)	Compound	Monitored ions in SIM		
				Target ion (m/z)	Reference ion 1 (m/z)	Reference ion 2 (m/z)
6.015			Endrin	263	265	317
6.067			Ethion	153	125	384
6.068			p,p'-DDD	165	212	320
6.083			Endosulfan II	195	241	337
6.083			Oxadixyl	163	120	132
6.123			p,p'-DDT	235	165	237
6.162			Triazophos	161	162	257
6.345	6.20-6.60	0.1	Propiconazole I	259	173	261
6.365			Propiconazole II	259	173	261
6.458			Endosulfan sulfate	387	272	274
6.548			Tebuconazole	125	250	163
6.848	6.60-7.25	0.17	Bifenthrin	181	165	166
6.902			Bromopropylate	341	183	185
6.903			Phosmet	160	133	161
6.943			Methoxychlor	227	212	228
7.017			Dicofol	139	251	253
7.201			Tetradifon	159	227	356
7.283	7.25-7.53	0.12	Pyriproxyfen	136	186	226
7.286			Phosalone	182	121	184
7.316			Azinphos methyl	160	104	132
7.401			lamda-Cyhalothrin	181	197	208
7.561	7.53-7.73	0.1	Mirex	272	237	274
7.574			Pyrazophos	221	232	265
7.609			Fenarimol	139	219	251
7.881	7.73-8.10	0.1	Permethrin	183	163	165
7.996			Pyridaben	147	117	132
8.04			Coumaphos	362	210	226
8.183	8.10-8.30	0.1	Cyfluthrin	163	165	226
8.335	8.30-8.53	0.1	Cypermethrin	163	165	181
8.787	8.53-9.50	0.1	Fenvalerate	125	167	225
8.856			Fluvalinate	250	252	502
8.88			Esfenvalerate	125	167	225
9.284			Azoxystrobin	344	372	388

En principio el método desarrollado cumple con las restricciones del sistema en cuanto al número de iones permitidos por ventana. Sin embargo, en la tabla anterior queda reflejada la fuerte dependencia con el tiempo de escaneo, el cual aumenta automáticamente con el incremento del número de iones por grupo. Este hecho convierte al *scan time* en un factor crítico a tener en cuenta en el desarrollo del método en modo SIM.

Dada la importancia del valor del *scan time*, parece razonable estudiar en profundidad el efecto que el diseño del método tiene sobre este valor y como se ve afectada la calidad de los datos cromatográficos. En un método *full scan* con un rango de masas 50-400 umas, resulta evidente la relación directa del *scan time* con la sensibilidad, como se ilustra en la **Figura II.3**. No obstante, aunque un mayor *scan time* ofrezca una mayor sensibilidad, el precio a pagar es un menor número de puntos por pico. En análisis cuantitativos, el número mínimo de puntos por pico aceptado para una adecuada representación y cuantificación suele ser de 8 a 10 (Maštovská & Lehotay, 2003). En este caso en particular, tiempos de escaneo de 0.5 s proporcionan únicamente 4 puntos/pico, por lo que se podría establecer que un tiempo de escaneo entre 0.1 y 0.2 segundos supondría un buen compromiso entre sensibilidad y número de puntos por pico.

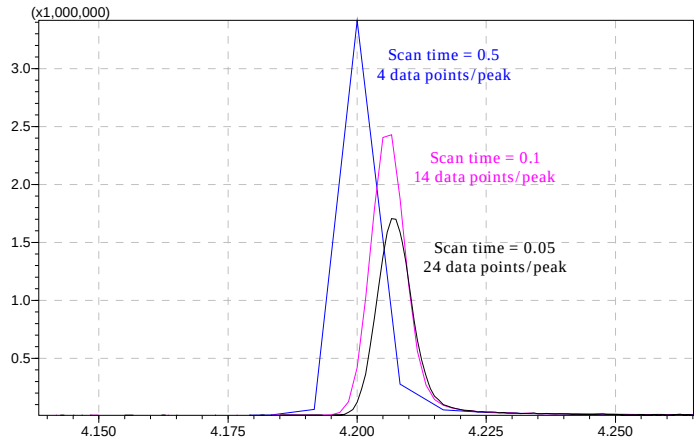

Figura II.3. Efecto del tiempo de escaneo en la sensibilidad y número de puntos por pico.

El efecto del *scan time* es todavía más complicado cuando se desarrolla un método SIM. Aunque la velocidad máxima del cuadrupolo simple empleado es de 10000 uma/s, ésta se puede reducir hasta 100 veces cuando la adquisición se realiza en SIM debido a ciertos parámetros como el *settling time*, de modo que la monitorización de un determinado número de iones concretos conlleva un mayor tiempo que la adquisición de un rango de masas continuo. Así, grupos SIM con más de 10 iones sufren un aumento automático del *scan time* (en principio fijado a 0.1 s), como puede observarse en la **Tabla II.2**, con la consiguiente pérdida de calidad en la forma de pico. Los compuestos más perjudicados en este sentido son aquellos comprendidos en las ventanas centrales (desde el minuto 4.056 al 6.162), adquiridos con tiempos de escaneo superiores a 0.4 s, presentando un número insuficiente de puntos por pico. La solución a estas limitaciones vendría del uso de un sistema que permita una mayor velocidad de escaneo, especialmente en modo SIM. En este sentido, el GC-MS Shimadzu QP2010 Ultra, versión mejorada del Shimadzu QP2010 Plus, es capaz de alcanzar los 20000 uma/s, con una adquisición en modo SIM hasta 5 veces más rápida, permitiendo monitorizar 50 iones de una misma ventana en 0.1 s. En la **Figura II.4** se muestra la comparación de la influencia del tiempo de escaneo con el número de puntos por pico en ambos sistemas, observándose la mejor forma de pico con la instrumentación Shimadzu QP2012 Ultra. Desafortunadamente, la adquisición de este equipo en nuestro grupo de investigación fue posterior a estas experiencias y, por tanto, la validación del método aún queda pendiente.

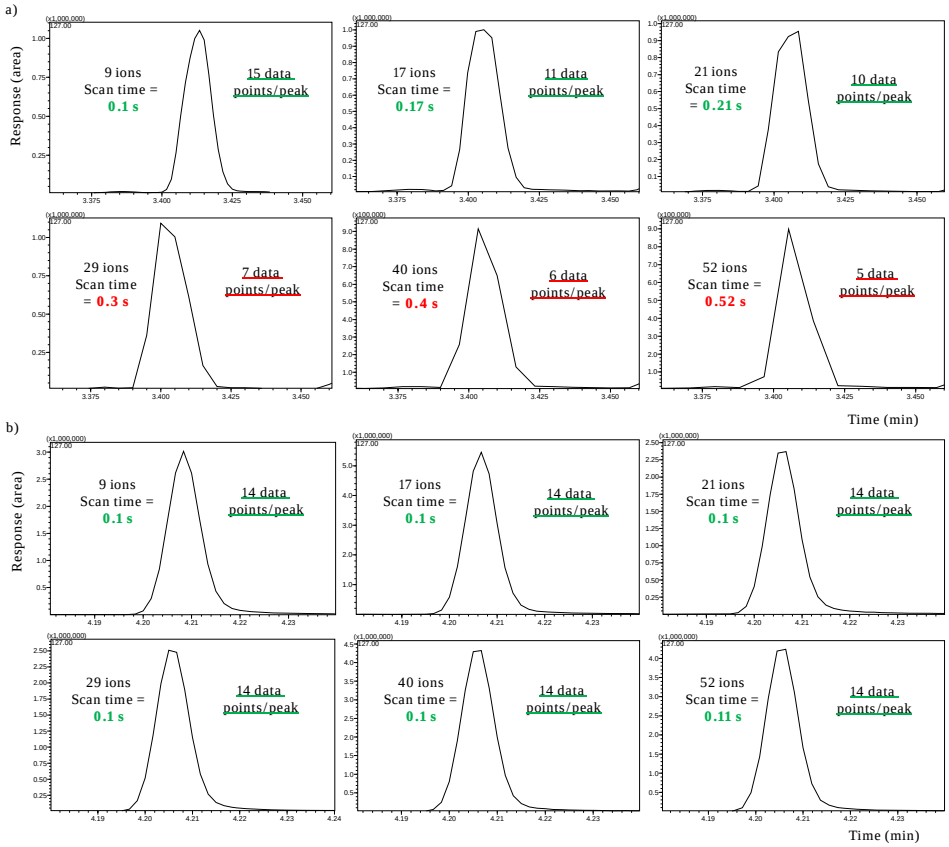

Figura II.4. Pico cromatográfico del pesticida mevinfos cuando se incluye en grupos SIM con diferente número de iones monitorizados. Equipo empleado: a) GC-MS Shimadzu QP2010 Plus; b) GC-MS Shimadzu QP2010 Ultra.

En caso de no disponer de equipos con mayor velocidad de escaneo, la reducción del número de compuestos a determinar se presenta como recurso final con el fin de adaptar una metodología *fast* GC-MS a las capacidades del sistema.

En el primer método desarrollado y validado (Artículo científico 1) se determina un total de 66 analitos (además de pesticidas se incluyen PAHs, PBDEs, PCBs y alquilfenoles) en muestras de agua en menos de 10 minutos (separación cromatográfica), con tiempos de escaneo comprendidos entre 0.1 y 0.22 s para los diferentes grupos SIM, en función del número de iones/compuestos.

Por otro lado, aprovechando la capacidad del sistema de trabajar en modo SIM y *full scan* simultáneos, en este primer artículo se evaluó el alcance de esta modalidad para realizar un análisis *target* y *non-target* simultáneo. Ello implica la adquisición, en cada ventana SIM, de un barrido completo de masas en un determinado rango además de los iones específicos seleccionados para cada analito y, por tanto, de nuevo, un aumento drástico del tiempo de escaneo. Considerando que las condiciones del método desarrollado para los 66 compuestos están en el límite de ser aptas para fines cuantitativos, los grupos SIM no admitirían la adquisición adicional de un *full scan* sin afectar negativamente a la forma de pico. La alternativa, nuevamente, es la reducción considerable del número de compuestos *target*. Cabe considerar, pues, si el análisis *non-target* merece sacrificar la cuantificación de ciertos analitos o analizar las muestras por duplicado en los modos SIM y *full scan* aplicados independientemente. Dado el corto tiempo de análisis, la segunda opción puede resultar igualmente ventajosa.

Las condiciones descritas en este primer artículo consolidan las bases para el desarrollo de las metodologías *fast* GC-MS comprendidas en los Artículos científicos 2 y 3, en las que se llegan a determinar un total de 56 pesticidas en menos de 9 minutos. En estos casos la atención se centra en un nuevo obstáculo, el efecto matriz, que en el Artículo científico 1 resultó prácticamente nulo para el análisis de aguas, pudiéndose emplear un calibrado preparado en solvente para la cuantificación de los analitos. Por el contrario, las muestras más complejas analizadas en los posteriores trabajos (frutas, vegetales y zumos) provocaron generalmente la exaltación de la señal de los analitos con respecto a la señal obtenida de los mismos en disolvente, por lo que el calibrado en matriz resultó necesario para una correcta cuantificación. El solo uso de patrones internos no resultó suficiente para corregir este efecto; la diferencia en las respuestas relativas se traduce en pendientes de las curvas de calibrado diferentes (mayores en calibrado en solvente), como puede observarse en la **Figura 2** del Artículo científico 2 (pág. 95). Ello puede deberse al hecho de utilizar únicamente 4 patrones internos para corregir a los más de 50 compuestos incluidos en los métodos en lugar de emplear patrones internos marcados isotópicamente de cada analito, que sería la situación ideal para corregir eficazmente el efecto matriz.

La influencia de la matriz también puede provocar un aumento del ruido y modificar las respuestas de los analitos en las diferentes muestras, condicionando el nivel mínimo del calibrado para cada analito, como se refleja en la **Tabla II.3**.

Tabla II.3. Niveles mínimos de concentración del calibrado (mg/kg) alcanzados para los pesticidas estudiados en el Artículo científico 2 en las diferentes matrices.

Compounds	Apple	Orange	Carrot	Tomato	Olive
Dichlorvos	5	5	5	1	5
Chlorpropham	5	10	20	50	20
Trifluralin	5	5	10	5	5
Phorate	5	10	10	5	5
alpha-HCH	5	10	10	5	10
Atrazine	5	10	1	50	n.e.
Hexachlorobenzene	5	5	5	5	5
Terbuthylazine	5	5	10	10	n.e.
beta-HCH	10	20	50	10	50
Propyzamide	10	20	10	20	50
Diazinon	10	20	20	5	20
Lindane	5	10	50	10	50
Pirimicarb	5	5	5	10	20
Chlorothalonil	20	n.e.	10	n.e.	n.e.
Metribuzin	5	20	50	n.e.	n.e.
Chlorpyriphos methyl	1	5	10	5	1
Parathion methyl	10	20	50	10	5
Alachlor	5	10	10	5	5
Heptachlor	10	20	10	5	20
Pirimiphos methyl	5	10	5	5	5
Fenitrothion	10	50	50	10	10
Malathion	10	50	50	20	20
Fenthion	20	20	50	10	20
Metholachlor	5	5	10	5	5
Chlorpyriphos	1	50	20	10	1
Parathion ethyl	20	20	50	n.e.	20
Aldrin	5	5	10	5	5
Cyprodinil	5	10	10	n.e.	5

Compounds	Apple	Orange	Carrot	Tomato	Olive
Pendimethalin	20	50	20	20	20
Chlofenvinphos	20	50	20	20	n.e.
Isodrin	10	20	20	10	20
Quinalphos	10	20	20	20	20
Tolylfluanid	10	n.e.	n.e.	n.e.	n.e.
Methidathion	10	10	n.e.	n.e.	50
trans-Chlordane	10	50	20	5	10
Endosulfan I	20	50	50	50	n.e.
p,p'-DDE	5	10	10	10	5
Buprofezin	10	50	50	20	50
Dieldrin	50	10	10	50	50
Endrin	50	10	50	50	50
Endosulfan II	10	10	10	10	n.e.
p,p'-DDD	10	20	20	20	50
Ethion	50	50	10	50	50
Oxadixyl	20	20	n.e.	n.e.	n.e.
Propiconazole I	50	50	50	10	n.e.
Propiconazole II	50	50	50	10	n.e.
p,p'-DDT	n.e.	n.e.	10	n.e.	50
Endosulfan sulfate	20	n.e.	n.e.	10	10
Bifenthrin	5	20	10	1	5
Phosmet	10	n.e.	n.e.	n.e.	n.e.
Methoxychlor	n.e.	n.e.	10	10	n.e.
Tetradifon	n.e.	50	n.e.	10	n.e.
Pyriproxyfen	n.e.	10	20	10	5
Fenarimol	5	10	n.e.	10	n.e.
Cypermethrin	10	10	n.e.	n.e.	n.e.
Fenvalerate	50	10	n.e.	n.e.	n.e.

n.e. not estimated due to matrix interferences and/or poor sensitivity.

No obstante, a pesar de la eficacia del calibrado en matriz para corregir la exaltación de la señal, el principal inconveniente en la cuantificación surgió ante la presencia de componentes de la matriz extraídos coeluyendo con los analitos de interés, como también se discute en la bibliografía (Kirchner *et al*., 2005b). En estas condiciones se requiere una alta selectividad por parte del MS con el fin de seleccionar cuidadosamente iones libres de interferencias en un proceso de optimización matriz-dependiente. Aunque el uso del cuadrupolo simple permitió resolver coeluciones entre analitos en las condiciones establecidas (con patrones en solvente), el modo SIM no resultó suficiente para algunas combinaciones matriz/analito (ver **Tabla 2** del Artículo científico 2, pág. 97). Cabe destacar que en

la mayoría de casos las interferencias, especialmente notables a niveles de concentración bajos, afectaron a uno de los dos iones de confirmación, permitiendo la correcta cuantificación del ión *target*. Los casos más drásticos se dieron en algunas matrices para determinados analitos que presentaron interferencias en sus tres iones, siendo inviable su sustitución por iones "libres" (7 compuestos de un total de 56 en las cuatro matrices).

Las limitaciones del cuadrupolo simple para el análisis de muestras complejas se pueden resolver mediante un aumento en la selectividad del MS como la que podría aportar el triple cuadrupolo o un TOF con medida de masas exacta.

Otra alternativa para evitar o reducir interferentes de la matriz sería proponer mejoras en la etapa del tratamiento de muestra. El método QuEChERS aplicado en el Artículo científico 2 incorpora una etapa de *clean-up* en la que a la muestra se le adiciona PSA con la finalidad de eliminar ácidos grasos, azúcares y ácidos orgánicos. Adicionalmente, el uso de C_{18} permite eliminar lípidos y esteroles, mejorando la purificación de matrices como la naranja y la aceituna. Sin embargo, este *clean-up* parece no ser siempre suficiente; una purificación posterior aplicando SPE podría suponer una solución para ciertos tipos de matrices problemáticas (Garrido Frenich, 2008), aunque con el consiguiente aumento del tiempo de análisis.

En el desarrollo de un método *fast* GC-MS es importante considerar el tiempo empleado en el tratamiento de muestra, ya que suele consumir una parte importante del tiempo total del análisis. A estos efectos, el método QuEChERS se consideró una buena opción para combinar con la cromatografía rápida, como se reporta en otros trabajos (Hercegová *et al.*, 2006), pero sin tomar medidas adicionales de purificación. Así mismo, la disponibilidad de un inyector PTV en el sistema empleado permitió la inyección directa de los extractos de ACN en el cromatógrafo, evitando el paso adicional de cambio de solvente que implicaría la inyección de estos extractos en modo *splitless*.

Como se discute en el Artículo científico 3, en el que se comparan los tratamientos de muestra QuEChERS, SPE y SPME para la determinación de

pesticidas en zumos, el método QuEChERS es el que permite extraer y analizar un mayor número de muestras en menor tiempo.

En cuanto a los interferentes coextraídos de la matriz estudiados en este último trabajo, la extracción SPE resultó ser la menos apropiada. Esta técnica, en cambio, se considera el tratamiento por excelencia para las muestras de agua, como demuestran los resultados obtenidos en el Artículo científico 1. La posibilidad de pre-concentrar los extractos mediante SPE supone una ventaja con respecto al método QuEChERS, al igual que la extracción SPME. Esta última permitió disminuir los LODs hasta 200 veces con respecto a la SPE y al QuEChERS para la extracción de pesticidas en zumos, pero el largo tiempo de extracción que implica reduce considerablemente el rendimiento por muestra.

Así, SPE y QuEChERS para la extracción de pesticidas en aguas y alimentos, respectivamente, resultaron los tratamientos óptimos para combinarse con *fast* GC-MS. A pesar de no incluir una etapa de pre-concentración de los extractos, los LOQs derivados del método QuEChERS fueron suficientes considerando la regulación actual en el campo alimentario, en la que los MRLs mínimos están fijados a 0.01 mg/kg.

La validación de las metodologías propuestas en los tres artículos presentados en este capítulo se llevó a cabo en función de la linealidad, exactitud, precisión, LODs, LOQs y capacidad de confirmación. Las características y parámetros que comportan la validación de métodos se encuentran bien resumidos en artículos científicos como los de Swartz & Krull, 2009 & 2011.

Los resultados obtenidos fueron satisfactorios para la mayoría de los compuestos estudiados, alcanzando bajos LODs y LOQs. Excepto para una pequeña minoría, los LODs para los contaminantes determinados en las distintas aguas del Artículo científico 1 oscilaron entre 0.1 y 10 ng/L. En el análisis de alimentos desarrollado en los Artículos científicos 2 y 3 se consiguieron LODs en un rango de

0.5-10 µg/kg y 0.5-10 µg/L. Los valores obtenidos para los tres tipos de matrices estudiadas se muestran de forma esquemática en la **Figura II.5**.

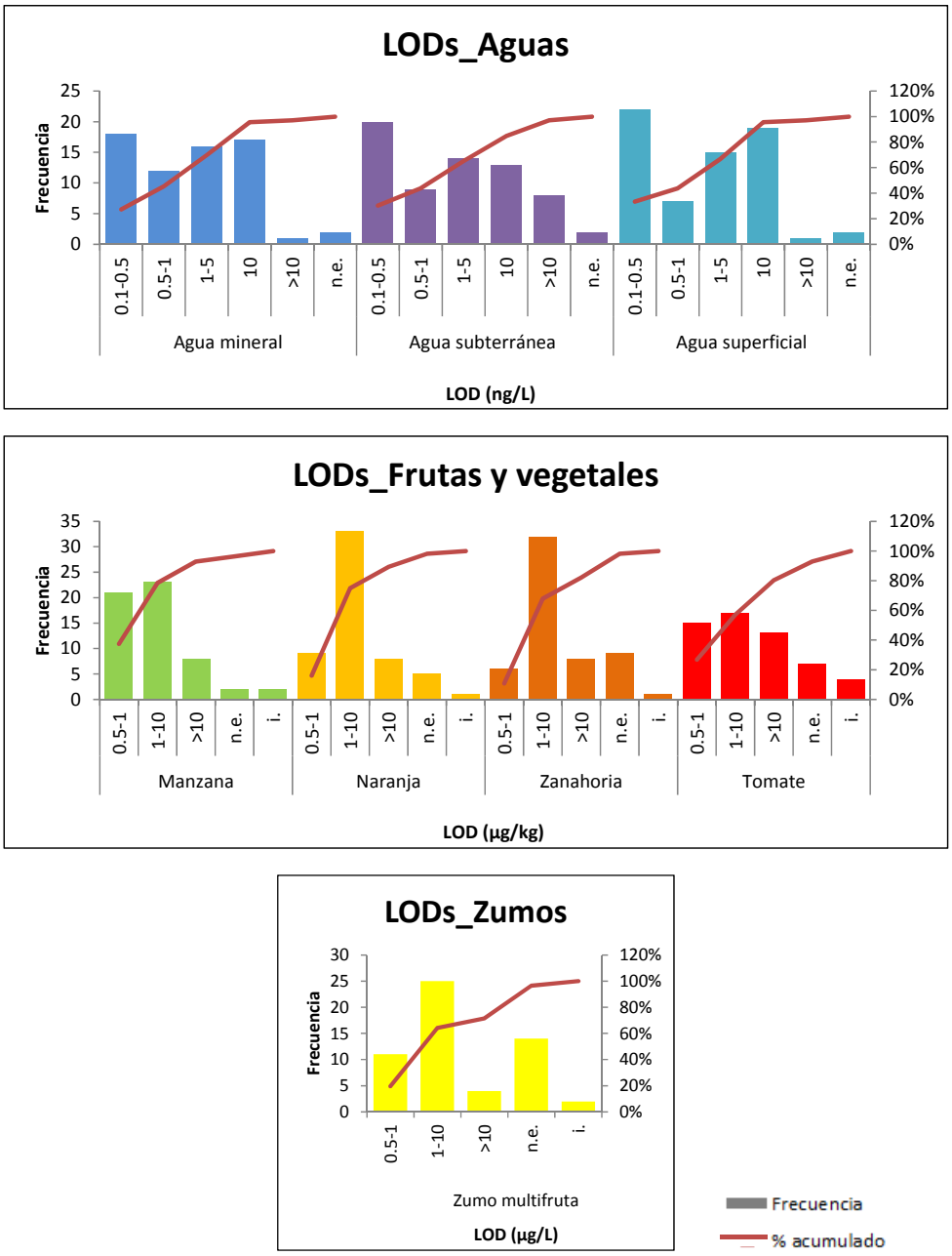

Figura II.5. Histogramas obtenidos a partir de los LOD en las diferentes matrices (n.e., no establecido; i., interferencias de la matriz con los analitos).

Cabe resaltar que la certeza de los resultados analíticos puede venir afectada por ciertos factores críticos a tener firmemente en cuenta, como son la naturaleza de los analitos (especialmente en métodos multiresiduo) y de los patrones internos empleados, la complejidad y co-extractos de la matriz, así como los niveles de concentración y los criterios de confirmación (Stoytcheva, 2011). En algunos casos, determinados analitos objeto de estudio no han podido ser validados por alguna de estas razones, como se discute en los artículos correspondientes.

En cuanto a la confirmación de los positivos en las muestras, existe cierta controversia con respecto a los criterios fijados por la *Commission Decision 2002/657/CE* y el *Guidance document SANCO/12571/2013*. Para los contaminantes estudiados se establece un mínimo de 3 puntos de identificación, que en modo SIM se traduce en la adquisición de 3 iones. Las relaciones entre las intensidades del ión *target* con los de referencia (*Q*/*q*) deben ajustarse a las teóricas obtenidas con patrones de referencia, para lo que se fijan tolerancias de desviación máximas permitidas que dependen del grado de similitud entre las intensidades de los iones, como se ilustra en la **Figura II.6**.

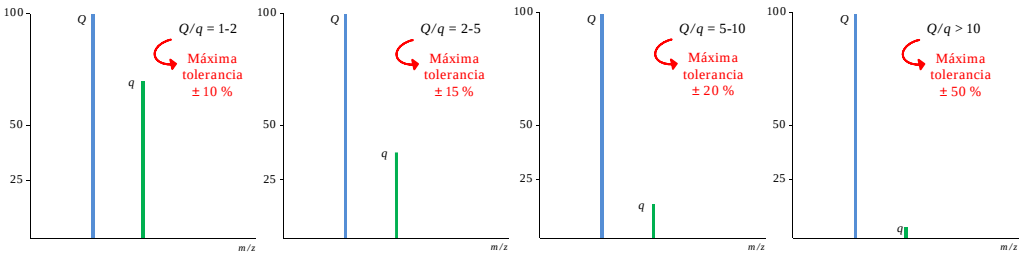

Figura II.6. Tolerancias máximas permitidas de intensidades relativas de iones en GC-MS, de acuerdo con la Decisión de la Comisión Europea 2002/657/CE y el Documento SANCO/12571/2013.

Considerando este criterio, parece razonable permitir una mayor desviación cuando la diferencia entre iones es mayor ya que, para una baja abundancia del ión de referencia, pequeñas diferencias en la intensidad del mismo pueden alterar significativamente el valor de la *Q*/*q*. Sin embargo, estudios recientes han

demostrado que no existe una relación entre la variabilidad de la *Q/q* y la intensidad relativa (Mol *et al.*, 2012), considerando demasiado estrictas las tolerancias establecidas en la legislación.

En los trabajos presentados en este capítulo, el cumplimiento de las *Q/q* resultó problemático en ciertas ocasiones. La similitud entre las intensidades de los iones no garantizó una menor desviación con respecto a las relaciones de referencia. Indistintamente, las relaciones entre los iones *target* y de referencia se vieron afectadas especialmente por el nivel de concentración, ya que a concentraciones bajas disminuye la calidad del espectro y las *Q/q* tienden a desviarse con respecto a las obtenidas a concentraciones mayores. Además, la influencia de la matriz es más notable a niveles bajos, perjudicando el cumplimiento de las *Q/q*. En algunos casos, la presencia de interferentes coextraídos de la matriz afectó notablemente a la relación de iones, requiriéndose la sustitución de los iones interferidos, como se ha comentado anteriormente.

Según la bibliografía consultada (Mol *et al.*, 2012 & 2013), son diversos los parámetros que pueden alterar los resultados cualitativos de los análisis: los iones seleccionados y el número; trabajar con respuestas absolutas o relativas; el nivel de concentración; el efecto matriz; la técnica de análisis (GC-MS(/MS) o LC-MS(/MS)); el analizador y fuente de ionización empleados; el tiempo de escaneo y el número de puntos por pico, y la estabilidad de la respuesta del detector durante el análisis.

Así, la capacidad de confirmación basada en el criterio de la Comisión Europea y la guía SANCO resultó demasiado rigurosa para aplicarse a los métodos desarrollados en este capítulo. En su lugar, se aplicó un criterio más realista basado en la presencia de los tres iones adquiridos y el cumplimiento, al menos, de una *Q/q*, al tiempo de retención correspondiente. La comparación de los valores obtenidos de las muestras se hizo con los resultantes de los patrones de referencia a niveles de concentración semejantes.

Con todas las ventajas y limitaciones consideradas en el uso del cuadrupolo simple en *fast* GC, se ha demostrado que la capacidad de este analizador puede ajustarse para la determinación de un número elevado de contaminantes en un corto tiempo de análisis, favoreciendo el empleo de una instrumentación poco compleja y relativamente económica.

Potencial de la nueva fuente de ionización APCI acoplada a GC-MS (analizador triple cuadrupolo) para la determinación de pesticidas y dioxinas en alimentos y medio ambiente

III. 1. Introducción

A pesar de la conocida eficacia de los diferentes analizadores utilizados en MS para determinadas aplicaciones, las últimas tendencias en análisis cuantitativos prefieren el uso de analizadores acoplados como el triple cuadrupolo, capaces de trabajar en modo MS/MS aumentando la selectividad y sensibilidad de los métodos analíticos (Hernández *et al.*, 2013; Kalachova *et al.*, 2013; Cervera *et al.*, 2010). La necesidad de disponer de este tipo de instrumentación más compleja se evidencia frente al análisis de muestras cuya composición puede interferir o dificultar la determinación de los analitos de interés.

En este sentido, como se ha expuesto en el capítulo II, el uso del cuadrupolo simple ofrece buenos resultados en el análisis de aguas, pero su selectividad se ve más limitada en el análisis de muestras más complejas como frutas y vegetales para ciertas combinaciones matriz/analito. Por su parte, el modo SRM con el que permite trabajar el analizador QqQ promete un notable aumento de la selectividad gracias a la adquisición de iones específicos que proceden de la fragmentación de un determinado ión precursor. Así, la presencia de iones comunes entre los analitos y la matriz disminuye considerablemente, facilitando el análisis de muestras complejas. Una de las claves del proceso reside en la elección del ión precursor adecuado.

Al igual que en modo SIM, la optimización de un método *target* en SRM parte de la selección del ión más específico y/o abundante para que actúe como precursor. Como se ha explicado en el capítulo I, tras la fragmentación del mismo a distintas energías de colisión y la adquisición del *product ion scan*, se seleccionan los iones producto más específicos y/o abundantes con el fin de encontrar transiciones sensibles y selectivas. En principio la selección del pico molecular como precursor resultaría la opción más específica, pero el modo de ionización EI empleado habitualmente en GC hace que ésta no sea siempre la opción más sensible. La elevada energía emitida en la fuente de EI favorece la fragmentación del $M^{+\bullet}$, por lo que los espectros de masas generados se caracterizan por la baja o nula abundancia del

mismo. En algunos casos la ausencia del $M^{+\bullet}$ induce a la selección de iones precursores de baja *m/z*, con la consiguiente disminución de la especificidad. Del mismo modo, una alta fragmentación genera en ocasiones espectros ricos en iones fragmento con baja intensidad, perjudicando a la sensibilidad de las transiciones MS/MS utilizadas. La capacidad de identificación también puede verse afectada por la similitud en los espectros de masas de algunos compuestos, especialmente aquellos que pertenecen a la misma familia química y no presentan transiciones MS/MS suficientemente específicas.

Ante estos casos problemáticos, la reciente comercialización de la fuente APCI para GC se presenta como una alternativa interesante. Aunque las fuentes de ionización a presión atmosférica son habituales en LC-MS, los primeros intentos de acoplamiento con GC datan de finales de la década de 1970 (Horning *et al*., 1977). En los años posteriores se publicaron algunos trabajos relacionados con algunas modificaciones (Korfmacher *et al*., 1987a & 1987b) pero, probablemente debido al elevado coste de la instrumentación específica, no fue hasta mediados de la pasada década cuando se reavivó el interés por el acoplamiento GC-(APCI) MS (McEwen & McKay, 2005; McEwen, 2007; Schiewek *et al*., 2008). Desde entonces se han desarrollado aplicaciones en diversos campos con los nuevos modelos comerciales (Portolés *et al*., 2010 & 2012; Hurtado-Fernández *et al*., 2013; Ballesteros-Gómez *et al*., 2013; Wachsmuth *et al*., 2011).

Así, algunos de los puntos débiles de las fuentes típicas utilizadas en GC –la excesiva fragmentación ocurrida en EI y el carácter selectivo de la CI (no útil para métodos multiresiduo)– vienen paliados con la ionización suave y universal que ofrece la APCI. La presencia del ión molecular, abundante en la mayoría de casos e incluso como pico base del espectro de masas, permite el desarrollo de métodos MS/MS más sensibles y selectivos.

El mecanismo de ionización que tiene lugar en la fuente APCI se basa en el empleo de N_2 como gas reactivo, dando lugar a procesos de transferencia de carga desde el plasma generado ($N_2^{+\cdot}$ y $N_4^{+\cdot}$) a las moléculas de analito. Simultáneamente, es posible otro mecanismo de reacción con las trazas de vapor de agua presentes en la

fuente, que generan la molécula protonada ($[M+H]^+$) mediante un proceso de protonación a partir del ión $[H3O^{+\cdot}]$. Ambos procesos se esquematizan en la **Figura III.1** (Portolés *et al.*, 2010). La adición de modificadores en la fuente, como el H_2O, puede favorecer la protonación de las moléculas de analito susceptibles a ello, haciendo aumentar la presencia de la molécula protonada en el espectro, lo que puede aportar sensibilidad adicional.

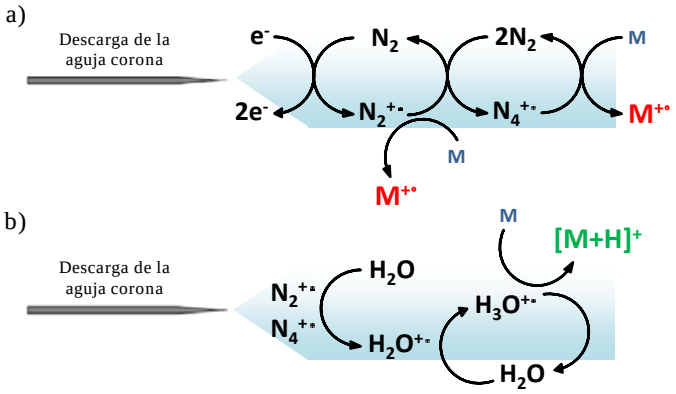

Figura III.1. Mecanismos de ionización en la fuente APCI. a) Condiciones de transferencia de carga desde el N_2 al analito (M). b) Condiciones de protonación.

El presente capítulo, pues, ha supuesto un doble salto respecto al anterior en cuanto al analizador (del uso del cuadrupolo simple al QqQ) y a la fuente de ionización (de EI a APCI). La instrumentación empleada consta de un GC Agilent 7890A acoplado a un triple cuadrupolo XevoTQ-S de Waters, con una fuente de ionización APCI. Además de la mejora de sensibilidad y selectividad inherente al modo MS/MS, en esta nueva generación de triples cuadrupolos, la sensibilidad y reproducibilidad no se ven tan fuertemente influenciadas por los tiempos de escaneo. En este caso el *dwell time* (tiempo empleado en la monitorización de un ión) puede alcanzar valores tan bajos como 0.01 s, permitiendo la adquisición de más de 40 iones o transiciones incluidos en una única ventana SRM sin perjudicar ni a la sensibilidad ni a la forma de pico.

Ante la novedad del acoplamiento GC-(APCI) MS/MS, el principal objetivo de los tres trabajos presentados en este capítulo se centra en la demostración de las capacidades de este nuevo sistema para la determinación de pesticidas y dioxinas en alimentos y muestras biológicas y medioambientales.

En el primero de ellos (Artículo Científico 4) se evalúan algunos casos en el campo de pesticidas y alimentos considerados problemáticos en EI por la ausencia o baja abundancia del pico molecular en el espectro de masas. Los 25 pesticidas estudiados se inyectaron previamente en un sistema GC-(EI) MS/MS con el fin de comparar los espectros con los obtenidos por APCI y evaluar la fragmentación del $M^{+\bullet}$. Así mismo, teniendo en cuenta los dos posibles mecanismos que tienen lugar en la fuente APCI, se estudió el efecto del uso de agua como modificador con el fin de exaltar la presencia de la molécula protonada. Los resultados obtenidos alentaron al desarrollo de un segundo trabajo para comprobar el potencial cuantitativo de este sistema en análisis de alimentos.

Así, en el Artículo Científico 5 se optimizó cuidadosamente un método SRM para 142 pesticidas tratando de seleccionar transiciones con el pico molecular o la molécula protonada como ión precursor. La validación del mismo se llevó a cabo en matrices de frutas y vegetales, resaltando el cumplimiento de las relaciones *q/Q* entre la transición de confirmación y la de cuantificación.

Mención especial merece el Artículo científico 6 por el aporte significativo que puede suponer la consecución de un método basado en GC-(APCI) MS/MS en el campo de las dioxinas. De entre los POPs contemplados en la Convención de Estocolmo, estos compuestos (incluyendo PCDDs y PCDFs) son los que implican mayores retos analíticos. Aunque sólo 7 PCDDs y 10 PCDFs (de los 75 y 135 congéneres que existen, respectivamente, de cada grupo) se consideran tóxicos y, por tanto, de carácter prioritario, su determinación por técnicas GC-MS suele resultar complicada tanto por las bajas concentraciones que suelen encontrarse en alimentos y medio ambiente (del orden de partes por trillón o cuatrillón, pero igualmente perniciosas), como por la presencia de interferentes a concentraciones elevadas. Por otro lado, la determinación individual de cada congénere es necesaria para establecer

el potencial tóxico adecuadamente, ya que este factor varía según la posición de los cloros en la molécula. A estos efectos, la WHO ha establecido el Factor de Equivalencia Tóxica (TEF) asignando toxicidades específicas a cada congénere (*http://www.who.int/foodsafety/chem/tef_update/en/*) en función del valor fijado para la dioxina más tóxica, la 2,3,7,8-TCDD, a la que se le asigna un TEF máximo de 1. La existencia de toxicidades relativas permite evaluar el riesgo de toxicidad global en una muestra según los congéneres detectados; de hecho, los niveles máximos permitidos establecidos por las directivas no están fijados para cada congénere independientemente sino para la suma de todos según el Equivalente Tóxico (TEQ). Este parámetro se calcula como el sumatorio de la concentración de cada congénere multiplicado por el correspondiente TEF:

$$TEQ = \sum_{i}^{n} ([Conc]_i \times TEF_i)$$

Los análisis requieren, pues, de técnicas altamente sensibles y selectivas para cumplir con los requisitos reglamentarios. Concretamente, los métodos oficiales descritos por la EPA (*EPA Method 1613*) y la Unión Europea (*EN 1948-1/2/3*) recomiendan el uso de GC acoplado a espectrometría de masas de alta resolución (HRMS) para una determinación exacta y precisa. En el caso de las dioxinas, la ionización por EI no produce una excesiva fragmentación, con lo que normalmente se seleccionan dos iones característicos del cluster molecular para su cuantificación y confirmación. El analizador de referencia en este campo es el sector magnético –basado en la separación de los iones por la acción de campos magnéticos–, ya que ofrece una elevada resolución (>150000) y sensibilidad capaz de alcanzar límites de detección a niveles de ultra-traza (hasta 3 fg) (Eljarrat & Barceló, 2002). Sin embargo, la complejidad de estos instrumentos y la necesidad de personal cualificado encarecen los análisis, sumado al tratamiento de muestra exhaustivo y a la cuantificación por dilución isotópica que requiere del uso de patrones de cada congénere marcados isotópicamente con ^{13}C. Alternativas más accesibles y económicas, como el uso del triple cuadrupolo, la trampa de iones o el TOF (siempre aplicando EI), se han reportado en la bibliografía pero únicamente para el análisis de muestras más concentradas (Bavel & Abad, 2008; Malavia *et al.*, 2007).

La aplicación de una metodología basada en GC-(APCI) MS/MS en el campo de las dioxinas podría suponer una alternativa comparable a la GC-(EI) HRMS tradicional. El trabajo al respecto presentado en esta Tesis Doctoral (Artículo Científico 6) reúne la colaboración de diferentes grupos de investigación con el objetivo de demostrar el potencial y la robustez del nuevo sistema GC-(APCI)QqQ MS/MS para la determinación de dioxinas en muestras alimentarias, biológicas y medioambientales.

III.2. Artículo científico 4

Improved gas chromatography-tandem mass spectrometry determination of pesticide residues making use of atmospheric pressure chemical ionization

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Improved gas chromatography-tandem mass spectrometry determination of pesticide residues making use of atmospheric pressure chemical ionization

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Abstract

The capabilities of a recently launched atmospheric pressure chemical ionization (APCI) source for mass spectrometry (MS) coupled to gas chromatography (GC) have been tested in order to evaluate its potential in pesticide residue analysis in fruits and vegetables. Twenty-five pesticides were selected due to their high fragmentation under electron ionization (EI), making that the molecular ion ($M^{+\bullet}$) is practically absent in their spectra. The fragmentation of these pesticides under APCI conditions was studied, with the result that $M^{+\bullet}$ was not only present but also highly abundant for most compounds, with noticeable differences in the fragmentation patterns in comparison with EI. Moreover, the addition of water as modifier was tested to promote the formation of protonated molecules ($[M+H]^+$). Under these conditions, $[M+H]^+$ became the base peak of the spectrum for the majority of compounds, thus leading to an increase of sensitivity in the subsequent GC–MS/MS method developed using triple quadrupole analyzer (QqQ). Highly satisfactory sensitivity and precision, in terms of repeatability, were reached and linearity was satisfactory in the range 0.01–100 ng/mL. The developed methodology was applied to apple, orange, tomato and carrot QuEChERS fortified extracts in order to evaluate the matrix effects. In summary, the soft and reproducible ionization in the APCI

source has greatly favored the formation of $[M+H]^+$ oppositely to EI where abundant fragmentation occurs and where the molecular ions have low abundance or are even absent in the mass spectrum. In this way, the use of APCI has facilitated the development of tandem MS methods based on the selection of abundant $[M+H]^+$ as precursor ion.

Keywords

Atmospheric pressure chemical ionization; Gas chromatography; Tandem mass spectrometry; Triple quadrupole; Pesticides.

1. INTRODUCTION

Gas and liquid chromatography (GC and LC) coupled to mass spectrometry (MS) are the techniques of choice in pesticide residue analysis (PRA) for a wide variety of sample matrices. The availability of different types of analyzers as single (Q) or triple (QqQ) quadrupole, time-of-flight (TOF) and ion trap detector (ITD) allows performing both quantitative and qualitative analysis with satisfactory sensitivity and selectivity [1-8]. GC–MS is commonly applied for non-polar, volatile and thermostable compounds. In some cases, a derivatization step is required to make them GC-amenable, especially for transformation products, which are often more polar and less-volatile than their parent compounds. On the contrary, LC–MS is more appropriate for non-GC amenable compounds, i.e. thermolabile, polar and (semi) non-volatile analytes. Due to the large diversity in polarity, volatility and stability of pesticides, nowadays it is compulsory to use both techniques in a complementary way. MS ion sources play an important role in PRA. Notable differences can be found in the ionization processes occurring in LC–MS and GC–MS.

The most common sources in LC–MS are those based on atmospheric pressure ionization, specifically electrospray ionization (ESI) and atmospheric pressure

chemical ionization (APCI) [9]. Due to the low fragmentation achieved by these ionization techniques, the presence of the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecule is common in the LC–MS spectra. On the contrary, ionization in GC–MS conventionally occurs under vacuum conditions: electron ionization (EI) and chemical ionization (CI). Undoubtedly, EI is the most commonly applied in the wide majority of GC–MS applications, including PRA. It is well known that this source produces high fragmentation of the molecules, leading in many cases to the absence of the molecular ion ($M^{+\bullet}$) in EI spectra. CI produces softer ionization. The reaction with a reagent gas occurs with an energy transfer that generally does not exceed 5 eV, so mass spectra exhibit less fragment ions than in EI. Better selectivity and sensitivity are achieved for some compounds under CI, as well as less matrix interferences, but this technique is quite restricted to specific analyte chemical classes [10-12] since it is not as universal ionization as EI. The interest in application of multiresidue methods including a wide variety and large number of compounds makes EI mode the most commonly applied in PRA. This is favored by its robustness and good reproducibility. In addition, EI spectra in full scan mode (under 70 eV) are available in commercial libraries, facilitating the identification of compounds by an easy search matching.

Many methods have been reported in PRA based on GC–(EI)MS, using single quadrupole under Selected Ion Monitoring (SIM) mode, or ion trap operating under tandem mass spectrometry (MS/MS) [3, 13, 14]. Recently, triple quadrupole analyzer (QqQ) operating in selected reaction monitoring (SRM) mode has received much attention due to its better performance for quantitative multiresidue analysis [15-19]. The selection of adequate precursor and product ions and the subsequent application of SRM mode enhance selectivity and sensitivity, minimizing or even eliminating matrix interferences. In this way, very low detection limits can be achieved, favored by the low chemical background noise in the chromatograms.

However, the extensive fragmentation due to the high energy transferred to the molecules during the ionization process produces little or no molecular ions for many pesticides, as for example organochlorine (OC) pesticides, organophosphorus (OP), pyrethroids and chloroacetanilides [20-22]. Besides, compounds belonging to the

same chemical family can show similar EI spectra; so the use of common ions/transitions can complicate the identification and quantification processes, especially if analytes are coeluting. When the molecular ion is absent or has very low abundance, it is necessary to select a (abundant) fragment ion as precursor. In addition to the loss of sensitivity, the specificity of the method can be also affected. Thus, the potential of tandem MS is lost.

Specific problematic cases, as the aforementioned, would require a soft and universal technique able to provide abundant molecular ions to be used as precursor ions in multiresidue GC–MS/MS analysis. Atmospheric pressure ionization in GC–MS was first introduced by Horning et al. [23]. Subsequent modifications were described in the 80s [24, 25], but the technique has not been implemented yet as a common routine analysis because of the high costs of the specialized instrumentation and the unavailability of easy interchangeable ionization sources. Recently, a new APCI source using a nitrogen purge gas has been developed and commercialized [26-28]. Although it has not been widely applied, it offers attractive analytical capabilities in GC–MS analysis. From first applications in 2009 until now, GC–APCI–TOF MS has been used in different fields, like pesticide residue analysis [29], pharmaceuticals development [30], profiling of phenolic compounds in oil [31] and methabolic profiling [32].

The aim of this paper is to evaluate the potential of the APCI source in GC–MS/MS using triple quadrupole analyzer. For this purpose, 25 pesticides have been selected in order to study their ionization behavior under atmospheric pressure conditions and to evaluate the potential advantages in comparison with EI ionization. Spectra obtained with both sources have been compared paying special attention to the presence and abundance of the molecular ion and/or the protonated molecule. Experiments under product ion scan mode and SRM have been performed, and the most specific and/or sensitive transitions have been selected in order to improve the performance of GC–MS/MS for some problematic pesticides, which suffer high fragmentation under EI. Different fruits and vegetable fortified extracts obtained after QuEChERS application (AOAC Official method) [33], and spiked with the 25

studied pesticides, have been analyzed in order to evaluate the applicability of the new ion source.

2. EXPERIMENTAL

2.1. Reagents

All pesticide standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock standard solutions (around 500 μg/mL) were prepared by dissolving reference standards in acetone and stored in a freezer at −20 °C. Working standard mixtures were prepared by volume dilution of stock solutions in hexane.

Hexane, acetone, acetonitrile (MeCN), toluene, glacial acetic acid (HAc), anhydrous $MgSO_4$ and anhydrous sodium acetate (NaAc) were purchased from Scharlab (Barcelona, Spain). All solvents were for pesticide residue analysis or HPLC grade. Two types of 2 mL micro-centrifuge tubes for QuEChERS d-SPE containing 50 mg PSA and 150 mg anhydrous $MgSO_4$, or 50 mg PSA, 150 mg anhydrous $MgSO_4$ and 50 mg C_{18}, were obtained from Teknokroma (Barcelona, Spain).

2.2. Instrumentation

2.2.1. GC-(EI) MS/MS

A GC system (Agilent 6890N, Palo Alto, USA) equipped with an autosampler (Agilent 7693) was coupled to a triple quadrupole (QqQ) mass spectrometer (Quattro Micro GC, Waters, Boston, USA), equipped with an EI source. A fused silica HP-5MS capillary column (length 30 m × I.D. 0.25 mm × d_f 0.25 μm) (J&W Scientific, Folson, CA, USA) was used for GC separation. Injector was operated in splitless mode, injecting 1 μL at 280 °C. The oven temperature was programmed as follows: 70 °C (1 min), 25 °C/min to 150 °C and 10 °C/min to 300 °C (3 min). Helium was used as carrier gas at a constant flow rate of 1 mL/min (linear velocity of 40 cm/s). The interface and source temperatures were set at 260 °C and 250 °C, respectively. In

order to prevent damage in the EI filament, a solvent delay of 4 min was selected. TargetLynx (a module of MassLynx) was used to process the data.

2.2.2. GC-(APCI) MS/MS

A GC system (Agilent 7890A, Palo Alto, CA, USA) equipped with an autosampler (Agilent 7693) and coupled to a triple quadrupole (QqQ) mass spectrometer (Xevo TQ-S, Waters Corporation, Manchester, UK), with an APCI source was used (the trade name is APGC). A fused silica DB-5MS capillary column (length 30 m × I.D. 0.25 mm × d_f 0.25 μm) (J&W Scientific, Folson, CA, USA) was used for GC separation. Injector was operated in splitless mode, injecting 1 μL at 280 °C. The oven temperature was programmed as follows: 70 °C (1 min), 25 °C/min to 150 °C and 10 °C/min to 300 °C (3 min). Helium was used as carrier gas at 2 mL/min, which corresponds to a linear velocity of 52 cm/s. A pulsed splitless injection was carried out using an initial pressure of 240 kPa.

The interface temperature was set to 310 °C using N_2 (from liquid N_2) as auxiliary gas at 250 L/h and as cone gas at 170 L/h, and N_2 (from gas cylinder quality 99.9990%) as make-up gas at 320 mL/min. The APCI corona pin was operated at 1.8 μA. The ionization process occurred within a closed ion volume, which enabled control over the protonation/charge transfer processes. The water, used as modifier when working under proton-transfer conditions, was placed in an uncapped vial, which was located within a specially designed holder placed in the source door. TargetLynx was used to process the data.

2.3. Sample treatment

Samples of apple, orange, tomato and carrot were purchased from a local market in Castellón (Spain). A sample treatment based on the well-known QuEChERS procedure was applied (AOAC official method 2007.01 [33]): 15 g of triturated sample were weighted in a 50 mL polypropylene centrifuge tube, mixed with 15 mL MeCN (with 1% HAc) and shaken by hand for 30 s. Then, 6 g anhydrous $MgSO_4$ and 1.5 g anhydrous NaAc were added and immediately shaken vigorously by

hand to prevent formation of $MgSO_4$ aglomerates. Then, the tube was centrifuged at 3000 rpm for 2 min.

For the cleanup step, 1 mL of the upper MeCN extract was poured into a d-SPE tube containing 150 mg $MgSO_4$ and 50 mg PSA (exceptionally, tubes used for orange extract purification contained 150 mg $MgSO_4$, 50 mg PSA and 50 mg C_{18}). The tubes were shaken on a Vortex for 30 s and centrifuged at 3000 rpm for 2 min. 500 µL of the extract were transferred into an evaporation graduated tube containing 1 mL of toluene and evaporated to approximately 300 µL under a gentle nitrogen stream at 50 °C. The extracts were fortified with standard pesticides at 10 ng/mL and adjusted to a final volume of 500 µL with toluene.

3. RESULTS AND DISCUSSION

3.1. Selection of pesticides

In order to fully demonstrate the capabilities of the APCI source, 25 pesticides were selected on the basis of their mass spectral behavior in the EI source. The compounds selected can be problematic for the development of GC–MS/MS methods under EI conditions, due to the high fragmentation suffered and the subsequent difficulty to select appropriate MS–MS transitions. These pesticides were divided into three groups as a function of their EI mass spectra:

- *Group 1*: compounds for which the absence of the $M^{+\bullet}$ in electron ionization spectra forces the selection of an abundant fragment ion (i.e. with lower m/z) as the precursor ion in MS/MS, leading to a possible loss in specificity. Representative examples are found in pesticides as pyriproxifen and buprofezin. In these cases, the fragmentation of the molecular ion $M^{+\bullet}$ (321 for pyriproxifen and 305 for buprofezin) leads to a EI spectrum mostly "dominated" by the presence of fragment ions with low m/z. Consequently,

the commonly selected transitions for them have M/2 or M/3 as precursor ions.

- *Group 2*: highly fragmented compounds, leading to EI spectra rich in fragment ions. Their mass spectra show many ions, all with poor intensity. Consequently, the transitions selected in MS/MS lead to a method that may not be sensitive enough to perform trace analysis at the low levels expected in the samples. Pesticides belonging to the endosulfan family exhibit this trend, as well as the OC pesticides aldrin, endrin and their isomers.

- *Group 3*: compounds whose EI spectra show strong similarity to other compounds, making then difficult to use selective and specific MS/MS transitions. This usually occurs with pesticides belonging to the same chemical family, as the OP pesticides mevinphos, dicrotophos, monocrotophos and phosphamidon. The same transitions, with m/z 127 as precursor ion, are commonly selected for these compounds since it is the base peak of the spectrum for all of them, which correspond to a part of the molecule common to OP family. In these cases, only retention times allow distinguishing each analyte in a SRM method. However, this situation becomes critical if compounds are nearly eluting, or even coeluting, as may occur with heptachlor epoxide B and oxychlordane. These pesticides cannot be included in the same GC-(EI) MS/MS method since they usually elute at the same chromatographic time and no specific transitions can be found for oxychlordane. This problem is even worse when trying to carry out a non-target screening analysis in scan mode, as the similarity in the mass spectra can lead to a doubtful identification.

3.2. Full scan experiments

Once the model analytes were selected, pesticide standards in solvent were used to study their ionization under APCI source. All APCI spectra were obtained, evaluated and compared with EI spectra. The behavior of the molecular ion of each analyte under both ionization conditions is summarized in **Table 1**.

Table 1. Behavior of the molecular ion of the selected pesticides under EI and APCI ionization modes and single quadrupole mass analysis. MS/MS transitions commonly used under EI mode are shown.

t_R	Compounds	Molecular formula	M	EI	APCI		APCI + H_2O	EI transitions	
				M+·	M+·	MH+	MH+		
4.70	Dichlorvos	$C_4H_7Cl_2O_4P$	220			+++	+++	185>93	109>79
5.97	Mevinphos	$C_7H_{13}O_6P$	224		++	+	++	127>109	192>127
6.96	Molinate	$C_9H_{17}NOS$	187	+		+++	+++	187>126	126>55
8.00	Dicrotophos	$C_8H_{16}NO_5P$	237	+	++	++	+++	127>109	127>95
8.24	Monocrotophos	$C_7H_{14}NO_5P$	223	+	++	++	+++	192>127	192>164
8.95	Terbufos	$C_9H_{21}O_2PS_3$	288				+	231>129	231>175
9.80	Phosphamidon	$C_{10}H_{19}ClNO_5P$	299		+	++	+++	127>109	264>127
9.76	Endosulfan ether	$C_9H_6Cl_6O$	340	+	+	++	++	272>237	239>204
9.94	Chlorpyriphos methyl	$C_7H_7Cl_3NO_3PS$	321			++	+++	288>93	197>169
10.77	Chlorpyriphos	$C_9H_{11}Cl_3NO_3PS$	349		+	+++	+++	199>171	316>260
10.85	Aldrin	$C_{12}H_8Cl_6$	362		+	+	++	261>191	263>193
11.39	Isodrin	$C_{12}H_8Cl_6$	362		++	++	++	193>157	195>123
11.56	Chlorfenvinphos	$C_{12}H_{14}Cl_3O_4P$	358			++	+++	267>159	323>267
11.56	Oxychlordane	$C_{10}H_4Cl_8O$	420		+	+	++	185>121	235>141
11.56	Heptachlor epoxide B	$C_{10}H_5Cl_7O$	386		+	+	++	355>265	351>261
12.23	Endosulfan I	$C_9H_6Cl_6O_3S$	404			++	++	239>204	272>237
12.72	Buprofezin	$C_{16}H_{23}N_3OS$	305	+	++	+++	+++	105>77	172>115
12.73	Dieldrin	$C_{12}H_8Cl_6O$	378	+	++	++	++	263>193	261>191
13.10	Endrin	$C_{12}H_8Cl_6O$	378		++	++	++	263>193	261>191
13.36	Ethion	$C_9H_{22}O_4P_2S_4$	384		+	+++	+++	231>129	231>175
14.01	Endosulfan sulfate	$C_9H_6Cl_6O_4S$	420		++		++	274>239	272>237
15.63	Azinphos methyl	$C_{10}H_{12}N_3O_3PS_2$	317				+	160>77	160>132
15.66	Pyriproxyfen	$C_{20}H_{19}NO_3$	321			++	+++	136>96	136>78
16.04	Fenarimol	$C_{17}H_{12}Cl_2N_2O$	330	+	++	+++	+++	251>139	219>107
16.17	Azinphos ethyl	$C_{12}H_{16}N_3O_3PS_2$	345				+	160>132	160>77

+, very small peak; ++, clearly identifiable peak (>20%); +++, base peak (or >80%).

As it can be seen, these analytes either do not show $M^{+\bullet}$ under EI or its abundance is very low. For these reason, most transitions in an EI-based SRM method use a fragment as precursor ion. In some occasions, this precursor ion has a low m/z value, as in the transition 105 > 77 used for buprofezin (M = 305). A clear improvement was observed when using APCI under charge-transfer conditions: several compounds (18 out of 25) exhibited $M^{+\bullet}$, although it was not the base peak of the spectrum in most cases. As an example, **Fig. 1** shows buprofezin spectra in the EI and APCI sources. The $M^{+\bullet}$ (305) was practically absent in the EI spectrum (**Fig. 1 a**),

while its abundance was around 30% in the APCI spectrum (**Fig. 1 b**). In addition, rather different fragmentation patterns were observed in EI and APCI.

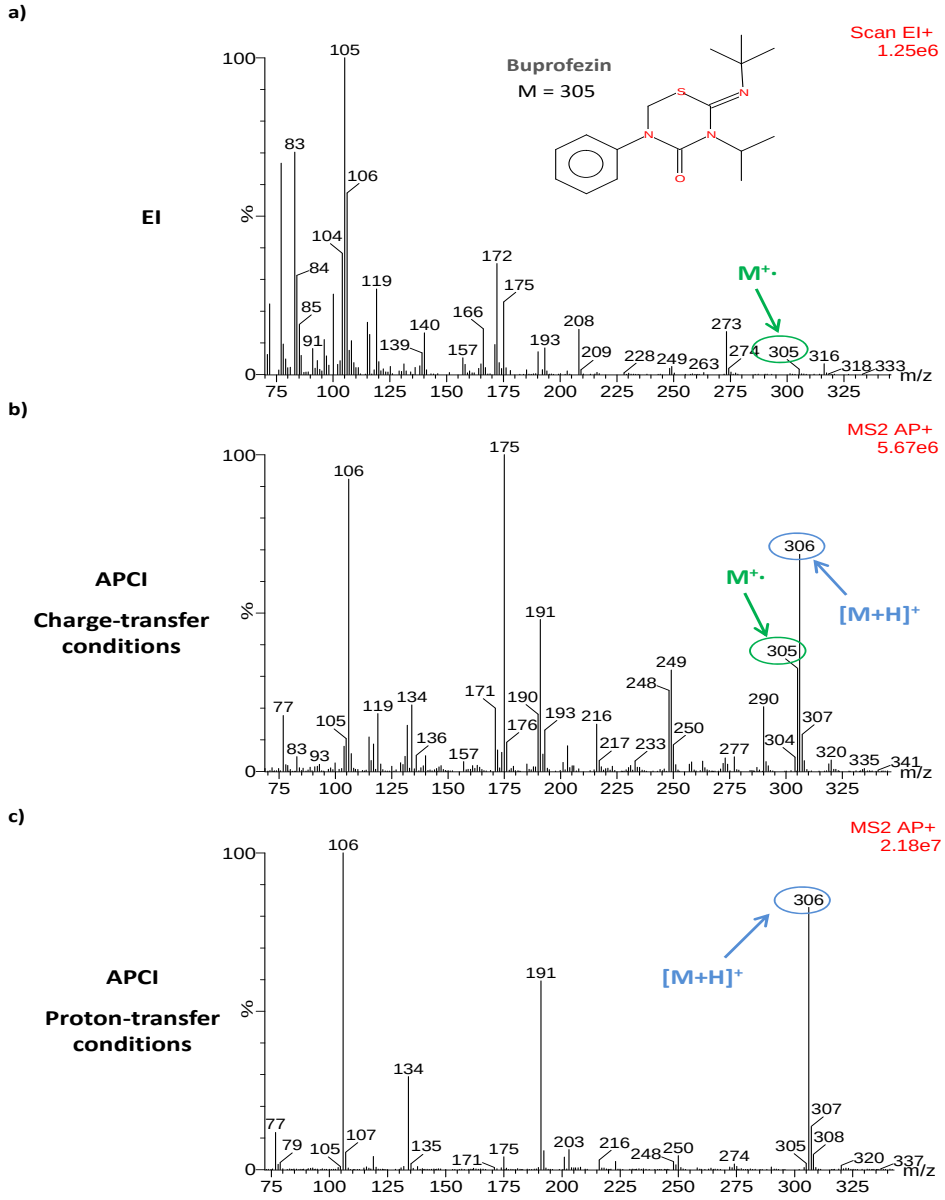

Fig. 1. Comparison of buprofezin spectrum using: (a) EI source, (b) APCI source under charge-transfer conditions and (c) APCI under proton-transfer conditions.

Most analytes (17 out of 25) presented the $[M+H]^+$ ion in the charge-transfer APCI spectrum, even together with $M^{+\bullet}$ (**Fig. 1b**). This behavior can be understood considering the two possible ionization mechanisms in APCI, as explained in a recent work [29]: charge transfer, yielding typically $M^{+\bullet}$, or protonation, which promotes the formation of the protonated molecule $[M+H]^+$. In the primary ionization event, the nitrogen plasma (N_2^+ and N_4^+) created by the corona discharge needle is the responsible of the analyte molecules ionization. The second mechanism takes place due to the presence of water vapor traces in the source, which reacts with the nitrogen plasma ions and leads to the generation of $H_3O^{+\bullet}$ that produces analyte $[M+H]^+$ ions by a proton transfer process.

Considering the observed tendency of pesticides to be protonated, an additional experiment was performed enhancing the proton-transfer conditions by introducing water as modifier in the APCI source. Better results than those obtained under charge-transfer conditions were obtained, since the $[M+H]^+$ was present for most compounds (21 out of 25). In addition, $[M+H]^+$ was commonly the peak base of a low fragmented spectrum. A representative example is shown in **Fig. 1c** (buprofezin), in which the APCI spectrum using water as modifier showed high abundance of $[M+H]^+$, and also low fragmentation. Moreover, some pesticides such as terbufos, azinphos methyl, azinphos ethyl, aldrin, oxychlordane and heptachlor epoxide B, which did not show an abundant $M^{+\bullet}$ (or $[M+H]^+$) with charge-transfer conditions, increased slightly their abundance under proton-transfer conditions.

It was concluded that the use of water as modifier improved the presence of the protonated molecule in the mass spectra of all the studied pesticides. The selection of $[M+H]^+$ as precursor ion would allow developing a SRM method with more specific transitions, solving problematic cases included in group 1. Thus, in the aforementioned example of buprofezin, the transition 105 > 77 could be replaced by another one using 306 ($[M+H]^+$) as precursor ion.

On the other hand, pesticides belonging to group 2 (e.g. endosulfan family), experimented a very different fragmentation behavior under both ionization modes. As it can be seen in **Fig. 2**, $M^{+\bullet}$ for endosulfan ether and endrin was practically

absent, and numerous fragment ions constituted the EI spectrum. On the contrary, the APCI spectrum was characterized by high abundance of the $[M+H]^+$ cluster and by low fragmentation, with $[M+H]^+$ being the base peak of the spectrum.

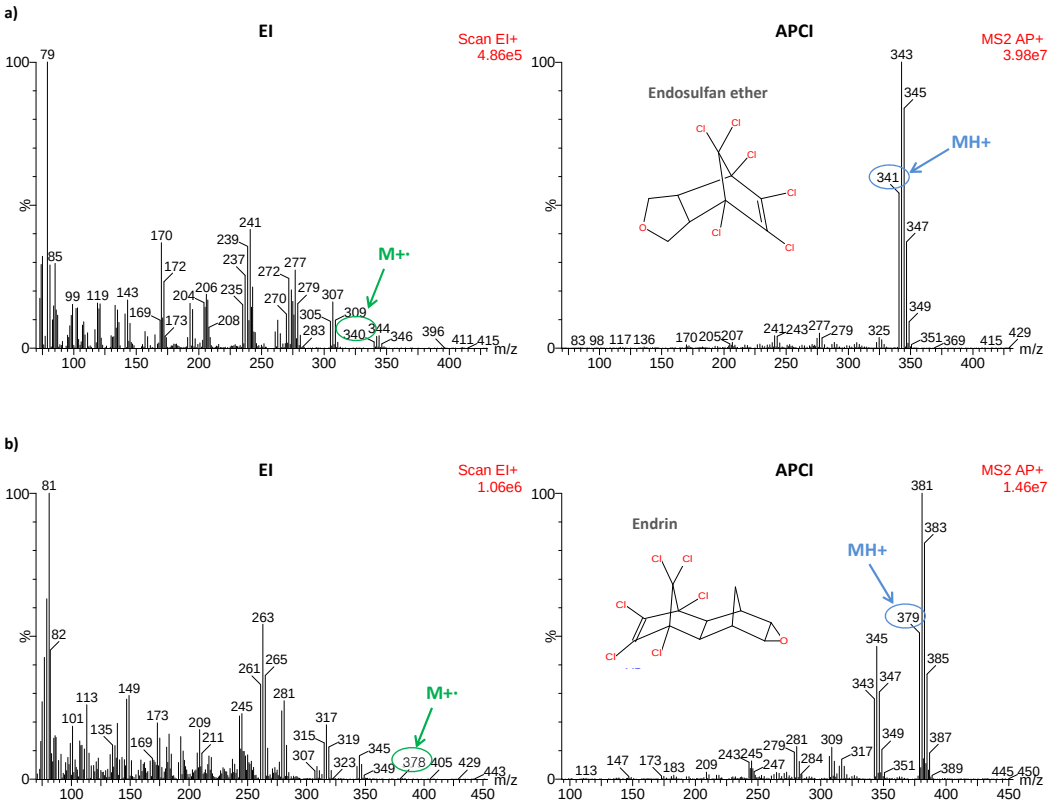

Fig. 2. Full scan spectra obtained under EI (top) and APCI using water as modifier (bottom) for: (a) endosulfan ether and (b) endrin.

In the last group of pesticides (group 3), common transitions have to be selected in EI. They would be spectrally resolved only if transitions coming from the molecular ion were used, as it makes the difference between the molecules. An example is shown in **Fig. 3** for mevinphos, dicrotophos, monocrotophos and phosphamidon.

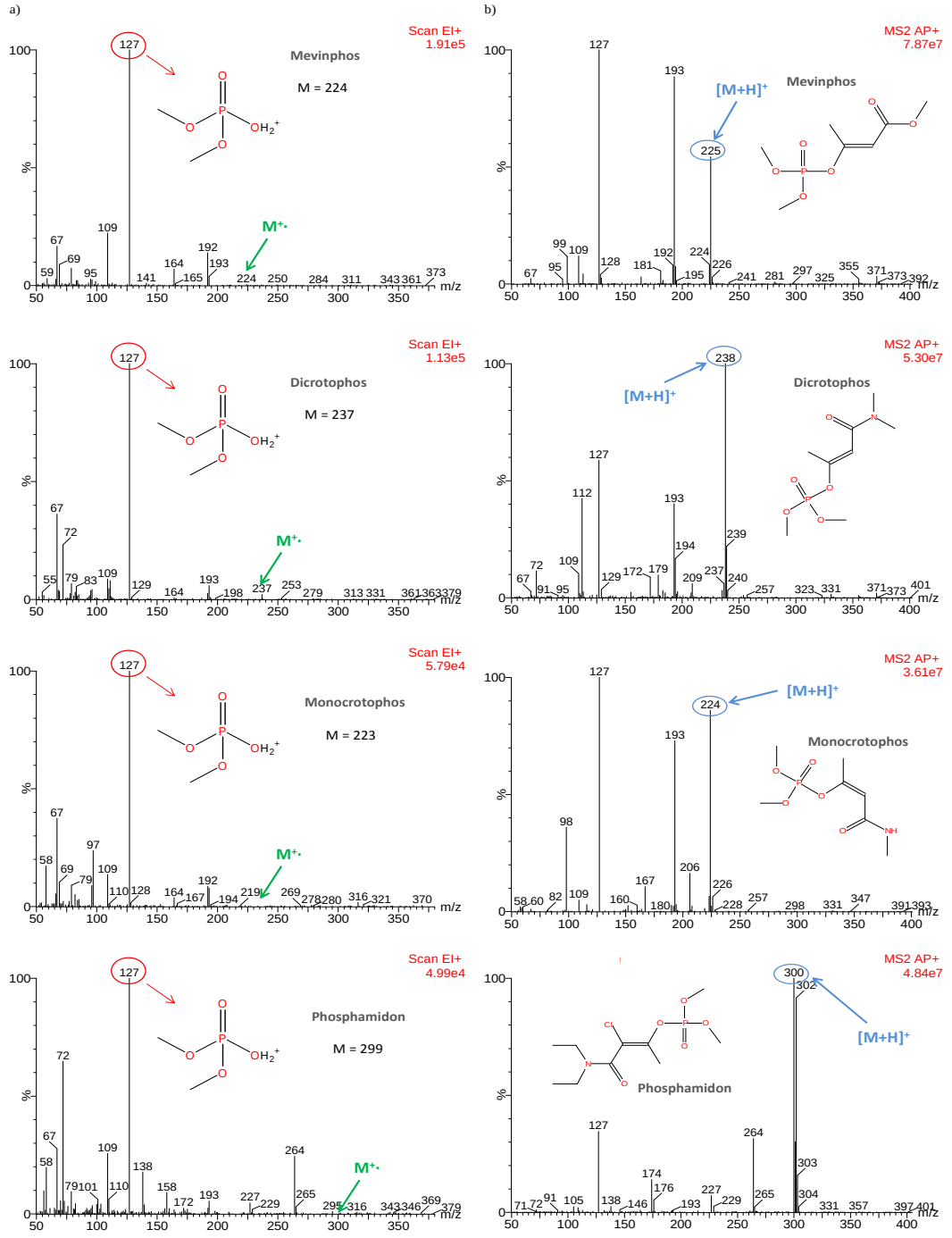

Fig. 3. Comparison of full scan spectra obtained under (a) EI and (b) APCI using water as modifier for the OP pesticides mevinphos, dicrotophos, monocrotophos and phosphamidon.

The EI spectra of these OP pesticides are characterized by the presence of the same fragment ion (m/z 127) as the base peak (**Fig. 3 a**). All transitions coming from 127 as parent ion are common to these four pesticides, as it corresponds to the same chemical structure. Selecting more specific ions (e.g. m/z 192, 193, 264) are possible and this would lead to more specific transitions but with a dramatic loss of sensitivity. However, in APCI (using water as modifier), all of them showed its corresponding $[M+H]^+$ with satisfactory sensitivity (**Fig. 3 b**) (it was the base peak of the spectrum for some of them); so specific transitions can be selected for each compound using the protonated molecule as precursor ion. Thus, pairs of compounds belonging to the group 3 could be also spectrally resolved by APCI selecting the $[M+H]^+$ as precursor ion.

3.3. Optimization of the cone voltage

Once the ions are formed in the APCI source, they are sampled through the skimmer cone by generating a voltage, which can be optimized for each ion. Small voltages can lead to poor ion sampling, and too high voltages can lead to fragmentation before reaching the mass analyzer. In the case of our APCI source, the nozzle of the skimmer cone is big enough to reduce the influence of the cone voltage. The effect of the cone voltage was studied in the range 10–50 V and, in all cases, values over 40 V led to a loss in sensitivity. As it is shown in **Table 2**, for the pesticides studied optimum values ranged from 10 to 40 V, although differences in sensitivity were small.

Table 2. Experimental conditions of the optimized GC–(APCI)MS/MS method using water as modifier. Quantifier (Q) and qualifier (qi) transitions.

t_R (min)	Window (min)	Compounds	Cone voltage (v)	MS/MS Transitions		Collision energy (eV)
4.7	3.0-5.0	Dichlorvos	10	Q	221 > 145	10
				q_1	221 > 113	30
				q_2	221 > 127	20
5.97	5.0-6.5	Mevinphos	30	Q	225 > 127	10
				q_1	225 > 113	30
				q_2	225 > 193	10
6.96	6.5-7.5	Molinate	20	Q	188 > 126	10
				q_1	188 > 98	20
				q_2	188 > 160	10
8	7.5-9.7	Dicrotophos	40	Q	238 > 112	10
				q_1	238 > 127	20
				q_2	238 > 193	10
8.24		Monocrotophos	20	Q	224 > 127	10
				q_1	224 > 113	30
				q_2	224 > 193	10
8.95		Terbufos	10	Q	187 > 131	10
				q_1	187 > 97	20
				q_2	187 > 113	20
9.8	9.7-10.5	Phosphamidon	40	Q	300 > 127	20
				q_1	300 > 174	10
				q_2	300 > 227	10
9.76		Endosulfan ether	30	Q	341 > 217	30
				q_1	341 > 170	30
				q_2	341 > 205	20
9.94		Chlorpyriphos methyl	40	Q	322 > 125	30
				q_1	322 > 212	30
				q_2	322 > 290	20
10.77	10.5-11.3	Chlorpyriphos	20	Q	350 > 198	20
				q_1	350 > 294	10
				q_2	350 > 322	10
10.85		Aldrin	30	Q	363 > 159	20
				q_1	363 > 215	20
				q_2	363 > 327	10
11.39	11.3-12.0	Isodrin	30	Q	363 > 159	20
				q_1	363 > 215	20
				q_2	363 > 327	10
11.56		Chlorfenvinphos	30	Q	359 > 170	30
				q_1	359 > 99	10
				q_2	359 > 205	20

Table 2 (continued).

t_R (min)	Window (min)	Compounds	Cone voltage (v)	MS/MS Transitions		Collision energy (eV)
11.56		Oxychlordane	10	Q	421 >151	20
				q_1	421 > 115	20
				q_2	421 > 285	30
11.56		Heptachlor epox B	20	Q	387 > 217	30
				q_1	387 > 251	20
				q_2	387 > 252	10
12.23	12.0-12.6	Endosulfan I	10	Q	405 > 323	10
				q_1	405 > 205	20
				q_2	405 > 217	30
12.72	12.6-13.0	Buprofezin	30	Q	306 > 106	20
				q_1	306 > 203	10
				q_2	306 > 250	10
12.73		Dieldrin	20	Q	379 > 325	10
				q_1	379 > 147	20
				q_2	379 > 261	20
13.1	13.0-13.8	Endrin	30	Q	379 > 343	10
				q_1	379 > 243	20
				q_2	379 > 244	20
13.36		Ethion	10	Q	385 > 125	20
				q_1	385 > 97	10
				q_2	385 > 143	30
14.01	13.8-14.6	Endosulfan sulfate	10	Q	323 > 217	30
				q_1	323 > 252	20
				q_2	323 > 287	10
15.63	14.6-21.0	Azinphos methyl	20	Q	261 > 125	20
				q_1	261 > 167	10
				q_2	261 > 183	10
15.66		Pyriproxyfen	10	Q	322 > 185	20
				q_1	322 > 129	30
				q_2	322 > 227	10
16.04		Fenarimol	40	Q	331 > 268	20
				q_1	331 > 139	30
				q_2	331 > 259	20
16.17		Azinphos ethyl	20	Q	289 > 137	20
				q_1	289 > 233	10
				q_2	289 > 261	10

3.4. Optimization of the flow rate of He

Carrier gas (He) flow rates of around 1 mL/min are typically applied in GC–(EI) MS since they provide a good compromise between column efficiency – according to the van Deemter equation for 0.25 mm internal diameter capillary columns used – and ionization efficiency of the EI source. However, when using an APCI source, higher carrier gas flow rates (2 mL/min) can be used without loss in ionization performance, thus allowing an improvement in detection response, enhancing resolution of critical pairs of components. APCI full scan chromatograms acquired at flow rates 1.2 and 2 mL/min, corresponding to linear velocities of 40 and 52 cm/s respectively, were evaluated and compared. As can be seen in **Fig. 4**, the reduction of peak width in the last eluting compounds using 2 mL/min led to an improvement of the resolution, as occurs with fenarimol and azinphos ethyl, which could not be chromatographically resolved using 1.2 mL/min as flow rate. Moreover, early eluting compounds as dichlorvos, dicrotophos and monocrotophos showed an improvement of the peak shape. No significant differences were observed on the chromatographic peaks for the rest of compounds. Thus, 2 mL/min was selected as carrier gas flow rate.

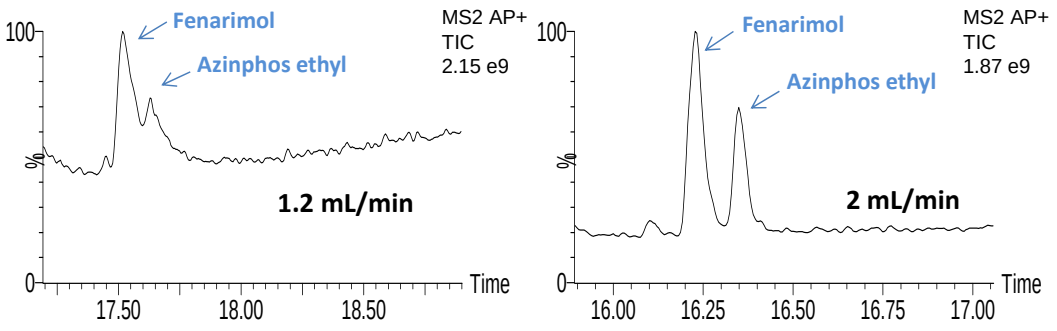

Fig. 4. Total ion GC–MS chromatogram of fenarimol and azinphos ethyl using 1.2 mL/min and 2 mL/min as flow rate.

3.5. Product ion scan experiments

Product ion scan experiments were performed in order to find selective transitions based on the use of $[M+H]^+$ as precursor ion. Moreover, as $[M+H]^+$ was the base peak of the mass spectrum for most compounds, satisfactory sensitivity was achieved in most cases. Product ion scan was performed at different collision energies (10, 20 and 30 eV) and the most sensitive transitions were selected for the development of the subsequent SRM method.

For those pesticides whose $[M+H]^+$ peak showed low abundance, as azinphos methyl or azinphos ethyl, an alternative precursor ion was also selected. This pair of pesticides represents another example of compounds included in group 3, so the selection of specific transitions had to be carefully studied when a precursor ion different than $[M+H]^+$ had to be selected. **Fig. S1 a** (Supplementary data) shows that the EI spectra of both pesticides are practically identical, with the absence of $M^{+\bullet}$; then the same transitions (normally coming from the fragment ion m/z 160) have to be used in conventional SRM methods for both compounds. On the contrary, different fragmentation patterns were observed in APCI spectra (**Fig. S1 b**, Supplementary data), so that the selection of different precursor ions is possible. In the case of azinphos ethyl, m/z 289 was selected as precursor ion since it was the most abundant non-common peak. However, the best precursor ion for azinphos methyl (expectedly m/z 261) was also present in the APCI spectrum of azinphos ethyl, so its selection could not ensure the desirable specificity in single MS. In order to study whether tandem MS improve specificity, product ion scan spectra from m/z 261 were compared for both pesticides with the result that different product ions were present (see **Fig. S2**, Supplementary data), allowing an adequate GC–MS/MS determination of both compounds. Thus, it is feasible using m/z 261 as precursor ion for azinphos methyl determination without losing specificity due to the presence of azinphos ethyl.

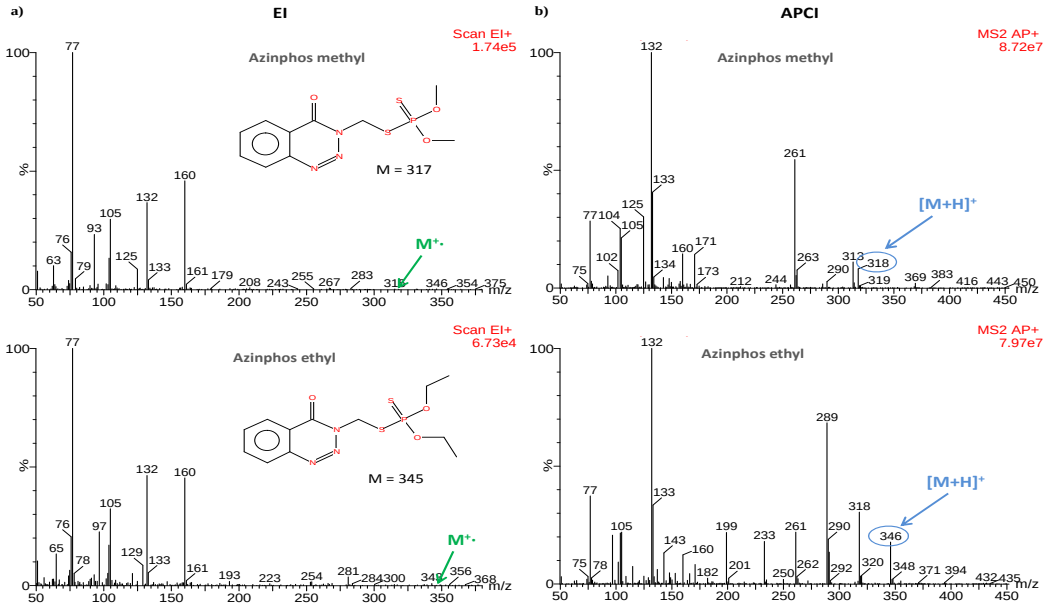

Fig. S1. Comparison of full scan spectra obtained under a) EI and b) APCI using water as modifier for azinphos methyl and azinphos ethyl.

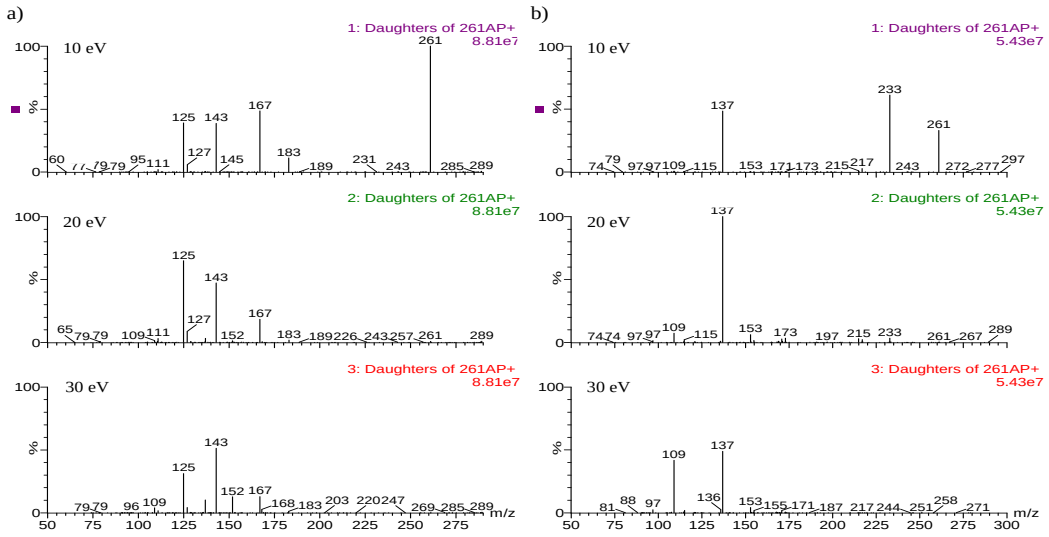

Fig. S2. Product ion scan spectra of 261 at different collision energies for: a) azinphos methyl and b) azinphos ethyl.

3.6. SRM method

Once selected the best combinations for precursor and product ions and established the optimum collision energies, a SRM method was developed. Experimental MS/MS parameters for each compound are shown in **Table 2**. Three transitions were chosen for each compound using $[M+H]^+$ as precursor ion for all of them with the exception of terbufos, endosulfan sulfate, azinphos methyl and azinphos ethyl, in which the selection of an alternative precursor ion improved method sensitivity, without compromising specificity.

In the new generations of triple quadrupole instruments (as the one used in this work), sensitivity and repeatability of response are not affected by the dwell time used during acquisition. In this work, dwell times as low as 10 ms were used without resolution and sensitivity losses. This value was automatically calculated by the software depending on the peak width, number of points per peak desired and number of transitions acquired simultaneously. Values from 10 to 250 ms were used to obtain 12 points per peak.

SRM chromatograms acquired under EI and APCI sources were compared to corroborate the potential of APCI to solve problematic cases. The pair oxychlordane and heptachlor epoxide B represents another case included in the group 3 since both pesticides have most transitions in common. Moreover, the coincidence in the retention times makes their simultaneous determination troublesome in a SRM method. As **Fig. 5 a** shows, when transition 235 > 141 (typical for oxychlordane) was acquired, a peak due to heptachlor epoxide B was also present using the EI source. However, the use of 421 > 151 (421 corresponds to the $[M+H]^+$ ion of oxychlordane) in APCI allows the determination of oxychlordane without interference from heptachlor epoxide B (**Fig. 5 b**).

A similar case was observed for chlorpyriphos methyl and chlorpyriphos ethyl. Transitions selected in EI for both pesticides are not specific. In contrast, transitions coming from $[M+H]^+$ in APCI source allowed their spectral differentiation, although this case was less critical since they were chromatographically resolved.

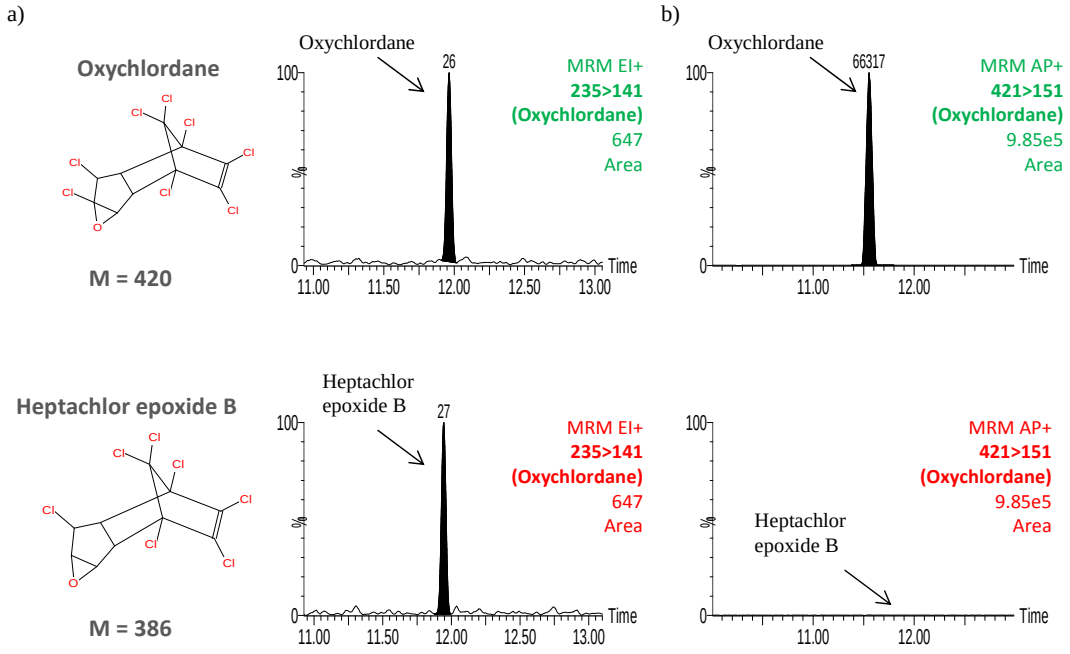

Fig. 5. Product ion scan spectra of 261 at different collision energies for: a) azinphos methyl and b) azinphos ethyl.

3.7. Instrumental analytical characteristics

The developed SRM method was applied to evaluate instrumental linearity and precision under APCI conditions using water as modifier. Linearity was studied by injecting standards in solvent (n = 2) in the range 0.1–100 ng/mL (corresponding to 0.1–100 µg/kg in sample). Most compounds showed a linear tendency from 0.1 to 100 ng/mL with r^2 > 0.99 and residuals lower than 30%.

Precision of the method was estimated from area responses obtained after repeated injections of a 0.5 ng/mL standard (n = 10) and expressed as repeatability in terms of relative standard deviation (R.S.D., %). Results were satisfactory since R.S.D. were lower than 20% for all compounds.

In order to study the applicability of the developed chromatographic method, apple, orange, tomato and carrot blank samples were extracted applying the QuEChERS method, and the extracts obtained were spiked at 10 ng/mL (corresponding to 10 μg/kg in sample) with all studied pesticides. Responses for spiked samples were compared to those of standards in solvent. Product ion spectra were evaluated in all the matrices and no significant differences in the fragmentation pattern were observed with respect to standards in solvent. Thus, it seemed that matrix effects did not affect to the fragmentation in the collision cell.

SRM chromatograms of all pesticides/matrix combinations were evaluated. No significant matrix effects were observed, although a little enhancement of the response in the sample extracts occurred. **Fig. 6** illustrates representative examples for ethion and fenarimol in tomato and carrot samples, respectively.

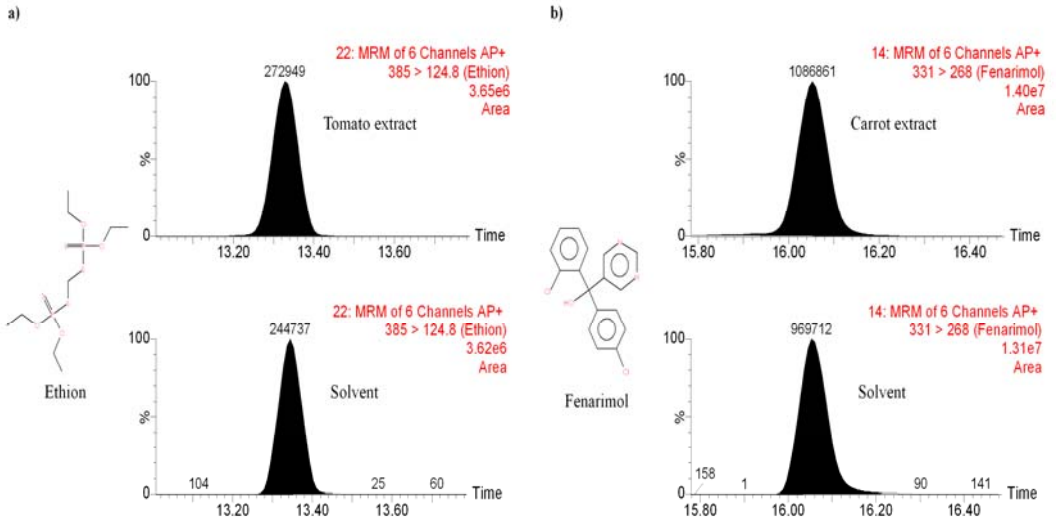

Fig. 6. SRM chromatograms for (a) ethion in spiked tomato extract (10 ng/mL) and in solvent standard (10 ng/mL) and (b) fenarimol in spiked carrot extract (10 ng/mL) and in solvent standard (10 ng/mL).

Satisfactory chromatographic peaks were obtained for all compounds at 10 ng/mL, as well as satisfactory sensitivity that led to an estimation of LOQ (calculated as 10 times S/N) between 0.02–2 μg/kg depending on the analyte and matrix under study. The good selectivity achieved in this case contrast with the results obtained in a previous work [34], in which ethion and fenarimol could not be adequately determined in these samples due to the heavy matrix interferences observed when applying a GC–MS SIM method (using a single quadrupole). Therefore, in the present work the use of GC–MS/MS combined with an APCI source provided a notable increase in sensitivity and selectivity.

4. CONCLUSIONS

Spectral fragmentation of 25 selected pesticides has been evaluated using a GC(APCI)–MS/MS system with triple quadrupole analyzer. The soft ionization process in the APCI source has favored the presence of the $M^{+\bullet}$ and/or $[M+H]^+$ ion in the spectrum of most pesticides, inversely to the pattern fragmentation typically observed under EI ionization mode. Moreover, the use of water as modifier has enhanced the sensitivity for $[M+H]^+$, which was the base peak of the spectrum in most cases. Thus, further experiments were performed under these conditions.

Pesticides under study were sorted into three model groups as a function of their fragmentation pattern under EI source. For the wide majority, APCI demonstrated a strong potential to improve the tandem MS determination in comparison with EI mode because of the possibility to select a more abundant and specific precursor ion in SRM mode. This occurred for highly fragmented pesticides, for which a fragment ion with low m/z should be used as precursor ion (group 1), as well as for those compounds which transitions showed poor sensitivity (group 2). The use of APCI allowed in these cases to select more specific and sensitive transitions using $[M+H]^+$ as precursor ion. Some pesticides (group 3) presented spectral fragmentation similar to other compounds, normally belonging to the same chemical

family, and APCI offered the possibility to spectrally resolve them selecting their respective $[M+H]^+$ as precursor ion.

Product ion scan experiments have been also performed to evaluate the fragmentation of the correspondent $[M+H]^+$. Abundant product ions were normally achieved, and this facilitated the application of tandem MS methods selecting $[M+H]^+$ as precursor ion. GC–MS/MS making use of APCI offers interesting features in comparison to EI source for quantitative multiresidue analysis, since transitions selected can be more specific and sensitive. Preliminary experiments on linearity and precision of the GC–(APCI)–MS/MS method have been performed with satisfactory results. The applicability of the method has been also tested in real samples comparing responses of fortified blank QuEChERS extracts and standards in solvent. Matrix effects have not been observed in terms of fragmentation behavior and no interferences have been found in SRM transitions.

Results obtained in this work are optimistic for future improvements of multiresidue quantitative analysis, although further experiments are still required to fully evaluate the potential of this approach in environmental applications and/or in food safety.

Acknowledgments

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III.3. Artículo científico 5

Application of gas chromatography–(triple quadrupole) mass spectrometry with atmospheric pressure chemical ionization for the determination of multiclass pesticides in fruits and vegetables

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Application of gas chromatography–(triple quadrupole) mass spectrometry with atmospheric pressure chemical ionization for the determination of multiclass pesticides in fruits and vegetables

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Abstract

A multi-residue method for the determination of 138 pesticide residues in fruits and vegetables has been developed using a new atmospheric pressure chemical ionization (APCI) source for coupling gas chromatography (GC) to tandem mass spectrometry (MS). Selected reaction monitoring (SRM) mode has been applied, acquiring three transitions for each compound. In contrast to the extensive fragmentation typically obtained in classical electron ionization (EI), the soft APCI ionization allowed the selection of highly abundant protonated molecules ($[M+H]^+$) as precursor ions for most compounds. This was favorable for both sensitivity and selectivity. Validation of the method was performed in which both quantitative and qualitative parameters were assessed using orange, tomato and carrot samples spiked at two levels, 0.01 and 0.1 mg/kg. The QuEChERS method was used for sample preparation, followed by a 10-fold dilution of the final acetonitrile extract with a mixture of hexane and acetone. Recovery and precision were satisfactory in the three matrices, at both concentration levels. Very low limits of detection (down 0.01 μg/kg

for the most sensitive compounds) were achieved. Ion ratios were consistent and identification according to EU criteria was possible in 80% (0.01 mg/kg) to 96% (0.1 mg/kg) of the pesticide/matrix combinations. The method was applied to the analysis of various fruits and vegetables from the Mediterranean region of Spain.

Keywords

Atmospheric pressure chemical ionization; Gas Chromatography; Tandem mass spectrometry; triple quadrupole; Pesticides; Fruit and vegetable analysis, validation, identification.

1. INTRODUCTION

The control of pesticide residues in food commodities is a requirement to verify compliance with regulatory limits set by the European Commission (EC 396/2005) to ensure good agricultural practice and food safety. Sensitive and robust analytical techniques are required that preferably cover various pesticide chemical classes with different physicochemical properties. A common analytical approach is to combine generic sample preparation techniques, with inherently low selectivity, with highly selective instrumental analysis.

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure is a popular generic sample preparation method for the extraction of pesticides from fruits and vegetables. It involves a rapid extraction using acetonitrile (MeCN) and a cleanup step based on dispersive-SPE (d-SPE) using a primary secondary amine (PSA) sorbent and anhydrous $MgSO_4$ to remove water [1] and [2]. Numerous applications have been successfully validated for a large number of pesticides in a variety of complex matrices [3], [4] and [5].

For a major part of the pesticides, liquid chromatography combined with mass spectrometry is considered as the method of choice [6]. However, many pesticides are

also amenable to gas chromatography (GC) coupled to mass spectrometry (MS) which makes it a valuable complementary technique, especially because it is the only option for certain pesticide classes and therefore has to be used anyway. Several quantitative applications have been described in literature using GC–MS with a single quadrupole analyzer operating in selected ion monitoring (SIM) [7], [8] and [9], especially for multi-residue analysis with a limited number of compounds. However, the determination of a larger number of analytes usually requires more selective techniques, as tandem mass spectrometry (MS/MS). The use of triple quadrupole (QqQ) working under selected reaction monitoring (SRM) improves selectivity, as well as sensitivity [10], [11], [12], [13] and [14].

In GC–MS/MS, electron ionization (EI) is by far the most widely used ionization technique because of its capability of ionizing virtually any organic compound. A rather strong fragmentation is inherent to EI. This is a disadvantage in GC–MS/MS because in many cases fragments have to be used as precursor ions which are then further fragmented to smaller product ions. This compromises both sensitivity and selectivity compared to LC–MS/MS where quasi molecular ions are obtained during ionization. Softer ionization modes such as chemical ionization (PCI, NCI) and supersonic molecular beam (cold EI) [15] are available for GC but these options have restrictions with respect to applicability and commercial availability, respectively. Atmospheric pressure chemical ionization (APCI), commonly used in LC–MS/MS, has been described as an alternative source for GC–MS and a way to couple GC to mass spectrometers initially developed for LC–MS [16] and [17]. Application studies including pharmaceutical development [18], profiling of phenolic compounds in oil [19], metabolic profiling [20] and pesticide residue analysis [21], most of them using GC–(APCI) TOF MS, can be found since 2009. Recently, we investigated the potential of APCI in GC–triple quadrupole MS for wide-scope pesticide residue analysis [22] and [23]. Compared to EI, little or no fragmentation occurs while compared to PCI/NCI the applicability to different classes of compounds was much wider. Besides the selectivity advantage arising from the ability to use the quasi-molecular ion as precursor ion, the sensitivity was also found to be substantially improved. This was partly due to the use of high-end MS/MS detectors

normally used for LC–MS/MS (in fact, by changing the source, the same MS/MS could be coupled to either LC or GC).

In the previous papers the emphasis was on the potential, ionization mechanisms, and features of GC–(APCI) MS/MS. In this work the focus is on applicability for routine wide-scope multi-residue analysis of pesticides in fruits and vegetable matrices, with emphasis on quantitative and qualitative performance. A GC–(APCI) MS/MS method for simultaneous detection of 138 pesticides (around 48 of them non LC-amenable) was set up including three transitions for each compound. Using a QuEChERS method for sample preparation, the method was validated for three matrices (orange, tomato, carrot). Matrix effects, linearity, accuracy, limits of quantification (LOQs) and limits of detection (LODs) were established. With respect to identification, compliance of ion ratios with EU criteria was assessed. Real samples were analyzed to test the method applicability, including orange, tomato, carrot and also apple, lettuce and courgette.

2. EXPERIMENTAL

2.1. Reagents

Pesticide standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock standard solutions (around 500 μg/mL) were prepared by dissolving reference standards in acetone and stored in a freezer at −20 °C. Working standard mixtures were prepared by volume dilution of stock solutions in hexane for preparation of matrix-matched calibrants and in acetone for sample fortification.

Hexane, acetone, acetonitrile (MeCN), toluene, glacial acetic acid (HAc), anhydrous $MgSO_4$ and anhydrous sodium acetate (NaAc) were purchased from Scharlab (Barcelona, Spain). Solvents used were of pesticide residue analysis or HPLC grade. Two types of 2 mL micro-centrifuge tubes for QuEChERS d-SPE containing 50 mg PSA and 150 mg anhydrous $MgSO_4$, or 50 mg PSA, 150 mg

anhydrous $MgSO_4$ and 50 mg C_{18}, were obtained from Teknokroma (Barcelona, Spain).

2.2. Sample material

Three types of sample matrices were used in the validation study: orange, tomato and carrot. Blank samples, used for the matrix-matched calibration, sample fortification and quality control, were obtained from organic cultivars (pesticide free).

Three different varieties from each food commodity were analyzed to investigate the presence of pesticides, all of them purchased from local markets in the Castellón province (Spain).

Apple, lettuce and courgette samples, also purchased from local markets, were analyzed to extend the method applicability.

2.3. Instrumentation

Data were acquired using a GC system (Agilent 7890A, Palo Alto, CA, USA) equipped with an autosampler (Agilent 7693) and coupled to a triple quadrupole (QqQ) mass spectrometer (Xevo TQ-S, Waters Corporation, Manchester, UK), operating in APCI mode. A fused silica DB-5MS capillary column (length 30 m × I.D. 0.25 mm × film 0.25 μm) (J&W Scientific, Folson, CA, USA) was used for GC separation. The injector was operated in splitless mode, injecting 1 μL at 280 °C. The oven temperature was programmed as follows: 70 °C (1 min), 15 °C/min to 150 °C and 10 °C/min to 300 °C (3 min). Helium was used as carrier gas in constant flow mode (2 mL/min). A pulsed splitless injection was carried out using an initial pressure of 240 kPa, maintained for 1 min, and then changed to a constant flow of 2 mL/min, which corresponded to a linear velocity of 52 cm/s. In the SRM method, automatic dwell time (values ranging from 3 to 63 ms) was applied in order to obtain 15 points per peak.

The interface temperature was set to 310 °C using N_2 as auxiliary gas at 250 L/h, a make-up gas at 300 mL/min and cone gas at 170 L/h. The APCI corona

discharge pin was operated at 1.8 μA. The water used as modifier when working under proton-transfer conditions was placed in an uncapped vial, which was located within a holder placed in the source door.

TargetLynx (a module of MassLynx) was used to handle and process the acquired data.

2.4. Sample treatment

The QuEChERS procedure applied was that proposed in the AOAC official method 2007.01 [2]: 15 g of sample (previously homogenized in a food chopper) were weighted in a 50 mL polypropylene centrifuge tube, mixed with 15 mL MeCN (with 1% HAc) and shaken by hand for 30 s. Then, 6 g anhydrous $MgSO_4$ and 1.5 g anhydrous NaAc were added and immediately shaken vigorously by hand to prevent formation of $MgSO_4$ agglomerates. Then, the tube was centrifuged at 3000 rpm for 2 min.

For the cleanup step, 1 mL of the upper MeCN extract was transferred into a d-SPE tube containing 150 mg $MgSO_4$ and 50 mg PSA (or 150 mg $MgSO_4$, 50 mg PSA and 50 mg C_{18} when oranges were extracted). The tubes were shaken on a Vortex for 30 s and centrifuged at 3000 rpm for 2 min. Finally, 50 μL of the extract (acetonitrile) were transferred into a 2 mL vial and diluted with 300 μL of hexane and 150 μL of acetone.

Matrix-matched standards were prepared for each sample matrix as follows: after the cleanup step, 50 μL of the MeCN extract obtained from a blank sample were mixed with 250 μL of hexane, 150 μL of acetone, and 50 μL of the pesticide standard solution in hexane at adequate concentration to obtain a calibration range of 0.1–100 ng/mL (corresponding to 1–1000 μg/kg in sample).

2.5. Validation study

The developed SRM method was validated using orange, carrot and tomato in order to evaluate linearity, recovery, precision, selectivity and LODs and LOQs.

Linearity was studied by injecting standards in hexane (n = 3) at eight concentration levels, 0.1, 0.5, 1, 5, 10, 25, 50 and 100 ng/mL, and was considered acceptable when regression coefficients were higher than 0.99 and residuals lower than 30%.

Accuracy was estimated from recovery experiments, by analyzing six replicates spiked at two levels (0.01 and 0.1 mg/kg). Precision was expressed as repeatability in terms of relative standard deviation (RSD, %) (n = 6) calculated for each fortification level.

The LOQ was defined as the lowest concentration level validated with satisfactory values of recovery (70–110%) and precision (RSD < 20%) [24].

The LOD, defined as the concentration corresponding to a signal-to-noise ratio of three, was estimated from the chromatogram of the matrix-matched standards at the lowest calibration level used for each compound.

The selectivity of the method was evaluated by verification of the absence of interfering peaks at the retention time of each compound in blank samples for the acquired MS/MS transitions.

The ratio of each of the two qualifier ions relative to the quantifier (calculated by dividing the lower by the higher response) were used to verify compliance with EU criteria [24] of the pesticides in the spiked samples and to confirm peak identity in real samples.

3. RESULTS AND DISCUSSION

3.1. GC–(APCI) MS/MS optimization

Optimization of the MS/MS conditions started by using pesticide standard solutions in hexane with the mass spectrometer operating in full scan mode to obtain the MS spectra. Experiments under N_2 and proton transfer conditions (using water as modifier) were performed. The proton transfer mechanism revealed a notable tendency for the majority of the studied pesticides to be protonated, since the $[M+H]^+$ was present for most compounds and frequently as the peak base of the spectrum, with very low fragmentation. Thus the use of water as modifier was considered for further experiments.

The cone voltage was studied in the range 5–40 V for all compounds and those values which result in higher sensitivity were selected for each pesticide (**Table 1**). The helium flow rate was set at a relatively high flow rate of 2 mL/min since this was found to be beneficial for peak shape and analysis speed in an earlier work [23] using a GC–(APCI) MS system.

To continue with MS/MS optimization, the base peak of the spectrum for each compound ($[M+H]^+$ in most cases) was selected as precursor ion (in some cases, two different precursor ions were chosen). The main goal was to develop a SRM method with three MS/MS transitions (the most sensitive ones) in order to carry out a reliable quantification and identification of the pesticides detected in samples. The fragmentation pattern of the precursor ions was studied through product ion scan experiments at different collision energies (10, 20 and 30 eV) and again the most sensitive transitions were selected for the final SRM method. **Table 1** shows the SRM transitions corresponding to both quantifier and the qualifier transitions monitored.

Table 1. Experimental conditions of the optimized GC–(APCI)MS/MS method using water as modifier. Quantifier (Q) and qualifier (q) transitions.

t_R (min)	Window (min)	Compounds	SRM Transitions		Collision energy (eV)	Cone voltage (V)
6.2	6-7.75	Hexachlorobutadiene	Q	*258* > 223	20	30
			q_1	*258* > 141	20	
			q_2	*258* > 188	30	
6.45		Dichlorvos	Q	*221* > 109	20	10
			q_1	*221* > 127	20	
			q_2	*221* > 145	10	
6.95		Carbofuran	Q	165 > 123	10	20
			q_1	165 > 105	30	
			q_2	165 > 137	10	
8.19	7.75-8.8	Mevinphos	Q	193 >127	10	30
			q_1	*225* > 127	10	
			q_2	*225* > 193	10	
8.45		Propham	Q	138 > 120	10	30
			q_1	138 > 77	30	
			q_2	138 > 92	10	
9	8.8-9.95	Carbaryl	Q	145 > 117	20	10
			q_1	145 > 115	20	
			q_2	144 > 115	20	
9.1		2-Phenylphenol	Q	*171* > 153	20	10
			q_1	*171* > 151	30	
			q_2	*171* > 152	30	
9.21		Pentachlorobenzene	Q	*248* > 213	30	40
			q_1	*248* > 142	30	
			q_2	*248* > 178	30	
9.3		Molinate	Q	*188* > 126	10	20
			q_1	*188* > 98	20	
			q_2	*188* > 160	10	
10.02	9.95-10.3	Propoxur	Q	*210* > 111	10	10
			q_1	*210* > 168	10	
			q_2	*210* > 135	10	
10.05		Propachlor	Q	*212* > 170	20	30
			q_1	*212* > 94	30	
			q_2	*212* > 106	30	
10.12		Demeton-s-methyl	Q	143 > 111	10	20
			q_1	143 > 125	10	
			q_2	143 > 127	10	
10.14		Diphenylamine	Q	*170* > 93	30	40
			q_1	*170* > 152	20	
			q_2	*170* > 153	20	
10.39	10.2-10.8	Atrazine deisopropyl	Q	*174* > 132	20	30
			q_1	*174* > 104	30	
			q_2	*174* > 146	20	
10.4		Chlorpropham	Q	172 > 154	10	40
			q_1	172 > 111	20	
			q_2	172 > 126	20	
10.49		Ethalfluralin	Q	*334* > 232	10	20
			q_1	*334* > 186	30	
			q_2	*334* > 300	20	
10.5		Terbumeton desethyl	Q	*198* > 142	20	20
			q_1	*198* > 86	30	
			q_2	*198* > 100	30	
10.5		Atrazine desethyl	Q	*188* > 146	20	10
			q_1	*188* > 104	30	
			q_2	*188* > 111	10	
10.65		Trifluraline	Q	*336* > 232	20	30
			q_1	*336* > 186	30	
			q_2	*336* > 202	30	

Table 1 (continued).

t_R (min)	Window (min)	Compounds	SRM Transitions		Collision energy (eV)	Cone voltage (V)
10.7	10.5-11.3	Terbutylazine desethyl	Q	*202* > 146	20	20
			q_1	*202* > 104	30	
			q_2	*202* > 116	30	
10.79		Cadusafos	Q	*271* > 131	10	30
			q_1	*271* > 97	10	
			q_2	*271* > 125	20	
10.87		Phorate	Q	*261* > 97	30	20
			q_1	*261* > 171	10	
			q_2	*261* > 199	10	
10.97		alpha-HCH	Q	181 > 146	20	10
			q_1	181 > 109	30	
			q_2	217 > 181	10	
11.12		Hexachlorobenzene	Q	*282* > 247	30	40
			q_1	*282* > 177	30	
			q_2	*282* > 212	30	
11.2		Dichloran	Q	*207* > 190	20	10
			q_1	*207* > 124	30	
			q_2	*207* > 160	20	
11.2		Dimethoate	Q	*230* > 125	20	30
			q_1	*230* > 171	10	
			q_2	*230* > 199	10	
11.25	11-12	Ethoxyquin	Q	218 > 174	30	30
			q_1	218 > 160	30	
			q_2	218 > 202	20	
11.27		Simazine	Q	202 > 132	20	30
			q_1	202 > 104	30	
			q_2	202 > 124	20	
11.38		Atrazine	Q	*216* > 174	20	10
			q_1	*216* > 104	30	
			q_2	*216* > 132	30	
11.47		beta-HCH	Q	181 > 146	20	10
			q_1	181 > 109	30	
			q_2	217 > 181	10	
11.49		Terbumeton	Q	*226* > 170	20	30
			q_1	*226* > 114	30	
			q_2	*226* > 142	30	
11.6	11.5-11.85	Dioxathion	Q	271 > 97	30	20
			q_1	271 > 125	10	
			q_2	271 > 141	20	
11.6		gamma-HCH	Q	181 > 146	20	10
			q_1	181 > 109	30	
			q_2	217 > 181	10	
11.66		Terbutylazine	Q	*230* > 174	20	20
			q_1	*230* > 104	30	
			q_2	*230* > 132	30	
11.67		Propetamphos	Q	222 > 138	10	20
			q_1	222 > 110	20	
			q_2	*282* > 138	20	
11.68		Cyanophos	Q	*244* > 125	30	5
			q_1	*244* > 134	30	
			q_2	*244* > 150	20	
11.68		Terbufos	Q	187 > 97	10	5
			q_1	187 > 131	20	
			q_2	187 > 159	10	
11.72		Propyzamide	Q	*256* > 190	10	30
			q_1	*256* > 145	30	
			q_2	*256* > 173	20	
11.9	11.7-12.25	Diazinon	Q	*305* > 169	30	40
			q_1	*305* > 153	30	
			q_2	*305* > 249	20	

Table 1 (continued).

t_R (min)	Window (min)	Compounds	SRM Transitions		Collision energy (eV)	Cone voltage (V)
12.01		Terbacil	Q	161 > 144	20	20
			q_1	161 > 118	20	
			q_2	161 > 143	20	
12.05		delta-HCH	Q	181 > 146	20	30
			q_1	181 > 109	30	
			q_2	217 > 181	10	
12.08		Tefluthrin	Q	177 > 127	20	5
			q_1	*419* > 177	10	
			q_2	*419* > 325	10	
12.14		Chlorothalonil	Q	*265* > 230	20	30
			q_1	*265* > 133	30	
			q_2	*265* > 211	20	
12.4	12.25-12.7	Pirimicarb	Q	*239* > 182	20	10
			q_1	*239* > 109	30	
			q_2	*239* > 138	30	
12.43		Endosulfan ether	Q	*341* > 217	30	30
			q_1	*341* > 170	30	
			q_2	*341* > 205	20	
12.62		Phosphamidon	Q	*300* > 127	20	40
			q_1	*300* > 174	10	
			q_2	*300* > 227	10	
12.62		Dichlofenthion	Q	*315* > 259	20	30
			q_1	*315* > 179	20	
			q_2	*315* > 287	10	
12.66	12.4-13.15	Metribuzin	Q	*215* > 187	20	40
			q_1	*215* > 145	20	
			q_2	*215* > 171	20	
12.79		Vinclozolin	Q	*286* > 242	10	5
			q_1	*286* > 164	30	
			q_2	*286* > 172	20	
12.8		Parathion methyl	Q	*264* > 232	20	20
			q_1	*264* > 125	30	
			q_2	*264* > 155	30	
12.8		Chlorpyrifos methyl	Q	*322* > 125	30	40
			q_1	*322* > 212	30	
			q_2	*322* > 290	20	
12.94		Alachlor	Q	238 > 162	20	30
			q_1	238 > 132	30	
			q_2	*270* > 147	30	
12.96		Heptachlor	Q	335 > 264	20	40
			q_1	335 > 230	30	
			q_2	335 > 299	10	
13.03		Metalaxyl	Q	*280* > 220	10	30
			q_1	*280* > 160	20	
			q_2	*280* > 192	10	
13.1	12.8-13.6	Methiocarb sulfone	Q	201 > 122	10	20
			q_1	201 > 91	30	
			q_2	201 > 107	30	
13.15		Demeton-s-methylsulfone	Q	*263* > 125	20	30
			q_1	*263* > 169	10	
			q_2	*263* > 231	10	
13.25		Terbutryn	Q	*242* > 186	20	40
			q_1	*242* > 116	30	
			q_2	*242* > 138	30	
13.3		Methiocarb	Q	*226* > 169	10	30
			q_1	*226* > 121	20	
			q_2	*226* > 122	30	
13.3		Fenitrothion	Q	*278* > 125	30	40
			q_1	*278* > 169	30	
			q_2	*278* > 200	20	

Table 1 (continued).

t_R (min)	Window (min)	Compounds	SRM Transitions		Collision energy (eV)	Cone voltage (V)
13.34		Pirimiphos methyl	Q	*306* > 125	30	5
			q_1	*306* > 109	30	
			q_2	*306* > 164	30	
13.5		Malathion	Q	*331* > 125	30	20
			q_1	*331* > 117	20	
			q_2	*331* > 211	10	
13.61	13.3-13.85	Aldrin	Q	*363* > 159	20	30
			q_1	*363* > 215	20	
			q_2	*363* > 327	10	
13.63		Metolachlor	Q	*284* > 252	20	20
			q_1	*284* > 134	30	
			q_2	*284* > 176	30	
13.66		Fenthion	Q	*279* > 247	10	20
			q_1	*279* > 105	20	
			q_2	*279* > 169	30	
13.68		Cyanazine	Q	*241* > 214	20	30
			q_1	*241* > 132	30	
			q_2	*241* > 205	20	
13.71		Chlorpyrifos	Q	*350* > 198	20	20
			q_1	*350* > 294	10	
			q_2	*350* > 322	10	
13.72		Parathion-ethyl	Q	*292* > 236	20	20
			q_1	*292* > 110	30	
			q_2	*292* > 123	30	
13.76		Triadimefon	Q	*294* > 197	10	40
			q_1	*294* > 129	20	
			q_2	*294* > 141	20	
13.76		4,4'-Dichloronbenzophenone	Q	*251* > 139	20	20
			q_1	*251* > 111	30	
			q_2	*251* > 129	30	
14.04	13.85-14.4	Bromophos methyl	Q	*365* > 125	20	30
			q_1	*365* > 211	30	
			q_2	*365* > 239	30	
14.15		Isodrin	Q	*363* > 159	20	30
			q_1	*363* > 215	20	
			q_2	*363* > 327	10	
14.16		Cyprodinil	Q	*226* > 118	30	40
			q_1	*226* > 133	30	
			q_2	*226* > 210	30	
14.3		Pendimethalin	Q	*282* > 212	10	20
			q_1	264 > 147	30	
			q_2	264 > 201	20	
14.35	14.1-14.6	Heptachlor epoxide B	Q	351 > 251	30	20
			q_1	351 > 217	20	
			q_2	351 > 287	10	
14.37		Oxychlordane	Q	*421* > 151	20	10
			q_1	*421* > 115	20	
			q_2	*421* > 285	30	
14.41		Tolyfluanid	Q	238 > 137	20	5
			q_1	238 > 110	30	
			q_2	238 > 122	30	
14.43		Heptachlor epoxide A	Q	351 > 251	30	20
			q_1	351 > 217	20	
			q_2	351 > 287	10	
14.46		Chlorfenvinphos	Q	*359* > 170	30	30
			q_1	*359* > 99	10	
			q_2	*359* > 205	20	
14.47		Fipronil	Q	*437* > 368	20	30
			q_1	*437* > 255	30	
			q_2	*437* > 315	30	

Table 1 (continued).

t_R (min)	Window (min)	Compounds	SRM Transitions		Collision energy (eV)	Cone voltage (V)
14.5	14.2-14.8	Captan	Q	264 > 236	10	30
			q_1	264 > 156	30	
			q_2	264 > 180	10	
14.52		Quinalphos	Q	*299* > 163	20	30
			q_1	*299* > 147	30	
			q_2	*299* > 271	10	
14.6		Folpet	Q	260 > 130	20	5
			q_1	260 > 102	30	
			q_2	260 > 232	10	
14.63		Procymidone	Q	*284* > 256	20	30
			q_1	*284* > 186	30	
			q_2	*284* > 228	20	
14.67		Triflumizole	Q	*346* > 278	10	10
			q_1	*346* > 206	20	
			q_2	*346* > 266	20	
14.79	14.4-15.3	Chinomethionate	Q	*235* > 175	20	30
			q_1	*235* > 104	30	
			q_2	*235* > 121	30	
14.8		Methidathion	Q	*303* > 145	10	10
			q_1	*303* > 125	20	
			q_2	*303* > 257	10	
14.8		trans-Chlordane	Q	371 > 264	30	10
			q_1	371 > 299	20	
			q_2	371 > 335	20	
14.82		Bromophos ethyl	Q	*393* > 337	20	10
			q_1	*393* > 162	30	
			q_2	*393* > 365	10	
15.01		Endosulfan I	Q	*405* > 323	10	5
			q_1	*405* > 217	30	
			q_2	*405* > 251	20	
15.14		Fenamiphos	Q	*304* > 217	20	40
			q_1	*304* > 202	30	
			q_2	*304* > 234	20	
15.17	15-15.8	Chlorfenson	Q	*303* > 159	10	5
			q_1	*303* > 111	10	
			q_2	*303* > 128	30	
15.32		Imazalil	Q	*297* > 159	20	10
			q_1	*297* > 109	20	
			q_2	*297* > 176	20	
15.36		Fludioxonil	Q	*248* > 127	30	30
			q_1	*248* > 154	20	
			q_2	*248* > 182	20	
15.43		*p*,*p'*-DDE	Q	*316* > 246	30	20
			q_1	*316* > 210	30	
			q_2	*316* > 281	20	
15.49		Dieldrin	Q	*379* > 325	10	20
			q_1	*379* > 254	30	
			q_2	*379* > 261	20	
15.57		Oxyfluorfen	Q	*362* > 316	10	30
			q_1	*362* > 237	20	
			q_2	*362* > 334	10	
15.61		Buprofezin	Q	*306* > 106	20	30
			q_1	*306* > 203	10	
			q_2	*306* > 250	10	
15.9	15.6-16.4	Endrin	Q	*379* > 343	10	30
			q_1	*379* > 243	20	
			q_2	*379* > 244	20	
16.05		Endosulfan II	Q	*405* > 323	10	30
			q_1	*405* > 217	30	
			q_2	*405* > 251	20	

Table 1 (continued).

t_R (min)	Window (min)	Compounds	SRM Transitions		Collision energy (eV)	Cone voltage (V)
16.18		*p,p'*-DDD	Q	235 > 165	20	5
			q_1	235 > 99	30	
			q_2	235 > 199	20	
16.25		Oxadixyl	Q	*279* > 219	10	5
			q_1	*279* > 117	30	
			q_2	*279* > 132	20	
16.27		Ethion	Q	*385* > 143	30	5
			q_1	*385* > 97	10	
			q_2	*385* > 125	20	
16.47	16.3-16.8	Sulprofos	Q	*323* > 139	20	10
			q_1	*323* > 155	30	
			q_2	*323* > 219	10	
16.62		Famphur	Q	*326* > 217	20	30
			q_1	*326* > 125	20	
			q_2	*326* > 152	30	
16.65		Carbofenothion	Q	*343* > 157	20	5
			q_1	*343* > 97	30	
			q_2	*343* > 121	30	
16.67		Carfentrazone ethyl	Q	*412* > 346	20	10
			q_1	*412* > 366	20	
			q_2	*412* > 384	10	
16.78	16.5-17	Propiconazole	Q	*342* > 159	20	30
			q_1	*342* > 187	20	
			q_2	*342* > 256	10	
16.8		Endosulfan sulfate	Q	323 > 217	30	10
			q_1	323 > 251	20	
			q_2	323 > 287	10	
16.84		Fenhexamid	Q	*302* > 143	30	30
			q_1	*302* > 142	30	
			q_2	*302* > 178	20	
16.85		*p,p'*-DDT	Q	235 > 165	20	5
			q_1	235 > 99	30	
			q_2	235 > 199	20	
17.17	16.9-17.9	Diflufenican	Q	*395* > 266	20	10
			q_1	*395* > 238	30	
			q_2	*395* > 246	30	
17.23		Captafol	Q	*348* > 312	10	10
			q_1	*348* > 117	30	
			q_2	*348* > 161	20	
17.27		Resmethrin	Q	*339* > 171	10	30
			q_1	*339* > 143	20	
			q_2	*339* > 293	10	
17.54		Iprodione	Q	*330* > 245	10	30
			q_1	*330* > 174	30	
			q_2	*330* > 288	10	
17.71		Fenoxycarb	Q	*302* > 256	10	40
			q_1	*302* > 183	20	
			q_2	*302* > 213	20	
17.71		Phosmet	Q	*318* > 160	10	20
			q_1	160 > 133	20	
			q_2	*318* > 133	30	
17.77		Bifenthrin	Q	181 > 165	20	10
			q_1	181 > 115	30	
			q_2	181 > 166	30	
17.86	17.6-18.7	Methoxychlor	Q	*345* > 213	20	10
			q_1	227 > 141	30	
			q_2	227 > 169	30	
18.22		Tetradifon	Q	*355* > 195	20	30
			q_1	*355* > 133	30	
			q_2	*355* > 167	20	

Table 1 (continued).

t_R (min)	Window (min)	Compounds	SRM Transitions		Collision energy (eV)	Cone voltage (V)
18.4		Azinphos methyl	Q	261 > 125	20	20
			q_1	261 > 167	10	
			q_2	261 > 183	10	
18.44		Leptophos	Q	*411* > 171	20	40
			q_1	*411* > 139	30	
			q_2	*411* > 379	20	
18.45		Pyriproxifen	Q	*322* > 185	20	10
			q_1	*322* > 129	30	
			q_2	*322* > 227	10	
18.55		lambda-Cyhalothrin	Q	*450* > 225	10	10
			q_1	*450* > 141	20	
			q_2	*450* > 157	30	
18.64	18.5-19.8	Mirex	Q	270 > 235	20	10
			q_1	270 > 117	30	
			q_2	270 > 141	30	
18.9		Acrinathrin	Q	428 > 401	20	10
			q_1	428 > 205	30	
			q_2	428 > 260	20	
18.88		Fenarimol	Q	*331* > 268	20	40
			q_1	*331* > 139	30	
			q_2	*331* > 259	20	
19.01		Azinphos ethyl	Q	289 > 137	20	20
			q_1	289 > 233	10	
			q_2	289 > 261	10	
19.43		Permethrin	Q	355 > 319	10	10
			q_1	391 > 183	30	
			q_2	391 > 355	10	
19.66		Coumaphos	Q	*363* > 227	30	30
			q_1	*363* > 211	30	
			q_2	*363* > 307	20	
20.09	19.7-20.35	Cyfluthrin	Q	*434* > 191	10	10
			q_1	*434* > 91	30	
			q_2	*434* > 127	30	
20.4	20.1-20.85	Cypermethrin	Q	*416* > 191	10	20
			q_1	*416* > 91	30	
			q_2	*416* > 127	30	
20.51		Flucythrinate	Q	412 > 219	30	5
			q_1	412 > 220	30	
			q_2	412 > 236	30	
20.59		Etofenprox	Q	359 > 183	20	10
			q_1	359 > 161	20	
			q_2	359 > 289	20	
21.21	20.85-21.6	Fenvalerate	Q	*419* > 225	10	10
			q_1	*420* > 125	10	
			q_2	*420* > 226	10	
21.38		tau-Fluvalinate	Q	*503* > 181	20	30
			q_1	*503* > 208	30	
			q_2	*503* > 250	20	
21.4		Esfenvalerate	Q	167 > 125	10	5
			q_1	167 > 99	30	
			q_2	167 > 139	10	
21.94	21.7-22.5	Deltamethrin	Q	*504* > 279	10	5
			q_1	*504* > 171	20	
			q_2	*504* > 200	30	
22.24		Azoxystrobin	Q	*404* > 372	10	20
			q_1	*404* > 329	30	
			q_2	*404* > 344	20	

Precursors corresponding to $M^{+\cdot}$ or $[M+H]^+$ are shown in italic.

3.2. Sample treatment optimization

With the QuEChERS sample preparation procedure, the final extract obtained is acetonitrile. The direct injection of the acetonitrile extract was considered less favorable. A (partial) solvent venting using a programmable temperature vaporizer injector could not be done with the GC system used, therefore a solvent exchange step was applied. Initially, in order to avoid evaporation until dryness, 1 mL of toluene was added to the 500 μL of the acetonitrile extract; evaporation until 300 μL using nitrogen stream was performed and then adjusted to 500 μL with toluene. In this way, no losses during the evaporation process were observed. However, the injection of the toluene extracts resulted in a dramatic loss of repeatability. Therefore, a solvent exchange into hexane was tested. In this case evaporation until dryness was unavoidable and the evaporation conditions had to be carefully optimized in order to avoid analyte losses. An evaporation system operating under vacuum was used, which allows a more controlled evaporation and at lower temperature compared with evaporation under nitrogen stream (miVac Modulator Concentrator, provided by Fisher Scientific S.A.S., Illkirch, France). The evaporation was carried out at 30 °C during approximately 30 minutes. However, no satisfactory results were obtained since some notable losses were observed in some analytes with low interday reproducibility.

Then, with the high sensitivity achieved in this GC–(APCI) MS/MS system in mind, the possibility of the direct dilution of the extract with hexane was considered. Standards in acetonitrile at 10 ng/mL were diluted with hexane (1/10), adding 20% of acetone to make the solution miscible. It is noteworthy that, in a multi-residue method that includes a large variety of compounds as in this work, the response of the most sensitive compounds are 1000 times higher than those ones with lower sensitivity. Consequently, dilution experiment led to a loss of some analytes that did not show enough sensitivity to be detected. A dilution of 1/5 with hexane (with 20% of acetone) was also tested but no considerable improvements with respect to the dilution 1/10 were observed for the less sensitive compounds, so this 1/10 dilution (with 20% of acetone) was selected for further experiments.

In this way, experiments were performed by diluting acetonitrile sample extracts fortified at 10 ng/mL (dilution 1/10 with hexane) and it revealed a significant improvement in peak shapes and sensitivity. In presence of matrix, a higher amount of acetone had to be added (30%) in order to keep the solution miscible. In conclusion, 50 μL of acetonitrile extract was mixed with 150 μL of acetone and 300 μL of hexane.

3.3. Matrix effect

Matrix effects for all matrices were checked by comparing responses of standards in the mixture acetonitrile, hexane and acetone (in the proportions described above), at 10 ng/mL, with the response of matrix-matched standards (prepared as described in the section "Sample treatment"), at the same concentration. An enhancement of the signal was observed for most compounds except in a few cases such as pyrethroids where a slight suppression occurred, which was in agreement with earlier observations [22]. Matrix effects observed under GC–(APCI) MS are the result of that occurring in the GC inlet (normally enhancement) and in the APCI source (normally suppression). The signal enhancement observed for most compounds can be attributed to that occurring in the GC liner. The matrix shields active sites in the liner and column, which reduces interaction of the analytes on these sites, and leads to enhanced analyte peaks. This effect is most pronounced for polar analytes (typically those with strong hydrogen bonding potential) [25]. Looking at those compounds for which this enhancement is not expected (e.g. hexachlorobenzene, HCHs, etc.), no suppression coming from the APCI source is observed. Thus, it can be concluded that matrix effect observed in GC–(APCI) MS system are mainly arising from the GC inlet and to a lesser extend to suppression from APCI source. For optimum peak shape and sensitivity, as in any GC-based pesticide residue analysis, matrix-matched calibration curves were necessary to perform accurate quantitative analysis.

3.4. Validation results

Validation of the method was performed in terms of trueness (recovery), precision, LODs, LOQs and selectivity. These parameters were evaluated in three types of matrices, orange, tomato and carrot.

Linearity was studied in the range 0.1–100 ng/mL using pure solvent standard solutions and adjusted to quadratic curves. Each concentration level was injected in triplicate. The regression coefficients were higher than 0.99 for all compounds over the whole range tested. As mentioned above, matrix-matched calibration was used for quantification purposes. In this case, in order to quantify properly, shorter ranges were selected depending on the concentration level to be quantified. In this way, residuals were better and lower than 30%.

Trueness and precision were evaluated by means of recovery experiments (n = 6) at two concentration levels (0.01 and 0.1 mg/kg) for each sample matrix. As can be observed in **Fig. 1**, the histograms show that most compounds presented satisfactory recoveries ranging between 70% and 120% for all the sample matrices at the two fortification levels, most of them between 70% and 110% (values are presented in **Table 1S**, Supplementary data). Thus, an LOQ of 0.01 mg/kg was demonstrated for most compounds. For the remaining compounds, acceptable results were obtained at 0.1 mg/kg (e.g. carbaryl in orange and carfentrazone-ethyl in carrot). For a limited number of compounds including molinate, propoxur and imazalil, the method was not suitable for the sample matrices and levels tested. Other compounds referred as problematic [26] and [27] as tolyfluanid, chlorothalonil and methiocarb sulfone, did not present satisfactory results in some matrices. RSDs lower than 10% were obtained for most analytes at both fortification levels, and even lower than 5%, as can be observed in **Fig. 2**.

Table S1. Average recovery (percent), R.S.D. (in parenthesis) and limits of quantification (LOQ) obtained after the application of the developed method to orange, tomato and carrot samples (n=6) fortified at two concentration levels.

Compounds	Orange				Tomato				Carrot			
	Fortification levels (µg/kg)		LOQ (µg/kg)	LOD (µg/kg)	Fortification levels (µg/kg)		LOQ (µg/kg)	LOD (µg/kg)	Fortification levels (µg/kg)		LOQ (µg/kg)	LOD (µg/kg)
	10	100			10	100			10	100		
Acrinathrin	97 (7)	101 (1)	10	0.3	90 (3)	101 (14)	10	0.1	99 (9)	107 (8)	10	0.09
Alachlor	92 (3)	99 (4)	10	0.09	100 (3)	85 (3)	10	0.07	97 (4)	103 (4)	10	0.05
Aldrin	56 (14)	78 (5)	100	1.2	91 (7)	81 (6)	10	4.3	80 (10)	77 (5)	10	3
Atrazine	93 (2)	100 (5)	10	0.2	96 (4)	75 (3)	10	0.17	112 (2)	97 (4)	10	0.1
Atrazine deisopropyl	104 (5)	117 (4)	10	1.8	118 (9)	92 (10)	10	7.5	120 (14)	113 (5)	10	10
Atrazine desethyl	100 (4)	99 (4)	10	0.25	95 (3)	76 (3)	10	0.52	118 (3)	92 (5)	10	0.43
Azinphos ethyl	-	91 (3)	100	1.2	-	83 (8)	100	4	-	120 (1)	100	5.5
Azinphos methyl	74 (15)	95 (2)	10	2.5	73 (2)	81 (7)	10	1.5	86 (11)	110 (7)	10	2.9
Azoxystrobin	110 (4)	109 (7)	10	0.03	106 (4)	83 (11)	10	0.06	100 (14)	88 (11)	10	0.14
Bifenthrin	87 (6)	86 (2)	10	0.2	85 (4)	75 (4)	10	0.38	101 (4)	70 (3)	10	0.43
Bromohos ethyl	120 (4)	120 (2)	10	0.02	108 (3)	102 (3)	10	0.05	105 (3)	107 (5)	10	0.04
Bromophos methyl	120 (1)	116 (1)	10	0.03	105 (2)	101 (2)	10	0.09	117 (4)	106 (4)	10	0.09
Buprofezin	91 (7)	86 (4)	10	0.33	83 (3)	74 (5)	10	0.3	98 (5)	93 (5)	10	0.42
Cadusafos	120 (8)	119 (6)	10	1.4	113 (10)	100 (5)	10	5	118 (7)	149 (4)	10	3.6
Captafol	87 (21)	109 (4)	10	1.15	70 (10)	72 (10)	10	1.5	98 (16)	113 (14)	10	1.5
Captan	67 (7)	72 (5)	100	1.3	106 (4)	73 (5)	10	1.2	89 (9)	87 (4)	10	6
Carbaryl	43 (51)	78 (8)	100	30	87 (21)	77 (8)	10	30	99 (12)	70 (5)	10	6
Carbofenothion	119 (8)	134 (3)	10	0.2	104 (6)	101 (5)	10	0.43	112 (3)	117 (7)	10	0.25
Carbofuran	91 (13)	98 (6)	10	4.3	105 (8)	113 (13)	10	1.7	117 (14)	95 (6)	10	0.88
Carfentrazone ethyl	120 (2)	113 (2)	10	0.02	117 (2)	113 (3)	10	0.06	136 (5)	118 (4)	100	0.2
Chinomethionate	80 (13)	94 (10)	10	0.88	85 (3)	67 (6)	10	0.6	104 (2)	81 (5)	10	0.47
trans-Chlordane	97 (14)	89 (10)	10	1	99 (4)	81 (5)	10	1	104 (14)	79 (8)	10	1.5
Chlorfenson	111 (7)	102 (8)	10	0.3	92 (2)	81 84)	10	0.25	82 (2)	96 (3)	10	0.3
Chlorfenvinphos	87 (7)	89 (2)	10	0.11	92 (4)	80 (3)	10	0.09	100 (6)	115 (6)	10	0.06
Chlorothalonil	113 (8)	95 (9)	10	2.5	>150 (6)	96 (14)	100	6.3	>150 (4)	>150 (5)	n.e	3.2
Chlorpropham	94 (7)	88 (8)	10	0.08	87 (3)	70 (5)	10	0.14	106 (3)	94 (4)	10	0.13
Chlorpyrifos	95 (5)	100 (4)	10	0.01	105 (11)	85 (2)	10	0.01	101 (4)	100 (3)	10	0.32
Chlorpyrifos methyl	91 (1)	96 (2)	10	0.01	96 (2)	81 (4)	10	0.03	98 (3)	106 (1)	10	0.08
Coumaphos	98 (4)	97 (1)	10	0.01	82 (3)	75 (6)	10	0.04	105 (9)	97 (5)	10	0.04
Cyanazine	96 (7)	101 (2)	10	0.02	91 (3)	85 (4)	10	0.06	113 (6)	119 (4)	10	0.19
Cyanophos	96 (5)	99 (3)	10	0.14	100 (3)	84 (4)	10	0.23	107 (4)	106 (3)	10	0.09
Cyfluthrin	92 (2)	101 (3)	10	0.08	96 (4)	87 (4)	10	0.03	109 (4)	111 (6)	10	0.02
lambda-Cyhalothrin	70 (7)	96 (2)	10	0.04	89 (3)	85 (3)	10	0.14	107 (5)	106 (2)	10	0.14
Cypermethrin	97 (3)	105 (4)	10	0.14	94 (4)	87 (6)	10	0.07	109 (3)	109 (6)	10	0.39
Cyprodinil	82 (13)	88 (8)	10	0.44	82 (7)	82 (6)	10	0.39	100 (3)	84 (5)	10	0.65
p,p'-DDD	107 (10)	118 (2)	10	0.21	80 (5)	74 (5)	10	0.54	105 (5)	96 (5)	10	0.5
p,p'-DDE	80 (3)	88 (6)	10	0.11	83 (4)	70 (3)	10	0.14	94 (5)	75 (4)	10	0.2
p,p'-DDT	90 (6)	109 (2)	10	3	74 (8)	71 (6)	10	2.2	88 (15)	80 (9)	10	4.3
Deltamethrin	94 (7)	101 (4)	10	0.19	117 (5)	87 (10)	10	0.79	73 (4)	70 (8)	10	4.6
Demeton-s-methyl	84 (4)	66 (4)	10	1.3	140 (7)	104 (5)	100	6.3	119 (7)	103 (4)	10	10
Demeton-s-methylsulfone	107 (5)	104 (3)	10	0.31	88 (7)	70 (9)	10	0.83	114 (12)	128 (9)	10	3
Diazinon	98 (4)	104 (3)	10	0.02	98 (2)	86 (4)	10	0.07	101 (3)	100 (4)	10	0.02
Dichlofenthion	93 (3)	95 (3)	10	0.02	87 (2)	79 (4)	10	0.05	113 (2)	81 (6)	10	0.3
Dichloran	95 (4)	97 (4)	10	0.08	96 (3)	78 (5)	10	0.14	115 (3)	95 (2)	10	0.17
4,4'-Dichlorobenzophenone	92 (4)	89 (2)	10	0.07	92 (2)	87 (4)	10	0.08	111 (2)	108 (3)	10	0.1
Dichlorvos	88 (4)	93 (2)	10	0.08	103 (2)	83 (4)	10	0.13	117 (4)	101 (3)	10	0.08
Dieldrin	91 (4)	94 (3)	10	0.3	91 (3)	76 (3)	10	1	106 (5)	88 (4)	10	1

Table S1 (continued).

Compounds	Orange				Tomato				Carrot			
	Fortification levels (µg/kg)		LOQ (µg/kg)	LOD (µg/kg)	Fortification levels (µg/kg)		LOQ (µg/kg)	LOD (µg/kg)	Fortification levels (µg/kg)		LOQ (µg/kg)	LOD (µg/kg)
	10	100			10	100			10	100		
Diflufenican	104 (4)	96 (2)	10	0.01	86 (4)	72 (1)	10	0.02	117 (2)	81 (5)	10	0.05
Dimethoate	100 (4)	102 (4)	10	0.04	97 (2)	74 (5)	10	0.07	121 (7)	133 (6)	10	0.21
Dioxathion	99 (4)	99 (1)	10	0.15	83 (2)	77 (6)	10	0.47	118 (4)	103 (5)	10	0.1
Diphenylamine	106 (6)	100 (3)	10	0.13	86 (6)	71 85)	10	0.3	117 (4)	93 (3)	10	0.21
Endosulfan eher	98 (4)	92 (3)	10	0.33	102 (4)	78 (3)	10	0.3	108 (4)	87 (6)	10	0.37
Endosulfan I	99 (5)	91 (5)	10	0.25	102 (6)	81 (4)	10	0.25	104 (6)	76 (4)	10	1.9
Endosulfan II	94 (12)	101 (17)	10	0.25	100 (10)	83 (2)	10	0.25	107 (3)	95 (4)	10	0.2
Endosulfan sulfate	93 (4)	90 (2)	10	0.13	90 (4)	83 (4)	10	0.19	101 (5)	114 (3)	10	0.17
Endrin	90 (7)	95 (4)	10	1.1	98 (4)	83 (2)	10	0.27	106 (5)	112 (6)	10	0.15
Esfenvalerate	114 (2)	116 (6)	10	0.5	79 (1)	87 (5)	10	0.13	97 (5)	109 (7)	10	0.2
Ethalfluralin	98 (6)	92 (10)	10	0.01	92 (4)	81 (4)	10	0.01	119(4)	88 (5)	10	0.02
Ethion	106 (8)	118 (10)	10	0.06	93 (4)	87 (3)	10	0.1	102 (4)	106 (7)	10	0.05
Ethoxiquin	116 (6)	118 (3)	10	0.71	84 (16)	71 (19)	10	0.24	-	3 (7)	n.e	0.54
Etofenprox	91 (4)	89 (2)	10	0.08	89 (3)	75 (4)	10	0.04	104 (3)	80 (4)	10	0.08
Famphur	99 (4)	103 (5)	10	0.01	93 (1)	88 (4)	10	0.02	115 (6)	105 (6)	10	0.02
Fenamiphos	105 (4)	98 (1)	10	0.02	89 (2)	70 (4)	10	0.03	119 (7)	113 (5)	10	0.1
Fenarimol	99 (5)	99 (3)	10	0.02	88 (2)	80 (4)	10	0.06	117 (19)	94 (4)	10	0.04
Fenhexamid	107 (8)	107 (3)	10	1.2	94 (13)	83 (7)	10	1.4	>150 (33)	>150 (4)	n.e	2.7
Fenitrothion	97 (3)	102 (2)	10	0.03	93 (2)	84 (5)	10	0.03	103 (6)	100 (2)	10	0.44
Fenoxycarb	103 (5)	106 (2)	10	0.65	97 (3)	85 (5)	10	1.4	114 (9)	119 (4)	10	1.9
Fenthion	103 (3)	109 (2)	10	0.02	83 (3)	80 (4)	10	0.03	102 (5)	100 (4)	10	0.06
Fenvalerate	102 (9)	114 (6)	10	0.94	70 (6)	76 (4)	10	0.48	94 (6)	120 (5)	10	1
Fipronil	103 (5)	114 (2)	10	0.01	94 (2)	93 (3)	10	0.01	120 (3)	108 (5)	10	0.01
Flucythrinate	104 (4)	102 (3)	10	0.05	88 (3)	82 (5)	10	0.17	116 (7)	106 (5)	10	0.12
Fludioxonil	87 (13)	82 (15)	10	0.17	120 (5)	76 (4)	10	0.41	109 (4)	89 (6)	10	0.83
Fluvalinate	125 (4)	124 (5)	10	0.11	93 (4)	83 (6)	10	0.09	110 (5)	117 (10)	10	0.08
Folpet	101 (5)	93 (5)	10	0.38	70 (7)	70 (3)	10	0.34	104 (7)	87 (4)	10	1.2
alpha-HCH	95 (16)	117 (3)	10	0.94	79 (5)	70 (9)	10	3	99 (13)	95 (9)	10	3
beta-HCH	108 (12)	113 (7)	10	3	70 (10)	81 (4)	10	10	98 (18)	86 (13)	10	3.9
delta-HCH	121 (8)	112 (7)	10	3	91 (6)	69(9)	10	5	90 (9)	94 (6)	10	4.9
gamma-HCH	99 (13)	117 (7)	10	3	96 (8)	76 (9)	10	5	103 (10)	90 (8)	10	5
Heptachlor	79 (7)	92 (4)	10	0.3	103 (5)	76 (7)	10	0.5	118 (6)	97 (3)	10	0.6
Heptachlor epoxide A	75 (12)	96 (3)	10	1.2	119 (9)	84 (10)	10	2.5	113 (15)	102 (7)	10	0.6
Heptachlor epoxide B	91 (15)	95 (3)	10	0.6	108 (6)	78 (5)	10	0.35	102 (8)	93 (5)	10	0.6
Hexachlorobenzene	95 (6)	76 (7)	10	2	83 (10)	86 (5)	10	1.8	88 (9)	70 (9)	10	3.3
Hexachlorobutadiene	72 (3)	98 (12)	10	0.22	73 (3)	78 (5)	10	0.39	92 (4)	89 (11)	10	0.68
Imazalil	57 (16)	18 (8)	n.e	1.3	58 (11)	8 (10)	n.e	2.2	60 (22)	21 (3)	n.e	3
Iprodione	100 (4)	103 (2)	10	0.1	100 (3)	83 (4)	10	0.17	104 (9)	112 (4)	10	0.17
Isodrin	75 (13)	80 (3)	10	0.68	89 (4)	74 (3)	10	2.1	103 (7)	75 (6)	10	0.5
Leptophos	89 (5)	92 (3)	10	0.04	81(2)	76 (5)	10	0.07	115 (3)	87 (7)	10	0.1
Malathion	101 (6)	111 (6)	10	0.07	99 (2)	91 (4)	10	0.08	105 (11)	110 (5)	10	0.07
Metalaxyl	98 (4)	101 (2)	10	0.04	93 (1)	72 (2)	10	0.04	120 (5)	108 (4)	10	0.08
Methidathion	84 (9)	107 (6)	10	0.56	102 (4)	96 (6)	10	0.71	110 (9)	120 (5)	10	1.5
Methiocarb	102 (4)	109 (3)	10	0.3	93 (2)	86 (7)	10	0.14	120 (7)	111 (6)	10	0.27
Methiocarb sulfone	100 (6)	98 (4)	10	0.06	102 (13)	83 (6)	10	0.2	>150 (54)	135 (4)	n.e	1.3
Methoxychlor	94 (2)	93 (3)	10	0.27	108 (2)	97 (3)	10	0.27	118 (4)	115 (5)	10	0.24
Metolachlor	95 (4)	106 (8)	10	0.63	93 (5)	84 (3)	10	0.58	103 (5)	108 (5)	10	1
Metribuzin	98 (3)	89 (4)	10	1.5	86 (5)	77 (5)	10	1.4	114 (3)	85 (5)	10	1.3

Table S1 (continued).

Compounds	Orange				Tomato				Carrot			
	Fortification levels (µg/kg)		LOQ (µg/kg)	LOD (µg/kg)	Fortification levels (µg/kg)		LOQ (µg/kg)	LOD (µg/kg)	Fortification levels (µg/kg)		LOQ (µg/kg)	LOD (µg/kg)
	10	100			10	100			10	100		
Mevinphos	96 (3)	84 (2)	10	0.04	92 (4)	80 (4)	10	0.07	119 (7)	119 (6)	10	0.11
Mirex	72 (9)	77 (10)	10	3	79 (11)	54 (7)	10	1.1	71 (11)	50 (8)	10	1.8
Molinate	>150 (2)	140 (1)	n.e	0.01	>150 (1)	127 (1)	n.e	0.01	>150 (3)	129 (2)	n.e	0.01
Oxadixyl	118 (19)	117 (17)	10	0.71	99 (2)	93 (2)	10	0.32	118 (8)	98 (5)	10	0.71
Oxychlordane	73 (8)	n. a.	10	0.68	90 (4)	n. a.	10	0.3	99 (5)	n. a.	10	1
Oxyfluorfen	93 (4)	103 (18)	10	0.01	97 (6)	80 (3)	10	0.02	111 (3)	98 (3)	10	0.02
Parathion ethyl	97 (5)	105 (5)	10	0.03	92 (2)	83 (4)	10	0.03	118 (3)	103 (3)	10	0.06
Parathion methyl	99 (3)	101 (3)	10	0.01	91 (3)	82 (5)	10	0.04	97 (5)	107 (3)	10	0.33
Pendimethalin	93 (4)	94 (4)	10	0.13	92 (2)	80 (2)	10	0.1	109 (4)	94 (3)	10	0.05
Pentachlorobenzene	87 (5)	100 (3)	10	0.09	83 (4)	70 (3)	10	0.18	85 (4)	86 (5)	10	0.09
Permethrin	103 (4)	94 (2)	10	1	89 (3)	108 (3)	10	1	86 (10)	70 (3)	10	0.79
2-Phenylphenol	102 (7)	120 (4)	10	0.05	115 (6)	114 (4)	10	0.08	105 (4)	95 (4)	10	0.03
Phorate	94 (10)	107 (4)	10	1.4	102 (12)	72 (5)	10	2.3	113 (11)	107 (3)	10	2.2
Phosmet	87 (13)	103 (3)	10	0.04	106 (3)	81 (10)	10	0.16	113 (7)	117 (9)	10	0.83
Phosphamidon	99 (4)	93 (1)	10	0.04	100 (2)	87 (2)	10	0.1	110 (5)	120 (7)	10	0.68
Pirimicarb	95 (6)	104 (6)	10	0.71	97 (5)	86(2)	10	0.6	120 (4)	104 (4)	10	0.75
Pirimiphos methyl	96 (3)	105 (2)	10	0.07	86 (3)	82 (5)	10	0.04	101 (1)	106 (4)	10	0.04
Procymidone	94 (5)	101 (8)	10	0.05	96 (2)	82 (4)	10	0.1	107 (2)	108 (3)	10	0.05
Propachlor	101 (2)	98 (3)	10	0.33	89 (2)	80 (3)	10	0.21	119 (3)	103 (4)	10	0.17
Propetamphos	95 (7)	96 (3)	10	0.44	86 (5)	70 (7)	10	0.56	102 (24)	102 (3)	10	0.16
Propham	80 (5)	82 (5)	10	0.19	86 (10)	81 (9)	10	0.6	110 (2)	99 (2)	10	0.1
Propiconazole	98 (2)	102 (7)	10	0.21	98 (6)	82 (3)	10	0.28	109 (3)	104 (4)	10	0.26
Propoxur	>150 (3)	>150 (3)	n.e	0.13	>150 (4)	>150 (2)	n.e	0.25	>150 (10)	>150 (7)	n.e	0.43
Propyzamide	95 (4)	95 (4)	10	0.03	98 (1)	85 (4)	10	0.1	100 (5)	94 (3)	10	0.27
Pyriproxifen	94 (3)	92 (3)	10	0.06	93 (3)	83 (3)	10	0.08	120 (5)	96 (3)	10	0.08
Quinalphos	98 (7)	105 (5)	10	0.04	94 (1)	85 (2)	10	0.11	104 (4)	108 (3)	10	0.08
Resmethrin	89 (3)	92 (2)	10	0.17	87 (3)	77 (3)	10	0.07	103 (6)	104 (5)	10	0.09
Simazine	112 (10)	105 (6)	10	0.13	88 (7)	72 (5)	10	0.79	116 (5)	100 (4)	10	0.83
Sulprofos	94 (4)	97 (3)	10	0.07	79 (3)	71 (3)	10	0.07	113 (3)	88 (5)	10	0.06
Tefluthrin	86 (4)	91 (1)	10	0.05	91 (4)	78 (3)	10	0.04	106 (2)	85 (3)	10	0.04
Terbacil	83 (8)	86 (8)	10	0.25	95 (5)	85 (4)	10	0.79	90 (7)	109 (8)	10	0.83
Terbufos	98 (9)	93 (3)	10	1.6	88 (11)	78 (5)	10	1.1	95 (7)	100 (4)	10	1.5
Terbumeton	93 (4)	86 (3)	10	0.19	93 (4)	71 (4)	10	0.15	112 (2)	104 (3)	10	0.12
Terbumeton desethyl	112 (4)	88 (2)	10	0.48	94 (3)	72 (2)	10	0.71	112 (2)	93 (5)	10	0.42
Terbutryn	126 (3)	123 (3)	10	0.07	107 (2)	90 (3)	10	0.04	114 (3)	103 (3)	10	0.06
Terbutylazine	94 (3)	107 (10)	10	0.06	90 (1)	73 (4)	10	0.07	109 (4)	114 (2)	10	0.06
Terbutylazine desethyl	103 (3)	97 (3)	10	0.01	94 (2)	71 (3)	10	0.13	117 (2)	105 (3)	10	0.13
Tetradifon	98 (5)	102 (3)	10	0.02	84 (4)	82 (4)	10	0.05	120 (4)	93 (6)	10	0.13
Tolyfluanid	77 (12)	66 (8)	10	0.25	12 (32)	49 (15)	n.e	0.21	60 (22)	110 (11)	100	0.23
Triadimefon	95 (3)	97 (2)	10	0.02	89 (2)	81 (3)	10	0.03	97 (2)	104 (3)	10	0.09
Triflumizole	97 (9)	96 (9)	10	0.06	90 (4)	76 (3)	10	0.11	104 (3)	105 (3)	10	0.21
Trifluralina	98 (2)	93 (3)	10	0.02	91 (2)	73 (5)	10	0.01	115 (2)	90 (2)	10	0.01
Vinclozolin	92 (3)	95 (5)	10	0.02	92 (3)	81 (3)	10	0.02	112 (2)	102 (2)	10	0.02

n.a. not available
n.e. LOQ not estimated as validation parameters at both fortification levels were not satisfactory
Underlined, not acceptable results

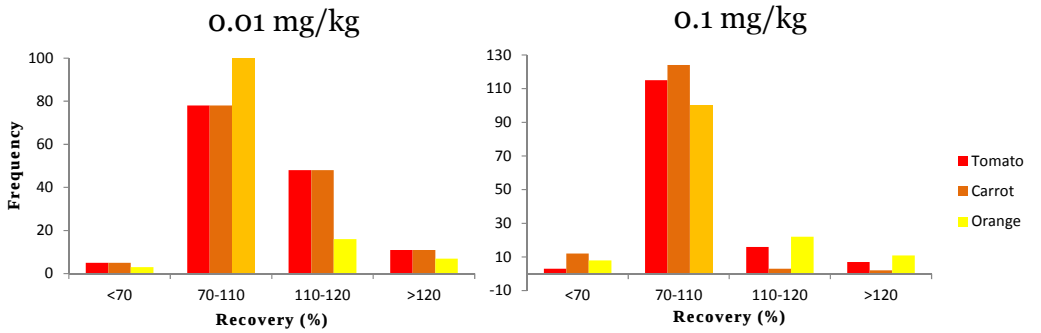

Fig. 1. Histograms obtained from the recovery experiments of the three sample matrices fortified at (a) 0.01 mg/kg and (b) 0.1 mg/kg.

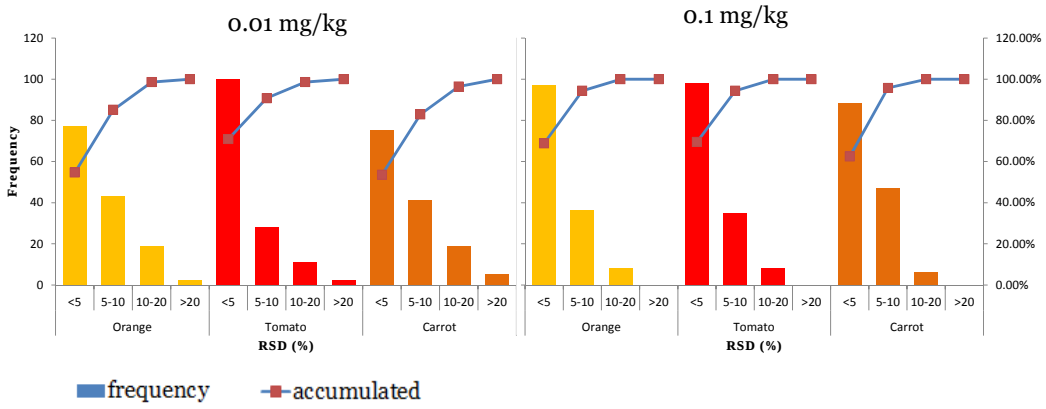

Fig. 2. Histograms obtained from the RSD values of the three sample matrices fortified at (a) 0.01 mg/kg and (b) 0.1 mg/kg.

Low LODs were obtained for all compounds since most of them ranged between 0.01 and 1 µg/kg in the three matrices (see **Fig. 3**). Only few values were higher than 1 µg/kg. **Fig. 4** shows four examples (selected from different LOD ranges showed in **Fig. 3**) for which signal-to-noise ratios were calculated from the lowest matrix-matched standard in orange samples and where LODs can be estimated by extrapolation.

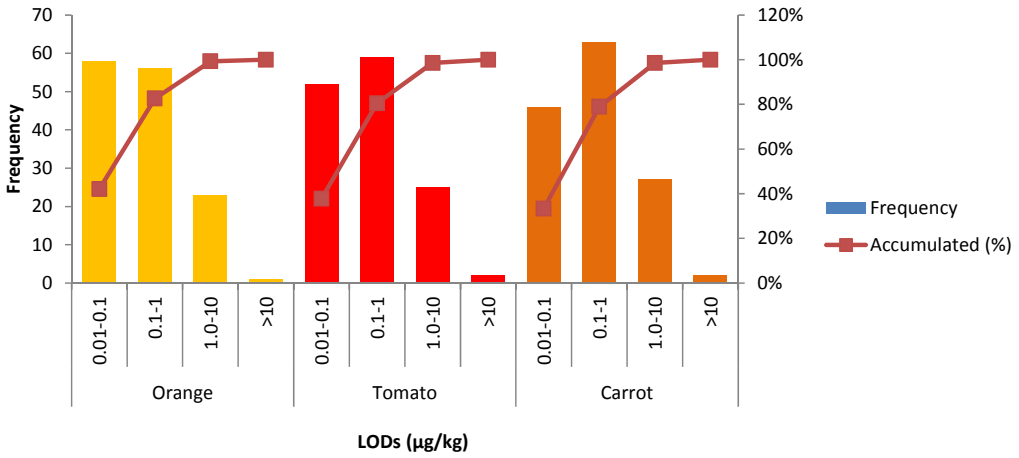

Fig. 3. Histograms obtained from the LOD values of the three sample matrices.

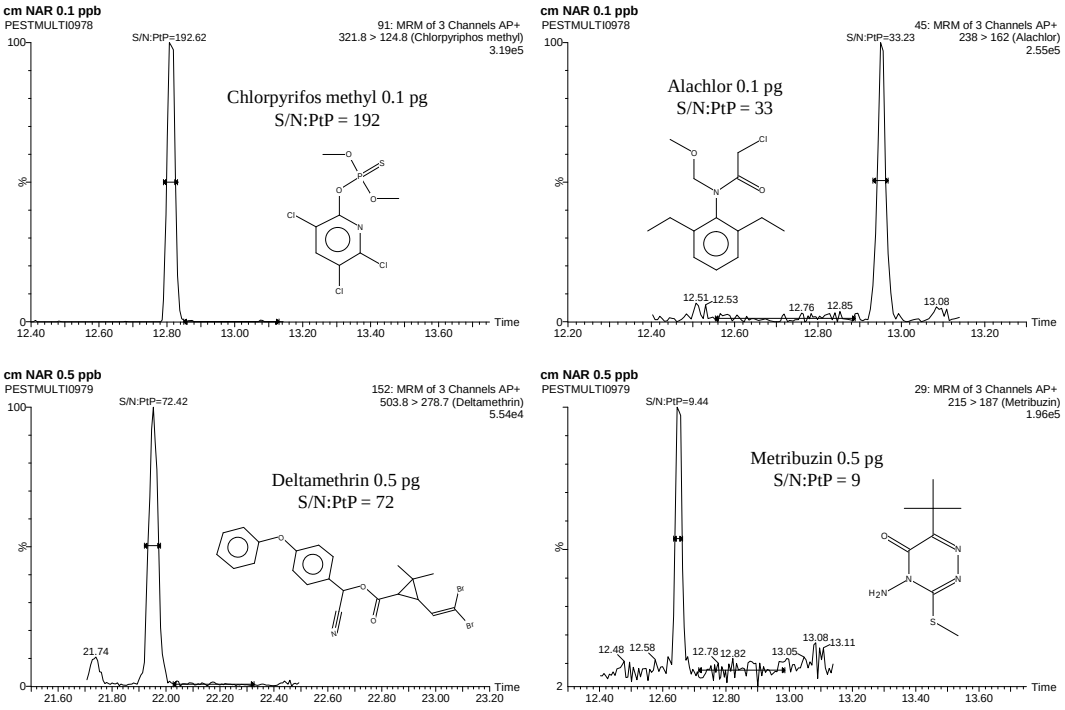

Fig. 4. GC–(APCI) MS/MS chromatogram of four pesticides from the lowest matrix-matched standard (0.1–0.5 ng/mL, corresponding to 0.1–0.5 pg on column) in orange samples. S/N:PtP: peak-to-peak signal-to-noise ratio.

The selectivity, as evaluated for each of the three specific SRM transitions measured, was satisfactory. GC–MS/MS chromatograms did not show interfering peaks at the analyte retention time for any of the pesticides investigated in this work.

3.5. Qualitative aspects: consistency of ion ratios and identification

With respect to the identification of pesticides in samples, criteria have been set for the ratio of the response obtained for the transitions measured [24]. Depending on the relative abundance of the two transitions, the ion ratio should be within 20–50% of the reference value. This aspect was evaluated in the validation for all pesticides, in each of the three matrices, at the two concentration levels. For each pesticide, two ion ratios were calculated: the first qualifier/quantifier (q1/Q) and the second qualifier/quantifier (q2/Q). The average ion ratio obtained for up to eight matrix-matched standards in the range of 0.1–100 ng/mL was used as reference ion ratio (values are included in **Table 2**). For the calculation of the average, signals with poor S/N and saturated signals were excluded. In general, the ion ratios for the different concentrations of the standards were very consistent (RSD <10% in most cases), even when the ion ratio was very unfavorable (<0.10).

For the spiked samples, the deviation of the individual ion ratios were calculated against the reference value and then compared with the maximum tolerable deviations according to the SANCO guideline [24]. In **Table 2**, for each pesticide, in each matrix and for each level (with n = 6), the number of ion-ratio compliances is given. Overall, the percentage of pesticides that met the ion ratio criterion for one ratio was 77–81% at 0.01 mg/kg, and 95–97% at 0.1 mg/kg, with not many differences between the three matrices tested. For 60–65% of the pesticides, the criterion was met for both ratios determined. The reason for not meeting the criteria generally was a too low sensitivity of one of the qualifier transitions measured. For the pesticide methidathion, no suitable qualifier transitions could be obtained and no adequate identification was possible.

Table 2. Study of the q/Q ratios and compliance with EU criteriaa for the three matrices studied at 0.01 and 0.1 mg/kg.*

Compound	Orange						Tomato						Carrot					
			ion ratio compliance (# out of 6)						ion ratio compliance (# out of 6)						ion ratio compliance (# out of 6)			
	q1/Q	q2/Q	0.01 q1/Q	0.1 q1/Q	0.01 q2/Q	0.1 q2/Q	q1/Q	q2/Q	0.01 q1/Q	0.1 q1/Q	0.01 q2/Q	0.1 q2/Q	q1/Q	q2/Q	0.01 q1/Q	0.1 q1/Q	0.01 q2/Q	0.1 q2/Q
Acrinathrin	0.951	0.63	6	6	6	6	0.831	0.598	5	6	6	6	0.844	0.651	5	6	6	6
Alachlor	0.999	0.057	6	6	6	6	0.987	0.045	6	6	6	6	0.999	0.046	6	6	6	6
Aldrin	0.949	0.836	4	6	4	6	0.998	0.867	3	6	4	5	1	0.835	3	6	3	6
Atrazine	0.397	0.099	6	6	6	6	0.339	0.099	6	6	6	6	0.329	0.089	6	6	6	6
Atrazine deisopropyl	0.534	0.548	5	6	5	6	0.529	0.481	5	6	6	6	0.518	0.47	6	6	6	6
Atrazine desethyl	0.2	0.002	6	6	0	6	0.178	0.001	6	6	0	5	0.195	0.001	6	6	0	6
Azinphos ethyl	0.552	0.534	0	6	0	6	0.597	0.469	0	6	0	6	0.595	0.471	0	4	0	6
Azinphos methyl	0.337	0.096	6	6	0	6	0.256	0.067	0	6	0	6	0.248	0.072	0	6	0	6
Azoxystrobin	0.344	0.31	6	6	6	6	0.391	0.304	6	6	6	6	0.406	0.289	6	6	6	6
Bifenthrin	0.932	0.056	6	6	6	6	0.973	0.055	6	6	6	6	0.982	0.054	6	6	6	6
Bromophos ethyl	0.654	0.445	6	6	6	6	0.597	0.376	6	6	6	6	0.564	0.358	6	6	6	6
Bromophos methyl	0.233	0.228	6	6	6	6	0.194	0.229	6	6	6	6	0.19	0.232	6	6	6	6
Buprofezin	0.16	0.316	5	6	3	0	0.112	0.176	5	6	4	6	0.126	0.149	6	5	6	0
Cadusafos	0.23	0.069	5	6	0	6	0.313	0.057	5	6	0	5	0.343	0.057	6	6	0	6
Captafol	0.492	0.452	6	6	6	6	0.461	0.409	5	6	6	6	0.447	0.393	4	6	5	6
Captan	0.483	0.271	0	6	5	6	0.335	0.227	0	6	2	6	-	0.216	-	-	2	6
Carbaryl	0.926	0.71	2	6	0	0	0.953	0.795	0	6	0	5	0.913	0.842	4	4	5	1
Carbofenothion	0.049	0.007	6	6	0	6	0.045	0.006	6	6	0	6	0.045	0.006	6	6	5	6
Carbofuran	0.054	0.006	0	6	0	5	0.061	0.007	0	6	0	4	0.058	0.006	1	6	5	6
Carfentrazone ethyl	0.329	0.305	6	6	6	6	0.471	0.356	6	6	6	6	0.457	0.337	6	6	6	6
Chinomethionate	0.602	0.412	6	5	6	5	0.566	0.375	5	6	6	6	0.563	0.354	6	6	6	6
trans-Chlordane	0.324	0.105	1	6	5	5	0.363	0.141	5	6	6	6	0.347	0.138	5	6	0	6
Chlorfenson	0.459	0.119	6	6	6	6	0.283	0.104	6	6	6	6	0.252	0.097	6	6	6	6
Chlorfenvinphos	0.853	0.284	6	6	6	6	0.941	0.228	6	6	6	6	0.93	0.23	6	6	6	6
Chlorothalonil	0.718	0.004	6	6	0	6	0.645	0.004	2	6	0	0	0.677	0.003	6	6	4	6
Chlorpropham	0.404	0.285	6	6	6	6	0.449	0.229	6	6	6	6	0.453	0.252	6	6	6	6
Chlorpyrifos	0.352	0.148	6	6	6	6	0.416	0.13	6	6	6	6	0.426	0.126	6	6	6	6
Chlorpyrifos methyl	0.44	0.155	6	6	6	6	0.459	0.146	6	6	6	6	0.438	0.137	6	6	6	6
Coumaphos	0.371	0.181	6	6	6	6	0.41	0.184	6	6	6	6	0.407	0.184	6	6	6	6
Cyanazine	0.117	0.093	6	6	6	6	0.101	0.091	6	6	6	6	0.099	0.096	6	6	6	6
Cyanophos	0.64	0.133	6	6	6	6	0.658	0.14	6	6	6	6	0.658	0.143	6	6	6	6
Cyfluthrin	0.335	0.307	6	6	6	6	0.346	0.315	6	6	3	6	0.344	0.304	6	6	6	6
lambda-Cyhalothrin	0.079	0.023	6	6	6	6	0.062	0.022	6	6	6	6	0.06	0.022	6	6	6	6
Cypermethrin	0.334	0.314	6	6	6	6	0.325	0.314	6	6	6	6	0.329	0.312	6	6	6	6
Cyprodinil	0.548	0.732	6	6	5	6	0.501	0.669	5	6	4	6	0.516	0.614	6	6	3	6
p,p'-DDD	0.214	0.028	6	6	0	6	0.276	0.02	5	6	6	6	0.268	0.02	5	6	0	6
p,p'-DDE	0.148	0.055	6	6	6	6	0.163	0.047	6	6	6	6	0.177	0.045	6	6	0	6
p,p'-DDT	0.29	0.017	6	6	0	6	0.258	0.018	0	6	0	6	0.267	0.017	0	6	0	6
Deltamethrin	0.131	0.142	6	6	6	6	0.131	0.132	4	6	0	6	0.132	0.128	4	6	2	6
Demeton-s-methyl	0.321	0.009	5	5	0	0	0.398	0.013	0	6	0	0	0.393	-	0	5	-	-
Demeton-s-methylsulfone	0.947	0.391	6	6	6	6	0.904	0.371	6	6	6	6	0.9	0.378	6	6	5	6
Diazinon	0.773	0.514	6	6	6	6	0.723	0.589	6	6	6	6	0.698	0.571	6	6	6	6
Dichlofenthion	0.462	0.917	6	6	6	6	0.362	0.898	6	6	6	6	0.341	0.9	6	6	6	6
Dichloran	0.842	0.912	6	6	6	6	0.906	0.781	6	6	6	6	0.874	0.729	6	6	6	6
4,4'-Dichlorobenzophenone	0.554	-	6	6	-	-	0.518	-	6	6	-	-	0.5	-	6	6	-	-
Dichlorvos	0.091	0.061	6	6	6	6	0.088	0.061	6	6	6	6	0.089	0.062	6	6	6	6
Dieldrin	0.774	0.689	6	6	6	6	0.761	0.748	6	6	6	6	0.789	0.721	5	6	6	6

Table 2 (continued).

Compound	Orange						Tomato						Carrot					
	q1/Q	q2/Q	ion ratio compliance (# out of 6)				q1/Q	q2/Q	ion ratio compliance (# out of 6)				q1/Q	q2/Q	ion ratio compliance (# out of 6)			
			0.01	0.1	0.01	0.1			0.01	0.1	0.01	0.1			0.01	0.1	0.01	0.1
			q1/Q	q1/Q	q2/Q	q2/Q			q1/Q	q1/Q	q2/Q	q2/Q			q1/Q	q1/Q	q2/Q	q2/Q
Diflufenican	0.177	0.136	6	6	6	6	0.156	0.107	6	6	6	6	0.164	0.106	6	6	6	6
Dimethoate	0.771	0.331	6	6	6	6	0.865	0.249	6	6	6	6	0.881	0.249	6	6	6	6
Dioxathion	0.341	0.125	6	6	6	6	0.305	0.146	6	6	5	6	0.281	0.131	6	6	6	6
Diphenylamine	0.09	0.071	6	6	6	6	0.076	0.079	6	6	6	6	0.076	0.079	6	6	6	6
Endosulfan ether	0.775	0.794	6	6	6	6	0.779	0.791	6	6	6	6	0.835	0.754	6	6	6	6
Endosulfan I	0.832	0.751	6	6	5	6	0.745	0.698	6	6	6	6	0.704	0.7	5	6	5	6
Endosulfan II	0.794	0.648	0	5	5	6	0.601	0.509	6	6	6	6	0.569	0.528	6	6	6	6
Endosulfan sulfate	0.733	0.554	6	6	6	6	0.757	0.61	6	6	6	6	0.751	0.592	6	6	6	6
Endrin	0.917	0.991	5	6	6	6	1.007	0.885	5	6	6	6	0.996	0.888	5	6	6	6
Esfenvalerate	-	-	-	-	-	-	0.047	0.032	3	6	4	6	0.062	0.031	6	6	6	6
Ethalfluralin	0.712	0.275	6	6	6	6	0.589	0.344	6	6	6	6	0.544	0.361	6	6	6	6
Ethion	0.327	0.041	6	6	6	6	0.285	0.024	6	6	6	6	0.289	0.022	6	6	6	6
Ethoxyquin	0.649	0.552	6	6	6	6	0.657	0.522	6	6	6	6	0.646	0.53	0	1	0	1
Etofenprox	0.305	0.109	6	6	6	6	0.334	0.109	6	6	6	6	0.345	0.115	6	6	6	6
Famphur	0.364	0.068	6	6	6	6	0.305	0.056	6	6	6	6	0.304	0.056	6	6	6	6
Fenamiphos	0.4	0.16	6	6	6	6	0.361	0.198	6	6	6	6	0.352	0.22	6	6	6	6
Fenarimol	0.736	0.343	6	6	6	6	0.623	0.305	6	6	6	6	0.643	0.304	6	6	6	6
Fenhexamid	0.615	0.498	6	6	6	6	0.658	0.478	6	6	6	6	0.656	0.481	6	6	6	6
Fenitrothion	0.371	0.27	6	6	6	6	0.388	0.259	6	6	6	6	0.372	0.253	6	6	6	6
Fenoxycarb	0.106	0.068	5	6	6	6	0.103	0.063	6	6	5	6	0.111	0.064	6	6	6	6
Fenthion	0.285	0.83	6	6	6	6	0.294	0.79	6	6	6	6	0.3	0.783	6	6	6	6
Fenvalerate	0.304	0.252	6	6	6	6	0.436	0.331	6	6	2	6	0.466	0.366	6	6	5	6
Fipronil	0.253	0.199	6	6	6	6	0.209	0.174	6	6	6	6	0.208	0.181	6	6	6	6
Flucythrinate	0.394	0.358	6	6	6	6	0.425	0.42	6	6	6	6	0.41	0.423	6	6	6	6
Fludioxonil	0.539	0.793	5	5	6	5	0.712	0.787	6	6	6	6	0.71	0.775	6	6	6	6
Fluvalinate	0.095	0.059	6	6	6	6	0.106	0.052	6	6	6	6	0.108	0.052	6	6	6	6
Folpet	0.45	0.488	6	6	6	6	0.456	0.422	6	6	6	6	0.447	0.407	6	6	6	6
alpha-HCH	0.578	0.597	4	6	4	6	0.675	0.61	4	6	0	5	0.744	0.561	4	6	2	6
beta-HCH	0.649	0.759	0	6	3	6	0.695	0.678	2	6	0	6	0.725	0.688	2	6	2	4
delta-HCH	0.579	0.617	3	5	1	6	0.6	0.586	0	6	0	4	0.627	0.596	0	6	0	6
gamma-HCH	0.628	0.485	3	6	5	6	0.707	0.411	4	6	0	6	0.722	0.391	3	6	0	5
Heptachlor	0.864	0.377	5	6	4	6	0.833	0.344	6	6	5	6	0.793	0.331	4	6	5	6
Heptachlor epoxide A	0.874	0.773	3	6	4	6	0.939	0.835	3	6	4	6	0.997	0.735	5	6	4	6
Heptachlor epoxide B	0.914	0.825	4	6	5	6	0.988	0.789	6	6	6	6	0.973	0.782	5	6	6	6
Hexachlorobenzene	0.951	0.084	4	6	0	6	0.881	0.052	5	6	0	0	0.807	0.049	5	6	0	5
Hexachlorobutadiene	0.923	0.446	6	6	6	6	0.977	0.359	6	6	6	6	0.994	0.336	6	6	5	6
Imazalil	0.33	0.288	2	6	0	6	0.315	0.349	0	3	0	6	0.308	0.352	0	6	0	0
Iprodione	0.149	0.097	6	6	6	6	0.169	0.099	6	6	6	6	0.161	0.089	6	6	6	6
Isodrin	0.956	0.875	4	6	5	6	0.922	0.874	6	6	6	6	0.828	0.81	5	6	6	6
Leptophos	0.252	0.12	6	6	6	6	0.255	0.109	6	6	6	6	0.255	0.109	6	6	6	6
Malathion	0.061	0.064	6	6	6	6	0.043	0.058	6	6	6	6	0.04	0.057	6	6	5	6
Metalaxyl	0.688	0.198	6	6	6	6	0.711	0.131	6	6	6	6	0.738	0.127	6	6	6	6
Methidathion	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methiocarb	0.894	0.139	6	6	6	6	0.917	0.155	6	6	6	6	0.864	0.155	6	6	6	6
Methiocarb sulfone	0.168	0.046	6	6	6	6	0.208	0.078	6	6	0	6	0.217	0.071	6	6	6	6
Methoxychlor	0.367	0.303	6	6	6	6	0.344	0.216	4	3	3	1	0.239	0.145	6	6	4	6
Metolachlor	0.397	0.244	6	6	6	6	0.366	0.196	6	6	6	6	0.357	0.185	6	6	6	6

Table 2 (continued).

Compound	Orange						Tomato						Carrot					
	q1/Q	q2/Q	ion ratio compliance (# out of 6)				q1/Q	q2/Q	ion ratio compliance (# out of 6)				q1/Q	q2/Q	ion ratio compliance (# out of 6)			
			0.01	0.1	0.01	0.1			0.01	0.1	0.01	0.1			0.01	0.1	0.01	0.1
			q1/Q	q1/Q	q2/Q	q2/Q			q1/Q	q1/Q	q2/Q	q2/Q			q1/Q	q1/Q	q2/Q	q2/Q
Metribuzin	0.25	0.153	3	6	0	6	0.287	0.131	6	6	6	6	0.305	0.136	4	6	5	6
Mevinphos	0.589	0.27	6	6	6	6	0.451	0.29	6	6	6	6	0.488	0.287	6	6	0	6
Mirex	0.265	0.283	3	6	0	6	0.258	0.248	0	5	0	4	0.238	0.23	0	5	0	4
Molinate	0.17	0.026	6	6	6	6	0.161	0.026	6	0	6	6	0.167	0.024	6	0	6	0
Oxadixyl	0.311	0.107	6	6	6	6	0.283	0.091	6	6	6	6	0.284	0.087	6	6	6	6
Oxychlordane	0.307	-	6	0	0	0	0.262	0.07	5	0	0	0	0.237	0.069	5	0	6	0
Oxyfluorfen	0.33	0.002	6	6	6	6	0.354	0.002	6	6	6	6	0.365	0.002	6	6	0	6
Parathion ethyl	0.521	0.463	6	6	6	6	0.451	0.37	6	6	6	6	0.434	0.382	6	6	6	6
Parathion methyl	0.86	0.288	6	6	6	6	0.755	0.248	6	6	6	6	0.716	0.232	6	6	6	6
Pendimethalin	0.263	0.199	6	6	6	6	0.222	0.174	6	6	6	6	0.223	0.158	6	6	6	6
Pentachlorobenzene	0.559	0.47	5	6	6	6	0.428	0.326	4	6	6	6	0.401	0.321	3	6	5	6
Permethrin	0.126	0.101	5	6	4	6	0.158	0.117	5	6	5	6	0.168	0.126	6	6	6	6
2-Phenylphenol	0.885	0.152	6	6	6	6	0.774	0.114	6	6	6	6	0.746	0.117	6	6	6	6
Phorate	0.847	0.538	5	6	3	6	0.893	0.862	5	6	3	6	0.893	0.856	5	6	4	6
Phosmet	0.232	0.255	6	5	5	6	0.213	0.231	5	6	5	6	0.236	0.207	6	6	5	6
Phosphamidon	0.565	0.684	6	6	6	6	0.532	0.575	6	6	6	6	0.492	0.552	6	6	6	6
Pirimicarb	0.179	0.067	6	6	6	6	0.204	0.063	6	6	6	6	0.202	0.062	6	6	6	6
Pirimiphos methyl	0.916	0.613	6	6	6	6	0.96	0.539	6	6	6	6	0.931	0.593	6	6	6	6
Procymidone	0.338	0.215	6	6	6	6	0.282	0.169	6	6	6	6	0.278	0.163	6	6	6	6
Propachlor	0.666	0.247	6	6	6	6	0.574	0.237	6	6	6	6	0.513	0.222	6	6	6	6
Propetamphos	0.264	0.251	6	6	6	6	0.245	0.213	6	6	6	6	0.247	0.215	4	6	5	6
Propham	0.318	0.12	3	6	6	6	0.272	0.144	0	6	6	6	0.223	0.129	6	6	6	6
Propiconazole	0.699	0.214	6	6	6	6	0.753	0.197	6	6	6	6	0.785	0.206	6	6	6	6
Propoxur	0.223	0.003	6	6	0	6	0.324	0.002	6	6	6	6	0.345	0.003	6	6	6	6
Propyzamide	0.716	0.428	6	6	6	6	0.764	0.444	6	6	6	6	0.783	0.447	6	6	6	6
Pyriproxifen	0.803	0.696	6	6	6	6	0.785	0.611	6	6	6	6	0.75	0.611	6	6	6	6
Quinalphos	0.619	0.617	6	6	6	6	0.654	0.559	6	6	6	6	0.636	0.576	6	6	6	6
Resmethrin	0.595	0.163	6	6	6	6	0.562	0.163	6	6	6	6	0.553	0.165	6	6	6	6
Simazine	0.832	0.481	5	6	5	6	0.838	0.452	6	6	6	6	0.811	0.41	6	6	6	6
Sulprofos	0.901	0.936	6	6	6	6	0.93	0.804	6	6	6	6	0.997	0.872	6	6	6	6
Tefluthrin	0.439	0.231	6	6	5	6	0.212	0.115	2	2	5	4	0.226	0.124	2	6	6	6
Terbacil	0.335	0.179	3	6	6	6	0.357	0.168	4	6	4	6	0.379	0.177	5	6	0	6
Terbufos	0.855	0.288	4	6	0	5	0.936	0.394	4	6	1	6	0.856	0.433	5	6	5	6
Terbumeton	0.066	0.06	6	6	6	6	0.063	0.06	6	6	0	6	0.062	0.053	6	6	5	6
Terbumeton desethyl	0.224	0.092	6	6	6	6	0.221	0.087	6	6	6	6	0.199	0.077	6	6	6	6
Terbutryn	0.071	0.05	6	6	6	6	0.068	0.046	6	6	6	6	0.064	0.047	6	6	6	6
Terbutylazine	0.192	0.08	6	6	6	6	0.176	0.083	6	6	6	6	0.173	0.081	6	6	6	6
Terbutylazine desethyl	0.161	-	6	6	-	-	0.142	-	6	6	-	-	0.141	-	6	6	-	-
Tetradifon	0.064	0.02	6	6	0	6	0.047	0.022	6	6	0	6	0.049	0.017	6	6	0	6
Tolyfluanid	0.055	0.041	6	6	5	6	0.05	0.041	5	6	5	6	0.057	0.041	6	6	6	6
Triadimefon	0.263	0.198	6	6	6	6	0.312	0.236	6	6	6	6	0.315	0.243	6	6	6	6
Triflumizole	0.065	0.028	6	6	6	6	0.054	0.022	6	6	6	6	0.056	0.022	6	6	6	6
Trifluraline	0.392	0.594	6	6	6	6	0.366	0.476	6	6	6	6	0.358	0.448	6	6	6	6
Vinclozolin	0.306	0.124	6	6	6	6	0.27	0.108	6	6	6	6	0.275	0.105	6	6	6	6

* ion ratio criteria according to SANCO/12495/2011

3.6. Application to real samples

In order to test the applicability of the developed method, three types of orange, tomato and carrot samples collected from local markets were analyzed. Moreover, the method was expanded for the analysis of three types of apple, lettuce and courgette, including a matrix-matched calibration for each sample matrix and a quality control at 0.05 mg/kg.

A total of 43 different pesticides were identified in the analyzed samples, most of them at levels well below 0.01 mg/kg and all under their corresponding MRLs. An overview of the detected pesticides is shown in **Fig. 5**.

Orange was the most contaminated sample and several positive findings were present in all the varieties analyzed. In tomato and carrot samples, pesticides were frequently detected but most of them below the LOQ. The different varieties of apple, lettuce and courgette did not present many positive findings, although those in apple samples were the most abundant. Among positive findings, only a small number were found above the LOQ (see **Table 3**). A concentration level around 1 mg/kg of the fungicide folpet was the most significant finding, detected in one of the apple varieties, although not exceeding its MRL (3 mg/kg). Captan and bifenthrin, which are commonly used in agricultural crops, were also detected at high levels in apple samples, between 0.1 and 0.5 mg/kg. The OP insecticide chlorpyrifos is also frequently used in apple and orange crops, for which concentrations between 0.03 and 0.1 mg/kg were found. The maximum positive findings in tomato samples were for the fungicide iprodione (around 0.1 mg/kg), whose presence is common in vegetable crops. The higher concentrations levels of pesticides found in carrot samples occurred for metalaxyl and cypermethrin around 0.1 mg/kg. Regarding courgette samples analyzed, no pesticides above 0.01 mg/kg were found.

Pesticide	Orange	Tomato	Carrot	Apple	Lettuce	Courgette
Diphenylamine						
Chlorpropham						
Terbumeton desethyl						
Terbutylazine desethyl						
Dimethoate						
Terbutylazine						
Chlorothalonil						
Phosphamidon						
Chlorpyrifos methyl						
Metalaxyl						
Methiocarb sulfone						
Methiocarb						
Chlorpyrifos						
Triadimefon						
4,4-Dichlorobenzophenone						
Cyprodinil						
Pendimethalin						
Fipronil						
Captan						
Folpet						
Procymidone						
Trifumizole						
Fenamiphos						
Fludioxonil						
p,p'-DDE						
Oxadixyl						
Sulprofos						
Famphur						
Propiconazole I						
Endosulfan sulfate						
Fenhexamid						
Propiconazole II						
Diflufenican						
Iprodione						
Phosmet						
Bifenthrin						
Pyriproxifen						
Fenarimol						
Coumaphos						
Cypermethrin						
Deltamethrin						
Azoxystrobin						

Fig. 5. List of detected pesticides in the different samples analyzed. Red color indicates the presence of the pesticide in the three varieties of the studied matrix and purple and green, the presence in two and one varieties, respectively.

Table 3. Concentrations of pesticides above the LOQ (mg/kg) detected in analyzed samples.

Pesticide	Orange			Tomato			Carrot			Apple			Lettuce		
	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3
Azoxystrobin								0.023							
Bifenthrin										0.11					
Captan										0.12	0.44				
Chlorpyrifos		0.11	0.035							0.028	0.042	0.059			
Chlorpyrifos methyl			0.013												
Cypermethrin									0.14						
Cyprodinil				0.015											
p,p'-DDE								0.035							
Fenhexamid					0.013										
Fludioxonil				0.011											
Folpet											1.3				
Iprodione				0.13	0.048	0.055									
Metalaxyl									0.13				0.013		
Pyriproxifen				0.024											

As regards identification, all detected pesticides were identified by the use of three transitions and the compliance of at least one q/Q ratio. Identification was problematic at low levels in a few compounds due to unfavorable q/Q ratios.

As an illustrative example, **Fig. 6** shows GC–(APCI) MS/MS chromatograms corresponding to three of the positive findings detected in analyzed samples: chlorpyriphos in apple (0.04 mg/kg), pyriproxyfen in tomato (0.02 mg/kg) and triadimefon in lettuce (below LOQ). A reliable identification of analytes in these samples was feasible by means of the experimental q/Q intensity ratios, even at those low concentration levels.

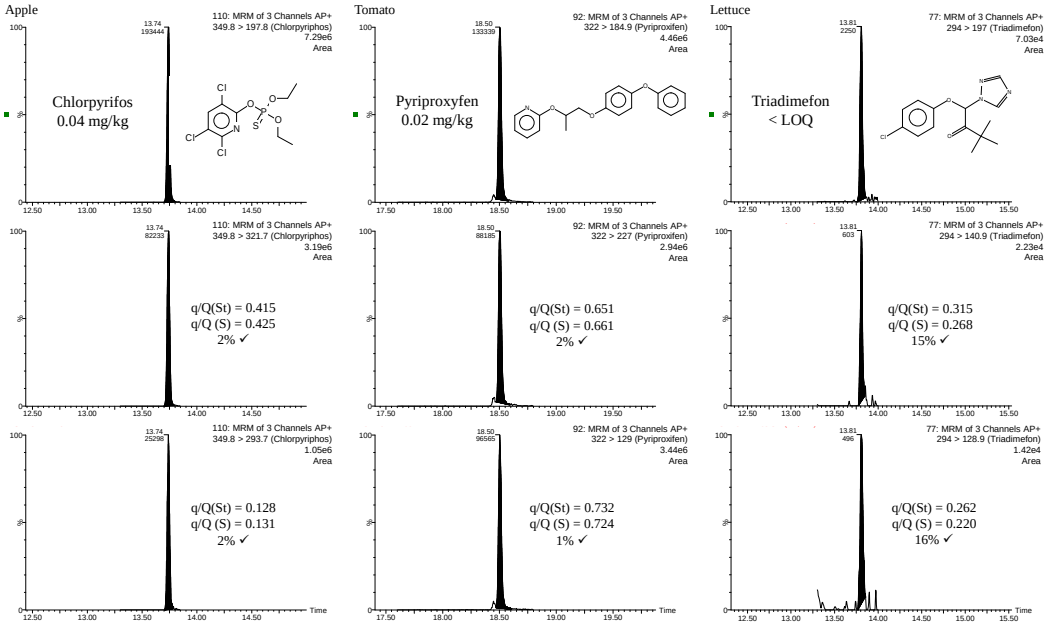

Fig. 6. GC–(APCI) MS/MS chromatograms for pesticides detected in apple, tomato and lettuce. (Q) quantification transition; (q) qualifier transition; (St) standard; (S) sample.

4. CONCLUSIONS

A multi-residue method for the determination of pesticide residues in fruit and vegetables was developed with satisfactory results using an innovative system based on an APCI source coupled to GC–(QqQ) MS/MS. The soft ionization allowed the use of the quasi-molecular ion as precursor in most cases contributing to an excellent selectivity and sensitivity. The high sensitivity (LODs of 1–100 fg on-column for most compounds) allowed dilution of QuEChERS extract by a factor of 10, without compromising method detection limits for most of the pesticides studied. The method was successfully validated for the simultaneous quantification and identification of 138 pesticides (three transitions each) in orange, tomato and carrot matrices at 0.01 and 0.1 mg/kg. This demonstrates the suitability of GC–(APCI) MS/MS for quantitative routine residue analysis. Analysis of fruit and vegetable samples allowed identifying and quantifying several pesticides like folpet, captan,

bifenthrin, chlorpyrifos, iprodione and chlorothalonil. In all cases, the concentration levels were below the MRLs set by the EU.

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III.4. Artículo científico 6

Atmospheric pressure chemical ionization tandem mass spectrometry (APGC/MS/MS) an alternative to high resolution mass spectrometry (HRGC/HRMS) for the determination of dioxins

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Atmospheric pressure chemical ionization tandem mass spectrometry (APGC/MS/MS) an alternative to high resolution mass spectrometry (HRGC/HRMS) for the determination of dioxins

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Abstract

The use of a new atmospheric pressure chemical ionization source for gas chromatography (APGC) coupled to tandem quadrupole mass spectrometer (MS/MS) as an alternative to high-resolution mass spectrometry (HRMS) for the determination of PCDDs/PCDFs is described. The potential of using atmospheric pressure chemical ionization (APCI) coupled to a tandem quadrupole analyzer has been validated for

the identification and quantification of dioxins and furans in different complex matrices. The main advantage of using the APCI source is the soft ionization at atmospheric pressure resulting in very limited fragmentation. APCI mass spectra are dominated by the molecular ion cluster, in contrast with the high energy ionization process under electron ionization (EI). The use of molecular ion as precursor in MS/MS enhances selectivity and consequently sensitivity by an increase in signal-to-noise ratios (S/N). For standard solutions of 2,3,7,8-TCDD injecting 10 fg in the splitless mode on a 30 m or 60 m, 0.25 mm I.D. and 25 μm film thickness low polarity capillary columns (DB-5MS type), S/N values greater than 10:1 were routinely obtained. Satisfactory linearity was achieved ($r^2 > 0.998$) for calibration curves ranging from 100 fg/μL to 1000 pg/μL. The results from a wide variety of complex samples, including certified and standard reference materials and samples from several QA/QC studies which were previously analyzed by EI/HRGC/HRMS, were compared with the results from the APGC/MS/MS system. Results between instruments showed good agreement both in individual congeners and toxic equivalence factors (TEQs). The data shows that the use of APGC in combination with MS/MS for the analysis of dioxins has the same potential in terms of sensitivity and selectivity as the traditional HRMS instrumentation used for this analysis. The APCI/MS/MS system as being a bench top system is however far more easy to use.

Keywords

PCDDs and PCDFs; Atmospheric pressuregas chromatography (APGC); Atmospheric pressure chemical ionization (APCI); Tandem quadrupole (MS/MS); Dioxins; High resolution mass spectrometry (HRMS).

INTRODUCTION

Within the UNEP program 'Assessment of Existing Capacity and Capacity Building Needs to Analyze POPs in Developing Countries' several activities were

undertaken during the period from 2006 to 2010. This program is focused on the development of analytical capacity for the POPs under the Stockholm Convention including several pesticides (DDT, chlordane, toxaphene) and industrial (by)-products (dioxins, PCBs). Recently brominated flame retardants (PBDE) and an organic fluor compound (PFOS) were added to the convention. One of the conclusions of the program was that it is quite a challenge to analyze all POPs in the sample types proposed for the global monitoring program (GMP) [1-2]. This is especially true for developing countries. One of the difficulties to develop a universal method for the Stockholm convention POPs is that both LC and GC have to be used for separation. Although among the newly added compounds to the Stockholm convention PFOS is routinely analyzed by LC/MS, the most problematic compounds on the Stockholm convention to be analyzed are dioxins. To achieve enough sensitivity and selectivity dioxins are routinely analyzed by using high resolution GC coupled to high resolution MS, instrumentation which is both expensive and complex elaborate to maintain.

Dioxins analysis is most commonly carried out using high resolution (HR) GC/MS using magnetic sector MS instrumentation operated in EI+ mode. This technique, even when used at lower ionization energies (~35 eV), results in significant fragmentation reducing the intensity of the molecular ion ($M^{\bullet+}$). Typical chemical ionization (CI) produces softer ionization, with an energy transfer that generally is lower and does not exceed 5 eV. Normally CI mass spectra exhibit less fragment ions than in EI. CI is typically produced under vacuum conditions using an ionization gas which fills the ion source. It is restricted to specific chemical classes [3-5] since it is not as universal ionization mechanism as EI if no extreme reagents are used. When using CI conditions for the ionization of dioxins often electron capture chemical ionization (NCI) occurs, resulting in loss of intensity of the molecular ion. The importance of an abundant molecular mass peak of the analyte is especially important for the development of MS/MS based methods. The selection of adequate precursor ions and the subsequent application of the MRM mode enhance selectivity and sensitivity, minimizing or even eliminating matrix interferences. When the molecular ion is absent or it has very low abundance, it might be necessary to select a

(abundant) fragment ion as precursor ion, with lower m/z and in most cases less compound characteristic. Thus, in addition to the loss of sensitivity, the specificity of the method can also be affected and losing the potential advantages of tandem MS. A soft and universal ionization technique able to provide abundant molecular ions to be used as precursor ions in multiresidue GC–MS analysis would be a step forward when using MS/MS. In this way, the low-energy (soft) ionization mechanism occurring in the APGC source generates spectral data typically rich in molecular or protonated molecule ion information [6]. Because of the reduced fragmentation when using APCI, the selection of the precursor ion is no longer a compromise between selectivity and sensitivity when developing a MS/MS based method.

Atmospheric pressure ionization in GC–MS was first introduced by Horning et al. [7] in the early 1970 using a ^{63}Ni corona discharge needle to form ions of the target compounds or different reagents [8]. Relatively soon after, this technique was used for the APCI analysis of dioxins by Horning et al. [9], Siegel and McKeown [10] and Mitchum et al. [11] who all used the ^{63}Ni source to ionize 2,3,7,8-TeCDD at atmospheric conditions. Also, using the ^{63}Ni source, Mitchum and Stalling [12] were able to analyze all 22 isomeric tetra-dioxins. The detection limits, however, were relatively high varying from 60 to 300 ppt and, although some selectivity was achieved by using different reagents, APCI at that time was by far less selective and less sensitive than high resolution GC/MS systems which became the preferred methodology for dioxin analysis [13]. Subsequent modifications were described in the 80s [14,15] but the technique was never implemented for common routine analysis.

Recently, APCI has made a revival when a new source using nitrogen purge gas was developed and commercialized [16-18]. Although not widely applied, it offers attractive analytical capabilities for GC–MS/MS analysis and has been used in different fields, including pesticide residue analysis [19], pharmaceuticals development [20], profiling of phenolic compounds in oil [21] and metabolic profiling [22]. APGC was recently successfully used for the analysis of more than 100 different pesticide residues by Portoles et al. [6] using an improved corona needle discharge source. APGC mass spectra for several of the POPs on the Stockholm convention

showed only limited or no fragmentation significantly enhancing the detection limits for compounds such as aldrin, dieldrin and endosulfan when compared to electron ionization (EI), where considerable fragmentation occurred and selection of ions for quantification in the SRM mode is difficult. Especially for POP MS/MS applications it is important to generate as much molecular ion of the most abundant ions of the chlorine cluster for subsequent fragmentation. For isotope dilution quantification using ^{13}C labeled standards, this is preferably the molecular ion.

This shows that the use of APGC is a possible way forward in the development of a universal detection system for all Stockholm convention POPs based on mass spectrometry. For dioxins, where high resolution GC/MS systems are often required to avoid inferences and to achieve the extreme low limit of detection (LOD) needed for food and feed [23], air or human samples, APGC could potentially be a very attractive alternative, opening the way for a universal mass detector for all POPs on the Stockholm convention including dioxins.

The aim of this paper is to demonstrate the capabilities of APGC/MS/MS (tandem quadrupole) using an APCI based ion source for the determination of dioxins in a variety of samples including environmental, air, human and food sample extracts.

MATERIAL AND METHODS

PCDD/F standards and samples

EPA-1613PAR and TF-TCDD-MXB standards solutions containing different mixtures of native PCDD/F congeners, EPA-1613LCS and EPA-1613ISS ^{13}C-labeled PCDD/F standards for sample preparation, as well as calibration standards 1613-EPACSL to 1613-EPACS5, with both native and ^{13}C-labeled PCDD/F and TCDD solution TF-TCDD-MXB, were all obtained from Wellington Laboratories (Guelph, Ontario, Canada). Certified reference materials BCR-607 (natural milk powder), BCR-677 (sewage sludge), BCR-490 and BCR-615 (fly ash) were acquired from the

Institute for Reference Materials and Measurements (IRMM), European Commission-Joint Research Centre (Geel, Belgium). Internal reference materials (spiked feed samples, human blood and naturally contaminated food and feed samples) for routine quality control/quality assurance (QA/QC) in the laboratories and different matrices from international inter-laboratory comparison studies (fish sample from the Interlaboratory Comparison on POPs in Food 2012 (13th Round), Norwegian Institute of Public Health (Oslo, Norway) and PT test samples from proficiency tests organized by the EU-RL for Dioxins and PCBs in Feed and Food) were also used to compare the results on different instruments. Additionally, several procedural blanks have been analyzed both by HRMS and APGC for all sample types. All blank levels were well below the amounts found in the samples.

Sample preparation

Sample treatment was performed following previously developed and validated methods [24] or standard methods [13] or based on analytical criteria of Commission Regulations (EU) No. 252/2012 and 152/2009 for the official control of food and feed [25]. Generally after sample extraction of the organic fraction, fat and polar interfering substances were removed by treating the n-hexane extracts with silica gel modified with sulfuric acid (44%) or gel permeation chromatography. Further sample purification was performed by an automated system (Power Prep™, FMS Inc., Waltham, MA, USA), or a manual clean up using an alumina oxide column eluted with hexane and a hexane/dichloromethane mixture or florisil column eluted with n-heptane and toluene followed by a carbon column eluted with hexane and toluene. The final extracts were either finally analyzed by APGC/MS/MS or both by APGC/MS/MS and HRGC/HRMS.

Instrumental analysis

APGC/MS/MS conditions

Four different APGC/MS/MS systems were used in this study in four different laboratories at Waters, Manchester, Great Britain; IUPA, Castellon, Spain; EURL for

Dioxins and PCBs, Freiburg, Germany and MTM, Örebro, Sweden. The basic set up of the instrument was similar in all cases. The GC system consisted of an Agilent 7890A (Agilent, Palo Alto, CA, USA) equipped with an auto sampler (Agilent 7693). The GC was coupled to a quadrupole mass spectrometer (Xevo TQ-S, Waters Corporation, Manchester, UK), equipped with an APGC ionization source. For dioxin analysis the GC was equipped with either a silica DB-5MS (UI) capillary column (60 m × 0.25 mm id. × film thickness 0.25 μm) (J&W Scientific, Folsom, CA, USA) or a BPX-5 capillary column (30 m × 0.25 mm i.d.,x 0.25 m) (SGE Analytical Science, Victoria, AUS). The injector was operated in splitless mode, injecting 1 μL at 280 °C at all instruments by pulsed splitless injection using an initial pressure of 240 kPa or injecting 5 μL in the PTV solvent vent mode (EURL, Freiburg). The interface temperature was set to 280-360 °C using N_2 (from gas cylinder, quality ≥ 99.9990%) as make-up gas at 150-370 mL/min in the constant flow mode depending on the instrument. As auxiliary gas N_2 (from liquid N_2, nitrogen generator or gas cylinder) was used at 250-300 L/h with tube to waste or 200 L/h without tube to waste. The cone gas was used to optimize the ionization, and set at 170-200 L/h when the comparison was made between all four instruments. The APCI corona pin was operated between 1.8 and 2.1 μA. The ionization process occurred within a closed ion volume, which enabled control over the protonation/charge transfer processes.

Quantitative analysis was performed in the multiple reaction monitoring mode (MRM) by monitoring two transitions for each of the native PCDD/F congeners and their corresponding ^{13}C-labeled analogues. The molecular ion [M•+] was always selected as precursor ion for all compounds (congeners and ^{13}C analogues) and fragmented by collision in the T-wave collision cell. The data were processed using the quantification application manager TargetLynx™ which automates data acquisition, processing and reporting for quantitative results. It incorporates a range of confirmatory checks that identify samples that fall outside user-specified or regulatory thresholds. A summary of the experimental conditions in the different laboratories is given in **Table 1**. The MS/MS conditions were taken from the literature [26,27] and optimized when needed and are given in **Table 2**.

Table 1. Experimental APGC/MS/MS conditions used by the different laboratories after optimization.

Instrumentation GC conditions	
Column:	DB-5MS 60 m x 0.25 mm, 0.25 µm BPx-5 30m x 0.25 mm, 0.25 µm
Carrier gas:	Helium at 1.4-2 mL/min
Injector mode:	Pulsed Splitless, 240- 450 kPa (1-2 mins) MMI Solvent Vent (50 kPa, 10 ml/min, 0.5 min)
Injector liner:	Single gooseneck splitless liner, 4mm Deactivated dimpled splittless liner, 2 mm
Column pneumatics:	Constant flow
Injection volume (µL):	1 ul splitless 5 ul MMI
Injector temperature (°C):	Splitless 280 ^{0}C MMI PTV mode (100 ^{0}C, 0.5 min; 340 ^{0}C, 20 min)
MS Conditions	
MS system:	Xevo TQ-S
Ionisation:	APGC with Dry N_2
Corona current:	1.5-2.1 µA
Source offset:	60-70 V
Cone Voltage:	30, 35 or 70 V
Source temperature:	150 ^{0}C
Cone gas flow:	170-200 L/h
Acquisition:	MRM
Collision gas:	Argon at 3.5-6.2 10^{-3} mbar
GC interface	280-360 ^{0}C
Aux gas flow	250-300 L/h 200 L/h (without tube to waste)
Make up gas	150-370 ml/min

Table 2. Multiple Reaction Monitoring (MRM) conditions for MS/MS method.

Compound	Precursor Ion	Product Ion	Collision Energy (eV)	Precursor Ion	Product Ion	Collision Energy (eV)
TCDF	304	241	40	306	243	40
^{13}C TCDF	316	252	40	318	254	40
TCDD	320	257	30	322	259	30
^{13}C TCDD	332	268	30	334	270	30
PCDF	338	275	40	340	277	40
^{13}C PCDF	350	286	40	352	288	40
PCDD	354	291	30	356	293	30
^{13}C PCDD	366	302	30	368	304	30
HxCDF	374	311	40	376	313	40
^{13}C HxCDF	386	322	40	388	324	40
HxCDD	390	327	30	392	329	30
^{13}C HxCDD	402	338	30	404	340	30
HpCDF	408	345	40	410	347	40
^{13}C HpCDF	420	356	40	422	358	40
HpCDD	424	361	30	426	363	30
^{13}C HpCDD	436	372	30	438	374	30
OCDF	442	379	40	444	381	40
^{13}C OCDD	470	406	30	472	408	30
OCDD	458	395	30	460	397	30

RESULTS AND DISCUSSION

Ionization optimization

The ionization in the APGC mode was optimized by using high concentration PCDD/PCDF standards (CS5, 200 ng/mL for TCDD) which were injected under full scan conditions. The ionization using APCI under charge-transfer conditions (no H_2O was added to the source) revealed an abundant presence of the molecular ion for all seventeen 2,3,7,8 chlorine substituted dioxins and furans. This is in good agreement with recent publications [6] explaining the ionization mechanism for APCI. The nitrogen plasma (N_2^+ and N_4^+) created by the corona discharge needle ionizes molecules yielding typically $M^{\bullet+}$. The protonated molecule $[M+H]^+$ can be also present in the spectrum due to the presence of water vapor traces in the source, this competing mechanism reduces the intensity of the molecular ion. The ionization under proton-transfer conditions was tested by introducing water as modifier in the

APCI source (an uncapped vial with water was placed in a specially designed holder placed in the source door). However, none of the tetra- to octa-chlorinated dioxins or furans showed much protonation, this is exemplified in **Figure 1** which shows the ionization behavior of OCDF with and without water added to the source. It can clearly be seen that ionization which is carried out only with N_2 (charge transfer) is more effective (in terms of response) and the m/z 444 precursor resulted in higher spectral abundance without using water as modifier and reducing proton transfer as much as possible. For the continuation of the development of a method for PCDD/DFs analysis, charge transfer conditions were used. A simple check to see if protonation occurs is the analysis of phenanthrene and comparing the abundance of m/z 178 (charge transfer) and m/z 179 (protonation), for this relatively easy protonated compound the protonated ion should not exceed 30%.

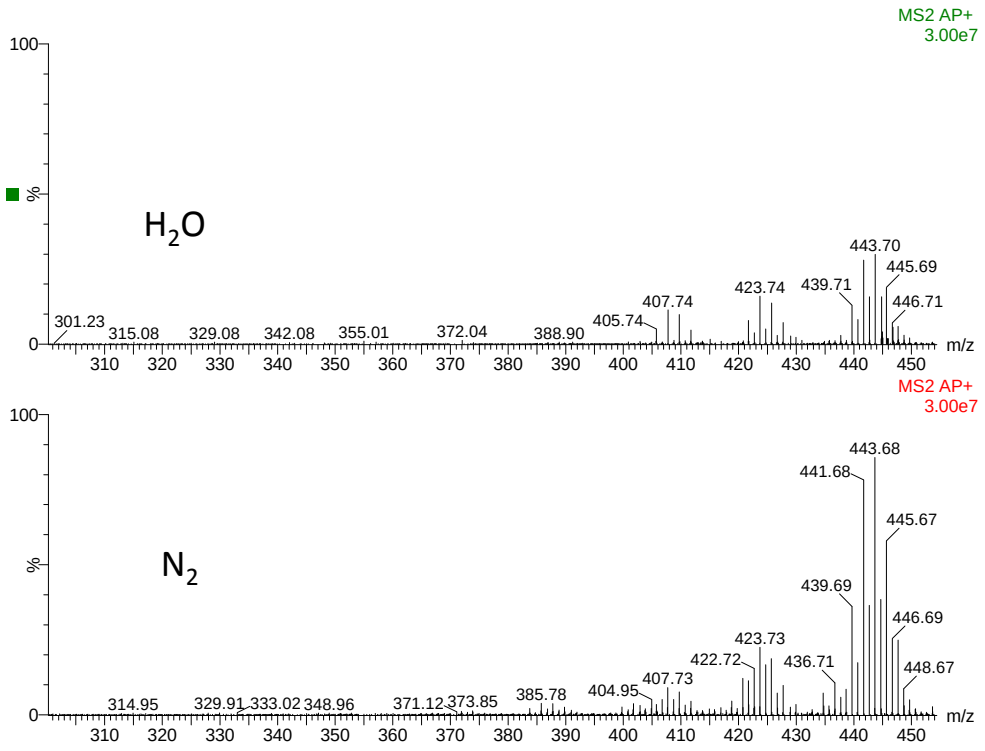

Figure 1. Comparison of the ionization characteristics of OCDF in the APGC source with (enhancing protonation) and without water (enhancing charge transfer) in the source.

Optimization of the cone voltage

The cone voltage was optimized for each PCDD/F by testing values between 20 and 70V in order to obtain the best sensitivity. Although no significant differences were observed, slightly higher response factors were obtained at 30V (especially compared with 70V) and thus a cone voltage of 30V was used for most of the experiments chosen. The optimization process is further illustrated in **Figure 2** where the relative response is given as a response surface diagram for the cone gas and the auxiliary gas. A clear optimum and stable region is seen between cone gas flow settings 170-225 L/h and auxiliary gas settings of a larger range (100-200 L/h). Both flows from different directions seem to influence the corona plasma and ion extraction. Too high values of the auxiliary gas flow did result in lower response for the target compounds. When removing the auxiliary to waste tube, lower auxiliary gas flows give better relative response illustrating the complexity of the optimization of the different gas flows into the source.

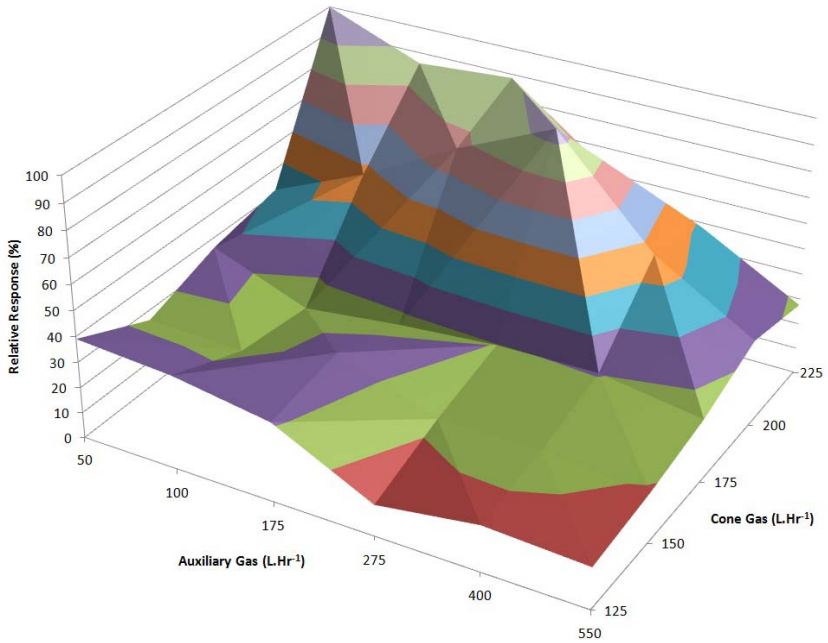

Figure 2. Response surface plot showing auxiliary gas (LHr^{-1}) versus cone gas (LHr^{-1}) optimization for PeCDD.

MRM method

Firstly, product ion scan experiments were performed in order to find selective transitions based on the use of M•+ as precursor ion. Different collision energies (10, 20, 30, 40 and 50 eV) were tested and the most sensitive transitions were selected for the development of the subsequent MRM method. Collision energies of 30 eV were selected for all the TCDDs and values of 40 eV were selected for the TCDFs. Lower energies led to nearly absence of product ions and too high energies led to excess fragmentation and therefore lower sensitivity. At low collision energies the product ion spectrum is dominated by the ^{35}Cl loss, but at the final optimum collision energies the transitions selected corresponded to the loss of [$CO^{35}Cl$] (**Figure S1**). This fragment is very specific for dioxins and furans. Collision energies of 30 eV were selected for all the PCDDs and values of 40 eV were selected for the PCDFs (**Table 2**) or 31 eV for both PCDDs and PCDFs. These collision energies are in agreement with GC/MS/MS collision energies using EI ionization [4,5].

Both automatic dwell time, set in order to obtain at least 15 points per peak (values ranging from 0.058 to 0.079 s) and a fixed dwell time of 0.1 ms were used in the different instruments tested. In all cases the chromatographic peak shape was considered adequate.

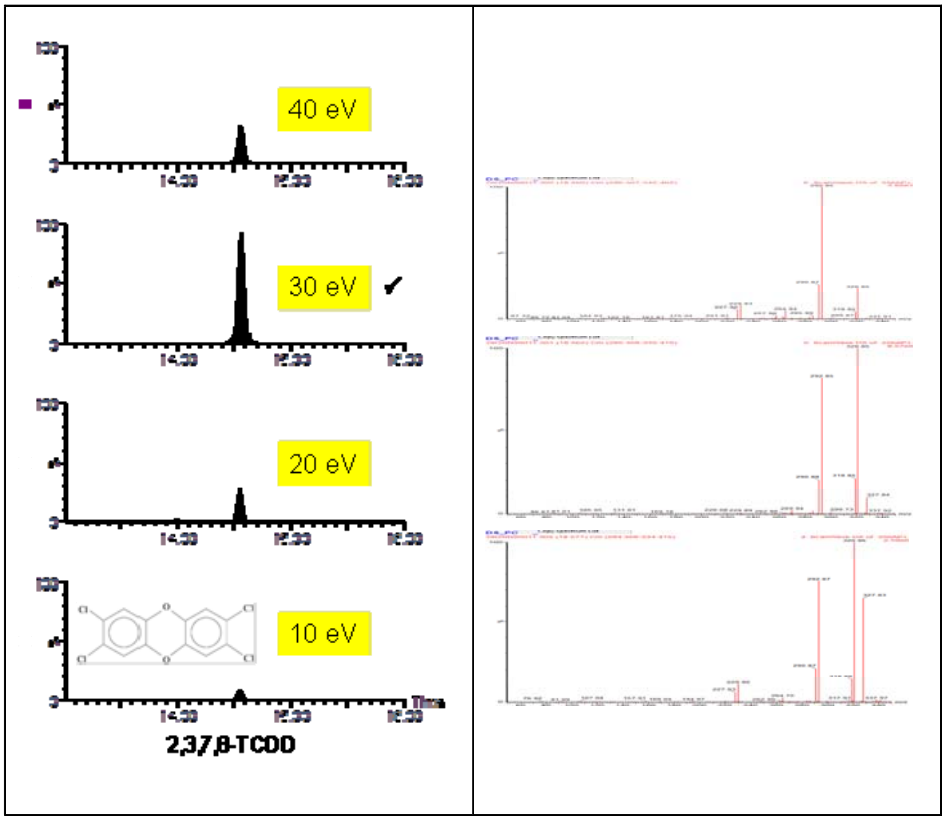

Figure S1. Effect of collision energy (eV) on the TCDD response for a standard solution and MS spectra for PeCDD illustrating different fragmentation at different collision energy.

Analytical parameters

The analytical performance was evaluated by calculating the detection limit, linearity, repeatability and reproducibility of the method in four different laboratories. These parameters are compared to the high resolution mass spectrometer, the instrumentation routinely used for the analysis of dioxins using standard method EPA1613 or EN 16215. The lowest calibration point for 2,3,7,8-TeCDD in this method contains 500 fg/µL (CS1), for the evaluation of high resolution instruments often a solution of 100 fg/µL is used at a S/N level > 100. After initial set up and tuning, a dilution of this test solution down to 10 fg was made. With this

solution S/N values of > 50 (**Figure 3**) were achieved in all four laboratories in most cases when optimum alignment of the corona needle was established in addition to the make-up and auxiliary gas flow conditions. Similar results were achieved using toluene or tetradecane as the injection solvent. The ultimate sensitivity was tested by using a mix of different TCDD congeners at concentrations of 2, 5, 10, 25, 50, and 100 fg/µL (TF-TCDD-MXB) of 1,3,6,8-TeCDD, 1,3,7,9-TeCDD, 1,3,7,8-TeCDD, 1,4,7,8-TeCDD, 1,2,3,4-TeCDD and 2,3,7,8-TeCDD, respectively, injected to evaluate the minimum amount of TCDD that could be detected. As can be seen from **Figure 4**, the lowest value which is visible just above noise level is 2 fg. Also the sensitivity for the other tetra- to octa- substituted PCDD/Fs was good for the 1/10 dilution of calibration solution CSL at 10, 50 and 100 fg/µL for TeCDD and TeCDF, PeCDD, PeCDF, HxCDF, HxCDD, HpCDD and HpCDF, and OCDD/OCDF, respectively. This is illustrated in **Figure 5** where the different MRM channels are given for this solution. All these results are impressive and comparable, or even better, than routinely achieved with high resolution magnetic sector GC/MS systems.

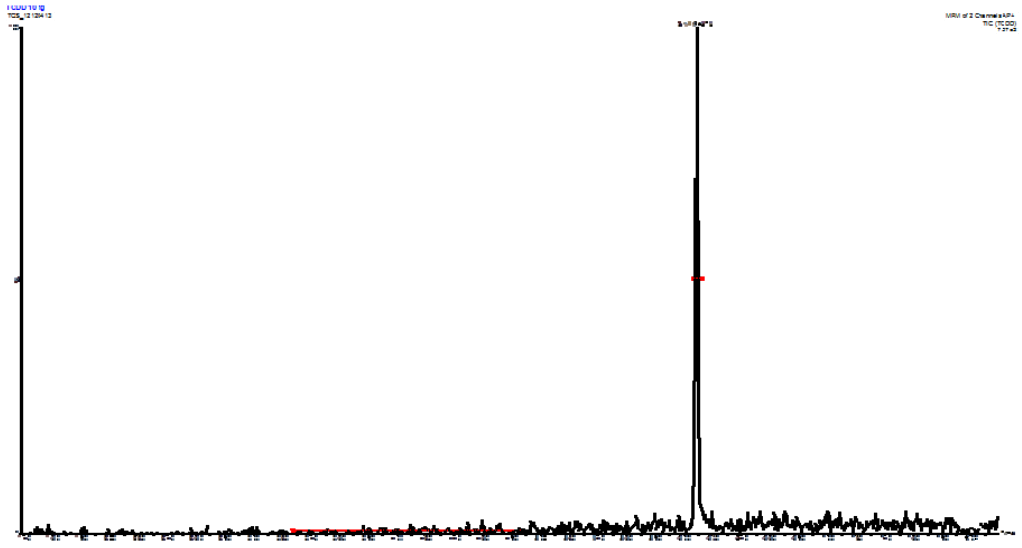

Figure 3. Injection of 1 µL of a 10 fg/µL solution of TCDD on a BPx-5 30 m x 0.25 mm, 0.25 µm column using the APGC conditions in Table 2.

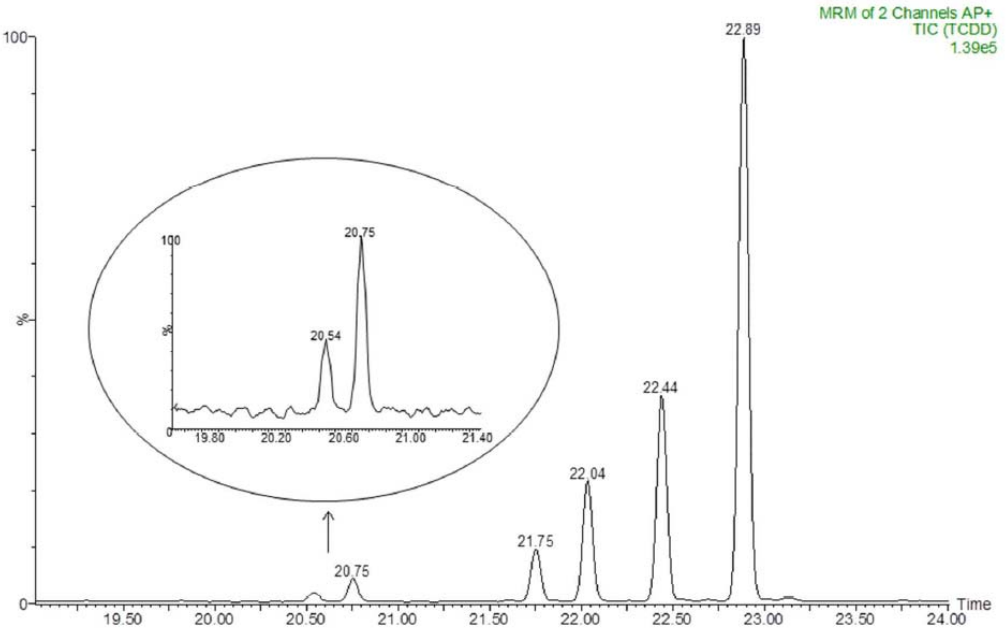

Figure 4. Sensitivity test after injection of 1 µL of a test solution containing 2-100 pg/µL of different TCDD isomers on a 60 m DB-5MS column (0.25 mm id x 0.25 um).

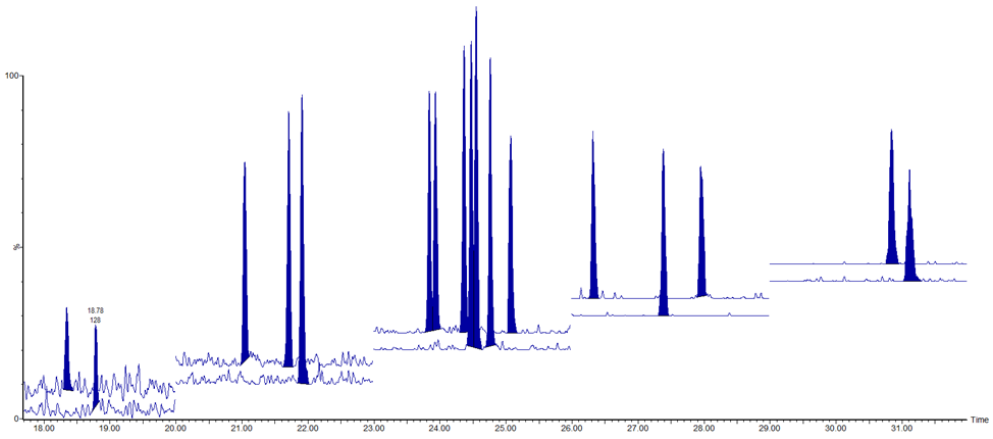

Figure 5. TeCDD and TeCDF at 10 fg, PeCDD, PeCDF, HxCDF, HxCDD, HpCDD, and HpCDF at 50fg and OCDD and OCDF at 100 fg, 1 µL injected see Table 2 for MRM conditions.

The linearity of the method was studied by analyzing the standard solutions (in triplicate) at six concentrations (CSL, CS0.5, CS1-CS4) ranging from 0.1 to 40 ng/mL for the Tetra PCDD/DFs, from 0.5 to 200 ng/mL for the Penta through Hepta PCDD/DFs and from 1.0 to 400 ng/mL for the Octa PCDD/DFs at the four different systems in four different laboratories. The linearity was satisfactory with the correlation coefficients (r^2) larger than 0.998. The RSD of the relative response factors (RRFs) as defined in standard methods EPA 1613 or EU 1948 were also satisfactory and all below 15% as specified in both methods. Based on area the repeatability was within 15% for the injection of 10 fg (n=3-10), and below 10% for all PCDD/DFs for the CSL standard against the corresponding ^{13}C standard (RRF).

An important criterion for the unequivocal identification of the PCDD/F congeners is the ion abundance ratio between the two monitored product ions, resulting from two different precursor ions. For quality control the ion abundance ratios can be compared with calculated or measured values. The calculated ratio depends on the relative abundance of the two selected precursor ions of the molecular ion [M•+] and the probability of the loss of [$CO^{35}Cl$] or [$CO^{37}Cl$] for formation of each product ion. It is only comparable with the measured ratios, if identical collision energy and collision gas pressure is applied for both transitions. The measured ion abundance ratios in calibration standards and sample extracts matched the calculated values within the QC limits of ± 15%, as derived from EPA 1613 for HRMS.

Besides the use of the signal-to-noise for the calculation of the limit of quantification (LOQ), these ion abundance ratios in combination with the relative response factors from calibration can be used as criteria to check the reliability of the results in the low concentration range. Based on maximum deviations of ± 15% of the calculated value for ion abundance ratios and deviations of ≤ 30% of the relative response factor of the mean value (with a CV ≤ 20% for the complete calibration) LOQs for 2,3,7,8-TCDD and 2,3,7,8-TCDF were obtained in the range of 10 – 30 fg on column for calibration standards.

Comparison APGC and high resolution GC/MS

Quality controls, certified reference materials

In order to test the capabilities of the developed method using GC-(APCI) MS/MS, several samples previously analyzed by HRMS were injected in the new system. Sample extracts from existing samples which previously had been run on a high resolution system were re-injected on the APGC/MS/MS system in three different laboratories, the EURL for Dioxins and PCBs in Germany, CSIC and IUPA in Spain and MTM in Sweden. A summary of the results based on TEQ [28] are given in **Figure 6** where the results of the different laboratories are given over a wide concentration range and a variety of different samples. The correlation between the results is very good and the relative difference $(X_{APGC}-X_{HRMS})/X_{HRMS}$ between the APGC results and the HRMS was less than 7% for all samples given in **Table 3**, when the APGC runs passed all QA/QC criteria in terms of chromatographic separation, linearity, S/N ratio and ion abundance ratio of selected transitions. In some cases loss of chromatography was seen which affected the results of the individual isomers. Also for some of the samples run on the shorter 30 m BPX-5, an overestimation of 1,2,3,7,8,9-HxCDF was seen. This is however not specific for the APGC but has also been seen for HRMS [29]. On average the results for the individual congeners were comparable with the HRMS results accept for levels just above the detection limit. These isomers however contribute very little to the total TEQ, and although the relative difference could be larger than 25%, this was not reflected in the total TEQ. More detailed congener specific data is given in the supplemental information where a comparison of results of analysis with APGC-MS/MS with results of GC-HRMS QC-charts for mixed animal fat, fish oil and hen's eggs sample is specified (**Figure S2**).

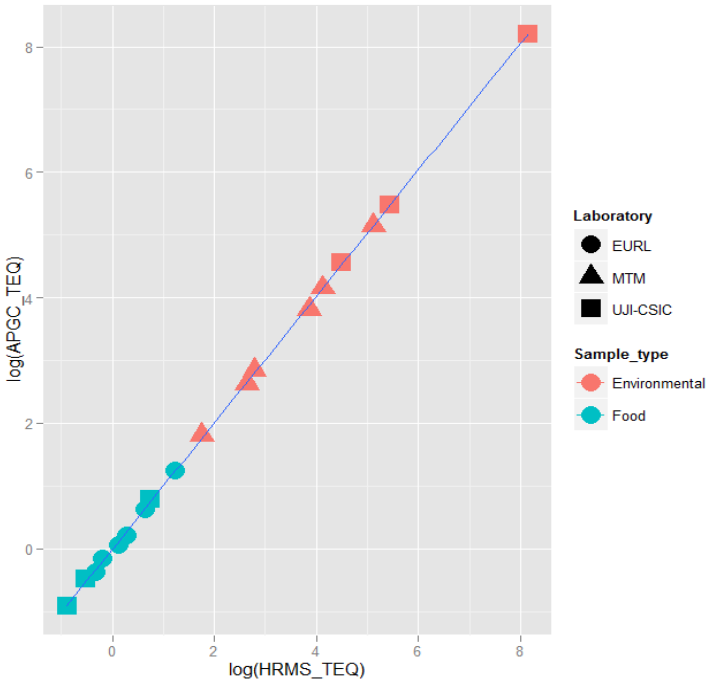

Figure 6. Comparison of APGC results and HRMS for different samples as described in the method and material section in three different laboratories.

Table 3. Comparison of results obtained for a variety of samples analyzed both by APGC/MS/MS and HRGC/HRMS. Data are given on WHO-PCDD/DF TEQ per sample.

	APGC	HRMS	
EURL	0.85	0.83	2%
(pg/g lipids)	0.69	0.72	-3%
	1.24	1.31	-6%
	1.07	1.14	-7%
	1.86	1.89	-2%
	3.46	3.39	2%
MTM	6.1	5.8	5%
(pg/PUF)	13.9	14.0	-1%
	45.6	47.7	-5%
	63.8	62.0	3%
	172	168	3%
	17.3	16.2	7%
CSIC/IUPA	2.19	2.12	3%
(pg/g)	0.40	0.41	-2%
	0.62	0.59	4%
	238	228	4%
	3640	3470	5%
	96.2	89.4	7%

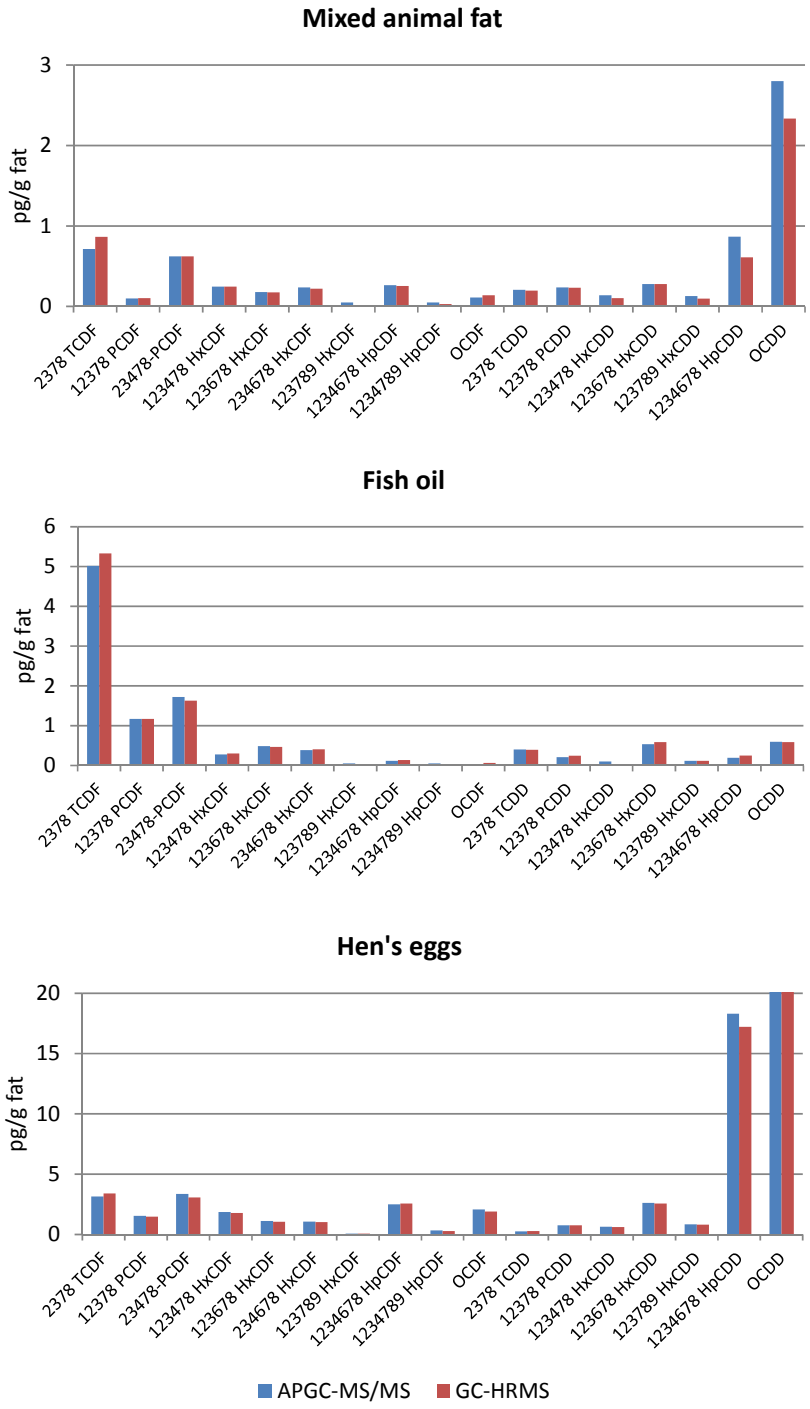

Figure S2. Comparison of results of analysis with APGC-MS/MS with results of GC-HRMS QC-charts for mixed animal fat, fish oil and hen's eggs sample.

CONCLUSIONS

The results of the APGC system are impressive and comparable with HRMS in selectivity and also in sensitivity (10 fg, S/N <50). Sensitivity of conventional GC/MS/MS systems has been lower than traditional HRMS. For monitoring purposes of all the POPs on the Stockholm Convention in complex samples such as air, human blood or milk included in UNEP global monitoring program this is a big step forward when the most difficult compound class can be analyzed on the same instrumentation, including difficult pesticides, brominated flame retardant but also persistent fluor compounds including PFOS connecting the instrument to (UP)LC.

Acknowledgments

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III. 5. Discusión de los resultados obtenidos

Los resultados mostrados en los tres artículos que componen este capítulo ofrecen una visión favorable y prometedora del nuevo sistema GC-(APCI) MS/MS para la determinación de contaminantes en alimentos y medio ambiente. La obtención de una sensibilidad y selectividad óptimas se basó en una cuidadosa optimización de las condiciones GC-MS, tratando de favorecer la presencia del pico molecular o de la molécula protonada en los espectros de masas de los compuestos estudiados.

El acoplamiento de GC a la nueva fuente APCI incorpora nuevos parámetros a considerar con respecto a los métodos convencionales por GC-(EI) MS tales como el voltaje de cono, la intensidad de la aguja corona o los distintos flujos de N_2 que llegan a la fuente. Concretamente, el voltaje de cono parece tener un efecto significativo en la fragmentación del pico molecular, como se indica en el Artículo científico 4. Este voltaje produce la transmisión de los iones generados en la fuente hacia el MS; si es demasiado pequeño puede que se pierdan iones durante el trayecto, mientras que un elevado valor puede provocar la fragmentación de los mismos antes de que lleguen al MS (fragmentación *in-source*). Este último efecto se ejemplifica en la **Figura III.2** para el pesticida etión, cuya fragmentación se evaluó en un rango de voltajes de cono entre 10 y 50 v.

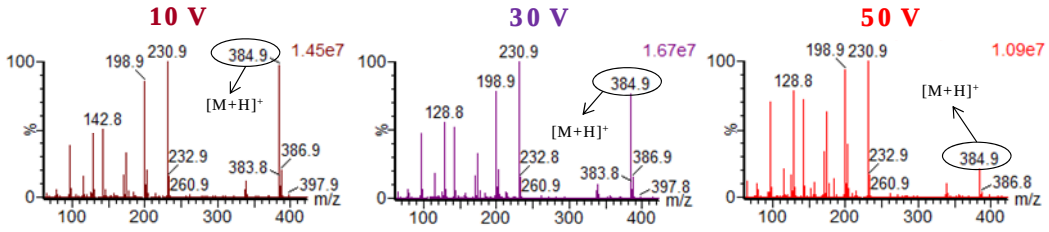

Figura III.2. Espectros de masa en APCI para el pesticida etión a diferentes voltajes de cono.

El comportamiento de la mayoría de pesticidas estudiados, incluyendo los del Artículo Científico 5, sigue esta misma tendencia, en la que las diferencias en sensibilidad de la molécula protonada son especialmente notorias a valores extremos. La respuesta de este voltaje también puede verse afectada por los flujos de N_2 que llegan a la fuente, como el gas auxiliar y el gas del cono. La influencia de los mismos sobre la respuesta cromatográfica se aprecia en la **Figura 2** del Artículo Científico 6 (pág. 251), en la que se puede observar la aparente complejidad de los efectos de dichos flujos.

Por otro lado, este nuevo sistema GC-(APCI) MS permite trabajar en un intervalo más amplio de flujo de gas portador que el habitual en GC-(EI) MS, ya que la ionización sigue siendo efectiva a flujos altos (Portolés *et al*., 2012), a diferencia de lo que ocurre en EI. De hecho, como se ilustra en la **Figura 4** del Artículo científico 4 (pág. 191) para el caso del fenarimol y el azinfos etil, un flujo de 2 mL/min ofreció una mayor resolución cromatográfica que la obtenida con 1 mL/min, además de una reducción del tiempo de análisis.

En cuanto al método de MS, resulta especialmente útil la posibilidad que ofrece este sistema de fijar un *dwell time* automático en función del número de puntos por pico deseados y el número de transiciones incluidas en una ventana SRM. Tiempos tan bajos como 3 ms se emplearon en la adquisición de algunos compuestos sin afectar negativamente a la sensibilidad y repetibilidad de las respuestas. Así mismo, dentro de cada ventana de adquisición también es posible aplicar un *dwell time* diferente para cada transición, ofreciendo una mayor versatilidad en función del comportamiento cromatográfico de cada analito. Aunque con dos transiciones sería suficiente para cumplir el mínimo de 3 puntos de identificación requeridos para la confirmación de cada compuesto según la directiva (*Commission Decision 2002/657/CE*), en los métodos desarrollados en los Artículos Científicos 4 y 5 se adquirieron hasta 3 transiciones con *dwell times* aceptables para una mayor fiabilidad en la identificación.

La presencia del ión molecular en los espectros de masas de la mayoría de compuestos estudiados en este capítulo ha marcado una notable diferencia con

respecto a la ionización por EI tal como se esperaba, gracias a la suave ionización que tiene lugar en la fuente APCI. Ello permitió resolver problemas de selectividad en ciertos pares de compuestos problemáticos como el oxiclordano y el heptaclor epóxido B (**Figura 5** del Artículo científico 4 (pág. 195)), o en los pesticidas organofosforados mevinfos, dicrotofos, monocrotofos y fosfamidon (**Figura 3** del Artículo científico 4 (pág. 187)), cada grupo con transiciones comunes en EI.

Merece resaltar igualmente el efecto del uso de agua como modificador, dispuesta en una cavidad especial dentro de la fuente APCI. Como se observa en la **Figura III.3**, la formación del ión quasi-molecular de la mayoría de los pesticidas seleccionados en el Artículo científico 4 se ve favorecida en estas condiciones, resultando en un aumento significativo de la sensibilidad con respecto al uso de EI y APCI en condiciones de transferencia de carga con N_2. Incluso en esta última situación sin uso de modificador, la presencia de trazas de agua en la fuente genera en algunos casos el $[M+H]^+$ en lugar del $M^{+\bullet}$, o una mezcla de ambos.

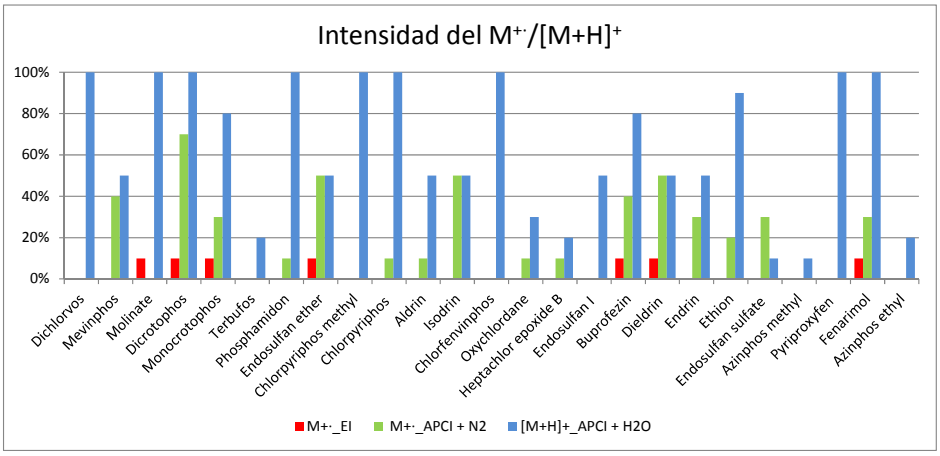

Figura III.3. Intensidad del pico molecular o de la molécula protonada en los espectros de masas obtenidos en EI y APCI, esta última en modo de transferencia de carga con N_2 y en condiciones de protonación.

La misma tendencia mostraron los pesticidas estudiados en el Artículo científico 5, por lo que la mayoría de transiciones SRM parten de la molécula protonada como ión precursor. Además de mejorar la selectividad, ello supuso un

aumento notable de la sensibilidad, reflejándose en los bajos límites de detección conseguidos (entre 0.01 y 1 μg/kg para la mayoría de compuestos en las tres matrices estudiadas).

Dado que no hay estricta necesidad de LODs tan bajos considerando que los mínimos MRLs establecidos por las directivas son del orden de 10 μg/kg, esta alta sensibilidad pudo aprovecharse para simplificar el tratamiento de muestra aplicado en el Artículo Científico 5. Como se ha comentado anteriormente en el capítulo I, la inyección directa al GC de los extractos de acetonitrilo derivados del QuEChERS requiere de un inyector PTV; en caso de contar únicamente con un *split/splitless* (como en el caso de este sistema), es conveniente un cambio de solvente a hexano o tolueno. La evaporación total de los extractos para re-disolverlos en hexano no resultó efectiva por la pérdida de algunos analitos. Por su parte, el cambio a tolueno también se descartó tras comprobar la baja reproducibilidad de las correspondientes respuestas. La alternativa, más rápida y simple, de diluir los extractos directamente con hexano se consideró teniendo en mente la alta sensibilidad conseguida (la adición de acetona fue necesaria para hacer miscible la mezcla hexano-acetonitrilo). Aunque el alto grado de dilución aplicado (1:10) no permitió eliminar completamente el efecto matriz, con ello se consiguió simplificar en gran medida el proceso de extracción.

El cumplimiento de las intensidades relativas de iones (*q/Q*) también se vio favorecido por la alta sensibilidad del método desarrollado en el Artículo Científico 5. Un elevado porcentaje de compuestos se validó de acuerdo a las normas descritas en la *Commission Decision 2002/657/CE* y en el *Guidance document SANCO/12495/201*, que establecen unas tolerancias máximas permitidas de *q/Q* en función de las intensidades relativas. A diferencia de las establecidas para el modo MS (indicadas en la discusión del capítulo II), en MS/MS las tolerancias son ligeramente más permisivas (ver **Figura III.4**), pero su cumplimiento puede resultar igualmente problemático a bajas concentraciones o ante la influencia de la matriz.

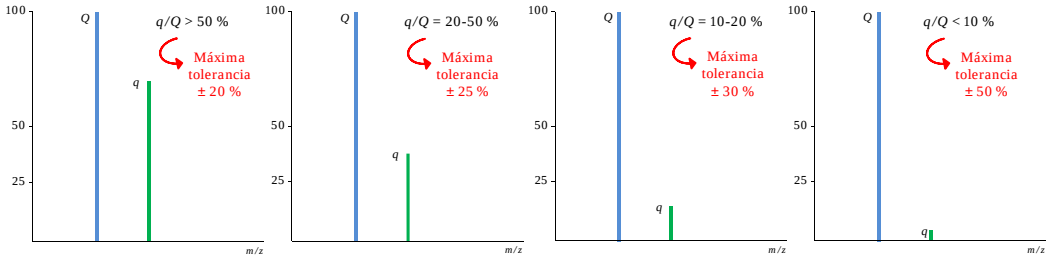

Figura III.4. Tolerancias máximas permitidas de intensidades relativas de iones en GC-MS/MS, de acuerdo con la Decisión de la Comisión Europea 2002/657/CE y el Documento SANCO/12495/2011.

Ante los estudios anteriormente mencionados que propugnan la nula relación entre la variabilidad de la *q/Q* y la intensidad relativa (Mol *et al.*, 2012 & 2013), el nuevo Documento SANCO/12571/2013 (*Guidance document SANCO/12571/2013*) establece una tolerancia común de 30% independientemente de las intensidades relativas, aunque sólo aplicable en el caso de MS/MS. Considerando este nuevo criterio, el porcentaje de compuestos incluidos en el presente método que cumplen satisfactoriamente las tolerancias aumenta aunque no notablemente, ya que la baja sensibilidad de los iones de referencia en algunos casos sigue repercutiendo al cumplimiento de las relaciones entre los iones (precisamente en aquellos con relaciones *q/Q* <10% donde el criterio pasa de 50% a 30%). De todas formas, en caso de no cumplirse la *q/Q* de una transición, el número de puntos de identificación requeridos para garantizar la confirmación de un positivo en el QqQ igualmente se cumplen con la otra transición adquirida.

La experiencia adquirida en el uso de la nueva fuente APCI en GC-MS/MS y los excelentes resultados obtenidos nos animaron a abordar un tema tan complejo y alejado de las líneas de investigación establecidas en nuestro grupo como es la determinación de dioxinas en muestras complejas. Este trabajo (Artículo científico 6) se ha desarrollado como una aportación de nuestro grupo de investigación al trabajo global conjunto realizado por los diferentes laboratorios participantes, expertos en el campo de las dioxinas.

Dada la estricta regulación de estos contaminantes, los laboratorios dedicados a su análisis siguen rigurosamente los criterios definidos por la EPA y la Unión Europea con el fin de garantizar una alta fiabilidad de los resultados. La optimización de las condiciones GC-MS/MS en nuestro laboratorio se llevó a cabo atendiendo a los requisitos mínimos exigidos en cuanto a límites de detección y cuantificación, selectividad y exactitud.

Los niveles mínimos detectables de PCDD/PCDFs en determinados tipos de muestras deben alcanzar valores en el rango de centenas de femtogramos (10^{-15} g). Así, 1 µL de una disolución estándar de PCDD/PCDFs disponible a 0.01 ng/L (equivalente a 10 fg) se inyectó en el GC-(APCI) MS/MS con el fin de evaluar su detección y repetibilidad. A pesar del bajo nivel inyectado, la relación S/N se mantuvo en valores aceptables para todos los congéneres, como puede observarse en el ejemplo de la **Figura III.5 a** para el pico cromatográfico correspondiente a la octaclorodibenzo-p-dioxina. La repetibilidad, expresada como desviación estándar relativa (RSD, %) de 10 réplicas, también resultó satisfactoria, obteniendo valores inferiores a 15% para todas las dioxinas y furanos. La inyección de un nivel 10 veces superior (10 réplicas de 0.1 ng/mL, (100 fg)) (**Figura III.5 b**) mejoró notablemente la RSD con valores entre 1 y 5%.

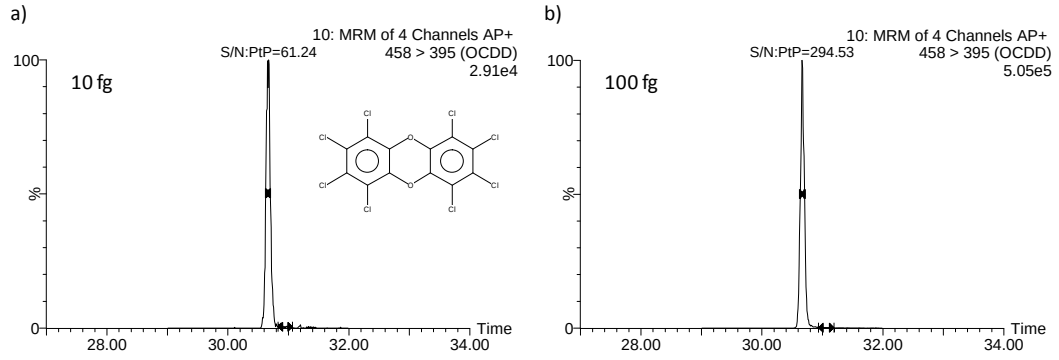

Figura III.5. Cromatogramas obtenidos por GC-(APCI) MS/MS para la octaclorodibenzo-p-dioxina. a) 0.01 ng/L (10 fg inyectados), (b) 0.1 ng/L (100 fg inyectados).

Esta alta sensibilidad conseguida en APCI se atribuye, en gran parte, a la presencia del ión molecular como pico base del espectro para la mayoría de congéneres, con muy baja fragmentación, a diferencia de la observada en EI (**Figura III.6**).

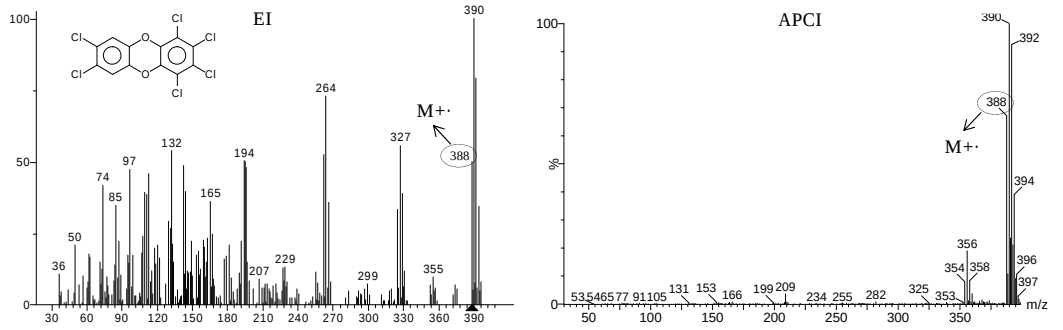

Figura III.6. Espectros de masas obtenidos por EI i APCI para la 1,2,3,4,7,8-hexaclorodibenzodioxina.

Así mismo, se llevó a cabo un test de sensibilidad a través de la inyección de un patrón con diferentes congéneres de tetradioxina a diferentes concentraciones. La mínima cantidad inyectada, 2 fg (correspondiente a la 1,3,6,8-TCDD), pudo ser detectada correctamente, como se aprecia en la **Figura III.7 a**. La comparación con el mismo patrón inyectado por GC-(EI) HRMS (**Figura III.7 b**) demuestra la alta sensibilidad del nuevo sistema GC-(APCI) MS/MS, equiparable con los métodos tradicionales empleados en este campo.

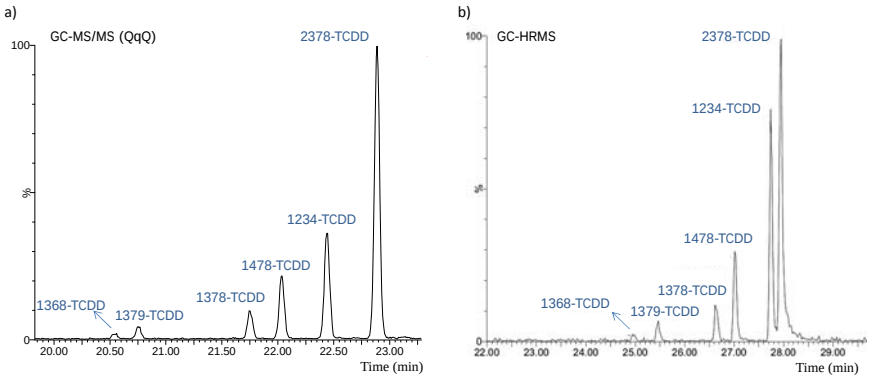

Figura III.7. Cromatogramas obtenidos por a) GC-(APCI) MS/MS y b) GC-(EI) HRMS para una mezcla de diferentes isómeros de tetraclorodioxina en el rango 2-100 pg/µL.

Otro aspecto importante en el desarrollo de un método analítico para dioxinas es que la resolución cromatográfica entre isómeros también debe cumplir con un mínimo de separación (un valle menor de 25% entre pico y pico), lo que se consiguió con una columna de 60 m.

Por su parte, la linealidad se estudió con disoluciones estándar conteniendo también los patrones de cada congénere marcados isotópicamente, por lo que se trabajó con respuestas relativas. Los rangos abarcados se fijaron de acuerdo al método 1613 definido por la EPA (0.1-40 ng/mL para la tetradioxina y tetrafurano, 1-400 ng/mL para la octadioxina y octafurano y 0.5-200 ng/mL para el resto) y se ajustaron a curvas lineales con coeficientes de regresión superiores a 0.999 en todos los casos. Con estas curvas se analizaron y cuantificaron extractos correspondientes a materiales de referencia certificados (leche en polvo, pescado, pienso, ceniza y fango), permitiendo evaluar la exactitud del método; como se ilustra en el ejemplo de la **Figura III.8** para el análisis de leche en polvo, se obtuvieron bajas desviaciones (<10%) con respecto a los valores certificados. Cabe destacar que el cumplimiento de las *q/Q* resultó satisfactorio, con desviaciones menores a 5% con respecto al valor medio obtenido de la recta de calibrado.

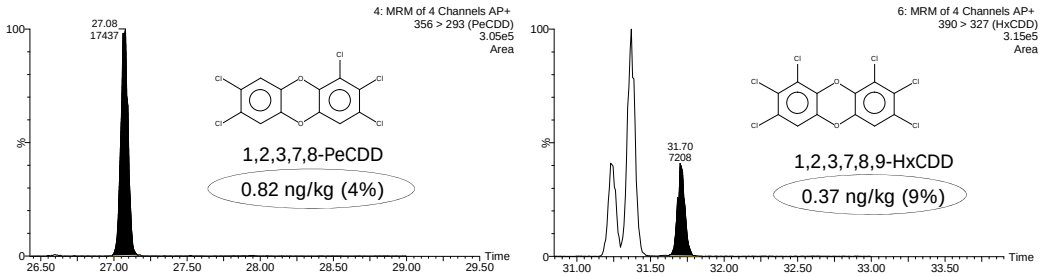

Figura III.8. Cromatogramas obtenidos por GC-(APCI) MS/MS para la 1,2,3,7,8-pentaclorodibenzofurano y la 1,2,3,7,8,9-hexaclorodibenzodioxina, ambas en muestras de leche en polvo detectadas a niveles de ng/kg (desviación con respecto al valor teórico entre paréntesis).

Así mismo, los diagramas *box plot* de la **Figura III.9** ejemplifican la correlación entre los resultados obtenidos por GC-(EI) HRMS, GC-(APCI) MS/MS y los valores de referencia en muestras de leche y fango. Como se observa, la mediana de las diferencias absolutas queda muy próxima a cero en todos los casos, con una ligera desviación de los valores respecto a los teóricos, tanto por HRMS como por MS/MS, siendo mejor la distribución entre los valores obtenidos por comparación de ambas técnicas.

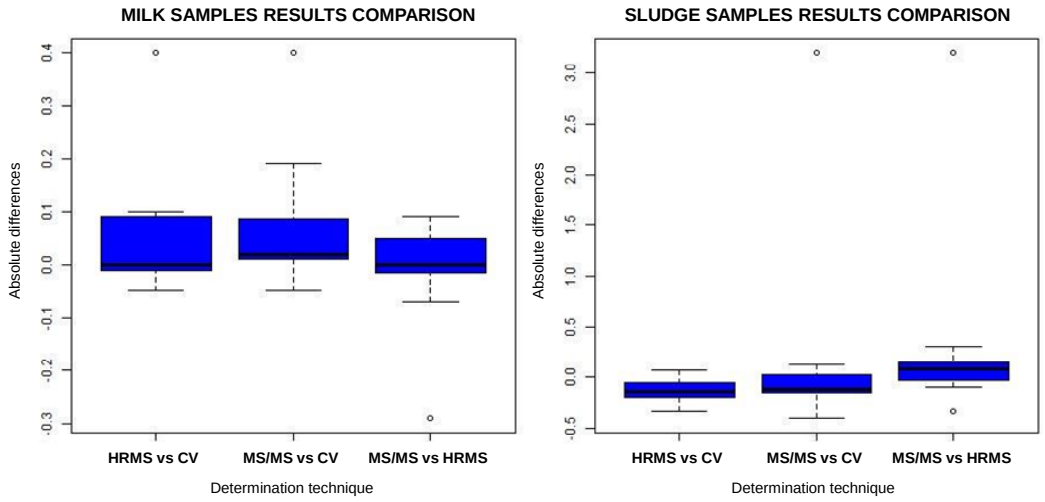

Figura III.9. Diagramas *plot box* de la comparación de los resultados obtenidos para todos los congéneres de las dioxinas y furanos estudiados, por las técnicas HRMS, MS/MS y los valores de referencia (CV), en muestras de leche y fango.

Por otro lado, la concordancia entre los resultados proporcionados por cada grupo participante en el mencionado trabajo queda reflejada en la **Figura 6** del Artículo Científico 6 (pág. 258), en la que la representación de los logaritmos de los valores de TEQ para HRMS y MS/MS genera una recta de pendiente 1, tanto para muestras ambientales como alimentarias.

A la vista de los resultados obtenidos en los tres artículos, este nuevo sistema GC-(APCI) MS/MS promete una mejora de los métodos clásicos empleando EI, suponiendo una alternativa atractiva para diversas aplicaciones.

Capítulo IV

Método de *screening* basado en GC-(EI)TOF MS, GC-(APCI)QTOF MS y UHPLC-(ESI)QTOF MS para la caracterización de sustancias desconocidas capaces de migrar desde el envase alimentario a simulantes de alimentos

IV. 1. Introducción

Además de los contaminantes de origen ambiental que pueden encontrarse en los alimentos (estudiados en los capítulos anteriores), otra fuente de contaminación potencial puede derivar del envasado de los mismos. Existen reglamentos al respecto que describen medidas específicas para asegurar las buenas prácticas de fabricación de materiales destinados a entrar en contacto con los alimentos (*Commission Regulation (EC) No 2023/2006*), incluyendo listas de las sustancias autorizadas para dicho proceso (*Real Decreto 847/2011*). Sin embargo, estos materiales no son suficientemente inertes y el contacto directo con el producto alimenticio puede propiciar la transferencia de sustancias desde el envase hasta el alimento, pudiendo alterar la composición y la toxicidad del mismo. Este fenómeno suele ocurrir por difusión y se conoce con el nombre de migración; el tiempo y la temperatura de contacto, la naturaleza del alimento y del material envasado, así como las características y concentración de los migrantes, son los factores que más afectan a este proceso (Arvanitoyannis & Kotsanopoulos, 2014).

Actualmente existe una gran diversidad de materiales disponibles para el envasado de alimentos adaptables a necesidades concretas. Entre ellos, los envases plásticos (solos o en combinación con otros materiales) son de los más empleados por su ligereza y flexibilidad y su amplia variedad de formulaciones. Constan de un polímero base de elevado peso molecular y otros compuestos tales como aditivos, residuos de polimerización o productos de degradación de bajo peso (<1000 Da), susceptibles de transferirse a los alimentos. A estos efectos, el Reglamento 10/2011 de la Unión Europea (*Commission Regulation (EU) No 10/2011*) establece unos límites de migración específica (SML) con el fin de controlar las cantidades de estas sustancias migrantes y evitar riesgos toxicológicos en los consumidores. Dicho reglamento recoge una larga lista de aditivos, monómeros y otras sustancias de partida, quedando excluidos los productos de polimerización y degradación, impurezas y otras sustancias no añadidas intencionadamente (NIAS). Los envases metálicos como las latas, fabricadas principalmente con hojalata y aluminio, también

son habituales para envasar líquidos y productos en conserva por su ligereza y hermeticidad. En este caso no existe un reglamento con SMLs para las posibles sustancias migrantes, aunque en el Reglamento 1935/2004 de la Unión Europea (*Regulation (EC) No 1935/2004*), los metales se incluyen entre uno de los grupos de materiales y objetos para los que pueden adoptarse medidas específicas.

Por otro lado, considerando la dificultad que supone el análisis de alimentos dada su matriz compleja, los ensayos de migración para la determinación de las sustancias capaces de migrar se llevan a cabo en medios que simulan la transferencia del material plástico al alimento. Estos medios o simulantes representan las propiedades físicoquímicas de los alimentos y quedan recogidos en la directiva 82/711/EEC (*Council Directive 82/711/EEC*), asignándose tal como se indica en la **Tabla IV.1**.

Tabla IV.1. Clasificación de los simulantes en función del tipo de alimento al que sustituyen en los ensayos de migración.

Simulante	Abreviatura	Tipo de alimento
Etanol 10%	Simulante A	Alimentos acuosos (pH > 4.5)
Ácido acético al 3%	Simulante B	Alimentos ácidos (pH < 4.5)
Etanol al 20%	Simulante C	Alimentos alcohólicos ≤ 20% Alimentos con cierto carácter lipofílico
Etanol al 50%	Simulante D1	Alimentos alcohólicos ≥ 20% Emulsiones grasa en agua Productos lácteos
Aceite vegetal	Simulante D2	Alimentos con grasa libre superficial
Tenax®	Simulante E	Alimentos secos

El isooctano y el etanol al 95% también están contemplados en dicha directiva como simulantes alternativos para materias grasas. En la misma se especifican a su vez las condiciones de duración y temperatura (del contacto envase-alimento) para efectuar los ensayos de migración.

La mayoría de estudios analíticos en este campo sobre la interacción envase-alimento se centran en el desarrollo de métodos *target* para la determinación de grupos específicos de sustancias reguladas por las directivas como plastificantes (Di Bella *et al.*, 2014; Pedersen *et al.*, 2008), estabilizantes (Gill *et al.*, 2010), antioxidantes (Noguerol-Cal *et al.*, 2007) o adhesivos (Canellas *et al.*, 2012; Aznar *et al.*, 2011). La identificación de sustancias desconocidas o NIAS en modo *non-target* plantea mayores dificultades, especialmente ante la falta de información en el etiquetado del envase por tratarse de sustancias imprevistas y por los bajos niveles de concentración esperados (Nerín *et al.*, 2013; Skjevrak *et al.*, 2005). En la literatura científica existe una notable carencia en cuanto al desarrollo de este tipo de métodos, especialmente para la identificación de migrantes envase-alimento. En este caso, una búsqueda de contaminantes más extensa, necesaria para asegurar el control alimentario, requiere de equipos sensibles y selectivos.

La investigación *non-target* llevada a cabo en el presente capítulo surge en el marco de un convenio de colaboración entre el centro tecnológico Ainia (Valencia) y la Universitat Jaume I para la realización del proyecto "Desarrollo de metodología basada en (Q)TOF (LC/MS y GC/MS) para la caracterización de sustancias desconocidas capaces de migrar del envase alimentario a simulantes de alimentos". Los ensayos de migración desde los envases alimentarios a los simulantes se llevaron a cabo en el centro Ainia, realizándose experiencias con los simulantes isooctano, Tenax® (poli(óxido de 2,6-difenil-p-fenileno)), etanol al 10% y ácido acético al 3%.

La tarea de nuestro laboratorio consistió en el desarrollo de métodos de amplio barrido (*screening*) para el análisis *non-target* de todas aquellas sustancias capaces de migrar desde envases plásticos y metálicos a simulantes alimentarios, incluidas aquellas no reguladas en las directivas. Con el fin de determinar el mayor número de compuestos posible, se aplicaron de forma complementaria las técnicas GC-MS y LC-MS. Se analizaron un total de 5 muestras de materiales plásticos en los 4 tipos de simulantes mencionados y 2 muestras de envases metálicos recubiertos en 2 simulantes, ácido acético 3% y etanol 20%, todas ellas junto con los correspondientes blancos. Cabe mencionar que este trabajo es el único de esta Tesis Doctoral donde se

ha hecho uso de la cromatografía líquida, concretamente de la de alta resolución (UHPLC), la cual representa una mejora con respecto a la LC en cuanto a rapidez, selectividad y sensibilidad gracias a la incorporación de sistemas capaces de soportar elevadas presiones, consiguiéndose picos más estrechos y con mayor resolución cromatográfica (Ibáñez *et al.*, 2008). Concretamente, las técnicas de análisis se han basado en GC-(EI)TOF MS, GC-(APCI)QTOF MS y UHPLC-(ESI)QTOF MS, cuya complejidad reside en el gran potencial que ofrecen los analizadores TOF y QTOF en análisis *non-target* gracias a su elevada resolución y exactitud de masa (Cajka & Hajslová, 2007; Ibáñez *et al.*, 2012). Así, es posible extraer iones en un rango de masas muy estrecho (*narrow window-extracted ion cromatograms*; nw-XICs) y disponer de información muy precisa acerca de la identidad de un pico, respaldada por la medida de masa exacta de los iones que componen el espectro de masas. Además, el uso del QTOF incrementa la fiabilidad de la identificación gracias a la posibilidad de fragmentar un ión precursor y hacer un barrido de los iones producto con elevada exactitud de masa (Portolés *et al.*, 2009). Por otro lado, el uso de las diferentes fuentes de ionización EI, APCI y ESI ha permitido cubrir una gran variedad de compuestos en un amplio rango de masas y polaridades y aprovechar de manera complementaria determinadas ventajas, como son la disponibilidad de librerías de espectros comerciales para EI, que facilitan la búsqueda de candidatos, y la obtención del pico molecular en APCI y ESI, cuya detección puede acotar la búsqueda y facilitar el proceso de identificación.

Con toda la información derivada de la aplicación de estas técnicas, el procesamiento de datos se ha basado en la ejecución de una estrategia mediante el uso de softwares específicos, en función de la técnica analítica aplicada. Las muestras en isooctano y Tenax® fueron analizadas por GC, empleando GC-(EI)TOF MS y GC-(APCI)QTOF MS. En primer lugar se procesaron los datos obtenidos con el TOF mediante la búsqueda automática en librerías de espectros y el estudio de la masa exacta de los fragmentos generados por EI, que derivó en una lista de posibles candidatos para ciertos picos detectados. Posteriormente, a partir de datos generados en el GC-(APCI)QTOF MS y aprovechando la suave ionización que tiene lugar en la fuente APCI, se procedió con la búsqueda del pico molecular/molécula protonada de

cada candidato con el fin de confirmar y/o descartar su presencia en las muestras. La adquisición simultánea de las funciones de alta y baja energía (modo MS^E) y la posibilidad de realizar experimentos en MS/MS empleando este sistema ha permitido así mismo estudiar la fragmentación, proporcionando información estructural valiosa.

Por otro lado, las muestras con etanol al 10% y ácido acético al 3% se analizaron por UHPLC-(ESI)QTOF MS en modo *post-target*, basado en un procedimiento desarrollado previamente en nuestro grupo de investigación (Díaz *et al.*, 2012). Se investigaron alrededor de 700 compuestos incluidos en una base de datos, generada a partir de las listas de sustancias reguladas, realizándose una búsqueda automática de la molécula protonada (ESI+) o desprotonada (ESI-). La fragmentación de los picos candidatos pudo ser estudiada del mismo modo aprovechando el potencial del QTOF MS.

El Artículo científico 7 que a continuación se adjunta se refiere únicamente al trabajo realizado por GC-(TOF y QTOF) MS para el análisis de las muestras procedentes de materiales plásticos. El procesamiento de las muestras analizadas por cromatografía de líquidos (tanto de plásticos como de envases metálicos) se describe en el correspondiente apartado de discusión del presente capítulo.

VI.2. Artículo científico 7

Analytical strategy based on the use of gas chromatography coupled to time-of-flight or hybrid quadrupole time-of-flight mass analyzers to investigate potential polymeric migrants into food simulants

Laura Cherta, Tania Portolés, Elena Pitarch, Joaquim Beltran, Francisco López, Carmen Calatayud, Begoña Company, Félix Hernández

Analytical and Bioanalytical Chemistry (2014), submitted

Analytical strategy based on the use of gas chromatography coupled to time-of-flight or hybrid quadrupole time-of-flight mass analyzers to investigate potential polymeric migrants into food simulants

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Abstract

Potential migrants that can come from material used for food packaging have been investigated in this work. Migration tests were carried out by using two food simulants, isooctane and Tenax®. An advanced analytical strategy for non-target analysis of these food simulants based on the use of gas chromatography (GC) coupled to high resolution mass spectrometry (HRMS) with two different ionization sources has been applied.

Initially, food simulants were analyzed by GC-time-of-flight (TOF) MS with electron ionization (EI) source, which provided a number of candidates based on peak identification using a library search and mass accurate measurements of selected ions. Then, a second analysis was performed using hybrid quadrupole (Q) TOF MS with an atmospheric pressure chemical ionization (APCI) source. This allowed to confirm/reject the candidates resulting from the GC-(EI)TOF screening by searching for the molecular ion and/or protonated molecule, which is the most frequently observed in APCI mass spectra due to the "soft" ionization occurring in this source. The fragmentation behavior of the tentative candidates was also studied

to obtain further evidence on compound identification. The identity confirmation was made by injecting commercial reference standards, which were acquired on the basis of the information obtained by GC-TOF MS and GC-QTOF MS analysis.

This analytical strategy was applied to the analysis of ten plastic material samples, which were subjected to migration assays with both food simulants, isooctane and Tenax®. The list of candidates from GC-(EI)TOF MS was considerably reduced after evaluating the data obtained by GC-(APCI)QTOF MS and 8 migrants could be finally identified in the samples.

Keywords

Gas chromatography; High resolution mass spectrometry; Hybrid quadrupole time-of-flight; Atmospheric pressure chemical ionization; Food packaging; Food simulants; Potential migrants.

INTRODUCTION

Plastic materials, widely used in the manufacture of food packaging, are prepared using polymers of high molecular mass and other starting substances, as monomers and additives, which are susceptible to migrate from the package food due to their low molecular mass (European Regulation No 10/2011). The migration of these substances into food in contact with the packaging is considered as a potential source of pollution because the migrants could alter the food composition, deteriorate the organoleptic properties and, even, incur a human health risk. The European Regulation No 1935/2004 about materials and articles intended to come into contact with food appeals for the Good Manufacturing Practice (Rg 2023/2006) and establishes the authorization process of substances. Specific measures for food-contact plastic materials are contemplated in the European Regulation No 10/2011 that establishes the specific migration limits (SML) in order to prevent the transfer of plastic constituents at harmful levels. Demonstration of compliance must be tested

using food simulants, which are assigned to simulate certain foodstuff according to their chemical properties. Several examples of studies performed to evaluate the main factors affecting migration to food by following the procedures for migration tests given in the Directive 82/711/EEC can be found in the literature (Canellas *et al.*, 2010; Vera *et al.*, 2011).

Special attention requires the non-regulated compounds that can be present in packaged food: the non-intentionally added substances (NIAS), which consist of impurities generated from manufacturing and/or degradation processes. The lack of information about the real composition of the final packaging complicates the identification of these compounds (Nerin *et al.*, 2013; Skjevrak *et al.*, 2005). The identification of NIAS and unknown compounds, usually expected at low concentration levels, requires considerable time and effort. Sensitive advanced analytical techniques are needed, especially when dealing with non-target analysis.

As it is well known, gas chromatography (GC) coupled to mass spectrometry (MS) has been the main analytical technique for the determination of non-polar, volatile and thermostable substances. In this way different mass analyzers have been used for the determination of potential migrants in food packaging materials, usually applying target methodologies (Alin *et al.*, 2011; Burman *et al.*, 2005; Fasano *et al.*, 2012; Simoneau *et al.*, 2012). Recent progress in analytical instrumentation has increased the use of time-of-flight (TOF) mass analyzers coupled to GC in different fields as environmental analysis, food safety and toxicology (Hernández *et al.*, 2007; Hajslova *et al.*, 2007; Meyer & Maurer, 2012). TOF MS provides a notable amount of chemical information and allows searching after MS acquisition for a high number of compounds, even without any previous information or analyte selection. Thus, TOF MS is a powerful technique for non-target analysis, due to the accurate-mass full-spectrum acquisition, which increases the identification efficiency, together with its good sensitivity in full scan acquisition (Cervera *et al.*, 2012; Hernández *et al.*, 2011). However, until now, very few applications using GC-TOF MS for the determination of migrants from food packaging materials have been reported based on a non-target approach (Nerín *et al.*, 2009).

Electron ionization (EI) is by far the most widely used in GC-MS based methods (including GC-TOF MS) because of its capability of ionizing virtually any organic compound in a robust and reproducible way (Koesukwiwat *et al.*, 2010; Lehotay *et al.*, 2011). Commercial standardized libraries including more than 200000 MS spectra under EI are available; so, as a first approach, the identification of unknown compounds can be performed by a simple search matching. However, the high fragmentation occurred under EI may complicate the finding of a conclusive library match, especially due to the spectral similarity between many substances and the absence/low abundance of the molecular ion ($M^{+\cdot}$) in most cases. Another limitation is that the use of nominal mass spectra from the databases may not be powerful enough for confirmation, so accurate mass confirmation has to be done in a subsequent step by specific software tools. Softer ionization sources, as chemical ionization (CI), can be used as a complement for the identification using GC-TOF MS (Portolés *et al.*, 2011), although it is quite restricted to specific chemical classes. The new commercially available atmospheric pressure chemical ionization (APCI) (commonly used in liquid chromatography-mass spectrometry) coupled to GC produces a soft and universal ionization, which makes it an attractive tool for food safety concerning food-contact materials (Domeño *et al.*, 2012; Canellas *et al.*, 2012). The potential of this source in a GC-QTOF MS system has already been demonstrated in pesticide residue analysis (Portolés *et al.*, 2010; Portolés *et al.*, 2014; Nácher-Mestre *et al.*, 2014), in which the presence of the molecular or quasi molecular ion notably facilitated a rapid and sensitive screening.

In this work, the interaction food-packaging material has been investigated in order to identify unknown substances capable to migrate from plastic materials to food simulants (isooctane and Tenax®) by using both GC-(EI)TOF MS and GC-(APCI)QTOF MS.

EXPERIMENTAL

Reagents

A total of 21 commercial analytical standards were used for confirmation purposes. Diethyl sulphide (CAS No 110-81-6), tetramethylurea (632-22-4), octamethylcyclotetrasiloxane (556-67-2), m-acethyl acetophenone (6781-42-6), p-acethylacetophenone (1009-61-6), 3-(methylthio)phenyl isothiocyanate (51333-80-3), guaiazulene (489-84-9) and cinchophen (132-60-5) were purchased from ABCR GmbH & Co. KG (Karlsruhe, Germany). Sigma-Aldrich (Madrid, Spain) provided the standards: ethyl p-tolylsulfide (622-63-9), butylated hydroxytoluene (97123-41-6), 5,6-dimethyl-2-aminobenzothiazole (29927-08-0), p-tolyldisulfide (103-19-5), di-n-octyl phthalate (117-84-0) and bis(2-ethylhexyl) phthalate (117-81-7). 2,4-di-tert-butyl-phenol (2,4-DTB) (CAS No 96-76-4), 2,4-di-tert-butyl-6-methylphenol (616-55-7), diisobutyl phthalate (84-69-5), dibutyl phthalate (84-74-2) and diisooctyl phthalate (27554-26-3) were acquired from Dr. Ehrenstorfer (Augsburg, Germany). 2,6-di-tert-butyl-p-benzoquinone (2,6-DTBQ) (719-22-2) was purchased from Chempur Co. (Karlsruhe, Germany) and 2-(methylthio)phenyl isothiocyanate (51333-75-6) was acquired from Fluorochem Co. (Glossop, United Kingdom).

Individual stock solutions (around 500 mg/L) were prepared by dissolving each solid reference standard in acetone and stored in a freezer at -20ºC. Each standard solution was volume diluted in hexane (to around 1 mg/L) for the individual injection into the chromatographic system.

Hexane and acetone, both for ultra-trace analysis grade, were purchased from Scharlab (Barcelona, Spain). Diethyl ether for residue analysis and Tenax® adsorbent (60-80 mesh) were acquired from Sigma-Aldrich and trimethylpentane (isooctane) (HPLC grade) was purchased from VWR Chemicals.

Sample treatment

The food simulants isooctane and polyoxide 2,6- diphenyl-p-phenylene (Tenax®) were used for performing the migration experiences. Five different samples obtained from the application of each simulant to five different plastic materials and the corresponding simulant blanks were analysed.

Migration into the simulant isooctane was carried out by exposing the plastic material in contact with the simulant under specific conditions (ten days at 60 ºC) established in the Appendix V, Chapter 2, Reg No 10/2011, and then transferred to a vial for the GC injection. The followed protocol is described in the regulation UNE-EN 13130-1.

Before the use of Tenax® as simulant, this chemical was cleaned with diethyl ether in a Soxhlet extractor for 6 h and dried in an oven for other 6 h. Then the migration test was performed by keeping the Tenax® in contact with the plastic material in a Petri dish and incubating it for 10 days at 60 ºC. Finally the analytes were manually extracted from the simulant with diethyl ether at room temperature.

Instrumentation

GC-(EI)TOF MS

An Agilent 6890N GC system (Palo Alto, CA, USA) coupled to a GCT TOF mass spectrometer (Waters Corporation, Manchester, UK) with an EI source (70 eV) was used. The instrument was operated under MassLynx version 4.1 (Waters Corporation). Sample injections were made using an Agilent 7683 autosampler.

The GC separation was performed using a fused-silica HP-5MS capillary column with a lenght of 30 m x 0.25 mm i.d. and a film thickness of 0.25 μm (J&W Scientific, Folson, CA, USA). Injector was operated in splitless mode, injecting 1 μL at 280 ºC. The oven temperature was programmed as follows: 60 ºC (1 min); 5 ºC/min to 300 ºC (2 min); total chromatographic time of 51 min. Helium was used as a carrier gas at constant flow of 1 mL/min.

The interface and ion source temperatures were both set to 250 ºC and a solvent delay of 3 min was selected. TOF MS was operated at 1 spectrum/s acquiring a mass range *m/z* 50-650 using a multi-channel plate voltage of 2800 V. TOF MS resolution was about 8500 (FWHM) at *m/z* 614. PFTBA, used for the daily mass calibration, was injected via syringe into the reference reservoir at 30 ºC for this purpose. Additionally, PFTBA was used as a lock mass correction for EI experiments (monitoring the ion with *m/z* 218.9856).

The application manager Chromalynx, a module of Masslynx 4.1 software, was used to investigate the presence of non-target (unknown) compounds in sample extracts. Library search was performed using the commercial NIST library.

GC-(APCI)QTOF MS

An Agilent 7890A GC system (Palo Alto, CA, USA) coupled to a quadrupole TOF mass spectrometer XevoG2 QTOF (Waters Corporation, Manchester, UK) with an APCI source was used. The instrument was operated under MassLynx version 4.1 (Waters Corporation). Sample injections were made using an Agilent 7683 autosampler.

The GC separation was performed using a fused silica DB-5 MS capillary column with a length of 30 m × 0.25 mm i.d. and a film thickness of 0.25 μm (J&W Scientific). The oven temperature was programmed as follows: 60 ºC (1 min); 5 ºC/min to 300 ºC (2 min). 1 μL was injected at 280 ºC under splitless mode. Helium was used as carrier gas at 1.2 mL/min.

The interface temperature was set to 310 ºC using N_2 as auxiliary gas at 150 L/h, make-up gas at 300 mL/min and cone gas at 16 L/h. The APCI corona pin was operated at 1.6 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 2.5 spectra/s acquiring a mass range *m/z* 50–650. TOF MS resolution was approximately 18000 (FWHM) at *m/z* 614. For MS^E

measurements, two alternating acquisition functions were used applying different collision energies: a low-energy function (LE), selecting 4 eV, and a high-energy function (HE). In the latter case a collision energy ramp (10-40 eV) rather than a fixed higher collision energy was used. PFTBA (Sigma Aldrich, Madrid, Spain) was used for the daily mass calibration. Internal calibration was performed using a background ion coming from the GC-column bleed as lock mass (protonated molecule of octamethylcyclotetrasiloxane, *m/z* 297.0830). MassFragment software (Waters) was used to justify the fragmentation behavior of the compounds detected. This software applies a bond disconnection approach to suggest possible structures for the fragment ions from a given molecule.

Data processing

GC-(EI)TOF MS

Analytical strategy to perform the non-target analysis from the accurate mass GC-(EI)TOF MS data was based on our previous work based on the screening and confirmation of organic pollutants in water (Hernández *et al.*, 2007; Portolés *et al.*, 2007).

The deconvolution package ChromaLynx Application Manager, a module of MassLynx software, was used to automatically process the data. Parameters such as scan width, spectra rejection factor or peak width at 5% height were previously defined. For every sample, this software detected all peaks that satisfied the established conditions and displayed their deconvoluted mass spectra. A library search was subsequently executed (NIST02 library) and a hit list with positive matches (library match >700) was generated. The formulae from these candidates were submitted to an Elemental Composition Calculator and the accurate mass measurements of the five most intense ions were evaluated for the confirmation/rejection of the finding. More than one identity fit with the experimental spectrum was expected (in terms of library match and accurate mass of main fragment ions –and molecular ion if this existed–).

In those cases where a component was found in both blank and samples, only those with a signal 10 times higher than that observed in the blank samples were considered as tentative candidates for further research.

GC-(APCI)QTOF MS

In order to confirm/reject previous tentative identifications performed by GC-(EI)TOF MS, samples were re-injected in the GC-(APCI)QTOF MS following the basis of our previous developed procedure (Portolés *et al*., 2010).

Taking profit of the soft ionization occurred in the APCI source, both the molecular ion and the protonated molecule ($[M+H]^+$) of the candidates proposed from the (EI)TOF MS data were searched by performing a narrow window-extracted ion chromatogram (nw-XIC, ±0.01 Da) in the (APCI)QTOF MS data. A chromatographic peak was expected at very similar retention time (approximately 1 min less than the value obtained in (EI)TOF MS).

The absence of a chromatographic peak when performing a nw-XIC at $M^{+\cdot}$ and/or $[M+H]^+$ did not involve the rejection although decreased the probability, since the APCI fragmentation degree depends on the compound nature and, although not as the common trend, the molecular ion can be lost in some cases under APCI conditions.

Further investigation on the fragmentation was performed by evaluating the MS^E acquisition, which provides two functions at low and high energy in the same injection. The low-energy function was used to investigate the presence of the molecular ion and/or protonated molecule, while the high-energy function was used to evaluate fragment ion information. Taking profit of the hybrid analyzer, tandem MS (MS/MS) experiments at different collision energies were also performed, in some cases, in order to improve the understanding of the fragmentation of the molecular ion or the protonated molecule, increasing reliability.

RESULTS AND DISCUSSION

The analytical non-target methodology proposed based on the combination of GC-(EI)TOF MS and GC-(APCI)QTOF MS was applied to 10 samples obtained from migration tests using isooctane and Tenax® as food simulants, and their corresponding blank samples.

In a first step, sample extracts were analyzed by using GC-(EI)TOF MS. In order to obtain spectra as pure as possible, a GC temperature program with a single soft temperature ramp was used to get a good chromatographic separation and reduce coelutions. Both library searching and accurate mass measurement of the five most intense ions were applied and tentative candidates were obtained. In order to confirm or reject those identifications, samples were re-analyzed by using GC-(APCI)QTOF MS. Searching for the molecular ion and the protonated molecule in the APCI mass spectra revealed essential information about the candidates proposed by (EI)TOF MS. Thus, in those cases where the absence of the molecular ion in the EI spectra made difficult the correct identification, molecular ion information obtained from the soft ionization occurred in the APCI source was useful.

After sample analysis, 18 detected peaks accomplished the established requirements of proposed strategy by (EI)TOF MS and (APCI)QTOF MS (**Table 1**). The number of the candidates obtained by (EI)TOF MS were reduced by approximately half after applying (APCI)QTOF MS (from a total of 63 candidates proposed by (EI)TOF for these 18 detected peaks, 36 were tentatively identified by (APCI)QTOF MS). However, in many cases, still more than one structure could justify the identity of a chromatographic peak due to the isomerism. As it can be seen in **Table 1**, discarding among those structures was not always feasible in spite of performing MS/MS experiments. Only the acquisition of commercial standards would ensure the unequivocal identity. After the injection of 21 available standards by GC-(APCI)QTOF MS, 8 compounds could be confirmed as positives and 3 identifications were rejected based on retention time and ionization and fragmentation behavior. The remaining detected peaks could not be finally confirmed

due to the lack of their corresponding commercial standards and they were considered as tentatively identified.

Table 1. Migrants detected in samples coming from the simulants isooctane and Tenax® after applying the combination of GC-(EI)TOF MS and GC-(APCI)QTOF MS. Confirmed compounds are shown in bold.

Rt (TOF) (min)	Rt (QTOF) (min)	CAS No	Candidates number by (EI)TOF MS	Candidates by (APCI)QTOF MS	Formula	Commercial standards	Status
5.55	**4.28**	**110-81-6**	2	**Diethyl disulfide**	**C4H10S2**	available	**positive**
7.04	5.96	632-22-4	1	Tetramethylurea	C5H12N2O	available	negative
7.26	6.3	556-67-2	3	Octamethylcyclotetrasiloxane	C8H24O4Si4	available	negative
13.96	12.98	622-63-9	6	Ethyl p-tolylsulfide	C9H12S	available	negative
		-		Benzene, 1-(ethylthio)-3-methyl-	C9H12S	n.a.	tentative
18.7	17.84	115754-89-7	5	2-(1-Hydroxycycloheptyl)-furan	C11H16O2	n.a.	tentative
18.8	**17.93**	**6781-42-6**	5	**m-Acethyl acetophenone**	**C10H10O2**	available	**positive**
		1009-61-6		p-Acethyl acetophenone	C10H10O2	available	negative
		1689-09-4		3,3-Dimethyl-2-benzofuran-1(3H)-one	C10H10O2	n.a.	negative
19.65	**18.81**	**719-22-2**	3	**2,6-di-tert-butyl-p-benzoquinone (2,6-DTBQ)**	**C14H20O2**	available	**positive**
20.68	**19.9**	**96-76-4**	6	**2,4-di-tert-butyl-phenol (2,4-DTB)**	**C14H22O**	available	**positive**
		1138-52-9		3,5-di-tert-butyl-phenol	C14H22O	n.a.	negative
		5875-45-6		2,5-di-tert-butyl-phenol	C14H22O	n.a.	negative
		50356-17-7		2,6-di-tert-butyl-phenol	C14H22O	n.a.	negative
20.75	19.98	97123-41-6	5	Butylated Hydroxytoluene	C15H24O	available	negative
		2934-07-8		2,4,6-Triisopropylphenol	C15H24O	n.a.	tentative
		616-55-7		2,4-Di-tert-butyl-6-methylphenol	C15H24O	available	negative
22.84	22.03	2254-94-6	4	2-Benzothiazolinethione, 3-methyl-	C8H7NS2	n.a.	tentative
		51333-80-3		3-(Methylthio)phenyl isothiocyanate	C8H7NS2	available	negative
		51333-75-6		2-(methylthio)phenyl isothiocyanate	C8H7NS2	available	negative
		64036-43-7		Benzothiazolethiol, 2-methyl-	C8H7NS2	n.a.	tentative
23.9	23.1	28291-69-2	2	2-(Ethylamino)-1,3-benzothiazole	C9H10N2S	n.a.	tentative
		29927-08-0		5,6-Dimethyl-2-aminobenzothiazole	C9H10N2S	available	negative
24.59	23.84	489-84-9	5	Guaiazulene	C15H18	available	negative
		483-78-3		Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	C15H18	n.a.	tentative
		489-77-0		6-Isopropyl-1,4-dimethylnaphthalene	C15H18	n.a.	tentative
28.55	**27.93**	**84-69-5**	8	**Diisobutyl phthalate**	**C16H22O4**	available	**positive**
		84-74-2		Dibutyl phthalate	C16H22O4	available	negative
		17851-53-5		1-Butyl 2-isobutyl phthalate	C16H22O4	n.a.	negative
30.46	**29.88**	**84-74-2**	-	**Dibutyl phthalate**	C16H22O4	available	**positive**
31.82	31.13	115725-44-5	1	Cyclic octaatomic sulfur	S8	n.a.	tentative
32.22	**31.67**	**103-19-5**	2	**p-Tolyldisulfide**	**C14H14S2**	available	**positive**
40.42	**40.34**	**117-81-7**	4	**Bis(2-ethylhexyl) phthalate (DEHP)**	**C24H38O4**	available	**positive**
		27554-26-3		Diisoctyl phthalate	C24H38O4	available	negative
		117-84-0		Di-n-octyl phthalate	C24H38O4	available	negative
42.32	42.32	132-60-5	1	Cinchophen	C16H11NO2	available	negative

n.a. not available

As an illustrative example of the methodology performed for the investigation of potential migrants in the samples studied, **Figure 1** shows a GC-(EI)TOF MS experimental accurate mass spectrum (A) of a detected peak found in three samples at 28.55 min, which presented a library match >700 for eight different candidate compounds (B-I). These spectra are all characterized by the absence of the M+· and the abundant presence of the *m/z* ion 149, whose structure can derive from any of the eight candidates with an accurate mass in accordance with the experimental value. Although some of the matched compounds have different molecular masses (see **Figure 1**), the high fragmentation degree observed in the experimental EI spectrum of the unknown compound did not allow assuring its molecular mass. Thus, none of the eight possible compounds could be discarded with this first approach using (EI)TOF MS.

The soft ionization provided by GC-(APCI)QTOF MS resulted crucial in order to investigate the mentioned example. When nw-XICs (±0.01 Da) were obtained for the different four *m/z* values corresponding to the eight protonated molecules proposed in **Figure 1** using their exact masses, only a chromatographic peak at $[M+H]^+$ 279.1596 was observed at the expected retention time 27.95 min (**Figure 2**). So, after evaluating the corresponding LE spectrum, the previous list of eight candidates was reduced to three compounds with molecular formula $C_{16}H_{22}O_4$ (MW=278.1518). The information derived from the HE did not reveal additional information about the fragmentation; neither MS/MS experiments could be helpful to find distinguishing fragments due to the isomerism between the three candidates. In order to guarantee the unequivocal confirmation, the available commercial standards were acquired and their injection under GC-(APCI)QTOF MS confirmed the peak identity as diisobutyl phthalate due to ionization, fragmentation and retention time accordance.

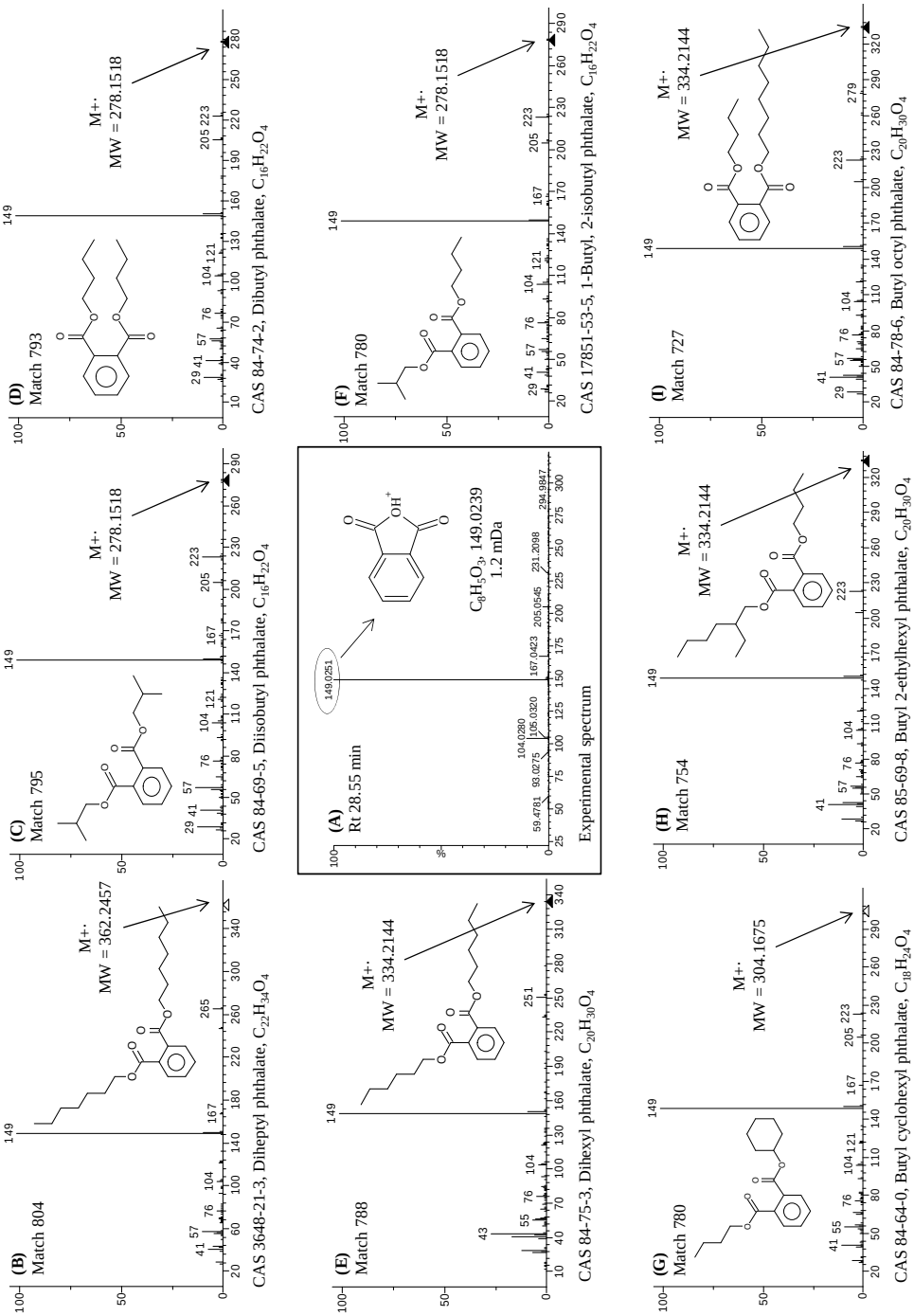

Figure 1. Theoretical mass spectra of the different candidates (B-I) that fit with the experimental spectrum (shown in the center, A) for a chromatographic peak obtained by GC-(EI)TOF MS.

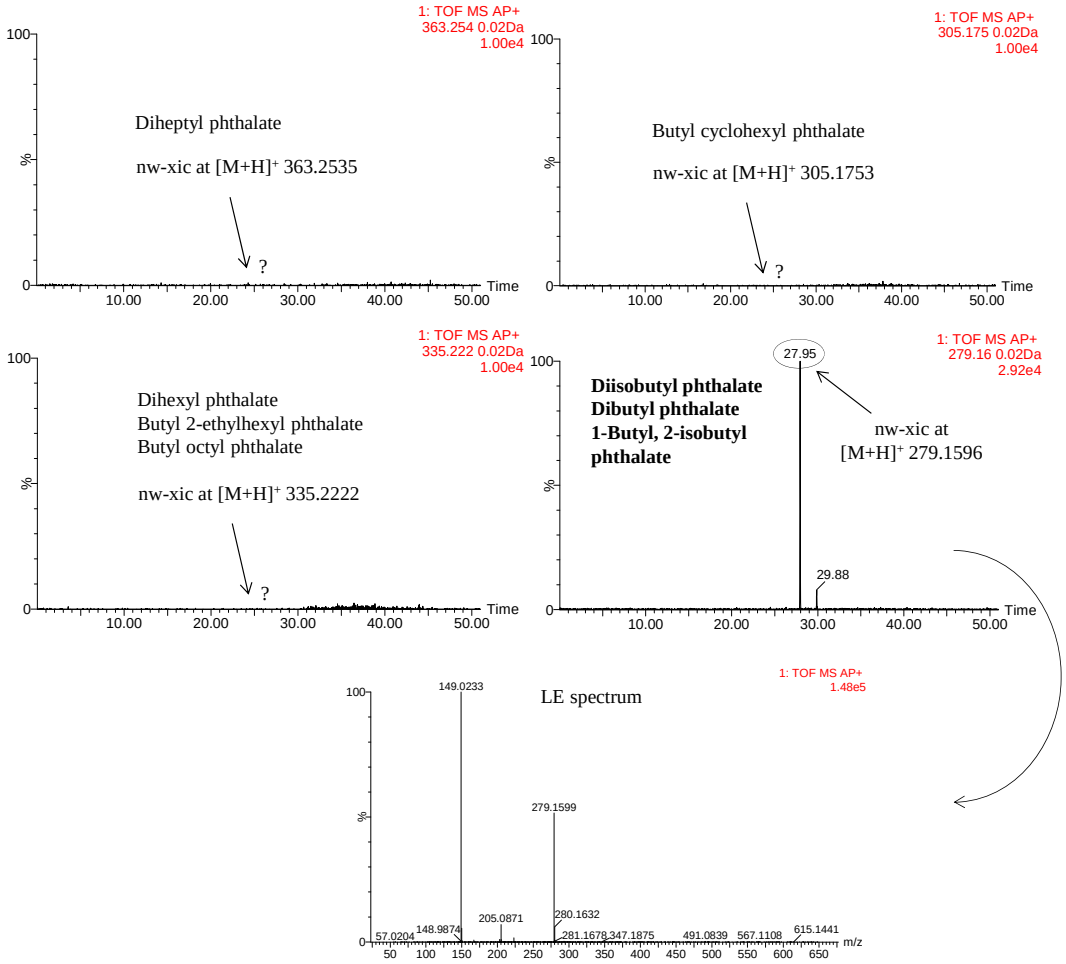

Figure 2. nw-XIC from the (APCI)QTOF MS data for the corresponding protonated molecule of the candidates in **Figure 1**. LE spectrum of the detected peak at 27.95 min.

Moreover, the aforementioned example gave more relevant information as an additional chromatographic peak at 29.88 min was observed in the nw-XIC at *m/z* ion 279.1596 by (APCI)QTOF MS (see **Figure 2**) and unnoticed by (EI)TOF MS. The LE and HE functions of this peak were identical to that at 27.95 min, probably corresponding to an isomer of the identified positive. Luckily, the injection of the commercial standards acquired confirmed the peak identity as dibutyl phthalate.

Figure 3 shows another singular example which proved the potential of the analytical strategy proposed. The experimental spectrum obtained from a chromatographic peak detected by GC-(EI)TOF MS in two samples (see **Figure 3 a**) presented a library match >750 with the theoretical spectra of two isomeric compounds, but EI fragmentation did not reveal significant information to distinguish between them. Then, the samples were analyzed by GC-(APCI)QTOF MS and the fragmentation under these conditions, and using water as modifier, provided the fragments 154.9992 and 91.0550 (see **Figure 3 b**). The structure proposed for those fragments only could be originated from the candidate p-tolyldisulfide. The injection of the commercial standard confirmed its identity.

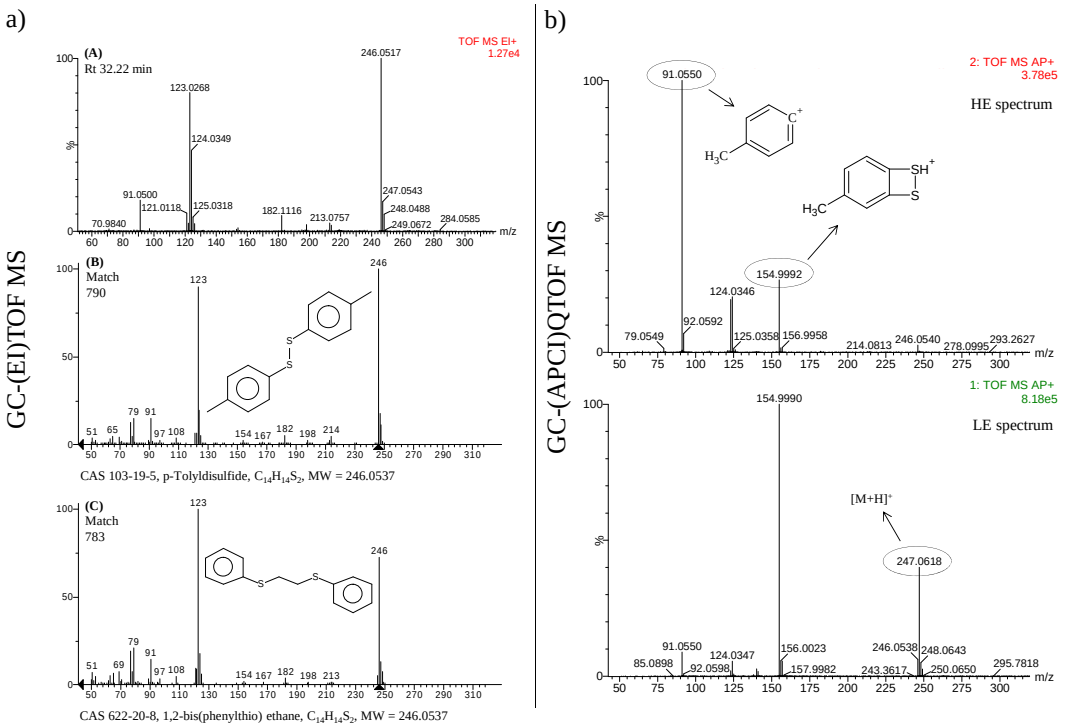

Figure 3. a) Experimental spectrum (A) obtained by GC-(EI)TOF MS for the peak at 32.22 min. Theoretical mass spectra (B-C) of the two candidates proposed for the unknown compound given in (A). b) Low and high energy spectra from the chromatographic peak obtained by GC-(APCI)QTOF MS for the unknown compound detected by GC-(EI)TOF MS.

As an example of the confirmation with the commercial standards, **Figure 4** shows a positive finding of an isomer of the di-tert-butyl phenol (DTB) in an isooctane sample. In this case, after performing the methodology developed based on GC-(EI)TOF MS and GC-(APCI)QTOF MS, four isomers were possible candidates for the chromatographic peak at the retention time 20.68 min (see **Table 1**). The commercial standards could not be acquired for three of them. Only 2,4-DTB was available and its confirmation could be expected as it is a common finding in plastic related studies. As it can be observed, the same retention time in the sample and in the standard solution (**Figure 4 a**) and the mass accuracy deviations calculated lower than 0.5 mDa (**Figure 4 b**) confirmed its identity.

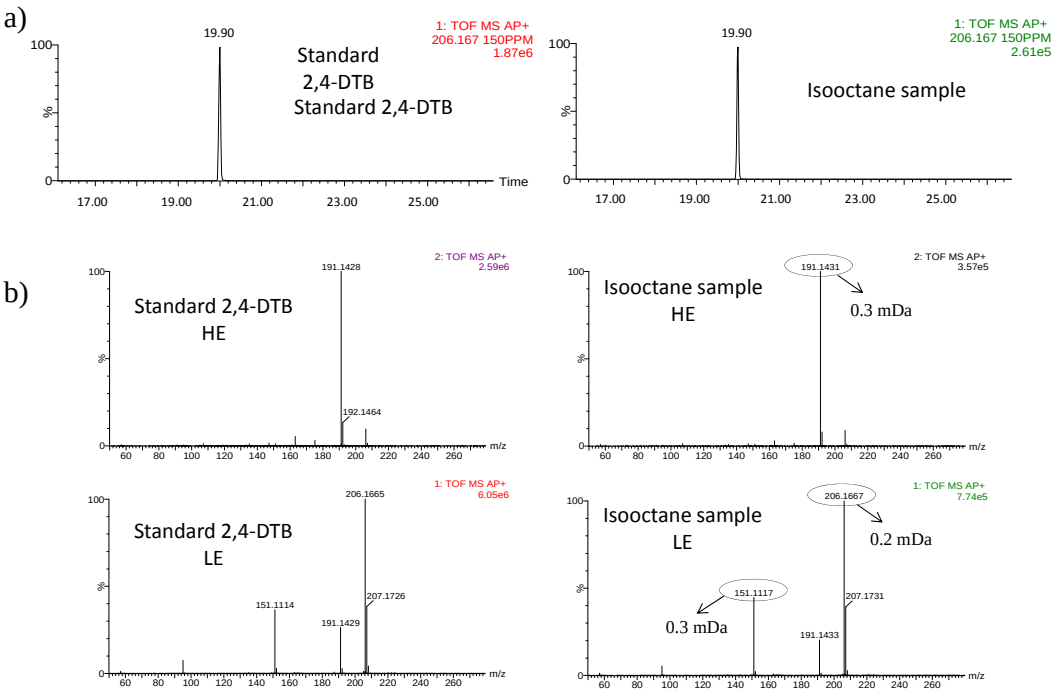

Figure 4. Positive finding of 2,4-DTB in an isooctane sample. a) GC-(APCI)QTOF MS nw-XICs chromatograms for 2,4-DTB in the sample and standard solution. b) Mass spectra at high and low energy functions for 2,4-DTB in the sample and standard solution.

As a summary of the results obtained, **Figure 5** shows the detection frequency of the potential migrants confirmed in the 10 samples analyzed coming from both simulants isooctane and Tenax®. Among positive findings, only DEHP [117-81-7] and dibutyl phthalate [84-74-2] are compounds regulated in the European Regulation No 10/2011, with their corresponding SML. They are common plasticizers which can be only used in articles containing non-fatty foods, according to the mentioned directive. Residues of these migrants are usually found in plastic bottled waters (Schmid *et al.*, 2008; Bach *et al.*, 2012; Al-Saleh *et al.*, 2011; Lee *et al.*, 2011) and can be also detected in food packaging materials (Fromme *et al.*, 2011; Aznar *et al.*, 2011). The rest of the identified compounds are non-regulated substances and there is no protocol on what should be done when NIAS are identified. The migrants more frequently detected were 2,4-DTB [96-76-4], present in all samples analyzed, and 2,6-DTBQ [719-22-2], identified in three and two samples coming from isooctane and Tenax®, respectively. Both compounds are common degradation products from the antioxidants Irganox 1010 and Irgafos 168 (Denberg *et al.*, 2009) and they are frequently detected as NIAS in migration studies (Félix *et al.*, 2012; Nerin *et al.*, 2013; Vera *et al.*, 2011; Skjevrak *et al.*, 2005). Diisobutyl phthalate [84-69-5], found in three samples, is a plasticizer commonly associated with printing inks and it has been also reported as NIAS in plastic films (Skjevrak *et al.*, 2005; Félix *et al.*, 2012). p-Tolyldisulphide [103-19-5], a rubber accelerator, was present in two Tenax® samples, as well as diethyl disulphide [110-81-6], which is a by-product of the commercial production of ethanethiol, an intermediate and starting material in manufacture of plastics. m-Acethyl acetophenone [6781-42-6] was identified in one sample coming from Tenax® but the lack of awareness in the literature makes difficult to know about their properties and migration from plastic materials.

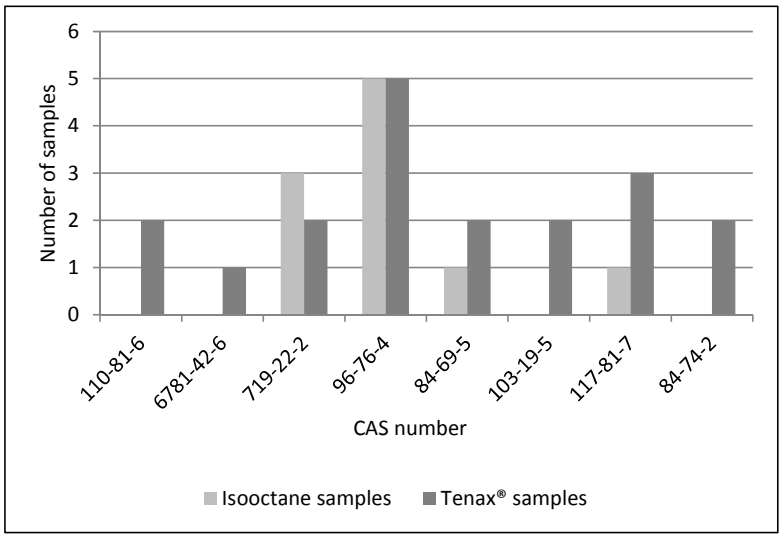

Figure 5. Frequency distribution of migrants confirmed in 10 samples analyzed by GC-(EI)TOF MS and GC-(APCI)QTOF MS after performing a migration study using the simulants isooctane and Tenax®.

CONCLUSIONS

An analytical strategy consisting on the combined used of GC-(EI)TOF MS and GC-(APCI)QTOF MS has been applied to perform a non-target analysis of samples coming from two food simulants, isooctane and Tenax®, which had been previously in contact with food packaging materials. The use of two different and complementary ionization sources (EI and APCI) has notably enhanced the identification potential.

Data processed from GC-(EI)TOF MS allowed detecting several migrants and assigning tentative formulae in accordance to library matches. However, the high fragmentation occurred under EI made doubtful the confirmation/rejection process based on the commercial library matches mainly due to the lack of information about the elemental composition. On the contrary, taking profit of the soft ionization provided by the APCI, the presence of the molecular or quasi molecular ion was used for the confirmation/rejection of the previous findings, facilitating a rapid and

sensitive screening. The fragmentation behavior could also be studied by evaluating the HE data (MS^E mode) and/or by performing MS/MS experiments in order to justify the formulae of each fragment proposed, which was feasible thanks to the use of a QTOF instrument.

Analysis by GC-(APCI)QTOF MS allowed reducing considerably the number of candidates previously proposed by GC-(EI)TOF MS. In many cases, around half of candidates from a detected peak by (EI)TOF could be rejected after searching for the molecular ion/protonated molecule in (APCI)QTOF and/or studying the fragmentation under these conditions. The unequivocal confirmation required the injection of the reference standards, although not all of them were commercially available. The injection of 21 standards allowed the confirmation of the identity of 8 migrants, from which DEHP and dibutyl phthalate are regulated in the European Regulation No 10/2011, and the rest considered as NIAS.

In most cases, the difficulty of arriving to conclusive results was evident in this kind of samples due to the extensive list of possible structures that are compatible with the data acquired, especially due to the isomeric nature of most candidates. Identification of unknowns is a challenge and, in addition, when standards are available their acquisition involves a considerable expense without ensuring conclusions, but the powerful combination of techniques applied in this work allowed a rapid screening that simplified and facilitated the identification process.

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IV. 3. Discusión de los resultados obtenidos

La investigación de compuestos desconocidos procedentes de materiales de envasado alimentario resulta de especial importancia ante la cantidad de contaminantes imprevistos que pueden migrar hasta el alimento. Muchos de estos compuestos no están regulados en las directivas, debido a la imposibilidad de conocer todas las impurezas y productos de degradación o descomposición, por lo que el desarrollo de métodos de análisis *non-target* debería contribuir a ampliar las listas incompletas de migrantes. No obstante, la identificación inequívoca en un análisis de desconocidos no resulta tarea sencilla y supone un consumo de tiempo y dinero considerables, ya que el tratamiento de los datos es laborioso y requiere la confirmación con patrones estándar comerciales. Las aplicaciones informáticas que realizan el procesamiento automático de los cromatogramas, como el ChromaLynx, un módulo del software MassLynx, permiten agilizar el trabajo, siendo necesario seguir una estrategia para el proceso de búsqueda e identificación, en función de la técnica analítica aplicada, como ya se ha comentado en la introducción de este capítulo.

Análisis por GC-(EI)TOF MS y GC-(APCI)QTOF MS

Las muestras en isooctano y Tenax® obtenidas a partir de estudios de migración en materiales plásticos fueron analizadas por GC-(TOF y QTOF) MS, como se describe en el Artículo Científico 7. El procesamiento de los datos con el software ChromaLynx simplificó el proceso de identificación de los picos detectados gracias a la obtención automática de los espectros de deconvolución y posterior comparación de los mismos con librerías espectrales teóricas. La disponibilidad de estas librerías de espectros por EI facilita la búsqueda de desconocidos ya que, tras el procesamiento, se generan listas de candidatos ordenados por porcentaje de analogía con un espectro teórico (*library match*). Aunque esta comparación se basa en la presencia o ausencia de los iones característicos de los espectros en masa nominal y no resulta demasiado específica o selectiva, permitió realizar un primer cribado de

gran utilidad para una primera aproximación a la identidad del compuesto desconocido.

Los casos más ambiguos surgieron entre compuestos pertenecientes a la misma familia o con fragmentos comunes en su estructura química, presentando una elevada similitud en sus espectros de masas. Además, la frecuente ausencia del pico molecular como consecuencia de la alta energía emitida en la fuente EI perjudicó a la selectividad, al igual que el predominio de fragmentos de baja m/z. La mayoría de los picos detectados en las muestras analizadas presentaron este problema al tratarse de moléculas de bajo peso molecular y altamente fragmentadas. Así, los correspondientes espectros de deconvolución generaron *library match* relativamente altos para un número considerable de compuestos, como en el ejemplo que se muestra en la **Figura 1** del Artículo científico 7 (pág. 295).

A partir de la lista de candidatos propuestos tras la búsqueda en la librería espectral, el ChromaLynx somete las fórmulas de los mismos al cálculo de la composición elemental, midiendo la masa exacta de los cinco iones más intensos. El error de masa calculado automáticamente para cada uno de estos iones permitió aplicar un criterio de confirmación o rechazo del analito en cuestión. Con esta elevada selectividad que confiere la exactitud de masa medida con el TOF, la lista inicial de candidatos se redujo notablemente en muchos casos.

El uso complementario de GC-QTOF MS con la fuente APCI supuso una ventaja adicional gracias a la presencia del pico molecular o de la molécula protonada en los espectros de masas, ya que en caso de localización de estos iones en un tiempo de retención similar al del pico estudiado por GC-(EI)TOF MS, la probabilidad de confirmación es mayor. Así, los nw-XICs a las masas exactas de los correspondientes $M^{+\bullet}$ o $[M+H]^+$ permitieron descartar más candidatos ante la ausencia de los mismos, como se ilustra en la **Figura 2** del Artículo científico 7 (pág. 296). La **Tabla 1** del mismo artículo (pág. 293) demuestra igualmente el potencial del uso del APCI-QTOF para descartar candidatos obtenidos por EI-TOF, en muchos casos hasta alrededor de la mitad de candidatos descartados.

No obstante, el proceso de identificación por GC-(TOF y QTOF) MS propuesto no resultó suficiente para muchos picos cuyos espectros, especialmente los detectados a niveles de concentración mayores, coincidían con el patrón de fragmentación típico de los hidrocarburos. Dada la extensa variedad de formulaciones y estructuras posibles y la elevada similitud en los espectros de masas de muchos de ellos (caracterizados por una alta fragmentación del pico molecular y la presencia de iones de baja relación *m/z*), la confirmación de estos compuestos no resultó factible. La búsqueda en librería de los espectros de deconvolución obtenidos por EI-TOF para todos estos picos generó un número elevado de candidatos (en general alcanos) con altos *match*, únicamente distinguibles entre ellos por el pico molecular. En este punto el uso del GC-(APCI)QTOF MS podría ser la clave para aproximarse a la identidad de estos compuestos. Sin embargo, la ionización en APCI para éstos no fue la esperada y ni el pico molecular ni la molécula protonada pudieron ser localizados (ni siquiera los fragmentos típicos observados en EI), al menos para los candidatos con *match* más elevados. La búsqueda por similitud en el tiempo de retención con los picos obtenidos por GC-(EI) TOF tampoco fue factible ya que, como se observa en la **Figura IV.1** para una misma muestra analizada por las dos técnicas, no se aprecia una tendencia similar en el cromatograma del APCI-QTOF que permita localizar los picos tan fácilmente como en el obtenido por EI-TOF.

En la bibliografía estos compuestos se suelen identificar conjuntamente como familia química (Simoneau *et al.*, 2002). Su identificación individual requiere información más precisa como la que podría obtenerse a partir del índice de Kovats, consistente en un índice de retención que describe el comportamiento de un compuesto en una columna cromatográfica en relación a una mezcla de n-alcanos en las mismas condiciones cromatográficas con el fin de predecir el tiempo de elución (Félix *et al.*, 2012; Nerín *et al.*, 2013).

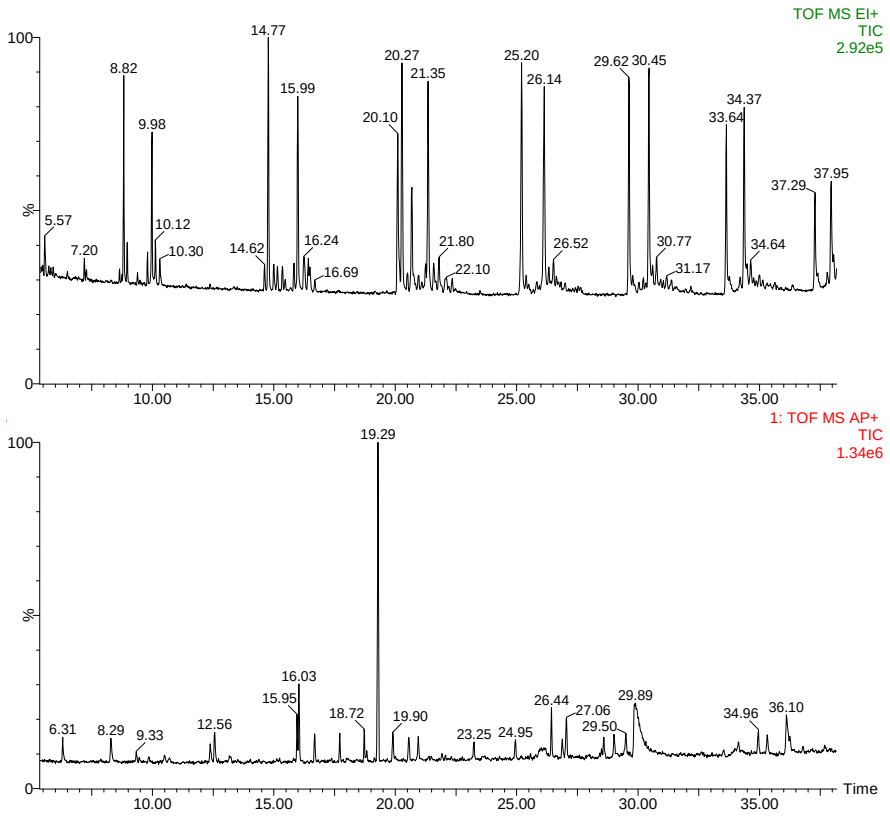


Figura IV.1. Cromatograma en *full scan* de una muestra en el simulante isooctano adquirido mediante GC-(EI)TOF MS (figura superior) y GC-(APCI)QTOF MS (figura inferior).

A pesar de la ausencia del pico molecular, el uso del GC-(APCI)QTOF MS también puede contribuir a la identificación de un compuesto con información relativa a la fragmentación. La función de alta energía adquirida en MS^E proporcionó información estructural útil en muchos casos, como en el ejemplo mostrado en la **Figura 3 b** del Artículo científico 7 (pág. 297), en el que la presencia de fragmentos característicos pudo justificar la identidad de uno de los dos candidatos tentativos. Herramientas como el MassFragment (aplicación del MassLynx) sirven de apoyo para estudiar la fragmentación y evaluar los errores de masa, calculados automáticamente, de los fragmentos propuestos.

La realización de experiencias adicionales en MS/MS suponen un paso más hacia una segura identificación gracias a la obtención de espectros con iones producto en masa exacta procedentes de la fragmentación del $M^{+\bullet}/[M+H]^+$. No obstante, la isomería existente entre candidatos para un mismo pico dificultó su distinción ante la imposibilidad de obtener fragmentos característicos o diferenciadores, también debido al bajo peso molecular de la mayoría de compuestos tentativos.

Llegados a este punto, la confirmación inequívoca únicamente puede darse con la inyección de los patrones estándar comerciales. Cabe destacar que de la lista de 63 candidatos inicialmente propuestos por EI-TOF para los 18 picos detectados, 27 pudieron ser descartados tras el uso de APCI-QTOF. Así, con la aplicación complementaria de estas técnicas, además de simplificar el proceso de identificación, la confirmación no requirió la adquisición de un número tan elevado de patrones comerciales.

Análisis por UHPLC-(ESI)QTOF MS

El estudio complementario realizado mediante UHPLC-(ESI)QTOF MS permitió ampliar el *screening* cubriendo un mayor rango de compuestos para las muestras procedentes de materiales plásticos en los simulantes ácido acético al 3% y etanol al 20%. También se analizaron mediante UHPLC dos muestras de envases metálicos recubiertos en los mismos simulantes.

A diferencia del análisis por GC-(EI) MS, la falta de librerías de espectros para LC-(ESI) MS dificulta la ejecución de un análisis *non-target*, por lo que la búsqueda de desconocidos partió de una base de datos creada en nuestro laboratorio (modo *post-target*) a partir de la lista de sustancias reguladas en el Reglamento 10/2011 de la Unión Europea (*Commission Regulation (EU) No 10/2011*). En esta base de datos se incluyeron alrededor de 70 plastificantes, 30 colorantes, 80 tintas de impresión, 250 monómeros y otras sustancias de partida y 250 aditivos y productos de polimerización. Así, la probabilidad de identificación de picos mediante LC-MS es menor, ya que la búsqueda inicial se centra únicamente en alrededor de 700 compuestos contenidos en la base de datos, a diferencia de los miles de compuestos

que pueden encontrarse en librerías electrónicas como la NIST, empelada en la metodología anterior por GC-MS, resultando inviable la detección de NIAS.

La adquisición *full scan* en modo MS^E se procesó con el ChromaLynx, capaz de buscar automáticamente la molécula protonada o desprotonada –en función del modo de ionización aplicado (ESI+ o ESI-, respectivamente)– a partir de la información incluida en la base de datos. Los cromatogramas se muestran en ventanas nw-XIC a las masas exactas del ión molecular (protonado o desprotonado) con el cálculo automático del error de masa, por lo que se pudo evaluar fácilmente la lista generada de candidatos, descartando aquellos con errores de masa superiores a 2 mDa. Para los picos considerados como tentativos se estudió la fragmentación mediante la evaluación de los espectros de masas de alta y baja energía, haciendo a la vez uso del MassFragment. En muchos casos, la baja sensibilidad obtenida, especialmente en la función de alta energía, dificultó el estudio de la fragmentación, disponiendo únicamente de la información relativa al pico molecular como apoyo para la identificación.

Para las muestras procedentes de materiales plásticos sólo se obtuvieron candidatos con la fragmentación justificada para cuatro picos cromatográficos. La lista de compuestos propuestos se muestra en la **Tabla IV.2**. Sin embargo, la posterior adquisición e inyección de los correspondientes patrones estándar, todos disponibles comercialmente, descartó la confirmación de la identidad de todos ellos. Dada la lista de compuestos incluidos en la base de datos, es posible que estos picos no identificados correspondan a isómeros de los candidatos propuestos no considerados en las directivas.

Tabla IV.2. Candidatos propuestos en las diferentes muestras procedentes de materiales plásticos en los simulantes ácido acético 3% y etanol 20% analizadas por UHPLC-(ESI)QTOF MS.

tR (min)	N° CAS	Candidatos	Fórmula	Patrón comercial	Estatus
1.19	2403-88-5	2,2,6,6-Tetrametil-4-piperidinol	$C_9H_{19}NO$	Disponible	Negativo
3.5	13811-50-2	1,3-Divinil-2-imidazolidinona	$C_7H_{10}N_2O$	Disponible	Negativo
3.87	52722-86-8	Hidroxietil tetrametilpiperidinol	$C_{11}H_{23}NO_2$	Disponible	Negativo
	2432-99-7	Ácido 11-aminoundecanoico	$C_{11}H_{23}NO_2$	Disponible	Negativo
9.68	693-57-2	Ácido 12-aminoundecanoico	$C_{12}H_{25}NO_2$	Disponible	Negativo

Por otro lado, en la **Tabla IV.3** se indican los candidatos propuestos para otros cuatro picos detectados en las muestras procedentes de envases metálicos. En este caso uno de ellos, la 2,4-diamino-6-fenil-1,3,5-triazina o benzoguanamina, pudo ser confirmado en una muestra en el simulante ácido acético al 3%.

Tabla IV.3. Candidatos propuestos en las diferentes muestras procedentes de envases metálicos en los simulantes ácido acético 3% y etanol 20% analizadas por UHPLC-(ESI)QTOF MS. En negrita se muestra el compuesto confirmado.

tR (min)	N° CAS	Candidatos	Fórmula	Patrón comercial	Estatus
3.06	120-47-8	Etilparabeno	$C_9H_{10}O_3$	Disponible	Negativo
4.2	**91-76-9**	**Benzoguanamina**	**$C_9H_9N_5$**	**Disponible**	**Positivo**
5.95	120-51-4	Bencil benzoato	$C_{14}H_{12}O_2$	Disponible	Negativo
6.78	94-13-3	Propilparabeno	$C_{10}H_{12}O_3$	Disponible	Negativo

Como se observa en la **Figura IV.2**, la confirmación de este compuesto se demostró por la similitud en el tiempo de retención cromatográfico y en el patrón de fragmentación (en las funciones de alta y baja energía) con el patrón estándar, obteniéndose bajos errores de masa en los iones fragmento procedentes de la función de alta energía (≤1 mDa).

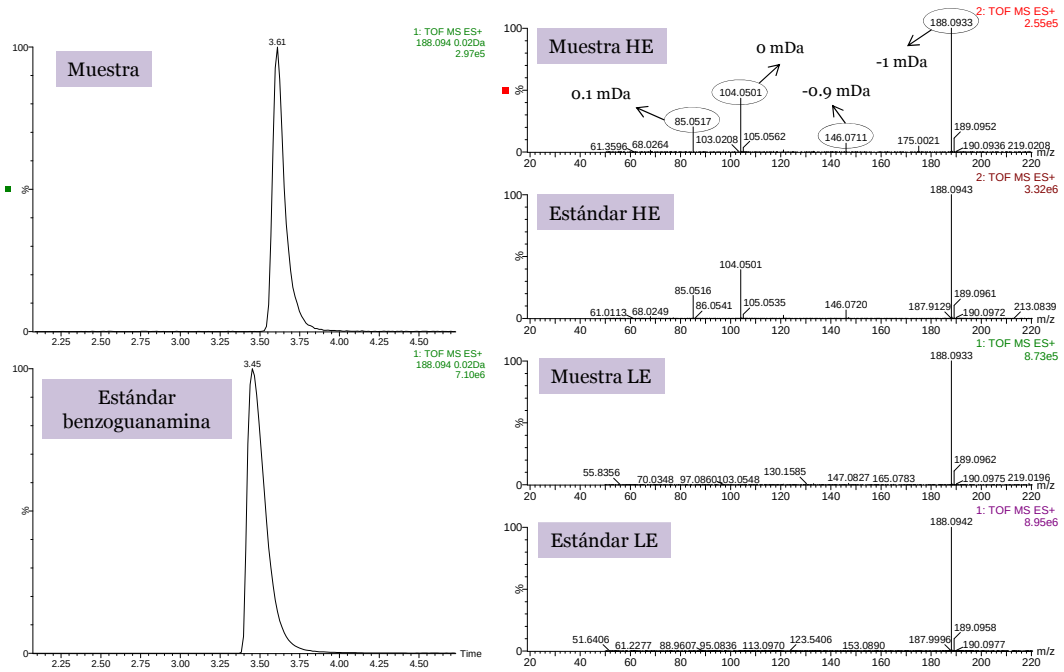
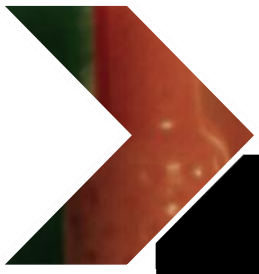


Figura IV.2. Cromatogramas obtenidos por UHPLC-QTOF MS (izda.) correspondientes al positivo benzoguanamina en una muestra procedente de envases metálicos en el simulante ácido acético 3%. Espectros de masas de las funciones de alta energía (HE) y baja energía (LE) (dcha.) para muestra y patrón.

Los derivados de la triazina como la benzoguanamina suelen emplearse como monómeros en la elaboración de resinas; así, su presencia en este tipo de envases metálicos recubiertos podría asociarse al uso de resinas como revestimiento para los mismos. El Reglamento 10/2011 de la Unión Europea (*Commission Regulation (EU) No 10/2011*) referente a materiales plásticos también se aplica a la categoría de revestimientos e incluye la regulación de esta sustancia, donde se especifica su autorización como monómero o sustancia de partida y no como aditivo o auxiliar para la producción de polímeros.

Considerando el bajo número de sustancias detectadas por LC y la limitación asociada al uso de una base de datos con un número determinado de compuestos, es

posible que los migrantes más abundantes sean aquellos no incluidos en las directivas. La identificación de sustancias desconocidas mediante LC supone un mayor reto comparado con GC ante la falta de librerías espectrales, aunque en ambos casos el análisis *non-target* entraña dificultades asociadas a la falta de información de las muestras (de los materiales empleados en el envasado), sobre todo por la presencia de sustancias imprevistas. El uso combinado de estas técnicas acopladas a analizadores poderosos como el TOF y QTOF supone una herramienta avanzada que ha aportado información valiosa relativa a la identidad de los compuestos capaces de migrar desde el envase alimentario.



Capítulo V

Conclusiones

Como conclusión general de los trabajos que componen esta Tesis Doctoral cabe resaltar la eficacia del acoplamiento GC-MS(/MS) para la determinación (detección, identificación y cuantificación) de contaminantes orgánicos en una amplia variedad de matrices alimentarias y medioambientales. La disponibilidad de diferentes analizadores compatibles con esta técnica le confiere una elevada versatilidad, permitiendo adaptarse a necesidades concretas. Así, el uso de analizadores como el cuadrupolo simple y el QqQ han resultado adecuados para aplicaciones cuantitativas, mientras que el TOF y QTOF han demostrado un gran potencial para fines cualitativos. Además, la reciente comercialización de la fuente APCI acoplada a GC-MS promete mejoras en cuanto a sensibilidad, selectividad y capacidad de confirmación con respecto a los métodos clásicos por EI, tanto en análisis *target* como en *non-target*.

Las conclusiones específicas que se pueden extraer de manera resumida son las siguientes:

1. La aplicación de *fast* GC-MS con un cuadrupolo simple como analizador en el análisis de aguas y alimentos ha permitido desarrollar y validar satisfactoriamente métodos capaces de determinar contaminantes orgánicos a bajos niveles de concentración (LODs: 0.1-10 ng/L en aguas, 0.5-10 μg/kg en frutas y 0.5-10 μg/L zumos). La alta sensibilidad conseguida ha venido favorecida por la adquisición en modo SIM, al igual que la selectividad del método con excepción de ciertas combinaciones matriz/analito, especialmente en el análisis de muestras de alimentos. Los métodos de extracción QuEChERS y SPE han demostrado ser tratamientos de muestra simples, rápidos y efectivos que, combinados con *fast* GC, permiten aumentar el rendimiento por muestra analizada.

2. El uso del cuadrupolo simple como analizador en *fast* GC ha resultado adecuado (aunque con algunas limitaciones) para el desarrollo de métodos basados en la determinación de alrededor de 60 analitos en menos de 10 minutos. Dada la fuerte dependencia del número de iones incluidos en los

grupos SIM sobre el tiempo de escaneo y, a su vez, sobre el número de puntos por pico, la optimización de las condiciones GC-MS requiere establecer un compromiso entre una buena resolución cromatográfica (para separar adecuadamente los grupos SIM) y un tiempo corto.

3. La nueva fuente APCI acoplada a GC-MS con analizador de triple cuadrupolo ha demostrado ser una herramienta eficaz para la determinación de pesticidas en alimentos. La alta sensibilidad (LODs obtenidos entre 0.01 y 1 μg/kg para la mayoría de compuestos en las diversas matrices estudiadas) y selectividad se han visto favorecidas por la presencia del ión molecular o quasi-molecular, el cual ha sido seleccionado como ión precursor en las transiciones SRM de la mayoría de los compuestos estudiados. Ello ha permitido el desarrollo y validación de un método multiresiduo para la determinación de 142 pesticidas en frutas y verduras en un tiempo cromatográfico corto (24 min). Cabe destacar que la dilución del extracto de ACN tras la aplicación del método QuEChERS (necesaria para la inyección en *splitless*) ha sido posible gracias a la elevada sensibilidad conseguida, simplificando el tratamiento de muestra.

4. Se ha comprobado que la aplicación de GC-(APCI)QqQ MS/MS en el campo de las dioxinas es comparable al uso de técnicas de alta resolución tradicionalmente empleadas para la determinación de estos contaminantes en muestras complejas a niveles del orden de femtogramos. El método desarrollado ha permitido la determinación de las 17 dioxinas y furanos incluidas en el método EPA-1613 con resultados satisfactorios.

5. Con el uso del QqQ, la capacidad de confirmación basada en el cumplimiento de las relaciones entre iones viene sobradamente cumplida con la adquisición de dos transiciones SRM. El uso del cuadrupolo simple se encuentra más limitado en este aspecto debido a su menor sensibilidad y selectividad, aunque el cumplimiento de al menos una relación Q/q se ha establecido como requisito para la confirmación de los compuestos estudiados.

6. El uso combinado de técnicas basadas en diferentes mecanismos de ionización como EI y APCI ha facilitado el *screening non-target* y la confirmación de sustancias migrantes desde material de envasado hasta simulantes de alimentos. La disponibilidad de librerías teóricas de espectros por EI y la medida de masa exacta proporcionada por el GC-(EI)TOF MS, junto con la información aportada por el GC-(APCI)QTOF MS sobre la masa molecular, ha resultado una combinación de gran utilidad para la identificación de compuestos desconocidos derivados del procesado de alimentos.

7. Con la aplicación complementaria de UHPLC-(ESI)QTOF MS a la estrategia desarrollada mediante GC-MS, se ha ampliado la investigación de contaminantes procedentes de materiales de envasado alimentario. Aunque la investigación de compuestos *non-target* sobre los que no se tiene información alguna resulta laboriosa, la disponibilidad de estas técnicas tan poderosas puede facilitar y simplificar en gran medida el proceso de identificación.

Conclusions

As a general conclusion from the work developed in this Doctoral Thesis is worth to highlight the efficiency of GC-MS(/MS) for the determination (detection, identification and quantification) of organic pollutants in a wide variety of food and environmental matrices. The availability of different analyzers compatible with this technique provides a high versatility to be adapted to specific requirements. Thus, the use of analyzers as the single and triple quadrupole has been adequate for quantitative applications, while the TOF and QTOF have shown a high potential for qualitative purposes. Moreover, the recent commercialization of the APCI source coupled to GC-MS is promising regarding sensitivity, selectivity and confirmation capability in comparison with the classical methods using EI, in both target and non-target analysis.

The specific conclusions can be summarized as following:

1. The application of fast GC-MS with a single quadrupole as analyzer in water and food analysis has allowed to satisfactorily develop and validate methods able to determine organic pollutants at low concentration levels (LODs: 0.1-10 ng/L in water, 0.5-10 μg/kg in fruits and 0.5-10 μg/L in juices). The high sensitivity achieved has been favored by the acquisition under SIM mode, as well as the method selectivity with the exception of some matrix/analyte combinations, especially in the analysis of food samples. It has been demonstrated that the extraction methods QuEChERS and SPE are simple, fast and effective sample treatments that, in combination with fast GC, allow enhancing the sample throughput.

2. The use of the single quadrupole as mass analyzer in fast GC has resulted adequate (although with some limitations) for the development of methods based on the determination of around 60 analytes in less than 10 minutes. Considering the strong dependence of the number of ions included in the SIM windows over the scan time and also over the number of points per peak, the optimization of the GC-MS conditions requires a compromise

between a good chromatographic resolution (in order to separate adequately the SIM groups) and a short time.

3. The new APCI source coupled to GC-MS with a triple quadrupole analyzer has shown to be an efficient tool for the determination of pesticides in food. The high sensitivity (LODs between 0.01 and 1 μg/kg for the majority of the compounds in the studied matrices) and selectivity have been favored by the presence of the molecular or quasi-molecular ion, which has been selected as precursor in the SRM transitions for most of the studied compounds. This has allowed the development and validation of a multiresidue method for the determination of 142 pesticides in fruits and vegetables in a short chromatographic time (24 min). It is worth to mention that the dilution of ACN QuEChERS extract (needed for splitless conventional injection) has been possible due to the high sensitivity obtained, which allowed simplifying the sample treatment.

4. It has been demonstrated that the application of GC-(APCI)QqQ MS/MS in the dioxins field is comparable to the use of high resolution techniques typically used for the determination of these pollutants in complex matrices at femtogram levels. The developed method has allowed the determination of the 17 dioxins and furans included in the EPA-1613 method with satisfactory results.

5. The confirmation capability based on the accomplishment of two ion ratios is fully achieved with the acquisition of two SRM transitions when using the QqQ. In this sense, the use of the single quadrupole is more limited due to the lower sensitivity and selectivity, although the accomplishment of at least one *Q/q* ratio has been established as requirement for the confirmation of the studied compounds.

6. The combined use of techniques based on different ionization mechanisms as EI and APCI has facilitated the non-target screening and the confirmation of migrating substances from packaging material to food

simulants. The availability of theoretical spectral libraries by EI and the accurate mass measurement provided by the GC-(EI)TOF MS, together with the information provided by GC-(APCI)QTOF MS about the molecular mass, has resulted a useful combination for the identification of unknown compounds derived from food packaging.

7. The complementary application of UHPLC-(ESI)QTOF MS to the strategy developed by GC-MS has allowed extending the investigation of pollutants coming from food packaging. Although the non-target investigation about unknown compounds is laborious, the availability of these powerful techniques can facilitate and simplify the identification process.

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Artículos científicos

ARTÍCULOS CIENTÍFICOS QUE COMPONEN LA TESIS DOCTORAL

Artículo científico 1

Multiclass determination of 66 organic micropollutants in environmental water samples by fast gas chromatography-mass spectrometry

Cherta L., Beltran J., Portolés T., Hernández F.

Analytical and Bioanalytical Chemistry (2012) 402, 2301-2314

Artículo científico 2

Application of fast gas chromatography-mass spectrometry in combination with the QuEChERS method for the determination of pesticide residues in fruits and vegetables

Cherta L., Beltran J., López F., Hernández F.

Food Analytical Methods (2013) 6, 1170-1187

Artículo científico 3

Comparison of simple and rapid extraction procedures for the determination of pesticide residues in fruit juices by fast gas chromatography-mass spectrometry

Cherta L., Beltran J., Pitarch E., Hernández F.

Food Analytical Methods (2013) 6, 1671-1684

Artículo científico 4

Improved gas chromatography-tandem mass spectrometry determination of pesticide residues making use of atmospheric pressure chemical ionization

Portolés T., Cherta L., Beltran J., Hernández F.

Journal of Chromatography A (2012) 1260, 183-192

Artículo científico 5

Application of gas chromatography-(triple quadrupole) mass spectrometry with atmospheric pressure chemical ionization for the determination of multiclass pesticides in fruits and vegetables

Cherta L., Portolés T., Beltran J., Pitarch E., Mol J.G.J., Hernández F.

Journal of Chromatography A (2013) 1314, 224-240

Artículo científico 6

Atmospheric pressure chemical ionization tandem mass spectrometry (APGC/MS/MS) an alternative to high resolution mass spectrometry (HRGC/HRMS) for the determination of dioxins

van Bavel B, Geng D., Cherta L., Nácher-Mestre J., Portolés T., Ábalos M., Sauló J., Abad E., Dunstan J., Jones R., Kotz A., Winterhalter H., Malisch R., Traag W., Hagberg J., Jogsten I.E., Beltran J., Hernández F.

Analytical Chemistry (2014), accepted for publication

Artículo científico 7

Analytical strategy based on the use of gas chromatography coupled to time-of-flight or hybrid quadrupole time-of-flight mass analyzers to investigate potential polymeric migrants into food simulants

Cherta L., Portolés T., Pitarch E., Beltran J., López F.J., Calatayud C., Company B., Hernández F.

Analytical and Bioanalytical Chemistry (2014), submitted

OTROS ARTÍCULOS RELACIONADOS

Enhancing MRM experiments in GC/MS/MS using APGC

Portolés T., Cherta L., Beltran J., Gledhill A., Hernández F.

Waters Application note

A validated method for the analysis of 142 pesticide residues using atmospheric pressure GC coupled with tandem quadrupole mass spectrometry

Cherta L., Portolés T., Beltran J., Pitarch E., Mol J.G.J., Hernández F., Roberts D., Rao R.

Waters Application note

Sugerencias para futuros trabajos

Considerando los resultados y conclusiones obtenidos en esta Tesis, se pueden sugerir algunas líneas de investigación futura que pueden resultar de interés:

- Avanzar en el desarrollo de métodos basados en *fast* GC-MS con analizadores suficientemente rápidos y selectivos que permitan la determinación de un mayor número de compuestos en un tiempo cromatográfico corto.

- Profundizar en el uso del nuevo sistema GC-(APCI)QqQ MS/MS para el desarrollo de métodos cuantitativos aplicados a matrices de origen medioambiental y alimentario de mayor complejidad.

- Extender el uso del GC-(APCI)QqQ MS/MS para la determinación de *dioxin-like* PCBs (PCBs semejantes a dioxinas) como alternativa a las técnicas de alta resolución empleadas en este campo.

- Avanzar en la investigación de las capacidades identificativas de GC-(TOF y QTOF) MS con las fuentes de ionización EI y APCI para los análisis *non-target* de compuestos desconocidos.

Suggestions for future work

Considering the results and conclusions obtained in this Thesis, some research lines of interest can be suggested:

- Progress in the development of analytical methods based on fast GC-MS with mass analyzers rapid and selective enough for the determination of a larger number of compounds in a short chromatographic time.

- Investigate the use of the new GC-(APCI)QqQ MS/MS system for the development of quantitative methods applied to environmental and food samples more complex or challenging.

- Widen the use of the GC-(APCI)QqQ MS/MS for the determination of dioxin-like PCBs as an alternative to high resolution techniques applied in this field.

- Progress in the investigation of identification capabilities of GC-(TOF and QTOF) MS with both ionization sources EI and APCI for non-target analysis.