INVESTIGACIÓN DE CONTAMINANTES ORGÁNICOS VOLÁTILES Y SEMIVOLÁTILES EN AGUAS Y VEGETALES MEDIANTE CROMATOGRAFÍA DE GASES-ESPECTROMETRÍA DE MASAS (TRIPLE CUADRUPOLO Y TIEMPO DE VUELO)

TESIS DOCTORAL

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Febrero 2015

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UNIVERSITAT JAUME·I

Universitat Jaume I

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Dr. Félix Hernández Hernández, Catedrático de Química Analítica, y **Dr. Joaquim Beltran Arandes**, Profesor Titular de Química Analítica, de la Universitat Jaume I de Castellón,

CERTIFICAN QUE: la **Tesis Doctoral** "Investigación de contaminantes orgánicos volátiles y semivolátiles en aguas y vegetales mediante cromatografía de gases-espectrometría de masas (triple cuadrupolo y tiempo de vuelo)" ha sido desarrollada bajo su dirección, en el área de Química Analítica del Departamento de Química Física y Analítica de la Universitat Jaume I de Castellón, por **Maria Inés Cervera Vidal.**

Lo que certificamos para los efectos oportunos en Castellón de la plana, a 6 de octubre de 2014.

Fdo. Dr. Félix Hernández Hernández **Fdo. Dr. Joaquim Beltran Arandes**

Este trabajo responde al compromiso adquirido con el Ministerio de Economía y Competitividad del Gobierno de España, por la concesión de una ayuda para la formación de personal investigador (FPI-MICINN) desde el 1 de septiembre de 2010 hasta el 1 de septiembre de 2014 (BES-2010-032412).

Maria Inés Cervera Vidal ha realizado una estancia breve de investigación en el *Institute for Biodiversity and Ecosystem Dynamics* (IBED) de la *Universiteit van Amsterdam* (UvA) en los Países Bajos, llevada a cabo desde el 3 de septiembre hasta el 21 de diciembre de 2012, gracias a una ayuda concedida por el Ministerio de Economía y Competitividad del Gobierno de España (Subprograma FPI-MICINN, Convocatoria 2011). El trabajo de investigación realizado llevó por título: "*Collection and chemical detection of volatiles emitted by chemically or biologically exposed tulip bulbs, using the analytical technique of headspace GC-quadrupole MS and GC-TOF MS for their determination*", bajo la supervisión del Prof. Dr. Pim de Voogt.

Los trabajos realizados en la presente memoria han sido en parte financiados por los proyectos del Ministerio de Ciencia e Innovación (CTQ2009-12347), de la Generalitat Valenciana (PROMETEO/2009/054; ISIC 2012/016), de Bancaixa (P1·1B2009-25) y de la Universitat Jaume I-Fundació Bancaixa (P1·1B2010-23).

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, directamente relacionados con el área de investigación: Dra. Patrizia Pelosi (*Researcher of National Institute of Health*, *Department of Environment and Primary Prevention*, *Pesticide Section*, *Rome, Italy*) and Dr. Johannes Gerardus Jacobus Mol (S*enior Scientist food safety analysis*, *group leader Natural Toxins and Pesticides*, RIKILT *Wageningen* UR, *the Netherlands*).

AGRAÏMENTS

Ha aplegat el moment del final, el moment on he de demostrar tot l'esforç emprat durant este temps en la investigació. Es per això que he d'agrair a tots l'ajuda rebuda en el dia a dia d'esta inoblidable etapa. De vegades eixa dedicació ha aplegat a donar bons fruits, d'altres no tant; però de cada moment he après alguna cosa que ha anat conformant la meua forma de ser. L'eufòria, l'esgotament, les alegries, els dubtes, les inquietuds, les satisfaccions, les celebracions, etc., son bons moments i sentiments que he viscut junt a tots vosaltres, que m'heu acompanyat en el llarg camí.

En primer lloc, voldria agrair als meus dos directors de Tesi, al Dr. Félix Hernández Hernández i al Dr. Joaquim Beltran Arandes. Félix, muchas gracias por tu confianza, por el esfuerzo empleado en mí y por todos tus consejos para sacar adelante nuestros trabajos, que han hecho que pueda estar aquí, ahora mismo, presentando mi Tesis Doctoral. Ximo, mil gràcies per tot el sacrifici i treball realitzat així com pel seguiment fet en cada moment, aconsellant-me com donar un pas més, aconseguint bons resultats. Als dos, moltes gràcies.

He d'agrair a Elena S. la seua ajuda durant el meu començament, però sobretot he d'agrair a Tania la seua implicació en mi durant la realització de tota la Tesi; xiques, gràcies per introduir-me de ple en el món de la cromatografia de gasos i la espectrometria de masses, quan eren prou desconegudes per a mi fins aquell moment. Gràcies com no a Elena P., per ajudar-me i formar part dels meus treballs. També vull agrair tot el aprés junt als meus companys de BPLs, Arantxa, Jose, Mercedes, Jaime i Edu, on he viscut una agradable etapa amb ells. Un lloc especial tenen per a mi Cris, Laura, Clara i Montse, en les que a més de compartir bons moments al treball he pogut compartir també grans moments fora d'ell, vivències,

viatges, estàncies, festes, congressos, etc., i que sempre recordaré. Gràcies amigues. Son molts els companys que he tingut tots estos anys i amb els que he pogut compartir mes o menys temps junts com María, Ana, Robert, Ramon, Angel, Emma, Carlos G., Miguel Angel, Ana Mª i Neus; d'altres que han passat per ací només per fer una estada, però ens han deixat un bon record; i també les més recents incorporacions Carlos, Richard, Rubén y Borja. A tots, gràcies.

Gràcies a la Agencia de Salut Pública de Barcelona per deixar-me utilitzar les seus instal·lacions per poder realitzar part del meu treball i a tot el personal per la seua bona acollida durant la meua estada.

I would like to thank Dr. Prof. Pim de Voogt for allowing me to perform a research stay in the IBED, at Universiteit van Amsterdam, in the Netherlands. I also would want to give my gratitude to the technician Frans van der Wielen for helping me in everything during my stay. He was and still is a good friend. Also, I must do a special mention for Dr. Prof. Maurice Sabelis, who is going through a very hard time, and his wife Iza Lesna for let me enter in the interesting field of the tulips. Thank you. I feel lucky to have had this great opportunity and I will never forget those 4 months of my life.

Gràcies als meus amics i amigues per estar sempre en mi i aguantar les meues explicacions. A ma mare i a mon pare els dec la paciència i la calma per afrontar cada situació; moltes gràcies per tot, no haguera sigut el mateix sense tot el vostre suport; en vosaltres ha sigut possible. En el meu germà veig la il·lusió per la investigació, seguint els meus passos, cosa que em fa feliç. Gràcies. Gràcies a ma "guela" i "güelita". I sobretot vull agrair ara i sempre al meu company de vida i amic, Antonio, per ajudar-me en cada moment, escoltar-me, consolar-me, recolzar-me i sempre fer-me la vida més fàcil. Per tot, a tots vosaltres, moltes gràcies.

Inés, Octubre 2014

RESUMEN

La presencia de contaminantes orgánicos en entornos no deseables y/o en cantidades mayores a las recomendadas es un asunto que preocupa actualmente, resultando necesario conocer los efectos nocivos que éstos pueden causar en la salud de la población. Para ello, se requiere una investigación profunda sobre los niveles de concentración de estos contaminantes en matrices de interés, como son los alimentos y las aguas, sobre todo si se trata de aguas de consumo humano. Estos estudios aparecen con el fin de abordar una visión global sobre la seguridad alimentaria y mantener un medio ambiente libre de contaminantes orgánicos, crucial para nuestra supervivencia. Entre los retos de la química analítica moderna se encuentran la investigación y elucidación de los contaminantes presentes en aguas y alimentos, que junto con la información de sus concentraciones, ayudan a encontrar las acciones adecuadas para combatir el problema de la contaminación del medio ambiente y mantener la seguridad alimentaria.

En la presente **Tesis Doctoral** se han abordado diferentes problemas analíticos relacionados con el análisis tanto de matrices de alimentos, tal como frutas y verduras sometidas comúnmente a la aplicación de productos fitosanitarios, como de matrices medioambientales. En todos los trabajos realizados se ha hecho uso de la cromatografía de gases acoplada a la espectrometría de masas (GC-MS) con diferentes analizadores (triple cuadrupolo

(QqQ), tiempo de vuelo (TOF) y cuadrupolo-tiempo de vuelo (QTOF)), en función de la complejidad y requisitos de los problemas abordados.

Tras presentar en el **Capítulo 1** una introducción general, en la que se resalta la importancia de realizar los análisis mencionados así como diferentes conceptos teóricos a tener en cuenta para el desarrollo y comprensión de la **Tesis**, en los capítulos posteriores se procede a mostrar los trabajos realizados, los cuales se han dividido en tres bloques diferenciados.

El **Capítulo 2** se centra en el uso del analizador QqQ MS, escogido para el desarrollo de métodos cuantitativos para la determinación de residuos de plaguicidas, tanto en matrices de carácter alimentario como medioambiental. Constancia de ello queda en el **Artículo científico 1**, en el que se realiza una detallada revisión de los métodos desarrollados mediante GC-MS/MS QqQ en la última década en dichos campos de aplicación. En el **Artículo científico 2** se muestra el desarrollo, validación y aplicación de un método cuantitativo para la determinación de 130 plaguicidas en diversos alimentos de origen vegetal, con la modalidad de trabajo de MS en tándem (MS/MS) que ofrece dicho analizador.

En el **Capítulo 3** se muestra el potencial del analizador TOF MS, simple o acoplado a un cuadrupolo (QTOF MS) para el análisis de residuos de plaguicidas (PRA), explorando diferentes enfoques. En el **Artículo científico 3** se desarrolla un método cuantitativo *target,* basado en GC-TOF MS, para una lista seleccionada de plaguicidas, el cual se validó en muestras de naranjas, tomates, zanahorias y manzanas. Su aplicabilidad se demostró con el análisis de muestras reales. Además, gracias a la existencia de librerías teóricas de espectros de masas adquiridos en modo de ionización electrónica (EI) y por comparación con los adquiridos, se realizó un análisis *non-target* de las muestras ya analizadas con el

objetivo de descubrir la presencia de otros compuestos no considerados inicialmente. La aproximación realizada en el **Artículo científico 4** fue diferente. Haciendo uso de GC-QTOF MS, con ionización química a presión atmosférica (APCI), se analizó un número considerable de muestras vegetales de distintos tipos (manzana, naranja, tomate, zanahoria, lechuga, calabacín, pimiento rojo y fresa). Mediante uso de QTOF MS en modo MS^E (adquisición simultánea del espectro en masa exacta a baja y alta energía de colisión), se aplicó un método de *screening* para unos 130 plaguicidas, para los cuales el método había sido previamente optimizado (ion *m/z* más abundante en la función de baja energía y fragmento *m/z* en la de alta energía). Tras la identificación de varios positivos en las muestras, se realizó una validación cuantitativa de todos ellos, procediendo seguidamente a cuantificarlos de forma retrospectiva en las muestras ya analizadas. Finalmente, se amplió el método de *screening* a 250 plaguicidas más, lo cual fue posible gracias a la posibilidad de utilizar para la detección el ion molecular/molécula protonada, muy abundante en el modo de ionización APCI (usando para ello la función de baja energía). Los positivos encontrados se estudiaron con mayor detalle intentando conocer, al menos de forma tentativa, la identidad del compuesto gracias a la información sobre iones fragmento aportada en la función de alta energía.

Por último, en el **Capítulo 4** se aborda un campo de trabajo diferente, aunque haciendo uso de las mismas técnicas analíticas. En este capítulo se usa GC-MS con analizadores QqQ y TOF MS para la investigación de contaminantes orgánicos volátiles en aguas y vegetales. En el **Artículo científico 5** se desarrolla un método para analitos volátiles en aguas, haciendo uso de una optimización multivariante para determinar las condiciones óptimas de la microextracción en fase sólida (SPME). La técnica instrumental utilizada para la optimización,

validación y aplicación a muestras de aguas superficiales y residuales fue GC-MS/MS QqQ. Finalmente, en el **Artículo científico 6** se realiza una investigación sobre los compuestos volátiles que emiten los bulbos de la flor del tulipán, como un trabajo colaborativo con el *Institute for Biodiversity and Ecosystem Dynamics* (IBED) de la *Universiteit van Amsterdam* (UvA) (*the Netherlands*). Se utilizó inicialmente la técnica GC-TOF MS, gracias a la ventaja de la adquisición espectral completa (*full scan*), llevando a cabo la búsqueda de dos compuestos concretos, el *Tulipalin A* y *α-methyl-γ-butyrolactone* por ser componentes ya conocidos de los tulipanes; realizando también una búsqueda *non-target* (software automatizado, comparando espectros de masas de EI) para descubrir sus componentes volátiles. Tras la detección e identificación de los posibles positivos, se creó un método cuantitativo mediante GC-MS/MS QqQ para su correcta cuantificación.

SUMMARY

INVESTIGATION OF VOLATILE AND SEMIVOLATILE ORGANIC POLLUTANTS IN WATER AND VEGETABLE SAMPLES BY GAS CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY (TRIPLE QUADRUPOLE AND TIME-OF-FLIGHT)

The presence of organic pollutants in different types of samples is a matter of current concern. It is nowadays more and more necessary to have data about the harmful effects that they can cause in the population's health. To this aim, a detailed investigation on concentration levels of these contaminants/residues in relevant matrices, as food and water (especially in drinking water) is required. Within the present challenges in analytical chemistry, the detection, reliable identification and accurate quantification of a large variety of organic contaminants in water and food is one of the most urgent responses. This information from analytical chemists is required to face current pollution and food safety problems.

In this **Doctoral Thesis**, different analytical problems have been studied related with the analysis of food matrices, as fruits and vegetables, commonly treated with pesticides, or environmental matrices. In every work presented in this **Thesis**, gas chromatography coupled to mass spectrometry (GC-MS) has been

applied making use of different analyzers (triple quadrupole (QqQ), time of flight (TOF) and quadrupole time-of-flight (QTOF)), depending on complexity and requirements of the situation presented.

In **Chapter 1**, a general introduction of the subject under research is presented, emphasizing the importance of the aforementioned analysis and describing different issues of interest to understand the work performed. The following chapters show the experimental work performed, divided in three different blocks.

Chapter 2 focuses on applications of QqQ analyzer in tandem MS methods. GC-MS/MS QqQ methodology has been developed in this part for quantification of pesticide residues in food and environmental matrices. **Scientific paper 1** presents a detailed revision of methods developed by QqQ MS and reported in the last decade in these fields. In **Scientific paper 2**, the development, validation and application of a quantitative method for determination of 130 pesticides in several vegetable matrices are shown, emphasizing the improvements in sensitivity and selectivity when using QqQ in tandem MS (MS/MS) mode. A detailed study on matrix effects is made in this work, followed by quantitative validation in different food matrices at very low concentration levels, establishing as LOQ objective 0.01 mg/kg.

In **Chapter 3**, the strong potential of TOF MS in pesticide residue analysis (PRA) has been demonstrated, as single analyzer or coupled to a quadrupole (QTOF MS), exploring target and non-target approaches. In **Scientific paper 3,** a quantitative target method based on GC-TOF MS has been developed for selected pesticides, with the validation being performed in oranges, tomatoes, carrots and apples. Its applicability was proved by analysing real-life samples. Additionally,

non-target analysis was carried out in order to detect other compounds not considered initially. This was possible thanks to the accurate-mass spectra obtained for the samples and by comparison with the theoretical mass spectral libraries available in electron ionization mode (EI). A different approach was applied in **Scientific paper 4**, where GC-QTOF MS with atmospheric pressure chemical ionization (APCI) was used. In this work, a large number of vegetables samples were analysed (apple, orange, tomato, carrot, lettuce, courgette, red pepper and strawberry) by applying a screening method for 130 pesticides. The screening was based on the use of QTOF MS in MS^E mode (simultaneous acquisition of mass spectra in accurate mass at low and high collision energy). Detection was based on the more abundant *m/z* ion (commonly the molecular ion/protonated molecule) at low energy function and identification was feasible using *m/z* fragment ions at the high energy function, all measured at accurate mass. After identification of several positives in the samples analysed, a quantitative validation was carried out for them, performing their quantification from the samples already analysed, in a retrospective way. The screening method was widened up to 250 pesticides more, which was easily made because detection was based on the presence of the molecular ion/protonated molecule, highly abundant in APCI mode (at low energy function). The positives found were studied in more detail in order to tentatively identify the compound detected, making use of the fragmentation information acquired at the high energy function.

Finally, in **Chapter 4** a different field of application was selected, although making use of the same analytical techniques. In this work, GC-MS was coupled to QqQ and TOF MS analyzers for investigation of volatile organic compounds in water and vegetables. In **Scientific paper 5**, a method for volatile contaminants in

water was developed, taking the advantage of multivariate optimization to establish the optimum conditions for solid-phase microextraction (SPME). GC-MS/MS QqQ was used for optimization, validation and analysis of surface and wastewater samples. In **Scientific paper 6** an investigation about volatile compounds emitted by tulip bulbs has been performed in a collaborative work with the Institute for Biodiversity and Ecosystem Dynamics (IBED) from *Universiteit van Amsterdam* (UvA) (the Netherlands). GC-TOF MS was initially used for screening of two compounds, Tulipalin A and α-methyl-γ-butyrolactone, known for being interesting components in tulips. Taking profit of the complete spectral acquisition information (accurate-mass full-spectrum acquisition) in TOF MS, a non-target searching was also applied to discover other unknown compounds that might be relevant in the aromatic profile. After detection and tentative identification of several compounds, a quantitative method by GC-MS/MS QqQ was developed in order to perform accurate and sensitive quantification.

ÍNDICE GENERAL

ÍNDICE DE ACRÓNIMOS

1-MCP	1-Metilciclopropeno
ACS	Sociedad americana de química
ADC	Convertidor de analógica a digital
AOAC	Asociación de químicos analíticos norteamericanos
APCI	Ionización química a presión atmosférica
API	Ionización a presión atmosférica
ASE	Extracción acelerada con disolventes
ASPB	Agencia de Salud Pública de Barcelona
BTEX	Benceno, Tolueno, Etilbenceno y Xilenos
CAR	Carboxeno
CEN	Comité europeo de normalización
CI	Ionización química
CID	Disociación inducida por colisión
CRS	Recondensación del solvente concurrente
DDD	Diclorodifenildicloroetano
DDE	Diclorodifenildicloroetileno
DDT	Diclorodifeniltricloroetano

DI	Inmersión directa
DRE	Mejora del rango dinámico
DVB	Divinilbenceno
d-SPE	Extracción en fase sólida dispersiva
ECD	Detector de captura de electrones
ECHA	Agencia europea de sustancias y preparados químicos
EFSA	Autoridad europea de seguridad alimentaria
EI	Ionización electrónica
EPA	Agencia de protección ambiental
EU	Unión Europea
FID	Detector de ionización de llama
FPD	Detección fotométrica de llama
FWHM	Anchura a media altura
GAP	Buenas prácticas agrícolas
GC	Cromatografía de gases
GCxGC	Cromatografía de gases bidimensional
GLP	Buenas prácticas de laboratorio
GPC	Cromatografía de exclusión por tamaños
HAc	Ácido acético
HCB	Hexaclorobenceno
HE	Alta energía

HF-LPME	Fibra hueca de microextracción en fase líquida
HPLC	Cromatografía de líquidos de alta resolución
HR	Alta resolución
HS	Alta velocidad
HS	Espacio de cabeza
IBED	Instituto para la biodiversidad y dinámica de los ecosistemas
ILIS	Patrón interno marcado isotópicamente
IP	Puntos de identificación
IS	Patrón interno
IT	Trampa de iones
IWW	Agua residual de influente
LC	Cromatografía de líquidos
LE	Baja energía
LLE	Extracción líquido-líquido
LLP	Partición líquido-líquido
LOD	Límite de detección
LOQ	Límite de cuantificación
LVI	Inyección de grandes volúmenes
m/z	Relación masa-carga
MAE	Extracción asistida por microondas
MAX	Intercambio aniónico de modo mixto

MCP	Detector de placa multicanal
MCX	Intercambio catiónico de modo mixto
MeCN	Acetonitrilo
$MgSO_4$	Sulfato de magnesio
MRL	Límite máximo de residuo
MS	Espectrometría de masas
MS/MS	Espectrometría de masas en tándem
MSPD	Dispersión en matriz en fase sólida
NaAC	Acetato sódico
NaCl	Cloruro sódico
NCI	Ionización química negativa
NPD	Detector nitrógeno-fósforo (termoiónico)
NPLC	Cromatografía de líquidos de fase normal
OC	Organoclorado
OP	Organofosforado
P&T	Purga y trampa
PAH	Hidrocarburo aromático policíclico
PDMS	Polidimetilsiloxano
PCB	Bifenilo policlorado
PCDD	Policlorodibenzodioxina
PCDF	Dibenzofurano policlorado

PCI	Ionización química positiva
PFC	Perfluorocarbono
PFE	Extracción de fluido presurizado
PFOS	Ácido perfluorooctanosulfónico
PFOSF	Fluoruro de perfluorooctano sulfonilo
PFTBA	Perfluorotri-n-butilamina
PLE	Extracción de fluidos presurizados
POP	Contaminante orgánico persistente
PRA	Análisis de residuos de plaguicidas
PSA	Amina primaria y secundaria
PTV	Vaporización de temperatura programable
Q	Cuadrupolo
QqQ	Triple cuadrupolo
QTOF	Cuadrupolo-tiempo de vuelo
QuEChERS	Rápido, fácil, barato, efectivo, robusto y seguro
REACH	Registro, evaluación, autorización y restricción de sustancias químicas
RPLC	Cromatografía de líquidos de fase inversa
Rpm	Revoluciones por minuto
RSD	Desviación estándar relativa
Rt	Tiempo de retención

S/N	Relación señal-ruido
SBSE	Extracción mediante barra agitadora magnética
SFE	Extracción con fluidos supercríticos
SIM	Monitorización selectiva de iones
SIR	Grabación del ion seleccionado
SPE	Extracción en fase sólida
SPME	Microextracción en fase sólida
SRM	Monitorización selectiva de transiciones
TDC	Convertidor de tiempo a digital
TLC	Cromatografía en capa fina
TOF	Tiempo de vuelo
TP	Producto de transformación
TPP	Trifenil fosfato
UAEE	Extracción por emulsificación asistida por ultrasonidos
UHPLC	Cromatografía de líquidos de ultra alta resolución
UJI	Universidad Jaume I
UvA	Universidad de Amsterdam
VOC	Compuesto orgánico volátil
XIC	Cromatograma de iones extraídos

INDEX OF ACRONYMS

1-MCP	1-Methylcyclopropene
ACS	American Chemical Society
ADC	Analogue-to-digital converter
AOAC	Association of Official Analytical Chemists
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Ionization
ASE	Accelerated Solvent Extraction
ASPB	Public health agency of Barcelona
BTEX	Benzene, Toluene, Ethyl benzene and Xylenes
CAR	Carboxen
CEN	European Committee for Standardization
CI	Chemical Ionization
CID	Collision Induced Dissociation
CSR	Concurrent solvent recondensation
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane

DI	Direct Immersion
DRE	Dynamic Range Enhancement
DVB	Divinylbenzene
d-SPE	Dispersive Solid Phase Extraction
ECD	Electron Capture Detector
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EI	Electron Ionization
EPA	Environmental Protection Agency
EU	European Union
FID	Flame Ionization Detector
FPD	Flame photometric detection
FWHM	Full Width at Half Maximum
GAP	Good Agricultural Practice
GC	Gas Chromatography
GCxGC	Two-dimensional chromatography
GLP	Good Laboratory Practice
GPC	Gel Permeation Chromatography
HAc	Acetic acid
HCB	Hexachlorobenzene
HE	High Energy

HF-LPME	Hollow Fiber Liquid Phase MicroExtraction
HPLC	High Pressure Liquid Chromatography
HR	High Resolution
HS	High Speed
HS	Headspace
IBED	Institute for Biodiversity and Ecosystem Dynamics
ILIS	Isotopically Labelled Internal Standard
IP	Identification points
IS	Internal Standard
IT	Ion Trap
IWW	Influent Waste Water
LC	Liquid Chromatography
LE	Low Energy
LLE	Liquid-liquid extraction
LLP	Liquid-liquid partition
LOD	Limit of Detection
LOQ	Limit of Quantification
LVI	Large Volume Injection
m/z	Mass to charge ratio
MAE	Microwave Assisted Extraction
MAX	Mixed-mode Anion Exchange

MCP	Multichannel Plate Detector
MCX	Mixed-mode Cation Exchange
MeCN	Acetonitrile
$MgSO_4$	Magnesium sulphate
MRL	Maximum Residue Level
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MSPD	Matrix Solid Phase Dispersion
NaAC	Sodium acetate
NaCl	Sodium chloride
NCI	Negative Chemical Ionization
NPD	Nitrogen-Phosphorus Detector
NPLC	Normal Phase Liquid Chromatography
OC	Organochlorine
OP	Organophosphorous
P&T	Purge and trap
PAH	Polycyclic aromatic hydrocarbon
PDMS	Polydimethylsiloxane
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-p-dioxin
PCDF	Polychlorinated dibenzofuran

PCI	Positive Chemical Ionization
PFC	Perfluorinated compound
PFE	Pressurized Fluid Extraction
PFOS	Perfluorooctane sulfonic acid
PFOSF	Perfluorooctane sulfonyl fluoride
PFTBA	Perfluorotri-n-butylamine
PLE	Pressurized Liquid Extraction
POP	Persistent Organic Pollutant
PRA	Pesticide Residue Analysis
PSA	Primary Secondary Amine
PTV	Programmable Temperature Vaporization
Q	Quadrupole
QqQ	Triple quadrupole
QTOF	Quadrupole time-of-flight
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
REACH	Registration, Evaluation, Authorisation and restriction of CHemical substances
RPLC	Reverse Phase Liquid Chromatography
Rpm	Revolutions per minute
RSD	Relative Standard Deviation
Rt	Retention time

S/N	Signal to noise ratio
SBSE	Stir Bar Sorptive Extraction
SFE	Supercritical Fluid Extraction
SIM	Selected Ion Monitoring
SIR	Selected Ion Recording
SPE	Solid Phase Extraction
SPME	Solid-phase microextraction
SRM	Selected Reaction Monitoring
TDC	Time-to-Digital Converter
TLC	Thin Layer Chromatography
TOF	Time-of-flight
TP	Transformation Product
TPP	Triphenyl phosphate
UAEE	Ultrasound-Assisted Emulsification Extraction
UHPLC	Ultra High Pressure Liquid Chromatography
UJI	University Jaume I
UvA	University of Amsterdam
VOC	Volatile Organic Compound
XIC	eXtracted Ion Chromatogram

OBJETIVOS

OBJETIVOS

El ***objetivo principal*** de la presente **Tesis Doctoral** es investigar las posibilidades analíticas que ofrece el acoplamiento de la técnica de cromatografía de gases con espectrometría de masas (GC-MS) haciendo uso de tres diferentes analizadores, triple cuadrupolo (QqQ), tiempo de vuelo (TOF) y cuadrupolo-tiempo de vuelo (QTOF); con el fin de aplicar su potencial al desarrollo de métodos analíticos avanzados, con alta sensibilidad y selectividad, que permitan una correcta detección, identificación y cuantificación de contaminantes orgánicos en el campo alimentario y medioambiental.

Los ***objetivos específicos*** planteados para alcanzar el objetivo general se detallan a continuación:

1. Examinar las aplicaciones reportadas en la bibliografía sobre uso de la técnica GC-MS para la determinación de residuos de plaguicidas en los campos de seguridad alimentaria y medioambiental desde sus inicios hasta la actualidad, poniendo especial énfasis en las aplicaciones del analizador de triple cuadrupolo.

2. Diseñar un método basado en GC-MS/MS QqQ para la determinación simultanea de un elevado número de plaguicidas con características físico-químicas variadas.

3. Validar de forma cuantitativa un método analítico moderno, con alta sensibilidad y selectividad, para la determinación de una larga lista de plaguicidas usando la técnica GC-MS/MS QqQ en matrices vegetales y demostrar su aplicabilidad en el análisis de muestras reales.

4. Explorar las capacidades del analizador TOF acoplado a GC, utilizando fuente de ionización electrónica, y demostrar su potencial para el análisis de residuos de plaguicidas (PRA) en matrices vegetales tanto en modo *target* como *non-target*.

5. Validar de forma cuantitativa un método analítico *target* desarrollado con la técnica GC-TOF MS para matrices vegetales y demostrar su aplicabilidad en el análisis de muestras reales.

6. Investigar la presencia de contaminantes orgánicos en muestras vegetales mediante un método de *screening* basado en GC-TOF MS, explorando las posibilidades de detección y/o identificación de los compuestos detectados gracias a la información aportada por dicha técnica (espectro completo con medidas de masa exacta).

7. Estudiar las ventajas que presenta la ionización a presión atmosférica (APCI) en GC-MS acoplada a un analizador híbrido de QTOF. Demostrar la aplicabilidad de la técnica GC-(APCI)QTOF MS para PRA en matrices vegetales.

8. Validar de forma cuantitativa un método analítico basado en GC-(APCI)QTOF MS para la determinación *target* de residuos de plaguicidas en matrices vegetales.

9. Estudiar la presencia de residuos de plaguicidas mediante *screening* en modo *post-target* basado en la búsqueda del ion molecular /molécula protonada, usando GC-(APCI)QTOF MS.

10. Optimizar de forma estadística multivariante los parámetros involucrados en la técnica de microextracción en fase sólida (SPME) de compuestos orgánicos volátiles (VOCs) de aguas.

11. Validar de forma cuantitativa un método analítico basado en GC-MS/MS QqQ para la determinación de VOCs en aguas y demostrar su aplicabilidad tras el análisis de muestras de aguas superficiales y residuales.

12. Utilizar las capacidades del GC-TOF MS para la identificación de VOCs presentes en muestras de bulbos de tulipán y desarrollar posteriormente un método cuantitativo, basado en GC-MS/MS QqQ, para los compuestos detectados en dichas muestras.

OBJECTIVES

OBJECTIVES

The ***main objective*** of this **Doctoral Thesis** is to investigate the analytical capabilities of gas chromatography coupled with mass spectrometry (GC-MS) using different analyzers, triple quadrupole (QqQ), time-of-flight (TOF) and quadrupole time-of-flight (QTOF), in order to apply their potential to the development of advanced analytical methods, with high sensitivity and selectivity, that allows the reliable detection, identification and quantification of organic contaminants in food safety and environmental pollution fields.

The ***specific objectives*** are detailed below:

1. Detailed revision in the literature of the GC-MS applications reported for determination of pesticide residues in food safety and environmental fields, from the early beginning to the present with special emphasis to the use of triple quadrupole analyzer.

2. Development of analytical methodology based on GC-MS/MS QqQ for the simultaneously determination of a large number of pesticides with different physicochemical properties in food matrices.

3. Quantitative validation of a sensitive and selective analytical methodology for determination of a large number of pesticides

using GC-MS/MS QqQ in several vegetable matrices and demonstration of its applicability with real samples analysis.

4. Explore TOF MS analyzer capabilities coupled to GC with electron ionization source, for pesticide residue analysis (PRA) in vegetable matrices in both target and non-target modes.

5. Quantitative validation of analytical target methodology based on GC-TOF MS for pesticide residues in vegetable matrices and applicability to real-life samples analysis.

6. Investigation of the presence of organic pollutants in vegetables matrices by application of a screening method based on GC-TOF MS, exploring the detection and/or identification possibilities of the compounds thanks to the information acquired with this technique (accurate-mass full scan spectra).

7. Study of the advantages of atmospheric pressure chemical ionization (APCI) in GC-MS coupled to a hybrid QTOF MS analyzer. Investigation of GC-(APCI)QTOF MS applications for PRA in vegetable matrices.

8. Quantitative validation of analytical methodology based on GC-(APCI)QTOF MS for target determination of pesticide residues in vegetable matrices.

9. Evaluation of the presence of pesticide residues by screening in post-target mode based on the searching of the molecular ion and/or the protonated molecule, using GC-(APCI)QTOF MS.

10. Multivariate statistical optimization of the main parameters involved in the solid phase microextraction (SPME) of volatile organic compounds (VOCs) in waters.

11. Quantitative validation of an analytical method based on GC-MS/MS QqQ for VOCs in waters and application to surface and wastewater samples analysis.

12. Evaluation of GC-TOF MS capabilities for identification of VOCs in tulip bulb samples and later development of a quantitative method, based on GC-QqQ MS/MS, for the compounds previously detected.

CAPÍTULO 1

INTRODUCCIÓN GENERAL

1. Importancia del control alimentario y medioambiental

En la actualidad, una de las preocupaciones que más afecta a la población es la referida a la salud. Entre los múltiples problemas de salud existentes, algunos tienen su origen en la aparición de ciertas dolencias sufridas tras el contacto con productos químicos. Las causas que pueden originarlas son muy variadas y en muchos casos desconocidas. Por ello, el uso de productos químicos (peligrosos) ha de estar bien documentado ya que, aunque la mayoría de estos productos se utilizan buscando beneficios en su aplicación y/o uso, no siempre se conocen de antemano sus efectos nocivos.

En 2007 entró en vigor la regulación internacional denominada REACH (*Registration, Evaluation, Authorisation and restriction of CHemical substances*) (Regulation (EC) No 1907/2006) que tenía como objetivo garantizar la salud humana y mejorar la protección del medio ambiente, llevando a cabo una mejor

clasificación de las propiedades intrínsecas de las sustancias químicas existentes en el mercado. En la central de datos de la ECHA (*European Chemicals Agency*) se encuentra recogida la información referida a la seguridad, a los riesgos asociados al uso y manipulación de forma correcta de productos químicos; así como para el caso de productos químicos peligrosos, la propuesta de alternativas adecuadas para su sustitución progresiva. Si en el año 2007 las enfermedades causadas por productos químicos eran el 1 % de todos los tipos de enfermedades en la Unión Europea (EU), se esperaba una reducción de un 10 % tras la aplicación del REACH, lo que resultaría en un 0.1 % del total. Este porcentaje, a primera vista insignificante, se podría traducir sin embargo en que se evitarían alrededor de 4500 muertes debidas a cáncer cada año. Debería ser posible observar estos beneficios a partir de los 10 años tras su aplicación, con lo cual sería esperable que los resultados fueran visibles a partir del año 2018 (European Commission REACH in brief, 2007).

En la 244ª edición de la reunión nacional de la Sociedad Americana de Química (ACS) (*Philadelphia,* 2012), se discutieron los peligros de ciertos productos químicos. Además, se trataron otros muchos temas, desde los riesgos de la agricultura biotecnológica hasta las implicaciones de incluir el *endosulfan* como contaminante orgánico persistente (POP) o el uso continuado del herbicida *diuron*. Así, en dicha reunión se concluyó que la población necesita sentirse segura y convencida de que se están controlando adecuadamente los riesgos medioambientales; además, se debe seguir trabajando para que las formas de acción para combatirlos, sean cada vez más accesibles. Estos ejemplos ilustran que la seguridad es una de las principales razones para la regulación de los productos químicos por todos los riesgos asociados, por lo que dichos compuestos deben

estar controlados y sometidos a estrictas regulaciones (Armbrust, 2013; Commission Regulation (EU) No 253/2011).

De entre todos los productos químicos, cabe destacar aquellos persistentes, como los POPs, que constituyen si cabe un mayor riesgo, ya que sus propiedades físicas y químicas combinadas hacen que una vez alcanzan el medioambiente permanezcan intactos durante largos periodos de tiempo; que se distribuyan ampliamente a través del medioambiente como resultado de procesos naturales; que se acumulen en el tejido graso de los organismos vivos, incluyendo humanos; y sobre todo porque son tóxicos tanto para los humanos como para los animales. Aunque en 2001 se adoptó "El convenio de Estocolmo" sobre POPs, no fue hasta el 2004 cuando éste se puso en vigor. Entonces, se creó una lista de 12 POPs incluyendo plaguicidas, productos químicos industriales y subproductos (*aldrin, chlordane,* DDT*, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, toxaphene, polychlorinated biphenyls* (PCBs), *polychlorinated dibenzo-p-dioxins (PCDD) y polychlorinated dibenzofurans* (PCDF)). Más tarde se revisó y en 2009 se incluyeron nuevos compuestos (*chlordecone, α-hexachlorocyclohexane, β-hexachlorocyclohexane, lindane, pentachlorobenzene, hexabromobiphenyl, hexabromodiphenyl ether, heptabromodiphenyl ether, perfluorooctane sulfonic acid* (PFOS), sus sales y *perfluorooctane sulfonyl fluoride (PFOSF), tetrabromodiphenyl ether* y *pentabromodiphenyl ether*); y más recientemente en 2011, también se incluyó el *endosulfan* (Stockholm Convention).

Ciertos grupos de compuestos químicos pueden presentar riesgos tanto para la seguridad ambiental como la alimentaria. Por lo que respecta a la alimentación, se pueden encontrar residuos de plaguicidas (>600), residuos de drogas veterinarias (>300) y contaminantes naturales como micotoxinas (>500),

toxinas de plantas (>500) y biotoxinas marinas (>100). Además, el número de contaminantes medioambientales que se puede encontrar es elevado (>1000) ya que pueden existir dioxinas, PCBs, hidrocarburos aromáticos policíclicos (PAHs), retardantes de llama, compuestos perfluorados (PFCs), biocidas, disruptores endocrinos, metales pesados, etc. Por último existen también cientos de compuestos peligrosos para la salud como derivados del procesamiento de muestras, de su uso fraudulento, procedentes de materiales en contacto con alimentos, etc.; como por ejemplo acrilamida, aminas heterocíclicas, furanos, desinfectantes o melanina, entre otros.

Por todos los riesgos asociados al gran número de productos químicos existentes se debe llevar a cabo un control analítico adecuado y por tanto es necesario conocer no solo la naturaleza sino también la magnitud (concentración) de los contaminantes/residuos encontrados, tanto en el entorno que nos rodea (medio ambiente) como en los alimentos que ingerimos en nuestra dieta.

1.1. Plaguicidas

Un plaguicida es una sustancia o mezcla de sustancias, naturales o sintéticas, formuladas para controlar o repeler cualquier plaga. El término plaga incluye tanto insectos como malas hierbas, microbios y algunos mamíferos, entre otros. Normalmente, los plaguicidas son sustancias químicas pero pueden ser también agentes biológicos, como virus o bacterias. La parte activa del formulado de un plaguicida es el llamado ingrediente activo y habitualmente es el fabricante quien lo formula y lo produce en forma de polvo, gránulos, polvo soluble, etc. junto con algunos adyuvantes que mejoran la retención del plaguicida y la

absorción en hojas o tallos (Tadeo, 2008). La comercialización de productos fitosanitarios está estrictamente regulada y controlada según la Regulación 1107/2009 (Regulation (EC) No 1107/2009).

Existen diferentes tipos de plaguicidas, que se clasifican en grandes grupos, según su uso. Cada uno de estos grandes grupos incluye una subclasificación dependiendo de su composición química:

- herbicidas, utilizados para matar malas hierbas y otras plantas que crecen en lugares no deseados. Las principales clases químicas incluyen amidas, ácidos benzoicos, carbamatos, nitrilos, nitroanilinas, organofosforados, ácidos fenoxi, piridinas y compuestos de amonio cuaternarios, piridazinas y piridazinonas, triazinas y ureas.
- insecticidas, se usan para matar insectos y otros artrópodos. Se pueden clasificar en benzoilureas, carbamatos, organoclorados, organofosforados y piretroides.
- fungicidas, empleados para luchar contra hongos. Azoles, benzimidazoles, ditiocarbamatos y morfolinas son las variedades químicas que podemos encontrar.
- acaricidas, para eliminar, controlar o prevenir la presencia o acción de los ácaros.
- molusquicidas, para controlar moluscos, como por ejemplo caracoles.
- nematicidas, para eliminar un parásito nematodo.
- rodenticidas, para matar, eliminar, repeler o atenuar la presencia o acción de los roedores.

Existe actualmente un número muy elevado de plaguicidas con usos registrados, que pueden estar presentes en numerosas matrices alimentarias,

biológicas y ambientales; por lo que resulta una tarea complicada tener un control total de cada una de las diferentes combinaciones plaguicida/matriz, de interés.

Los primeros usos de plaguicidas datan de tiempos remotos, con las primeras aplicaciones de elementos como el azufre en polvo o compuestos tóxicos como el arsénico, el mercurio y el plomo para proteger los cultivos. A lo largo de la historia, los plaguicidas han ido creciendo en número y mejorando sus fórmulas para crear compuestos más efectivos en el momento de su aplicación. Por ejemplo, el DDT, descubierto en 1939, empezó a ser el insecticida más utilizado en el mundo gracias a su efectividad y ayudó a controlar enfermedades como la malaria, transmitida a través de mosquitos y que en ese momento estaba causando una elevada mortalidad. Su toxicidad no fue realmente evaluada hasta que la bióloga Rachel Carson dio la voz de alarma en su libro *Silent Spring* en el año 1962. En él advirtió sobre el uso extensivo del DDT, el cual estaba causando la muerte, aparte de insectos, de animales de una mayor magnitud. El DDT fue finalmente prohibido en 1972.

En un *review* recientemente publicado (MacFarlane, 2013) sobre seguridad y salud en el trabajo, se evaluaron los efectos asociados a la exposición de plaguicidas en la salud de trabajadores del sector agrícola. Estos efectos pueden variar dependiendo del plaguicida considerado y del tipo y extensión de la exposición; bien sea a través de la piel, oral o por rutas de inhalación, aunque la exposición dermal es la más relevante en estos casos. Los efectos agudos incluyen irritación de piel y ojos o irritación respiratoria; y los efectos crónicos pueden incluir problemas neurológicos y efectos en la salud mental, efectos mutagénicos y reproductivos, efectos endocrinos y cáncer. Aunque se están realizando esfuerzos a través de distintas regulaciones para evitar estos problemas, todavía se

requiere un mayor control y concienciación de los trabajadores en el uso adecuado de equipos de protección personal.

En cuanto a la aplicación de plaguicidas a los cultivos es necesario establecer unos márgenes de tiempo entre la aplicación del plaguicida y la recogida del producto acorde a la cantidad aplicada y al tipo de plaguicida; ya que el incumplimiento de estos plazos se puede traducir en la presencia de residuos en los productos recolectados a niveles superiores de los recomendados. Los residuos de plaguicidas se definen como las sustancias activas, los metabolitos y los productos de degradación o de reacción de sustancias activas utilizadas actualmente o con anterioridad en productos fitosanitarios que están presentes en alimentos, productos agrícolas o alimentos para animales, incluidos en particular aquellos cuya presencia pueda deberse a su uso en la protección de cultivos, en veterinaria y como biocidas (Regulation (EC) No 396/2005).

1.1.1. Seguridad alimentaria

La política de seguridad alimentaria actual (Regulation (EC) No 178/2002) establece unos principios aplicados al análisis y a la prevención de riesgos, a la protección de los intereses de los consumidores, así como a la libre circulación de productos seguros y de calidad tanto en el mercado interior como en el comercio con terceros países. Es por ello que el control de la seguridad alimentaria ha de garantizar la salud de los consumidores con alimentos seguros, sanos y saludables a través de actuaciones a lo largo de la cadena alimentaria enfocadas a eliminar o minimizar posibles peligros para la salud. Así, la misión de la Autoridad Europea de Seguridad Alimentaria (EFSA) consiste en emitir dictámenes y prestar apoyo

científico y técnico en todos los ámbitos que tienen un impacto sobre la seguridad alimentaria. Dicha autoridad es una fuente independiente de información y es responsable de mantener informado al público en general sobre los riesgos.

La Unión Europea ha establecido los "límites máximos de residuos" (MRLs) de plaguicidas permitidos en alimentos con el objetivo de minimizar la exposición del consumidor (Regulation (EC) No 396/2005). Los MRLs se establecen en base a ensayos realizados en cumplimiento con las buenas prácticas agrícolas (GAP) en el marco de estudios realizados conforme a las buenas prácticas de laboratorio (GLP) de modo que se asegure que los residuos no suponen un riesgo inaceptable para la salud humana. Además, estos niveles están también sujetos a los requisitos que exige el comercio internacional con productos agrícolas (Appendix I (7039/VI/95) of Directive 91/414/EEC)

Los MRLs se establecen para alimentos, los cuales se clasifican en 12 grandes grupos, según las matrices con características similares (Regulation (EC) No 396/2005):

1. Fruta fresca o congelada y frutos secos
2. Vegetales frescos o congelados
3. Legumbres secas
4. Semillas y frutas oleaginosas
5. Cereales
6. Te, café, infusiones de hierbas y cacao
7. Lúpulo
8. Especias
9. Plantas de azúcar
10. Productos de origen animal

11. Pescado, productos de pescado, marisco, moluscos y otros productos alimenticios marinos o de agua dulce
12. Cultivos utilizados para alimento animal

Para controlar el cumplimiento de los MRLs es necesario realizar análisis periódicos en los alimentos, aplicando métodos analíticos fiables y rigurosamente validados en laboratorios especializados que dispongan de la acreditación ISO 17025. Los valores establecidos pueden estar sometidos a cambios cuando existan nuevos datos sobre toxicidad y/o más información. Los MRLs deben ser establecidos a unos niveles tan bajos como sean posibles para proteger la salud del consumidor, pero deben ser compatibles con las prácticas agrícolas. En ausencia de ensayos de residuos de plaguicidas bajo GLPs, se puede establecer como MRL un valor por defecto igual al límite de determinación analítica (generalmente, 0.01 mg/kg) (Regulation (EC) No 396/2005; Commission Regulation (EC) No 283/2013).

Los métodos requeridos para el control de residuos de plaguicidas deben de ser adecuados para su determinación al nivel de los MRLs establecidos. Generalmente, han de llegar a un límite de cuantificación (LOQ) de 0.01 mg/kg aunque en casos justificados puede ser suficiente que cumplan con el nivel más bajo de MRL establecido para su respectivo grupo. Para productos clasificados por la SANCO/825/00/rev.8 como difíciles de analizar (grano de café, grano de cacao, infusiones, lúpulo, especias, té y tabaco), el LOQ del método debe llegar a un 50 % del MRL, a menos que ese MRL se haya establecido al LOQ (Guidance document SANCO/825/00/rev.8). La investigación analítica es un elemento imprescindible dentro de la política de seguridad alimentaria ya que se requieren métodos analíticos fiables y robustos para determinar los residuos de plaguicidas,

y controlar si se encuentran dentro de los límites establecidos y si cumplen con la vigente legislación.

En los últimos años han surgido varias alertas sanitarias relacionadas con la aparición de contaminantes en productos alimenticios, algunas de las cuales están relacionadas con la presencia de plaguicidas no autorizados. Otra situación que ocurre en ocasiones es el no cumplimiento de los MRL, por encontrarse los residuos por encima de las concentraciones máximas permisibles.

1.1.2. Seguridad ambiental

La presencia de contaminantes orgánicos en el medio ambiente conlleva problemas de contaminación del aire, agua o suelos, y su origen puede proceder de diversas fuentes. Por ejemplo, en un campo donde se aplican plaguicidas, éstos suelen llegar con mucha facilidad al suelo y finalmente a las aguas superficiales, por ejemplo mediante arrastre por escorrentía superficial. La contaminación de las aguas por plaguicidas representa una amenaza para el medio acuático y en casos extremos puede provocar efectos como toxicidad aguda y crónica en organismos acuáticos, acumulación de contaminantes en el ecosistema y pérdida de hábitats y biodiversidad, lo que también puede suponer una amenaza para la salud humana. La directiva europea relativa a la política de aguas (Directive 2013/39/UE) engloba una serie de contaminantes orgánicos, entre los que se encuentran regulados unos 20 plaguicidas, considerando los casos como *endosulfan, hexachlorobenzene, hexachlorobutadiene, hexachlorocyclohexane, trifluralin y dicofol* como sustancias peligrosas prioritarias.

El número de sustancias prioritarias legisladas en el ámbito europeo de la política de aguas no es muy elevado de momento (45 en total), pero ello no implica que no deban controlarse otros muchos contaminantes, tanto en las aguas superficiales como en otras matrices medioambientales. Con toda seguridad, esta lista inicial se irá ampliando en un futuro próximo y la legislación irá avanzando a medida que se disponga de más información sobre la presencia de contaminantes en el medio ambiente y de los riesgos asociados para la salud. Dentro del grupo de plaguicidas, los herbicidas son los que se encuentran con más frecuencia en el medio ambiente acuático. Los estudios más recientes se centran en el análisis de sus productos de transformación (TPs), ya que debido a su hidrolisis, oxidación, biodegradación o fotólisis pueden estar presentes en mayor concentración que el plaguicida precursor (Richardson, 2011).

Aunque hay beneficios evidentes en el uso de plaguicidas para propósitos agrícolas, también existen efectos adversos, tanto para el medioambiente como para la salud humana, por ejemplo, tras la ingestión de productos contaminados. Por ello, se requiere el desarrollo de métodos analíticos fiables para la detección de plaguicidas, así como para la confirmación de su identidad y la cuantificación de cada uno de ellos en las matrices de interés.

1.2. Compuestos orgánicos volátiles

Los compuestos orgánicos volátiles (VOCs) son un amplio grupo de contaminantes importantes en cuanto a su control, para asegurar así la salud de la población. Se trata de compuestos orgánicos que se evaporan a temperatura

ambiente y a presión atmosférica, generando vapores. Éstos pueden ser, por una parte precursores del ozono ambiental (troposférico) tras su reacción con óxidos de nitrógeno (presentes en la atmósfera) y luz solar formando así el *smog* fotoquímico; y por otra parte pueden ser a la vez destructores del ozono estratosférico por la degradación de la capa de ozono con compuestos como 1,1,1-tricloroetano o tetracloruro de carbono. En su composición, además de carbono, se pueden encontrar elementos como hidrógeno, flúor, oxígeno, cloro, bromo, nitrógeno o azufre.

Al ser compuestos que se evaporan rápidamente a la atmósfera originan problemas de contaminación atmosférica, pudiendo suponer un riesgo para la salud, sobre todo por inhalación. Su elevada liposolubilidad hace que presenten una gran afinidad por las grasas y por ello se acumulen en los tejidos grasos del cuerpo humano. Además, son altamente inflamables, con lo que pueden arder con facilidad en contacto con el aire. En cuanto a su toxicidad, ésta varía en función de cada compuesto y de las condiciones de su exposición. Así pues, los efectos que tienen para la salud difieren mucho entre ellos y se pueden clasificar en tres grupos dependiendo de su peligrosidad. Existen los extremadamente peligrosos (benceno, cloruro de vinilo, 1,2-dicloroetano y azufre), los de clase A que pueden causar daños significativos al medio ambiente (acetaldehído, anilina, tetracloruro de carbono, etc.) y los de clase B que tienen un menor impacto en el medio ambiente (acetona, etanol, combustibles fósiles, etc.). Si la exposición es a corto plazo, pueden causar irritación de ojos, garganta, nariz; nauseas, dolor de cabeza, vómito de sangre, reacciones alérgicas, hinchazón, mareos, dolores estomacales e intestinales, fatiga y manchas en la piel; y a largo plazo pueden dañar hígado, riñones, sistema nervioso central o incluso ser carcinogénicos (EPA, US).

Los VOCs pueden proceder de diversas fuentes, que pueden ser tanto naturales como artificiales. Así, se pueden encontrar en la atmósfera como resultado de la descomposición de la materia orgánica (metano), por los rumiantes como las vacas (metano) y también pueden proceder de emisiones generadas por los vegetales. Por lo que respecta al origen artificial, son muchas las posibles fuentes de VOCs: pinturas y lacas, decapantes, artículos de limpieza, disolventes, repelentes de polillas, aromatizantes de aire, materiales empleados en maderas, sustancias en aerosol, disolventes de grasa, productos de uso automotor, disolventes para la industria de lavado en seco, cosméticos, plásticos, etc.

1.2.1. Problemática ambiental

Por los riesgos descritos anteriormente, hay una evidente necesidad de controlar los VOCs, sobre todo, aquellos que proceden de fuentes artificiales y cumplir así con las directivas para garantizar la salud de la población.

La principal vía de contaminación de estos compuestos es a través del aire. La directiva europea vigente (publicada en 1999 y modificada en 2004) (Council Directive 1999/13/EC; Directive 2004/42/CE) regula el control de la emisión de estos compuestos volátiles debido al uso de disolventes orgánicos en ciertas actividades e instalaciones. El interés por conocer la contaminación del aire es creciente, como por ejemplo la producida por la emisión de plantas de tratamiento de residuos plásticos (Tsai, 2009) o por la emisión de aplicaciones de pintura y procesos de impresión (Yuan, 2010). Sin embargo, las concentraciones de algunos VOCs en el interior de las casas pueden llegar a ser muy superiores (hasta 10 veces

más) que en el exterior, sobre todo si las casas están situadas en entornos industriales, donde los VOCs se encuentran en una mayor proporción (EPA, US).

Otros casos de contaminación por VOCs se producen cuando éstos son vertidos o utilizados de forma indebida. Aunque una parte importante se evapora, también pueden depositarse en el suelo, y por acción de la lluvia, del agua o de la nieve fundida, pueden llegar a lixiviar a través del suelo llegando a las aguas subterráneas e incluso llegar a contaminar los suministros de aguas potables (Chary, 2012). En la directiva sobre política de aguas (Directive 2013/39/EU) quedan regulados los VOCs que podrían provocar la contaminación de las aguas superficiales. De este modo los compuestos incluidos en esta directiva son benceno, cloroalcanos C_{10-13}, 1,2-dicloroetano, diclorometano, triclorobencenos y triclorometano.

2. Técnicas analíticas para la determinación de contaminantes orgánicos

Una de las técnicas analíticas por excelencia es la cromatografía, nacida a principios de 1900 de manos del botánico ruso Michael Tswett (considerado como el padre de la cromatografía) cuando describió la separación de pigmentos de plantas mediante cromatografía líquida (LC) a través de una columna de vidrio rellena de carbonato de calcio y alúmina. No fue hasta mediados de los años 50 cuando se inició la cromatografía moderna con una publicación sobre el uso de gas como fase móvil en la separación de ácidos grasos volátiles (James, 1952), generalizándose entonces los métodos cromatográficos. Años más tarde se describió un proceso cromatográfico con una columna de relleno para la cromatografía de gases (GC), explicando la difusión y el proceso de transferencia de masas (van Deemter, 1956). La entonces nueva técnica de GC era la más utilizada por ser simple, rápida y capaz de separar materiales volátiles que eran imposibles de separar mediante destilación. Tiempo después fue lógico pensar en la aplicación de los exitosos resultados obtenidos para GC, a la técnica más antigua, la LC. En 1963 se publicó un artículo (Giddings, 1963) con las condiciones de operación de LC análogas a las utilizadas con GC, dando lugar a una revolución, por lograr un nivel de eficiencia comparable entre ambas. Entre las nuevas condiciones para operar con columnas de LC se encontraba la necesidad de una elevada presión de trabajo, lo cual llevó al resurgimiento de la LC, esta vez denominada HPLC, *high pressure liquid chromatography.* La evolución de las técnicas a lo largo del tiempo ha hecho que ya poco tenga que ver la cromatografía inicial referida a una coloración con la que actualmente conocemos. Además, la mayoría de instrumentación cromatográfica actual está

equipada con detectores, de captura de electrones (ECD), termoiónico (NPD), de ionización de llama (FID); y de forma más reciente con espectrómetros de masas (MS) que les hace capaces de realizar medidas exactas y fiables de sus componentes (Miller, 2005).

2.1. Cromatografía de líquidos

La cromatografía de líquidos se basa en la distinta afinidad que presentan los compuestos contenidos en una muestra entre dos fases conocidas, la estacionaria y la móvil, que en HPLC se trata de un líquido bajo elevada presión (hasta $4x10^7$ Pa); con el fin de asegurar un ritmo de flujo constante y una cromatografía reproducible. Por otro lado la fase estacionaria está empaquetada en el interior de una columna de acero, la cual soporta las elevadas presiones necesarias para llevar a cabo la separación. De este modo la separación ocurre cuando los componentes de la mezcla interactúan a diferentes grados con las fases y por lo tanto se mueven a diferentes velocidades desde la posición donde se ha introducido la muestra hasta donde los cuales son detectados. Así, el trabajo de desarrollo de metodologías con LC se centra en la optimización de las propiedades de ambas fases para moverse entre los dos extremos y alcanzar de este modo la separación deseada (Ardrey, 2003). La técnica de LC es la más adecuada para la separación de compuestos polares, poco volátiles y termolábiles.

Dependiendo del tipo de columnas utilizadas, se pueden distinguir distintos tipos de cromatografía de líquidos. Así pues, la cromatografía líquida en fase inversa (RPLC) es utilizada para la separación de compuestos con una polaridad media-alta con fases estacionarias apolares (C_8, C_{18}…). La cromatografía

líquida en fase normal (NPLC) es utilizada para compuestos más apolares pero sus aplicaciones actuales son escasas. La cromatografía líquida se puede clasificar también en función del tamaño de partícula de la fase estacionaria. Comúnmente en HPLC el tamaño de partícula de las columnas utilizadas es ≥3.5 μm, y en el caso de encontrar tamaños < 2 μm, la cromatografía se denomina de ultra alta resolución (UHPLC).

2.2. Cromatografía de gases

En cromatografía de gases la separación sucede por la interacción del analito entre la fase estacionaria contenida en el interior de la columna cromatográfica y un gas, la fase móvil, en función de las diferentes propiedades físicas y químicas de los componentes. Por ello, en el proceso de separación de los constituyentes de la muestra, cada analito se moverá desde el inyector hacia el detector a una velocidad distinta.

Existen parámetros importantes a optimizar para cada tipo de combinación analito/muestra, con el fin de llevar a cabo la separación de forma específica y adecuada. Así pues, se debe considerar principalmente la naturaleza de los analitos para realizar la correcta elección de la fase estacionaria y de las temperaturas de trabajo. La GC es la técnica preferida para compuestos volátiles o semi-volátiles, apolares y termoestables (Miller, 2005). Así, para que los analitos sean compatibles han de cumplir que, aparte de ser térmicamente estables a las temperaturas de trabajo seleccionadas, deben de tener unas presiones de vapor de 10^{-6} Pa o más en el puerto de inyección y en la columna. Además, no deben ser ni muy polares, ni muy ácidos, ni muy básicos, ni altamente reactivos, ya que

podrían adherirse fuertemente a las superficies o a otras sustancias, lo que provocaría problemas para ser analizados mediante GC (Budde, 2001).

A diferencia de lo que ocurre en LC, la introducción de la muestra en GC se puede realizar utilizando diferentes sistemas y/o modos de inyección: s*plit/splitless*, *on-column* o vaporización de temperatura programable (PTV). Esta etapa es una de las más importantes ya que si la muestra no es transferida a la columna de forma adecuada, los resultados obtenidos podrían no tener validez. Su elección normalmente está unida a la limitación de su disponibilidad en el sistema cromatográfico utilizado.

Tanto para el modo *split* como para *splitless* se utiliza el mismo inyector denominado de *split/splitless*. La muestra se introduce en un tubo de evaporación llamado *liner,* a una temperatura elevada, entre 200-300 °C con el propósito de mezclar los analitos en fase vapor con el gas portador y transferirlos a continuación al interior de la columna.

Cuando la muestra es inyectada en modo *split*, únicamente se está introduciendo en la columna una fracción seleccionada de ella, eliminándose la mayor parte junto con gas portador a través de una válvula. Uno de los principales motivos de su uso es intentar solucionar el problema de la sobrecarga de muestra en la fase estacionaria, siendo especialmente útil en muestras con una elevada concentración de analito. Por otro lado, este modo de inyección presenta la desventaja de la posible discriminación de los diferentes componentes de la muestra, sobre todo en aquellas donde los analitos posean un amplio rango de puntos de ebullición (Hübschmann, 2001; Grob, 2004).

Si se desea la total transferencia de la muestra al interior de la columna (sobre el 95 %), se utiliza el modo de inyección *splitless*, haciendo uso del mismo sistema que en modo *split* pero con la válvula de purga completamente cerrada durante los 30-60 segundos posteriores a la inyección. Es el modo más utilizado para intentar mejorar los límites de detección, sobre todo cuando se está trabajando a niveles de traza, con muestras conteniendo bajas concentraciones de analito. Debido a que la muestra vaporizada permanece un mayor tiempo en el inyector, existe un cierto riesgo en cuanto a la descomposición termal o catalítica de los compuestos más lábiles así como posibles adsorciones irreversibles en el *liner* (Hübschmann, 2001; Grob, 2004).

Otro tipo de inyección, conocida como *on-column*, es una técnica donde toda la muestra entra directamente en la columna, pero sin una cámara previa de vaporización. Ésta entra de forma líquida directamente a la columna, donde se realiza la vaporización con el programa de temperaturas del horno. Es la mejor técnica si se quiere llevar a cabo una perfecta cuantificación; aunque si se trata de muestras muy sucias, al llegar todo el volumen inyectado a la columna, podrían existir problemas de contaminación de la columna y de posibles adsorciones. Estos problemas, en el mejor de los casos producirían ensanchamientos y deformación de los picos cromatográficos, aunque se podrían reducir y/o controlar mediante el uso de una pre-columna sin fase denominada *retention gap*.

Finalmente, otro de los sistemas de inyección comúnmente utilizados es el PTV, donde la muestra es introducida a una temperatura baja (debe corresponder al punto de ebullición del disolvente a 10^5 Pa de presión) en un inyector tipo *split/splitless* con ciertas modificaciones técnicas relativas al control de temperatura del mismo. Permite aplicar un programa de temperaturas en el

sistema para mejorar la transferencia de la muestra al interior de la columna, con la ventaja adicional de poder inyectar grandes volúmenes de muestra (hasta cientos de μL) (Grob, 2004).

Uno de los parámetros con más influencia para conseguir una buena separación cromatográfica de los componentes de la muestra es el referido a la fase estacionaria, es decir, a la elección adecuada del tipo de columna cromatográfica. Las columnas capilares de sílice fundida, actualmente utilizadas, son unos tubos abiertos revestidos en el interior con una fase estacionaria polimérica y por el exterior con un polímero de poliimida. Las ventajas que ofrecen estas columnas con respecto a las columnas de relleno utilizadas en los inicios de la GC son elevada resolución, reducción del tiempo de análisis, menor cantidad de muestra requerida y aumento de la sensibilidad. Existen muchas fases estacionarias en el mercado, disponibles con diferentes dimensiones, especialmente en cuanto a espesor de fase estacionaria. Para la correcta elección se deben tener en cuenta las características físico-químicas de los compuestos que se deseen separar. A pesar de la gran variedad existente, las más utilizadas en análisis por GC-MS son las que contienen un 100 % *methyl polysiloxane*, un 5 % *phenyl*- 95 % *dimethyl polysiloxane*, *cyanopolysiloxane* o *polyethylene glycol*, cubriendo así un amplio rango de polaridades. Para aplicaciones más específicas han ido apareciendo otros tipos de fases, como por ejemplo aquella a la que hace referencia la agencia de protección ambiental (EPA) en el método 502.2 para el análisis concreto de VOCs en aguas y que suelen tener una fase 6 % *cyanopropylphenyl*- 94 % *methylpolysiloxane* (EPA, US method 502.2; Grob, 2004).

3. La espectrometría de masas acoplada a la cromatografía de gases

En el campo de análisis tanto ambiental como alimentario, el acoplamiento de las técnicas cromatográficas a la espectrometría de masas ha supuesto un gran avance en cuanto a las técnicas de análisis si lo comparamos con otro tipo de detectores más convencionales como ECD, NPD, FID, etc., sobre todo por lo que respecta a mejoras obtenidas en la sensibilidad, y fundamentalmente en la selectividad.

3.1. Tipos de analizadores de masas

El analizador es la parte del espectrómetro de masas que produce una separación de los iones en base a su relación masa/carga (*m/z*) utilizando diferentes tipos de corrientes eléctricas y/o campos magnéticos. Con el paso de los años han ido apareciendo diferentes tipos, basados en diferentes principios, y con ventajas y/o características específicas, que se adaptan al objetivo y a las condiciones de trabajo. Así, los parámetros más importantes a tener en cuenta para la correcta elección de la instrumentación son el tipo de muestra, complejidad de las matrices, niveles de concentración, familia de analitos, información demandada, etc.

3.1.1. Cuadrupolo

El cuadrupolo (*Q*) es un tipo de analizador consistente en cuatro barras metálicas colocadas de forma paralela entre sí, generando cuatro polos eléctricos. Cuando los iones se encuentran en el interior del cuadrupolo, éstos viajan a través de él sometidos a los campos eléctricos generados entre los polos. Por su modo de trabajo, solo los iones con una cierta *m/z* alcanzan el detector a una proporción definida de voltajes; mientras que los otros iones describen trayectorias inestables y colisionan con los polos o son eliminados por el sistema de vacío.

El analizador de cuadrupolo permite trabajar en dos modos. El primero de ellos es aquel que realiza un barrido de masas completo para así obtener la información espectral completa (*scan*). La sensibilidad trabajando en este modo suele ser reducida por lo que, para incrementarla habría que limitar la cantidad de información adquirida. Por ello, el modo de trabajo SIM (*selected ion monitoring*) selecciona diferentes iones y adquiere únicamente el ion o iones fragmento específicos del compuesto. Así se evita la adquisición de señales correspondientes a iones de compuestos no deseados que podrían interferir de forma negativa en el estudio de los espectros de los analitos seleccionados. La principal ventaja de este modo de trabajo reside en la importante mejora de la sensibilidad, aunque a coste de una importante pérdida de la capacidad de identificación al reducirse de forma drástica la información espectral registrada. Aunque este tipo de analizadores presenta una resolución de masa unidad, son muchas las aplicaciones que se han llevado a cabo con él para la determinación de contaminantes orgánicos tanto en el campo medioambiental como alimentario, con la correcta identificación y/o cuantificación de los analitos *target* seleccionados en el método (Pang, 2006; Stefanelli, 2009; Barrek, 2009; Gómez, 2009; Covaci 2010).

3.1.2. Trampa de iones

El analizador de trampa de iones (*ion trap*, IT) basa su separación en el principio del almacenamiento de los iones dentro de un campo cuadrupolar tridimensional. Los iones son extraídos del analizador en orden creciente del valor de *m/z* cada vez, por expulsión resonante, para obtener un barrido espectral, registrado como espectro de masas. Así, los iones en fase gas se forman (externamente en un fuente de ionización o internamente en el propio volumen del analizador) y después pueden ser confinados en la trampa durante largos períodos de tiempo por la acción de campos eléctricos y/o radiofrecuencias. La habilidad para almacenar de forma selectiva los iones proporciona una mejora en la sensibilidad si lo comparamos con el cuadrupolo, trabajando en modo *scan*. Los iones atrapados en el interior de la trampa (mediante aplicación de voltajes y radiofrecuencias adecuados) se pueden utilizar para realizar *a posteriori* experimentos con ellos, bien sea para su expulsión directa hacia el detector (obtención de espectro de MS en modo *scan*) o bien sea para realizar un proceso de asilamiento de un determinado ion y posterior fragmentación por colisión; ya que con este tipo de analizadores existe la posibilidad de trabajar en modo de espectrometría de masas en tándem (MS/MS). Esta técnica funciona expulsando de la trampa todos los iones, excepto aquellos que se desean utilizar como iones precursores, y que mediante su aceleración y consiguiente colisión con un gas (He), se fragmentan. Sin embargo, el IT tiene la limitación en su capacidad de resolución, ya que presenta resolución de masa unidad. Otra desventaja es aquella relacionada con la alta probabilidad existente de la producción de interacciones ion-molécula durante el tiempo de residencia de los iones en la cavidad, desde que se producen hasta que son detectados. A los analizadores de IT se les denomina espectrómetros de masas en tándem en el tiempo. Aunque los instrumentos más

modernos han mejorado algunas de estas limitaciones, todavía hoy existe el problema en los análisis de compuestos a muy baja concentración y en matrices muy sucias, ya que la trampa se llena de iones derivados de la matriz y no dejan mucho "espacio" para el reducido número de iones del analito. A pesar de estas restricciones, su capacidad de realizar MS^n permite el aislamiento secuencial y en etapas de los iones precursores, la fragmentación en el interior de la trampa y el barrido de masas en el mismo espacio y en función del tiempo. Por todo ello su uso ha sido también bastante amplio en el análisis medioambiental y alimentario (Watson, 2007; Tadeo, 2008; Helaleh, 2005; Cortés-Aguado, 2008; Fernandes 2011; Fernandes 2012; Assoumani, 2014).

3.1.3. Triple cuadrupolo

El analizador de triple cuadrupolo (QqQ) es un espectrómetro de masas que consta de dos cuadrupolos (Q_1, Q_2) acoplados en serie con otro cuadrupolo (q) situado entre ellos que actúa como celda de colisión, permitiendo trabajar en modo de MS/MS. Éste, es el modo de análisis óptimo para este tipo de analizadores, desde el punto de vista de la sensibilidad. QqQ MS presenta una gran versatilidad, ya que puede operar en cuatro modos de trabajo MS/MS diferentes: *product ion scan, precursor ion scan, neutral loss* y *selected reaction monitoring* (SRM). Éste último modo, SRM, permite realizar en un solo paso, la identificación y la cuantificación de los analitos, con una elevada sensibilidad y selectividad en una única inyección.

Para trabajar en modo SRM, se selecciona en el primer cuadrupolo, el ion precursor de interés, eliminando así los iones que tienen un *m/z* diferente del

seleccionado, ya que no pueden atravesar el primer cuadrupolo (Q_1). En la celda de colisión se aplican los voltajes adecuados para generar los iones producto característicos, a partir de los iones precursores, gracias a la colisión de los iones acelerados con un gas de colisión no reactivo (Ar, He o N_2). Este proceso es conocido como disociación inducida por colisión (CID). Así, los iones productos generados son transferidos al segundo cuadrupolo (Q_2) donde de nuevo solo se selecciona un *m/z* específico mientras que los otros son desestabilizados y eliminados. Esta forma de trabajo realiza un doble filtro de masas, la cual reduce de forma drástica el ruido de fondo al mismo tiempo que se incrementa la selectividad.

Puede que uno de los inconvenientes que presenta la técnica sea que el número de transiciones detectadas en un análisis es limitado porque requiere de una optimización previa de los analitos que se deseen analizar (modo *target*). Su propio diseño de trabajo supone una limitación en cuanto a la determinación o detección de compuestos presentes en las muestras que no han sido previamente seleccionados, ya que se podría crear un problema tras la pérdida de información espectral, al no ser adquirida por completo.

A pesar de todo, esta técnica ha sido una de las más utilizadas en la pasada década, aplicada en el análisis de matrices alimentarias y medioambientales. Ha permitido la detección y cuantificación de un elevado número de analitos con distintas características físico-químicas (métodos multiclase), con una elevada sensibilidad, selectividad y especificidad, con un amplio rango dinámico lineal y una excelente repetibilidad (la precisión de las áreas de los picos es normalmente mejor del 10 %) (Walorczyk, 2007; Vidal, 2010; Labadie, 2010; Feo, 2011; Koesukwiwat, 2011; Camino-Sánchez, 2012; Kalachova, 2013).

3.1.4. Tiempo de vuelo

Debido a sus características intrínsecas de funcionamiento y fundamento, el analizador de tiempo de vuelo (*time-of-flight*, TOF) ofrece una ventaja con respecto a los analizadores descritos anteriormente, ya que la información espectral se adquiere de forma completa (*full scan*) y permite detectar tanto compuestos (*target*) como confirmar la identidad de posibles compuestos hasta el momento desconocidos (*non-target*) con una elevada sensibilidad.

La separación de los iones en un analizador de TOF se consigue por el diferente tiempo que tardan en recorrer el espacio de vuelo de un tubo con campo libre; basado en el hecho de que la velocidad del ion es dependiente de la relación *m/z*. En los diseños más recientes, los iones que son generados en la fuente de ionización son inicialmente acelerados ortogonalmente para conseguir paquetes discretos con una energía cinética determinada, los cuales son dirigidos hacia el analizador de masas utilizando un gradiente de un campo eléctrico orientado ortogonalmente al haz de iones. Los iones son detectados generalmente usando un detector de placa multicanal (MCP) y se transforma la señal analógica a una señal digital mediante un convertidor TDC (*time-to-digital converter*) o por un ADC (*analogue-to-digital converter*) (Tadeo, 2008).

TOF MS ofrece dos tipos de espectrómetros de masas diferenciados. La primera aproximación consiste en instrumentos con el analizador trabajando en condiciones de alta resolución pero con una moderada velocidad de adquisición, es decir *high resolution* (HR-TOF MS). Por otro lado, están los instrumentos con las características opuestas, con una resolución unidad pero con una elevada velocidad de adquisición, *high speed* (HS-TOF MS). Ambos tienen un elevado potencial por lo que respecta a su aplicación. Así, el HS-TOF MS es especialmente

adecuado para trabajar acoplado con GC ultrarrápida o con GC x GC (Cajka, 2007). Por otro lado, HR-TOF MS ofrece una elevada resolución y buena exactitud de masa. Este equipo consigue reducir la contribución de las interferencias isobáricas permitiendo la evaluación de los datos, con una ventana de error de masa reducida, lo cual mejora la detectabilidad de los analitos. Estos analizadores de HR-TOF MS ofrecen comúnmente un poder de resolución elevado, de unos 7000 FWHM (*full width at half maximum*), aunque con instrumentaciones más recientes, incluso se pueden obtener valores más elevados. La principal desventaja que acompaña a este tipo de analizadores, es que presenta un reducido rango dinámico lineal comparado con otros analizadores de MS más tradicionales aunque no impide el uso de este tipo de analizadores tanto en métodos cualitativos como cuantitativos. De hecho con las nuevas instrumentaciones del mercado, este aspecto se ha mejorado incluyendo el sistema DRE (*dynamic range enhancement*) que ofrece hasta 4 órdenes de magnitud para medidas de masa exacta y la posibilidad de realizar cuantificaciones fiables de matrices complejas (Hernández, 2007; Leandro, 2007; Mastovska, 2010; Portolés, 2011; Kaufmann, 2012).

3.1.5. Otros analizadores

Otro tipo de analizador de masas de alta resolución es por ejemplo, el Sector Magnético, que basa su separación en la desviación del continuo haz de iones por impulsos en un campo magnético debido a las fuerzas de Lorentz (Gross, 2004). Éste se ha acoplado tradicionalmente a GC, encontrando un campo de trabajo típico por ejemplo en la determinación de dioxinas y PCBs en muestras de alimentos y ambientales.

El Orbitrap es otro analizador de HR, recientemente desarrollado y acoplado a LC, que presenta una elevada resolución y exactitud de masa. Permite el confinamiento de los iones sobre un electrodo que tiene forma de huso con un electrodo cilíndrico, donde se miden las relaciones *m/z* basadas en la frecuencia del movimiento, dependiendo de la masa a la cual los iones se someten a oscilaciones harmónicas durante el periodo de atrapamiento. Las frecuencias son medidas por la adquisición de imágenes de dominio de tiempo de la corriente transitoria con la consecuente aplicación de un proceso de transformada de Fourier para obtener el espectro de masas (Botitsi, 2011).

A parte de los mencionados anteriormente, existen analizadores híbridos que se han conseguido tras acoplar diferentes analizadores combinando sus propiedades y creando así un instrumento todavía más poderoso. Es el caso del acoplamiento entre algunas de las técnicas ya comentadas, como un cuadrupolo acoplado con un TOF, un QTOF; o una trampa de iones acoplado a un Orbitrap, un LTQ-Orbitrap, entre otros.

Desde los primeros inicios de la espectrometría de masas hasta la actualidad se han ido mejorando las técnicas y por ello han ido apareciendo analizadores cada vez mejores, con mejor sensibilidad, mejor resolución… Así, por ejemplo, en un reciente *review* publicado sobre aplicaciones de la espectrometría de masas en el campo de la seguridad alimentaria, de 26 referencias relacionadas con el análisis por GC, 7 reportaron su uso con un analizador de cuadrupolo, 4 con analizador de IT, 5 lo hicieron con QqQ y finalmente el TOF fue el analizador más utilizado con 10 referencias reportadas de su uso (Wang, 2013).

3.2. Fuentes de ionización

El acoplamiento del sistema cromatográfico al espectrómetro de masas en GC se realiza mediante una interfase, que consta simplemente de una línea de transferencia conteniendo la columna capilar en su interior. Ésta se calienta a una temperatura lo suficientemente alta (entre 200- 300 °C) para asegurar la completa transferencia de los analitos desde la columna hacia el interior de la fuente de ionización de modo que no se retrasen en este punto por condensación. El caso de LC es diferente, ya que son las propias interfaces las que actúan como fuentes de ionización y además difieren en ciertos casos de las utilizadas para GC.

Durante la realización de la **Tesis Doctoral** que se presenta, únicamente se ha utilizado la cromatografía de gases para llevar a cabo los trabajos, por lo que la siguiente explicación es solo referida a aquellas fuentes de ionización compatibles con la mencionada técnica.

3.2.1. Ionización electrónica

La ionización electrónica (EI) es la técnica de ionización más comúnmente utilizada hasta el momento en el acoplamiento GC-MS. La energía necesaria para ionizar y fragmentar las moléculas en la fase gas se adquiere por la interacción con un haz de electrones producidos desde un filamento caliente y acelerados por un campo eléctrico, mientras la fuente de ionización se mantiene a baja presión (aprox. 10^{-4} Pa). Los electrones son atraídos hacia un ánodo (*trap*) situado en la parte contraria de la cámara de ionización donde está el filamento o el cátodo mediante un voltaje (en ocasiones regulable) que genera electrones con una

energía de 70 eV. Uno de los principales motivos para elegir este valor es que no hay ningún átomo o molécula que no pueda ser ionizado a esta energía; en cambio a 15 eV gases como He, Ne, Ar, H_2 y N_2 no podrían ionizarse. Otro motivo es que a 70 eV aproximadamente, la longitud de onda de De Broglie de los electrones coincide con la longitud de los enlaces típicos de moléculas orgánicas (aproximadamente 0,14 nm) y la transferencia de energía a las moléculas orgánicas se maximiza, dando lugar a una ionización más fuerte y por consiguiente a la fragmentación. A energías más altas, la longitud de onda de De Broglie de los electrones es menor que las longitudes de enlace de los analitos, por tanto las moléculas se convierten en "transparentes" a la vista de los electrones y disminuye la eficiencia de ionización. Este valor es también elegido porque la eficiencia de la curva de ionización es mayor y entre 60-80 eV la energía de los electrones varia muy poco. Además se asegura una mejor reproducibilidad del espectro de forma que nos permita una mejor comparación de los espectros de masas obtenidos en diferentes espectrómetros de masas, así como también con los espectros de las librerías teóricas comerciales (Gross, 2004).

Los filamentos utilizados en la ionización electrónica trabajan a temperaturas mayores de 1500 °C e irradian calor a la fuente de ionización. El recinto de la fuente de iones está construido de acero inoxidable y se mantiene a una temperatura de unos 200-300 °C para evitar la condensación de los analitos y la posible acumulación de restos en las paredes de la fuente. Bajo estas condiciones, la colisión de las moléculas estables de la fase vapor, con las paredes del metal caliente puede causar descomposición termal de los analitos y/u otras sustancias de la muestra. Los restos de carbono y otros productos de reacción no volátiles se van acumulando gradualmente en las paredes de la fuente y pueden producir iones que se traducen en el ruido de fondo del espectro de masas y

pueden contribuir a la variabilidad que a veces se observa entre diferentes espectros de masas del mismo analito. Esta contaminación de la fuente puede causar también una pérdida de resolución y sensibilidad (Budde, 2001).

Las identidad de cada molécula se puede deducir del patrón de fragmentación del ion molecular ($M^{+\cdot}$), bajo este modo de ionización. Así, la interpretación de los resultados de los espectros obtenidos se puede hacer mediante comparación con los espectros de masas de las librerías teóricas comerciales existentes, ya que ayudan en la identificación del compuesto. El mecanismo que sufren los analitos para dar lugar a su espectro de masas está basado en el comportamiento de la molécula neutra que absorbe energía durante la interacción de los electrones ionizantes con su nube de electrones, originando los distintos iones fragmento con distintas intensidades. Por ejemplo, las moléculas de un analito interaccionan con un electrón que tiene 70 eV de energía cinética y a través de la interacción absorbe aproximadamente 14 eV como energía interna, la cual rápidamente causa la expulsión de uno de los electrones del analito, quedando el primer electrón con una energía residual de orden de 56 eV de energía cinética. Cuanto mayor sea la energía absorbida desde el electrón ionizante, mayor será la tendencia para el ion molecular naciente a descomponerse en iones fragmento. Por consiguiente, es predecible que el lugar de ionización más probable sea aquel donde los electrones estén más débilmente unidos (Watson, 2007). En la **Figura 1** se puede observar el mecanismo de la ionización que tiene lugar en el interior de la fuente de EI.

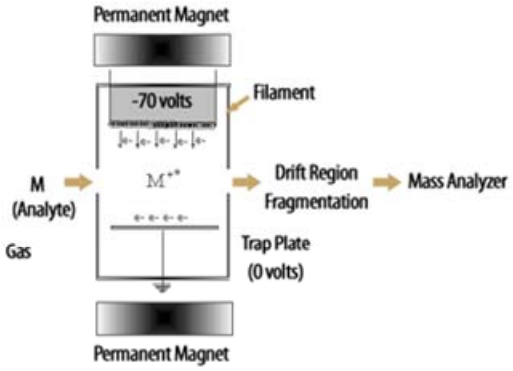

Figura 1. Fuente de ionización electrónica

3.2.2. Ionización química

El término ionización química engloba todas las técnicas de ionización suave, que a diferencia de la EI, suponen una reacción química exotérmica en la fase gas, llevada a cabo por un gas en base a sus iones reactivos. Se pueden formar tantos productos como iones estables positivos o negativos existan, dependiendo del proceso predominante. Los iones cuasi-moleculares se pueden definir como aquellos iones asociados a la masa molecular formados tras la aplicación de técnicas de ionización suave, como por ejemplo $[M+H]^+$, $[M-H]^+$, etc.

Las técnicas de ionización química logran la ionización del analito sin transferir demasiada energía al ion molecular naciente, lo que significa que hay poca fragmentación; pudiéndose obtener así el ion molecular intacto del analito. Se trata de una reacción entre la molécula y el gas reactivo, rico en protones normalmente. En ionización química positiva (PCI), el resultado es la formación de abundantes iones aducto, donde comúnmente el más abundante es el ion

molecular protonado, $[M+H]^+$. En la **Figura 2** se puede observar el proceso esquematizado de la PCI. En cambio, en la ionización química negativa (NCI) el principal proceso que se lleva a cabo es la captura de electrones, generando por tanto la especie M^-.

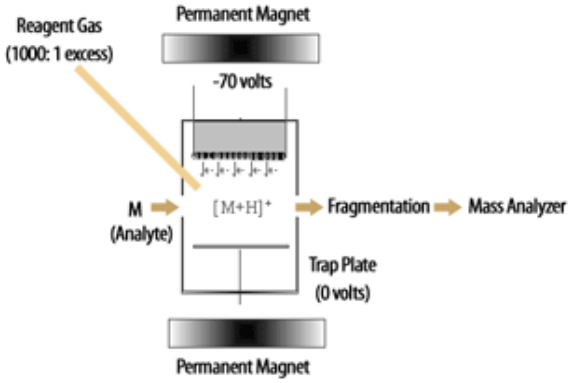

Figura 2. Fuente de ionización química positiva

En la técnica de ionización química original (la llamada CI), el gas reactivo necesita una cierta presión en la fuente para lograr una elevada concentración de iones reactivo y minimizar la posible ionización directa de los analitos por ionización electrónica. En ocasiones, CI puede tener problemas de reproducibilidad al establecer esta presión óptima del gas reactivo en la fuente de ionización. Recientemente se ha desarrollado una variación de esta técnica con el nombre de ionización a presión atmosférica (*atmospheric pressure ionization*, API), que actualmente conocemos como APCI (*atmospheric pressure chemical ionization*). La principal divergencia con CI es que, como su propio nombre indica, opera a presión atmosférica y además se diferencia de las anteriores con

respecto a mejoras obtenidas en la sensibilidad. El proceso de APCI se lleva a cabo tal como se indica en el esquema gráfico de la **Figura 3**.

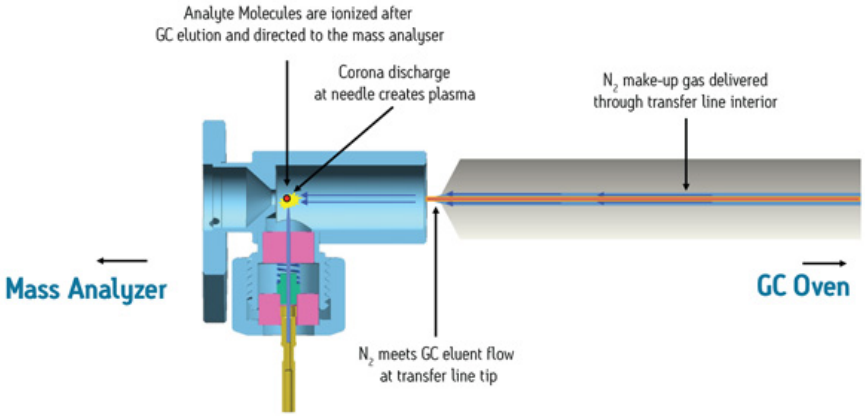

Figura 3. Fuente de ionización química a presión atmosférica

Tanto CI como APCI pueden utilizarse combinadas con cualquier tipo de analizador y aunque CI solo se puede acoplar a cromatógrafos de gases, la técnica de APCI viene con la ventaja de que se puede acoplar tanto a LC como a GC.

Este tipo de ionización, APCI, engloba dos mecanismos de reacción diferentes, teniendo en cuenta que éstas tienen lugar a presión atmosférica. En primer lugar se puede presentar el mecanismo de transferencia de carga, donde el ion $M^{+\cdot}$ es el ion principal en el espectro de masas. En segundo lugar puede existir el mecanismo de protonación, donde el ion principal será $[M+H]^+$, promovido normalmente por la adición de modificadores en el interior de la fuente de ionización como agua o metanol. Así, de este modo se induce la ionización de compuestos que *a priori* no serían ionizados. Este mecanismo de protonación ocurre sobretodo en compuestos que presentan un grupo ácido y que por ello tienen más tendencia a captar un protón en esa posición y presentar su molécula

protonada como ion más abundante. En cambio existen otros tipos de compuestos con los que incluso con la adición del modificador siguen presentando como pico base su ion molecular, $M^{+\cdot}$. Dependiendo del comportamiento que tienen los compuestos tras ser sometidos a la APCI, se pueden encontrar hasta 6 grupos diferentes según ha reportado Portolés *et al*. en un estudio detallado sobre la actuación de dicha fuente de ionización (Portolés, 2010).

APCI, al tener la posibilidad de acoplarse tanto a LC como a GC con diferentes analizadores es una buena herramienta de análisis, considerando además que es una de las fuentes de ionización incluida en las técnicas de análisis de mayor sensibilidad desarrolladas hasta el momento. Debido a su ionización suave, se obtiene una baja fragmentación con la ventaja de la obtención del ion molecular o la molécula protonada (ayudada por la adición de modificadores) como pico base. Este hecho ayuda en la determinación de contaminantes orgánicos en cuanto a la creación de los métodos analíticos, por varios aspectos. Una de las ventajas se puede encontrar en el proceso de creación de un método de *screening,* ya que simplemente examinando la presencia de la relación *m/z* ($[M+H]^+$ o $M^{+\cdot}$) en el espectro de masas adquirido, se puede detectar dicho analito y además combinado con analizadores de masa exacta sería una herramienta muy útil para la elucidación de la identidad de compuestos desconocidos. Por otro lado, APCI también mejora el desarrollo de métodos SRM en MS/MS, especialmente en aquellos casos con moléculas que por EI sufren una extensiva fragmentación. En el proceso de elección del ion precursor (normalmente el pico base) en APCI los iones presentan una mayor relación señal ruido (S/N) y mejor selectividad, por lo que las transiciones adquiridas serán más intensas y ofrecerán una mejora en la detección, sobre todo en el caso de compuestos presentes en las muestras a niveles de traza (Watson, 2007; Portolés, 2010; Hurtado-Fernández, 2013).

4. Tratamiento de muestras en la determinación de contaminantes orgánicos

4.1. Matrices vegetales

Las técnicas de extracción ideales serían aquellas que permitiesen extraer los analitos de las muestras sin arrastrar los componentes de la matriz, que podrían ocasionar interferencias en el análisis. Las técnicas que se pueden utilizar para la extracción de los diferentes tipos de compuestos en matrices de interés alimentario, son muy variadas, especialmente para productos vegetales.

La extracción directa de muestras sólidas con disolventes orgánicos es una técnica muy común, escogiendo los disolventes en función de la combinación matriz/analito a estudiar. Los disolventes que más se utilizan en análisis de residuos de plaguicidas son el acetonitrilo, utilizado en el método "universal" QuEChERS (*Quick, Easy, Cheap, Effective, Rugged and Safe*); la acetona, que favorece la extracción tanto de compuestos polares como apolares; el acetato de etilo, más apolar que los mencionados anteriormente, que aunque no extrae tan eficazmente los plaguicidas más polares presenta la ventaja de obtener unos extractos más limpios ya que se extraen menos componentes de la matriz (pero sí lípidos y ceras); y finalmente el metanol con un uso menos frecuente que los anteriores (Tadeo,2008; Huang, 2007; Nguyen, 2008).

Una parte fundamental en los procesos de extracción con disolventes es la energía aplicada sobre el sistema. Una posibilidad con gran aceptación es la extracción asistida por microondas, MAE, que utiliza microondas para calentar un disolvente en contacto con la muestra en un recipiente cerrado para extraer los

analitos de forma rápida y eficaz, con la ventaja de necesitar un menor tiempo de extracción y menos disolvente, comparado con una extracción convencional. Por otra parte, se ha descrito el inconveniente de la necesidad de una etapa extensiva de *clean-up* (Tadeo, 2008; Gfrerer, 2004). La extracción con fluido presurizado (PFE), técnica más conocida como extracción acelerada con solventes (ASE), utiliza disolventes para realizar la extracción a unas temperaturas y presiones elevadas para incrementar la velocidad de solubilización del analito en el disolvente, aumentando la eficacia de extracción de los disolventes y evitando la ebullición (Fernández Moreno, 2006; Gfrerer, 2004).

Un caso especial es la extracción con fluidos supercríticos (SFE), proceso en el cual se utiliza como disolvente un fluido supercrítico, que posee propiedades de gas y de líquido al mismo tiempo, ofreciendo mejores solubilidades, menores viscosidades y una elevada difusividad, permitiendo así una extracción más eficiente (Rissato, 2005).

Tras la aplicación de la etapa de extracción con disolventes, en muchas ocasiones es necesario realizar un *clean-up* posterior para conseguir extractos más limpios, con menor presencia de componentes de la matriz, evitando en la medida de lo posible problemas tanto en la cuantificación como en aspectos relacionados con la estabilidad/robustez instrumental. Para fines de *clean-up* es común aplicar técnicas como la cromatografía de permeación en gel (GPC), que separa las partículas en función de su tamaño y es muy adecuada para la eliminación de pigmentos de elevado peso molecular y material lipídico (Fernández Moreno, 2006; Huang, 2007). Otra técnica utilizada frecuentemente en matrices vegetales es la extracción en fase sólida (SPE), aunque es mucho más popular para fines de pre-concentración/*clean-up* en matrices acuosas (Huang, 2007).

Otro grupo de técnicas de extracción para muestras vegetales es el basado en el uso de adsorbentes sólidos. De entre todas las posibles aproximaciones, cabe destacar la microextracción en fase sólida (SPME), la extracción mediante barras agitadoras magnéticas (SBSE) o la extracción mediante dispersión en matriz en fase sólida (MSPD).

La SPME consiste en la extracción de los analitos mediante la adsorción de éstos sobre una fase estacionaria que puede variar en cuanto a dimensiones y tipo y que se encuentra recubriendo una fibra de sílice fundida. Esta técnica presenta dos modalidades de trabajo, dependiendo de dónde se encuentre situada la fibra en el momento de la extracción; bien en contacto con la muestra por inmersión directa (DI) para el caso de matrices acuosas (o zumos) o bien situada en el espacio de cabeza (HS) para compuestos más volátiles, tanto en matrices sólidas como líquidas. Esta técnica presenta la ventaja de no utilizar disolventes orgánicos para la extracción, ni tampoco para el modo HS en la elución, ya que ésta se suele llevar a cabo por desorción térmica en el propio sistema de inyección del cromatógrafo de gases (Beltrán, 2003; Sang, 2013; Natangelo, 2002).

Una técnica similar pero un poco más reciente que la SPME, es la SBSE, donde la extracción tiene lugar sobre una barra magnética agitadora recubierta con la fase estacionaria. Aunque el fundamento es similar a la SPME, presenta un aumento en la cantidad de fase estacionaria, lo que supone una ventaja con respecto a la detección de analitos que se encuentran a muy bajos niveles (Maggi, 2008).

Otra forma de extracción habitual en la determinación de plaguicidas en matrices vegetales es la MSPD, que se basa en la mezcla de la muestra con un sorbente (C_{18}, C_8, Florisil, sílice aminopropil derivatizado, etc.) y disolvente

seleccionado, para realizar de forma combinada la extracción de los analitos de la muestra al sorbente y a continuación al disolvente (Libin, 2006).

Uno de los métodos más populares en análisis de residuos de plaguicidas (PRA) en matrices vegetales combina varias de las técnicas de extracción y *clean-up* descritas anteriormente. Es conocido como método QuEChERS y sus siglas en inglés indican que es un método rápido, fácil, barato, efectivo, robusto y seguro. Con todos estos adjetivos es fácil entender porque se ha extendido su uso en el campo del análisis de matrices vegetales. Existen diferentes versiones, partiendo de la original que fue publicada en el año 2003 (Anastassiades, 2003). En el método original se usaba acetonitrilo como disolvente para la extracción seguido de la adición de $MgSO_4$ y NaCl. La posterior eliminación del agua y la etapa de *clean-up* se realizaban con una extracción en fase sólida dispersiva (d-SPE) con $MgSO_4$ y una amina primaria y secundaria (PSA). Entre las diferentes modificaciones/versiones del método QuEChERS destacan sobretodo dos de ellas, que son las más ampliamente utilizadas. En primer lugar, se encuentra el método oficial 2007.01 de la asociación de químicos analíticos norteamericanos (AOAC), en el cual se usa un tampón de acetato para ajustar el medio a un pH aproximado de 4.8, permitiendo la extracción de ciertos plaguicidas problemáticos como *folpet, dichlofluanid, chlorothalonil* o *pymetrozine.* La partición líquido-líquido se lleva a cabo con $MgSO_4$ y NaAc y la d-SPE se mejora al añadir el sorbente C_{18} al $MgSO_4$ y PSA presentes en el antiguo método, ya que ayuda a eliminar lípidos (Lehotay, 2005). Por otro lado, está el método estandarizado 15662 del comité europeo de normalización (CEN) el cual utiliza sales de citrato como tampón a un pH ligeramente superior, entre 5 y 5.5 (Payá, 2007), adicionalmente a las sales utilizadas comúnmente en el método original para la partición líquido-líquido. Las diferentes versiones publicadas persiguen la mejora en la extracción de ciertos

plaguicidas en matrices problemáticas. En general se obtienen buenos resultados para un elevado número de plaguicidas y matrices vegetales, sobre todo con las dos versiones modificadas principales, indicadas anteriormente (Lehotay, 2010).

4.2. Matrices acuosas

En referencia al análisis de muestras acuosas, se pueden encontrar por ejemplo, aguas de distintos tipos en las que las concentraciones de los analitos son usualmente muy bajas (niveles de (sub) ppb). Con la etapa de extracción, se persigue la pre-concentración de estos analitos, al tiempo que éstos se separan de la matriz acuosa. También puede tener lugar una cierta etapa de purificación, en función del sistema aplicado.

La técnica que más se utiliza en matrices acuosas cuando los analitos son semivolatiles o no-volátiles es la SPE (Pitarch, 2007; Pitarch, 2010), la cual permite extraer los analitos, pre-concentrarlos y realizar un *clean-up* de la muestra en única etapa. La SPE se basa en la retención de los analitos en un sorbente a través del cual pasa la muestra. Posteriormente, los analitos se eluyen con disolventes orgánicos adecuados. Este sorbente se encuentra incluido en un cartucho relleno y dependiendo de las distintas características físico-químicas de los compuestos a estudiar se selecciona el más adecuado, principalmente teniendo en cuenta la polaridad y las características ácido-base. De entre todas las fases existentes, las de C_{18} son las más adecuadas para compuestos apolares o de polaridad moderada, las de base polimérica (ej. Oasis HLB) presentan buenos resultados para compuestos de polaridad media-alta y para el caso de sustancias muy polares las de carbón activado serían las más adecuadas. Por otro lado, para el

caso de sustancias iónicas, los sorbentes de intercambio iónico serían los más apropiados, por ejemplo, de retención mixta de fase reversa (intercambio catiónico (MCX) o intercambio aniónico (MAX)). La gran diversidad de adsorbentes es uno de los puntos fuertes de la SPE, ya que permite cubrir un amplio rango de polaridades. Este tipo de extracciones se puede realizar en modo *off-line* (Bijlsma, 2009) en una etapa diferenciada a la de la inyección en el sistema cromatográfico, o bien en modo *on-line*, con un proceso que puede automatizarse mediante el uso de válvulas de alta presión, lo que le confiere la ventaja de una menor manipulación de muestra y por tanto mayor reproducibilidad, así como mayor velocidad del proceso (Hernández, 2005; Marín, 2006).

Otra técnica bastante utilizada desde hace años es la extracción líquido-líquido (LLE), basada en la distinta distribución de los compuestos orgánicos entre dos fases, la acuosa y la orgánica. Así, la polaridad del disolvente orgánico determina en buena medida la distribución de los analitos entre ambas fases. Esta técnica se ha utilizado con disolventes orgánicos muy diversos en la extracción de contaminantes orgánicos de matrices acuosas (Robles-Molina, 2013).

Tradicionalmente, SPE y LLE han sido y siguen siendo las técnicas más empleadas para la extracción de contaminantes orgánicos en muestras acuosas. En comparación, la SPE mejora algunos de los inconvenientes típicos de la LLE, como la separación de fases incompletas (emulsiones) o la necesidad de usar material de vidrio relativamente caro y frágil; pero sobre todo por el uso de elevadas cantidades de disolvente.

Técnicas como la SBSE o la SPME son también utilizadas en este ámbito ya que no utilizan prácticamente disolventes orgánicos (Cavaliere, 2012; Camino-

Sánchez, 2013). Entre éstas, la técnica SPME se ha usado con éxito y con mayor frecuencia en PRA para matrices acuosas (Beltrán, 2000; Hernández, 2000).

Cuando los compuestos de interés presentan un carácter más volátil, se utilizan técnicas como la purga y trampa (P&T), que se basa en la extracción de los analitos desde la muestra a una fase gaseosa y su arrastre mediante un gas hacia un sorbente, donde son retenidos. Posteriormente, los analitos atrapados son desorbidos mediante un disolvente (Liu, 2009; Ikem, 2009) o térmicamente (Bernier, 1999). La SPME en la modalidad de espacio de cabeza es una técnica muy utilizada para este tipo de compuestos en matrices acuosas donde los analitos en fase gas son adsorbidos en la fibra que puede ser a continuación desorbida, térmicamente, directamente en el inyector del GC sin la utilización de ningún disolvente (Fries, 2006; Niri, 2008; Pecoraino, 2008).

En los trabajos que forman parte de la presente **Tesis Doctoral** se ha empleado GC-MS para la determinación de contaminantes orgánicos. En cada capítulo se muestran los diferentes métodos desarrollados con la utilización de diferentes analizadores de masas. En el **Capítulo 2** se describen las investigaciones realizadas en el campo del análisis de residuos de plaguicidas utilizando GC-MS/MS con QqQ como analizador. En el **Capítulo 3** se explora el potencial tanto del TOF como del QTOF como analizadores de masas para el *screening*/análisis de residuos de plaguicidas en matrices vegetales. Por último, en el **Capítulo 4** se hace uso tanto del QqQ MS como del TOF MS para la investigación de contaminantes volátiles orgánicos en aguas y vegetales.

En cuanto al tratamiento de muestra, generalmente se ha basado en la extracción de plaguicidas en matrices vegetales, bien mediante ASE, como en el **Artículo científico 2,** o bien mediante el método QuEChERS en su versión tamponada con acetato, para los **Artículos científicos 3 y 4**. Por otro lado, el **Capítulo 4** trata sobre compuestos volátiles orgánicos, por lo que en los artículos incluidos (**Artículos científicos 5 y 6**) se ha hecho uso de técnicas más específicas para ellos como la SPME y la P&T, respectivamente.

CAPÍTULO 2

DETERMINACIÓN DE RESIDUOS DE PLAGUICIDAS MEDIANTE CROMATOGRAFÍA DE GASES ACOPLADA A ESPECTROMETRÍA DE MASAS EN TÁNDEM (TRIPLE CUADRUPOLO)

1. Introducción

La seguridad alimentaria es un aspecto de elevada importancia en la sociedad actual ya que afecta de forma directa a la salud de los consumidores. Es evidente la necesidad de realizar un control riguroso de los alimentos que se van a consumir y tener el mayor conocimiento posible sobre su composición, para poder disponer en todo momento de productos de elevada calidad, nutritivos y seguros para la sociedad. La EFSA, agencia europea responsable de llevar a cabo esta tarea, ayuda a controlar los posibles riesgos, adopta y/o revisa la legislación europea sobre alimentos o piensos, interviene en los procesos de aprobación de sustancias reguladas, como plaguicidas o aditivos alimentarios, y fomenta el desarrollo de nuevas políticas y marcos regulatorios en el campo de la nutrición.

Los alimentos, que en último lugar llegan al consumidor, provienen de fuentes muy variadas y por tanto se requiere un control minucioso durante toda la

cadena de producción alimentaria. La posible presencia de residuos tóxicos y contaminantes no deseados, a niveles mayores de los permitidos por la legislación, así como la existencia de otros factores como los biológicos pueden poner en peligro la salud del consumidor. Las legislaciones sobre contaminantes en alimentos se encuentran siempre en proceso de actualización para garantizar un elevado nivel de seguridad. Periódicamente se incluyen nuevos contaminantes que requieren una mayor atención a la luz de los avances en investigación. Cabe mencionar que entre los diversos contaminantes que se pueden encontrar en los productos alimentarios, los plaguicidas ocupan un lugar destacado, por su aplicación directa a productos vegetales y por su presencia habitual en muchos alimentos.

Debido a su potencial peligrosidad, se han establecido normas básicas con respecto al uso y comercialización de los mismos, bajo la premisa de que el uso de plaguicidas no debe tener efectos nocivos sobre los seres humanos ni sobre los animales (Council Directive 91/414/EEC). Debe tenerse en cuenta que, debido al extensivo uso de estos productos químicos durante la cadena de producción de los alimentos, la población está expuesta a ciertos niveles de plaguicidas a través de sus dietas. La Unión Europea ha regulado el uso de plaguicidas, por lo que todos los Estados Miembros deben aplicar los mismos procedimientos de evaluación y criterios de autorización de productos fitosanitarios. Todo ello con el fin de minimizar el nivel de exposición a estos contaminantes, tanto por parte del operador que los aplica en el campo, como a nivel ambiental y alimentario. Tras la aplicación de estos plaguicidas, con fines de conservación o mejora de los productos destinados a la alimentación, es habitual encontrar residuos de estos procesos. La definición de residuo de plaguicida puede incluir tanto a las sustancias activas, como a los metabolitos y productos de degradación.

En el Reglamento del 23 de febrero de 2005 (Regulation (EC) No 396/2005) se establecen los MRLs permitidos en alimentos o en piensos de origen vegetal y animal. Todos ellos quedan establecidos a unos niveles adecuados para proteger al consumidor, de modo que el residuo no suponga un riesgo inaceptable para la salud humana. Estos niveles son establecidos en base a ensayos de campo en cumplimiento con las buenas prácticas agrícolas (GAP) en el marco de estudios realizados conforme a las buenas prácticas de laboratorio (GLP). En ausencia de estudios de residuos, y siempre y cuando la materia activa esté autorizada por la EU, se establece un nivel por defecto (normalmente 0.01 mg/kg), coincidiendo con el límite de determinación analítica. Los métodos analíticos requeridos para el control de estas sustancias han de ser capaces de detectar residuos a estos bajos niveles. Es por ello que se necesita una elevada sensibilidad y selectividad, además de ofrecer una confirmación inequívoca de la identidad del residuo detectado y, por supuesto, ser capaces de realizar una cuantificación apropiada y precisa para cualquiera de los compuestos incluidos en el método analítico (Martínez-del-Río, 2013).

Los análisis de residuos de plaguicidas se suelen llevar a cabo actualmente mediante técnicas cromatográficas acopladas a espectrometría de masas. Dependiendo de la naturaleza de cada analito se debe escoger la técnica cromatográfica más conveniente. Así, entre los compuestos adecuados para análisis mediante LC se incluyen analitos con polaridad media o alta. En cambio, los compuestos más indicados para GC son aquellos que presentan baja polaridad y volatilidad intermedia o elevada. Ambas técnicas son complementarias entre sí, ya que para abordar el análisis completo de plaguicidas en un amplio rango de polaridades y volatilidades se requeriría el uso de ambas. En el caso de la presente

Tesis Doctoral todos los trabajos se han desarrollado mediante GC-MS, por lo que nos referiremos a esta técnica a partir de este punto.

En el campo de los alimentos, se han reportado y aún se siguen reportando numerosas aplicaciones de GC-MS con analizador de cuadrupolo. La mayoría de estos análisis se basan en modo SIM, con la adquisición únicamente de las *m/z* seleccionadas, lo cual mejora en gran medida la sensibilidad del método comparado con la adquisición en modo *scan* (Banerjee, 2013; Cherta, 2013). En términos de sensibilidad, GC-MS/MS IT presenta unas propiedades similares al GC-Q MS en SIM, aunque IT mejora notablemente las condiciones confirmatorias al trabajar en MS en tándem (Mezcua, 2009). El analizador IT ha sido ampliamente utilizado en métodos GC-MS/MS, pero en los últimos años ha perdido notoriedad frente al QqQ como analizador de masas en tándem. En general, las técnicas de MS/MS son muy utilizadas en PRA, ya que realizan un segundo filtro de los iones seleccionados lo que se traduce en un incremento notable de la selectividad, por la reducción de la contribución de interferencias isobáricas; produciendo también un incremento considerable de la sensibilidad y una disminución de los límites de detección. Además, la selectividad conseguida con la monitorización del paso desde un ion precursor a un ion producto característico produce un mayor grado de confidencia en cuanto a la confirmación de la identidad. Los analizadores IT tienen una elevada eficiencia como resultado de que los iones precursores y producto permanecen en una única trampa de iones, sin tener que transportarse de una cámara a otra, evitando así posibles pérdidas. Además, ofrecen información valiosa para una mejor confirmación de los positivos encontrados en las muestras gracias a la posibilidad de realizar experiencias de MS^n (Garrido, 2008).

El QqQ, en modo MS/MS, presenta la posibilidad de seleccionar en el primer cuadrupolo un ion precursor, fragmentarlo en la celda de colisión, y monitorizar solo los fragmentos resultantes seleccionados en el segundo cuadrupolo, creando finalmente una transición SRM específica de cada analito (Tadeo, 2008). Tanto IT como QqQ presentan indudables mejoras en la selectividad y sensibilidad, pero éstas son más importantes con el analizador QqQ, por su mejor relación señal-ruido. El uso de QqQ suele producir una disminución de la señal absoluta de los iones característicos del analito, por la rotura debida a la energía de colisión aplicada. Afortunadamente, esta disminución es mucho mayor en la señal debida al ruido químico, generando una gran aumento de sensibilidad. Por todo ello, su aplicación en el campo del PRA ha sido creciente en los últimos años, por ser una excelente herramienta, fiable y altamente sensible, tanto en el campo medioambiental como en la seguridad alimentaria, incluyendo la adecuada identificación y cuantificación de los analitos en una única inyección. Por todos estos motivos, GC-MS/MS QqQ se ha convertido en la técnica de elección para la determinación de plaguicidas (semi) volátiles y poco polares en la mayoría de laboratorios de rutina (Walorczyk, 2007; Garrido Frenich, 2010).

En el presente capítulo, se exploran las posibilidades de la técnica GC-MS/MS QqQ, se revisan sus aplicaciones en PRA y se desarrolla un método SRM para un elevado número de plaguicidas. Así, en el **Artículo científico 1** se muestra la revisión de las aplicaciones de la cromatografía de gases acoplada a la espectrometría de masas con analizador de triple cuadrupolo en PRA en el campo de la seguridad alimentaria y medioambiental. En él se recogen sus usos desde los inicios del triple cuadrupolo acoplado a la GC, hace aproximadamente una década, hasta la actualidad, haciendo hincapié en diferentes aspectos de interés como la preparación de muestra, el tipo de analito o el modo de análisis, entre otros.

Además, se enfatiza la importancia de esta técnica en este campo de trabajo y las ventajas que aporta en cuanto a su mayor sensibilidad y selectividad, además de su robustez.

En el **Artículo científico 2**, se desarrolla y se valida un método multiresidual mediante cromatografía de gases acoplada a espectrometría de masas con analizador de triple cuadrupolo, para la determinación de 130 plaguicidas, incluyendo diferentes familias químicas, en diversos tipos de frutas y verduras. Se presta especial atención al estudio del efecto matriz sobre numerosas matrices alimentarias. La parte del tratamiento de muestra se realizó en los laboratorios de la Agencia de Salud Pública de Barcelona, siguiendo los procedimientos normalizados de trabajo de dicho laboratorio.

2. Artículo científico 1

Analytical Methods

RSC Publishing

CRITICAL REVIEW

The role of GC-MS/MS with triple quadrupole in pesticide residue analysis in food and the environment

Cite this: *Anal. Methods*, 2013, **5**, 5875

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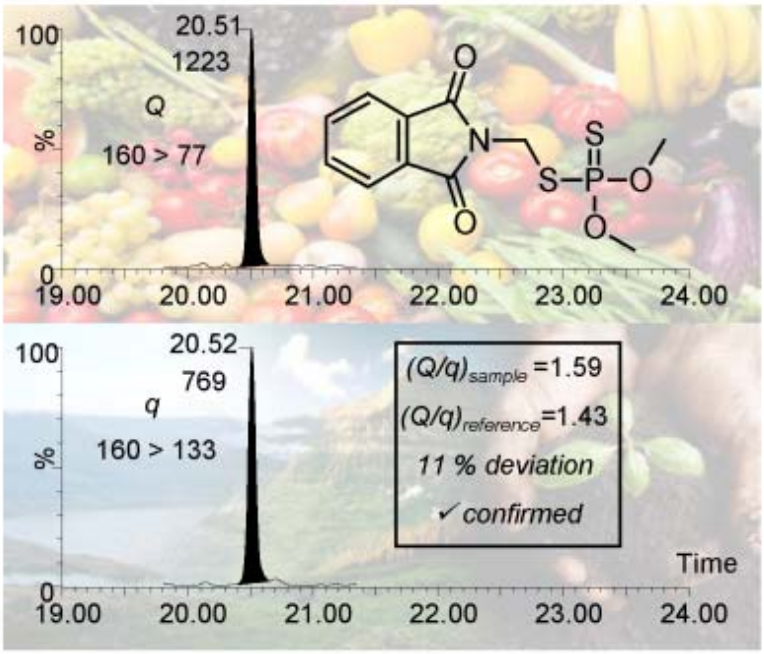
Gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) using a triple quadrupole (QqQ) analyzer has in the last few years become a powerful technique for the determination of pesticide residues due to its robustness, and excellent sensitivity and selectivity. This review gives an overview of currently published applications of GC-MS/MS with a QqQ analyzer for pesticide residue analysis of different food and environmental sample matrices. This technique allows the reliable quantification and identification of low pesticide concentrations for non-polar (semi) volatile compounds belonging to different chemical families. It has allowed a notable improvement of methods performance in comparison with the traditional GC methods with single stage quadrupole MS.

Received 4th July 2013
Accepted 7th August 2013

DOI: 10.1039/c3ay41104d

www.rsc.org/methods



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Analytical Methods, 5, 5875-5894, ***2013***

The role of GC-MS/MS with triple quadrupole in pesticide residue analysis in food and the environment

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Abstract

Gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) using a triple quadrupole (QqQ) analyzer has in the last few years become a powerful technique for the determination of pesticide residues due to its robustness, and excellent sensitivity and selectivity. This review gives an overview of currently published applications of GC-MS/MS with a QqQ analyzer for pesticide residue analysis of different food and environmental sample matrices. This technique allows the reliable quantification and identification of low pesticide concentrations for non-polar (semi) volatile compounds belonging to different chemical families. It has allowed a notable improvement of methods performance in comparison with the traditional GC methods with single stage quadrupole MS.

1. Introduction

Pesticide residue analysis (PRA) of food and environmental materials has become an important specialized field of modern analytical chemistry. The necessity of advanced analytical methods for its application in monitoring programs that ensure food-safety and environmentally responsible agricultural practices has been frequently highlighted. Reliable and sensitive analytical methods able to reach the low limits of quantification (LOQ) required by the legislation are needed. In most cases, LOQs lower than 0.01 mg kg^{-1} in food and lower than 0.1 μg L^{-1} in water are needed for monitoring purposes, where the reliable identification and quantification of hundreds of pesticide residues in many different matrices is normally pursued. In recent years, new developments in sample preparation and instrumentation, especially dealing with chromatographic techniques coupled to mass spectrometry (MS) or tandem MS, have allowed the high quality standards required from a qualitative and quantitative point of view in PRA to be achieved.

In the past decades, gas chromatography coupled to mass spectrometry (GC-MS) methods have been mostly based on selected ion monitoring (SIM) or full scan modes, evolving from single quadrupole (Q) to ion trap (IT) analysers. The first papers dealing with PRA by GC-MS can be traced back to 1970's when the determination of a reduced number of pesticides was carried out using packed column GC systems coupled to mass spectrometers with single quadrupole analyzers. In a recent review [1], it has been reported that the single quadrupole is still the most used analyzer in combination with GC. Similarly, Botitsi *et al.* [2] showed that during the period of 2006 to 2009, 26 out of the 47 reviewed papers that employed GC-MS for the determination of pesticides in food and water were

based on single quadrupole analysis. According to data reported, only 9 papers dealt with the use of triple quadrupole (QqQ), the rest being methods based on IT and time-of-flight (TOF) analyzers. In the review article from Andreu and Picó [1] on PRA in biota, 18 out of 24 papers reviewed dealt with the use of single quadrupole GC-MS.

Despite the wide existing applications, methods based on the use of single quadrupole instruments suffer from low sensitivity when working in the full scan mode. The sensitivity can be improved by working in the SIM mode, but the identification potential and the non-target/retrospective analysis capabilities are sacrificed. After development of the single quadrupole, the next step in the evolution of mass analyzers in pesticide residues analysis (without eliminating the use of the single quadrupole) was the increased use of ion trap mass spectrometers that allowed full spectra based methods to be developed with suitable sensitivity (similar to that obtained by a quadrupole in the SIM mode) in a single run. A number of papers have been published demonstrating the capability of ion trap analyzers for carrying out tandem MS experiments, improving sensitivity and selectivity, but losing the non-target capabilities. The fact that the MS/MS working mode of an ion trap is a product ion scan results in the co-elution of several analytes, or sample matrix components and notably reduces sensitivity and the number of points across the chromatographic peak. In the late 90s, the introduction of GC-TOF MS resulted in an improvement of full scan based methods and a step forward in non-target analysis. TOF MS is able to provide full spectrum acquisition data at high sensitivity. High-speed (HS) TOF MS, with a fast data acquisition rate (up to 500 spectra per second), is an excellent technique for GC × GC MS detection, for which the data acquisition speed is the most limiting factor. On the contrary, mainly coupled to 1D-GC and with lower data

acquisition rates (20–25 Hz) and a narrower dynamic range, high-resolution (HR) TOF MS provides sensitive full spectrum data with high mass accuracy, allowing the resolution of peaks from closely related interfering matrix components. In the last years, many papers can be found dealing with the determination of pesticides in food and environmental samples by GC × GC-(HS) TOF MS [3,4] but much less related with GC (HR) TOF MS [5,6]. The most important limitation of TOF MS is related to its low sensitivity which can make it troublesome to reach the required limits of detection (LOD) and, in the case of the HR TOF, also the peak saturation problems that occur in short dynamic ranges.

One of the major developments in the field of PRA has been the commercialization of liquid chromatography (LC) coupled to QqQ MS systems, which has benefited greatly from the high sensitivity and selectivity of tandem MS in the selected reaction monitoring (SRM) mode. Thus, QqQ has been the analyzer most used in LC-based methods in the 2006–2009 period [2], although this fact was not such evident for GC-based methods, which have suffered a notable delay in the wide acceptance of this analyzer in comparison to LC-MS/MS. In fact, LC-QqQ MS/MS started to be applied in PRA in the early 90's, while GC-QqQ MS/MS was applied around 15 years later. The first publications, between 2003 and 2005, reported the use of GC-QqQ MS/MS in food matrices like tobacco, oil, baby food or cucumber [7–10] and in human fat [11].

Several papers have been published on the comparison of different analyzers in GC-MS. Mezcua *et al.* [12] compared GC-Q MS and GC-IT MS(/MS) for the determination of insecticides in vegetables indicating that no significant differences were found in terms of sensitivity, although IT under MS/MS conditions was superior to GC-Q MS in the identification capability. Garrido *et*

al. [13] made an interesting comparison between two MS/MS systems, QqQ and IT, concluding that intraday-precision was similar for both, but interday-precision was found to be worst in the case of QqQ. In contrast, better linearity ranges were achieved for QqQ together with lower matrix effects especially for dirty samples *e.g.* fat containing samples. Additionally, a larger number of compounds can be included in a single injection in QqQ (SRM mode), although regarding identification capabilities IT in the tandem MS mode gives more information for a better confirmation of positive findings due to the MS^n possibilities.

Several ionization techniques have been used in GC-MS over time [14,15], electron ionization (EI) being the most popular and widely used. EI offers valuable information on the molecule structure (several fragment ions and, in some cases, molecular ions). It is a robust and universal ionization source that generates highly reproducible spectra that can be searched in available commercial spectral libraries for the identification of non-target compounds. However, EI generates highly extensive fragmentation. In some cases, it leads to mass spectra without abundant/intense characteristic peaks (*e.g.* molecular ion), and the sensitivity obtained can be poor. In those cases, alternative approaches, such as chemical ionization (CI), which can be applied in both the positive or negative mode allow mass spectra to be obtained with a predominant molecular ion peak and low fragmentation. CI has been applied in PRA, especially for the determination of organohalogenated pesticides due to its better sensitivity and selectivity for some of these compounds [16,17]. However, there is a low number of applications compared to EI, and it is not possible to carry out spectral library searching as it is not commercially available for CI.

A promising source is atmospheric pressure chemical ionization (APCI), which opens a new perspective in the development of GC-MS/MS methods. This source has been recently tested in PRA and offers very attractive features for compounds that suffer extensive fragmentation in the EI mode [18,19].

2. GC-MS/MS applications in food analysis

Nowadays, the control of pesticide residues in food commodities has become a requirement for compliance with the legislation, ensuring safety of the population and international and national trade. The determination of GC-amenable pesticides in food samples by using tandem MS with a QqQ analyzer has emerged in the last decade as a valuable approach, which allows higher selectivity and sensitivity and minimizes or even removes most chromatographic interferences. This section reviews the papers published in the last ten years related to the determination of pesticide residues in food samples by GC-QqQ MS/MS (***Table 1***). The most relevant aspects related to the studied pesticides, types of matrices, sample preparation procedures and analytical measurements are discussed in the present review.

The different physicochemical properties of the pesticide chemical classes increase the difficulty when developing a simultaneous analytical method for multiresidue analysis of food commodities. Thus, analytical methodologies based on GC-QqQ MS/MS for the determination of pesticides from the same family are quite common, *e.g.* for the determination of organochlorine (OC) [8,20,21], organophosphorous (OP) pesticides [22–24], or of pyrethroids [19,25] in food commodities. Some particular examples are the GC-QqQ MS/MS method

developed by Le Faouder *et al.* [26] just for fipronil, or by Peruga *et al.* for chlorothalonil [27]. Other authors have included two families of pesticides, typically OC and OP [28–32]. However, the majority of applications (around 70 %) deal with multiresidue methods for multiclass pesticides in food samples [7,9,10,16–18,33–58], the most adequate strategy available for monitoring purposes that minimizes time, costs, reagents, labors and hazards in order to obtain rapid analytical results in response to urgent demands. Moreover, most multiclass methods published include more than one hundred pesticides in their target list, among insecticides, herbicides, acaricides, fungicides, *etc.* [10,33,35–38,40,41,43,44,46–52,57]. In this respect, remarkable papers are those published by Okihashi *et al.* [35] and by Banerjee *et al.* [57], who have developed analytical methodology for the determination of up to 260 and 349 pesticides in fruits and vegetables, respectively.

In PRA, the term Food includes a wide range of treated products, fruits, vegetables, grains and other commodities. Even after being washed, stored, processed and prepared, some pesticide residues may remain in both fresh products and processed foods. From the overview of the applications shown in ***Table 1***, it can be seen that fruits and vegetables are the most frequent samples analyzed [10,16–19,24,27,32–37,39,41,44,46,48–50,52,54,55,57,59].Other matrices analyzed are oils and fats [8,22,23,38,40], cereals [38,39,43,59], muscles and livers [28–30,58],tobacco [7,42], ginseng [47,56], animal feeds [20,26], milk [25,26], eggs [31], wine [51], mussels [21], baby food [9] and fruit-based soft drinks [53].

Table 1. Pesticide residue analysis in food commodities by GC-(QqQ)MS/MS[a]

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg^{-1})	Number SRM transitions	Confirmation criteria	Comments
2003, J. Haib *et al.*[7]	Tobacco	Tobacco	42 multiclass pesticides (NCI mode)	PLE (acetone) and SPE clean-up (Florisil, silica gel, GCB)	(NCI)MS/MS	50	1	None	
			26 multiclass pesticides (EI mode)		(EI)MS/MS				
2005, K. Patel *et al.*[8]	Pork and fish, olive and hydrogenated vegetable oil	10 hydrogenated vegetable oils	19 OC pesticides	GPC	(EI)MS/MS	10	2	Acquisition of 2 SRM transitions	Matrix-matched standards
2005, C. C. Leandro *et al.*[9]	Baby food of fruit and rice; fish and pasta; and potato and pork	Baby food of vegetable lasagne; chicken, leek and sweet corn risotto; spaghetti with tomatoes and mozzarella; vegetable and chicken risotto; apple and vanilla dessert; mango and banana with yogurt; tropical fruit salad; banana and peach dessert; creamy rice breakfast, and apple and blueberry	12 multiclass pesticides and TPs	QuEChERS method with d-SPE clean-up ($MgSO_4$, C_{18}, PSA)	(EI)MS/MS	1	2	Ion ratio within the expected range for standards	PTV injection (8 μL) Matrix-matched standards
2005, A. Garrido Frenich *et al.*[10]	Cucumber	2 cucumbers	130 multiclass pesticides	Solvent extraction (ethyl acetate, Na_2SO_4)	(EI)MS/MS	10	2-3	Relative intensity ratios (tolerance of ± 20% compared with standards)	PTV injection (10 μL) 1st injection: screening (1 SRM) 2nd injection: confirmation and quantification (2–3 SRM)

Table 1. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg−1)	Number SRM transitions	Confirmation criteria	Comments
2006 (a), A. Garrido Frenich *et al.*[28]	Chicken, pork and lamb muscle	10 chickens, 10 pigs and 10 lambs	45 OC and OP pesticides	Solvent extraction (ethyl acetate, Na_2SO_4) and GPC clean-up	(EI)MS/MS	50	2- 3	Relative intensity ratios (Decision 2002/657/EC)	PTV injection (10 μL) Matrix-matched standards
2006, J. L. Martínez Vidal *et al.*[33]	Cucumber	(15 samples) green bean, pepper, cucumber and tomato	130 multiclass pesticides	Solvent extraction (ethyl acetate, Na_2SO_4)	(EI)MS/MS	25	2-3	Relative intensity ratios (tolerance ± 20% compared with standards)	PTV injection (10 μL) 1st injection: screening (1 SRM) 2nd injection: confirmation and quantification (2–3 SRM) Matrix-matched standards
2006, S. Walorczyk and B. Gnusowski[34]	Tomato and onion	6 tomatoes	78 multiclass pesticides	Solvent extraction (ethyl acetate, Na_2SO_4) and clean-up (aminopropyl, $MgSO_4$)	(EI)MS (EI)MS/MS	200	1–2	A purity search factor above 600 (GC-MS)	Matrix-matched standard
2006(b), A. Garrido-Frenich *et al.*[29]	—	Chicken muscle	6 OC and OP pesticides	Solvent extraction (ethyl acetate, Na_2SO_4) and GPC clean-up	(EI)MS/MS	—	2-3	None	Based on 2006(a), *Garrido Frenich et al.*
2007, A. Garrido Frenich *et al.*[30]	Chicken liver	8 chicken, 8 pork and 9 lamb livers	34 OC and OP pesticides	Solvent extraction (ethyl acetate, Na_2SO_4) and GPC clean-up	(EI)MS/MS	25	2–3	4 IPs (Decision 2002/657/EC)	PTV injection (10 μL) Matrix-matched standards

Table 1. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2007, M. Okihashi *et al.*[35]	Carrot, banana and grapefruit	(173 samples) fruits, vegetables and rice	260 multiclass pesticides	Solvent extraction (acetonitrile, $MgSO_4$, NaCl) and SPE clean-up (GCB/PSA)	(EI)MS/MS	20	2	Evaluation of relative intensity ratios	Matrix-matched standards Solvent exchange to acetone
2007, Le Faouder *et al.*[26]	Plant, milk and mineral feed	Plants, milk and mineral feeds	Fipronil and 4 metabolites	Solvent extraction (ethyl acetate) and SPE clean-up (Florisil, Atoll)	(EI)MS/MS	0.025 μg L^{-1} (milk) 50 (plants)	2	Evaluation of relative intensities in-between two transitions	—
2007 (a), P. Plaza Bolaños*et al.*[36]	Strawberry	20 strawberries	151 multiclass pesticides	QuEChERS method with d-SPE clean-up (PSA, $MgSO_4$)	(EI)MS/MS (EI)MS(SIM)	11.5	2–3	4 IPs (Decision 2002/657/EC)	PTV injection (10 μL) Matrix-matched standards
2007, T. Pihlström *et al.*[37]	Grape, carrot, lettuce and orange	Orange and lemon	122 multiclass pesticides	Solvent extraction (ethyl acetate, Na_2SO_4, $NaHCO_3$)	(EI)MS/MS	10	2 (for most frequent pesticides) 1(for less frequent pesticides)	None	1st injection: screening (1 SRM) 2nd injection: confirmation and quantification(2 SRM) Matrix-matched standards

Table 1. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2007, S. Walorczyk[38]	Wheat grain, barley, rye, bean, maize and animal feed based on rapeseed oil	(136 samples) cereal grain, bran, maize and by-products of rapeseed oil manufacture	122 multiclass pesticides	Citrate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, C_{18}, PSA)	(EI)MS/MS	50 10 (only wheat and grain)	2	2 SRM transitions (Decision 2002/657/EC)	Matrix-matched standards. Solvent exchange to toluene
2007, P. Plaza Bolaños*et al.*[31]	Egg	20 eggs	38 OC and OP pesticides	MSPD extraction (C_{18}, $MgSO_4$) and SPE clean-up (Florisil)	(EI)MS/MS	15	2–3	3 IPs (Decision 2002/657/EC)	PTV injection (10 μL) Matrix-matched standards
2007, P. Payá*et al.*[39]	Lemon, raisin, wheat flour and cucumber	—	38 multiclass pesticides	Citrate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, PSA)	(EI)MS/MS	5 (cucumber and lemon) 10 (raisin and flour)	2–3	Maximum acceptable deviation in the relative intensity ion ratios (SANCO/10232/2006)	PTV injection (3 μL) Matrix-matched standards
2007, A. Garrido Frenich *et al.*[40]	Olive oil	8 olive oils	100 multiclass pesticides	LLE (hexane–acetone) and GPC clean-up	(EI)MS/MS	12	2–3	Relative intensity ratios (Decision 2002/657/EC)	PTV injection (10 μL) Matrix-matched standards.
2008, J. L. Fernández Moreno *et al.*[41]	Cucumber and orange	24 cucumbers, 19 tomatoes, 11 aubergines, pepper, 44 beans, 14 courgettes and 10 oranges	142 multiclass pesticides	Acetate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, PSA)	(EI)MS (EI)MS/MS	11.5	2–3	A minimum of 3 IPs (Decision 2002/657/EC)	PTV injection (10 μL) Matrix-matched standards

Table 1. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2008, J. M. Lee *et al.*[42]	Tobacco	—	49 multiclass pesticides	Citrate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, C_{18}, PSA, GCB)	(EI)MS/MS	500	1	None	Matrix-matched standards
2008, E. Fuentes *et al.*[22]	Olive oil	8 olive and 2 avocado oils	9 OP pesticides	MAE (acetonitrile–dichloromethane) and SPE clean-up (ENVICarb)	(EI)MS/MS	30	2	Acquisition of 2 SRM transitions	PTV injection (2 μL) in split mode Matrix-matched standards
2008 (a), S. Walorczyk[43]	Wheat grain and feed mixture	Cereal grain, bran, whole ears, straw, hay and feed mixtures (145 in total); and 11 malts, 2 dried beans, potato starch and dried parsley root.	140 multiclass pesticides and 4 DPs	Citrate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, C_{18}, PSA)	(EI)MS/MS	10	2	A minimum of 3 IPs (Decision 2002/657/EC)	PTV injection (5 μL) Matrix-matched standards Solvent exchange to toluene
2008 (b), S. Walorczyk[44]	Lettuce	14 lettuces, 34 cabbages and 18 leeks	129 multiclass pesticides	Citrate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, PSA, GCB)	(EI)MS/MS	5	2	Relative intensity ratios (Decision 2002/657/EC)	PTV injection (5 μL) Matrix-matched standards Solvent exchange to toluene
2009, A. Garrido Frenich *et al.*[45]	Cucumber and orange	2 cucumbers and 2 oranges	12 multiclass pesticides	Solvent extraction (ethyl acetate, Na_2SO_4)	(EI)MS/MS	—	2–3	1st injection: 1 SRM transition (to detect positives). 2nd injection: 2–3 SRM transitions (to confirm)	PTV injection (10 μL) Matrix-matched standards

Table 1. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (µg kg−1)	Number SRM transitions	Confirmation criteria	Comments
2009, E. Fuentes *et al.*[23]	Olive and avocado oil	20 olive and 4 avocado oils	9 OP pesticides	APMAE and SPE clean-up (ENVI-C_{18})	(EI)MS/MS	25	2	Acquisition of 2 SRM transitions	PTV injection (2 µL)
2010, Wong*et al.*[46]	Bell pepper, broccoli, cantaloupe, carrot, onion, orange, peach, potato, spinach and tomato	Bell pepper, carrot, peach, spinach and tomato	167 multiclass pesticides and metabolites.	QuEChERS method with d-SPE clean-up (1st: C_{18}, $MgSO_4$; 2nd: PSA, GCB, $MgSO_4$)	(EI)MS/MS	10	2	Relative intensity ratios (Decision 2002/657/EC)	Matrix-matched standards Solvent exchange to toluene
2010 (b), Wong *et al.*[47]	Dried powdered ginseng	12 dried ginseng products	168 multiclass pesticides and metabolites	Solvent extraction (acetonitrile, acetone–cyclohexane–ethyl acetate) and SPE clean-up (C_8, GCB, PSA, Na_2SO_4)	(EI)MS/MS	25	2	Relative intensity ratios (Decision 2002/657/EC)	Matrix-matched standards Solvent exchange to toluene
2010, M. I. Cervera *et al.*[48]	Orange, nectarine and spinach	2 oranges, 2 nectarines and 2 spinach plants	130 multiclass pesticides	PLE (ethyl acetate) and GPC clean-up	(EI)MS/MS	10	2	Relative intensity ratios (Decision 2002/657/EC)	Study of matrix effect Matrix-matched standards
2010, L.-J. Qu *et al.*[24]	Leek	10 leeks	20 OP pesticides	MAE and acetate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, PSA, GCB)	(EI)MS/MS	10	2	None	Solvent exchange to hexane

Table 1. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2010, V. Nardelli *et al.*[20]	Fish feed	37 fish feeds	16 OC pesticides	LLE (hexane/acetonitrile) and SPE clean-up (silicagel-SCX)	(EI)MS/MS	20	2	A minimum of 3 IPs (Decision 2002/657/EC) and evaluation of the relative intensity ratios (tolerance of ±20% compared with standards)	No difference between calibration curves in solvent and in matrix
2010, Q. Lin*et al.*[59]	Corn, potato and cabbage	Corn, potato and cabbage	15 carbamates and chloroacetanilide pesticides	MSPD extraction (neutral alumina, acetone)	(EI)MS/MS	10	2	None	Matrix-matched standards
2011, F. J. Camino-Sánchez *et al.*[49]	Tomato, pepper, lettuce, cucumber, aubergines, courgettes, melon, watermelon and apple	944 tomatoes, 80 peppers, 10 lettuces, 189 cucumbers, aubergines, 61 courgettes, 60 melons, 78 watermelons and 18 apples	121 multiclass pesticides	Citrate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, PSA)	(EI)MS/MS	10	2	Relative intensity ratios (Decision 2002/657/EC)	PTV injection (6 μL) Matrix-matched standards Method accredited (UNE-EN ISO/IEC 17025:2005)
2011, Walorczyk & Drozdzynski[50]	Carrot, tomato and strawberry	78 apples, 73 tomatoes, 64 strawberries, 60 cucumbers, 58 currants, 57 mushrooms, 46 carrots, 37 peppers, 25 pears, 24 onions and 19 gooseberries	140 multiclass pesticides	Citrate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, PSA)	(EI)MS/MS	10	2	Relative intensity ratios (Decision 2002/657/EC)	PTV injection (5 μL) Matrix-matched standards Solvent exchange to toluene

Table 1. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2011, M. L. Feo *et al.*[25]	Milk	—	12 pyrethroid pesticides	Solvent extraction (hexane–dichloromethane) and SPE clean-up (C_{18}, basic alumina)	(NCI)MS/MS	25	2	Acquisition of 2 SRM transitions	PTV injection (3 μL) Standards in solvent
2011, J. Dong *et al.*[17]	Cabbage and apple	2 pumpkins, ginger roots, carrots, burdock plants, shallots, spinach plants, garlic bulbs, peaches, grapes and apricots	82 multiclass pesticides	Acetate buffering QuEChERS method modified with d-SPE clean-up (C_{18}, $MgSO_4$, PSA)	(NCI)MS/MS	10	2	Acquisition of 2 SRM transitions	PTV injection (10 μL) Matrix-matched standards
2011, Y.-G. Zhao *et al.*[32]	Cabbage	3 cabbages, kidney beans, spinach plants, lettuces and aubergines	29 OC and OP pesticides	Citrate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, TEPA-NCM)	(EI)MS/MS	2	2–3	None	Solvent exchange to acetone Standards in solvent
2011, S. Walorczyk *et al.*[51]	Red, white and rosé wine	22 red, 5 white and 3 rosé wines	161 multiclass pesticides	Citrate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, PSA and C_{18})	(EI)MS/MS	10 (red wine) 50 (white and rosé wine)	2	None	PTV injection (5 μL) Study of matrix effect Matrix-matched standards Solvent exchange to toluene

Table 1. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2011, J. Sánchez-Avila*et al.*[21]	Mussel	5 mussels	12 OC pesticides, isomers and DPs	Solvent extraction and SPE clean-up (Florisil)	(EI)MS/MS	200	2	Relative intensity ratios (Decision 2002/657/EC)	—
2011, U. Koesukwiwat *et al.*[52]	Broccoli, cantaloupe, lemon and sweet potato	—	150 multiclass pesticides	Acetate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, PSA, C_{18}, GCB)	(EI)MS/MS	10	2	None	PTV injection (5 μL) Matrix-matched standards
2011, J. Robles-Molina *et al.*[53]	Fruit-based soft drink	18 orange and 8 lemon flavoured soft drinks	32 multiclass pesticides	HS-SPME (polyacrylate fiber)	(EI)MS/MS	100 ng L^{-1}	3	Accomplishment of the ion ratio criterion (SANCO/10684/2009)	Matrix-matched standards
2011, J. Zhao*et al.*[54]	Cranberry	Cranberry	78 multiclass pesticides	QuEChERS method modified (H_2O, NaCl, Na_2SO_4, acetone–hexane) with d-SPE clean-up (PSA, GCB, $MgSO_4$)	(EI)MS/MS	10	2	None	Standards in solvent
2012, S. Walorczyk[55]	Apple, blackcurrant, carrot, huckleberry, strawberry and tomato	Apples, barley malt, blackcurrants, carrots, clementines, grapes, leek, plums, rapeseed, rucola, strawberries and tomatoes	36 multiclass pesticides	Citrate buffering QuEChERS method with d-SPE clean-up ($MgSO_4$, PSA, C_{18}, GCB)	(EI)MS/MS	—	2	None	CSR injection (5–20 μL) Solvent exchange to toluene

Table 1. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2012, K.-G. Lee and E.-K. Jo[56]	Ginseng	118 fresh, 24 red and 10 dried ginsengs	32 multiclass pesticides	Solvent extraction (acetonitrile, NaCl) and SPE clean-up (Florisil)	(EI)MS/MS	30	2	None	Standards in solvent
2012, N. Belmonte Valles *et al.*[16]	Tomato, apple and orange	20 tomatoes, apples, pears, oranges and green beans	53 multiclass pesticides	Solvent extraction (ethyl acetate, NaCl and $MgSO_4$)	(NCI)MS/MS	1	2	The accomplishment ion ratio criterion (SANCO/10684/2009)	—
2012, Banerjee K *et al.*[57]	Grape, pomegranate, onion, okra, tomato	10 grapes, pomegranates, onions, okra, tomatoes	349 multiclass pesticides	QuEChERs method modified with d-SPE clean-up ($MgSO_4$, PSA, GCB)	(EI)MS/MS	5	2	None	PTV injection (5 μL) Matrix matched standards
2012 (a), T. Portolés *et al.*[18]	—	Apples, oranges, tomatoes and carrots	25 multiclass pesticides	Acetate buffering QuEChERS method modified with d-SPE clean-up (C_{18}, $MgSO_4$, PSA)	(APCI)MS/MSE I)MS/MS	—	2	None	Solvent exchange to toluene
2012 (b), T. Portolés *et al.*[19]	—	Apples, oranges, tomatoes and carrots	8 pyrethroid pesticides	Acetate buffering QuEChERS method modified with d-SPE clean-up (C_{18}, $MgSO_4$, PSA)	(APCI)MS/MS (EI)MS/MS	—	At least 3	None	Solvent exchange to toluene

Table 1. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg−1)	Number SRM transitions	Confirmation criteria	Comments
2013, A. Peruga *et al.*[27]	Courgette, leek, tomato, strawberry, orange	Courgette	Chlorothalonil	Solvent extraction (acetone) and SPE clean-up (Oasis HLB)	(EI) MS/MS	10	2	The accomplishment of ion ratio criterion (SANCO/10684/2009)	EDTA added before extraction as preservative. Matrix matched standards
2013, Sapozhnikova Y and Lehotay S. J.[58]	Catfish muscle	21 catfish samples	18 multiclass pesticides	QuEChERs method with d-SPE clean-up ($MgSO_4$, Z-Sep Plus)	(EI)MS/MS	1	2	None	PTV injection (5 μL) Study of matrix effect

a **DP, Degradation Product; OC, Organochlorine; OP, Organophosphorous; TP, Transformation Product; EI, Electron Ionization; IP, Identification Point; NCI, Negative Chemical Ionization; SRM, Selected Reaction Monitoring; APMAE, Atmospheric Pressure Microwave-Assisted Extraction; CSR, Concurrent Solvent Recondensation; d-SPE, dispersive Solid Phase Extraction; GPC, Gel Permeation Chromatography; HS-SPME, HeadSpace Solid Phase Microextraction; LLE, Liquid–Liquid Extraction; MAE, Microwave Assisted Extraction; MSPD, Matrix Solid Phase Dispersion; PLE, Pressurized Liquid Extraction; PTV, Programmable Temperature Vaporizing; SPE, Solid Phase Extraction; GCB, Graphitized Carbon Black; PSA, Primary Secondary Amine; TEPA-NCM, TetraEthylPentAmine-Nano-Composite Material.**

GC-MS/MS methods for pesticide residues include the extraction of the analytes from the matrix, appropriate clean-up of the raw extracts and final measurement. The most used approach for extraction of pesticides from food samples is nowadays the QuEChERS procedure (Quick, Easy, Cheap, Effective, Rugged and Safe), which has been widely reported in the literature [60]. Some variations of the original method have led to two modified methods: the acetate buffered method (AOAC official Method 2007.1) [61] and the citrate buffered method (CEN Standard Method EN 15662) [39]. The QuEChERS procedure in combination with GC-QqQ MS/MS is one of the preferred approaches at present for residue determination of GC-amenable pesticides. Among the methods reported, it can be mentioned that those of Leandro *et al.* [9], Plaza Bolaños *et al.* [36] and Wong *et al.* [46], used the original method without modifications. In contrast, other authors obtained good results using the acetate buffered version [17–19,41,52], although citrate modification seems to be more used in this field. In fact, the citrate buffered version has become the most applied extraction method in pesticide residue analysis in food by GC-QqQ MS/MS [32,38,39,42–44,49–51,55]. In these methods, a subsequent clean-up step is applied based on dispersive-solid phase extraction (d-SPE) using different sorbents, such as primary secondary amine (PSA), C_{18}, Z-Sep Plus and/or graphitized carbon black (GCB), depending on the complexity of the matrix.

Obviously, other sample preparation procedures have also been applied. Some authors performed a simple extraction with solvents such as ethyl acetate [10,16,26,28–30,33,34,37,45,48], acetone [7,27], acetonitrile [35,47,56]; or mixtures hexane–acetone [40], hexane–acetonitrile [20] or hexane–dichloromethane [25]. Most of the reported procedures require the application of an additional clean-up step to remove interferences and also to improve detection limits. Gel permeation

chromatography (GPC) [28–30,40,48] and solid phase extraction (SPE) [7,20–23,25,26,31,35,47,56] have been commonly applied for this purpose. As an exception, Robles-Molina *et al.* [53] proposed a method consisting of a solventless sample treatment procedure based on headspace solid-phase microextraction (HS-SPME) for the determination of target pesticides in fruit-based soft drinks.

After sample preparation, the final extract is commonly injected into the inlet system with a classical split/splitless injector. In some cases, the final extracting solvent is not appropriate for injection into the split/splitless system, due to the high volume-expansion coefficient during vaporization. Solvent exchange prior to chromatographic injection to an adequate solvent such as toluene [18,19,38,43,44,46,47,50,51,55], acetone [32,35] or hexane [24] could be a good choice to solve this problem. Another option is the use of programmable temperature vaporizing (PTV), which is also employed to improve the limits of detection in PRA as it allows the injection sample volumes higher than the typical ones (1–2 μL) in a split/splitless injector [9,10,17,25,28,30,31,33,36,39–41,43–45,49–52,57,58,62]. Apart from PTV, other large volume injection (LVI) systems are on-column injection or concurrent solvent recondensation (CSR) injection [55].

Regarding to the GC-QqQ MS/MS measurement (***Table 1***), EI is the most used ionization mode for the determination of pesticides in food, although the use of negative CI (NCI) has also been reported [7,16,17,25]. For some compounds, with highly electronegative elements, such as halogen, oxygen, *etc.*, the use of the NCI mode usually provides better sensitivity and selectivity. The use of the QqQ allows selected reaction monitoring (SRM) to be applied, one of the most selective and sensitive approaches for simultaneous quantification and confirmation in PRA, when adequate precursor and product ions are selected. In

this way, most matrix interferences are minimized, or even eliminated, improving the selectivity and the sensitivity, and reaching low detections limits due to the lower chemical noise in the chromatograms.

In general, the criteria used for confirmation/identification of positive samples are not treated in detail in most of the papers published. Some authors do not mention this issue, and only acquire one SRM transition for each analyte without mentioning any confirmation criteria [7,37,42]. Other authors propose the acquisition of two SRM transitions, but do not mention which criteria are applied to consider positive confirmation [18,24,29,32,37,51,52,54–59]. Some authors [8,17,22,23,25] have used the mere presence of a second SRM transition as confirmation criteria for positives in the samples. However, the European Commission Decision 2002/657/EC [63] implements the concept of identification points (IPs). In the case of MS/MS determination, 1 identification point is earned from a precursor ion, and 1.5 identification points are earned from a resulting product ion. For the unequivocal confirmation of the identity of compounds at least 3 and 4 identification points are required for legal and banned substances, respectively [43].This has been applied by several authors such as Garrido Frenich *et al.* [30], Plaza Bolaños *et al.* [31,36], Walorcyzk [38,43] and Fernández Moreno *et al.* [41] in their work. Nowadays, the most widely accepted approach is based on the presence of chromatographic peaks at the two (or three [53]) transitions acquired, together with agreement of the R_t and the evaluation of the intensity ratio between the quantification (*Q*) and the confirmation (*q*) transition, and comparison with those of the reference standard within the maximum tolerances established by the European Commission Decision 2002/657/EC [21,28,40,44,46–50] and the SANCO [64] guidelines [16,27,39,53].

Modern QqQ instruments allow the simultaneous acquisition of two or more transitions in just a single GC analysis. However, some publications reported [10,33,37] performed a sequential approach with two sample injections: the first for rapid screening, with acquisition of only one transition, and the second for the confirmation and quantification of the compounds previously detected in positive samples, with acquisition of 2 or 3 transitions. As a particular example, Fuentes *et al.* [22,23] determined OP pesticides in olive oil by GC with flame photometric detection (GC-FPD) and the identity of residues in positive samples was confirmed through GC-QqQ MS/MS analysis by acquiring two transitions.

Once the identity of the analyte in the sample has been confirmed, quantification of pesticide residues is normally the next objective. Different approaches have been reported for quantification of pesticide residues in food, commonly considered as a complex matrix. Matrix-matched standards calibration is a good option for quantification of analytes affected by matrix effects, and it has been widely applied in PRA of food commodities [8,9,17,22,27–31,33–53,57,59]. Cervera *et al.* [48] studied the matrix effect of several food matrices (orange, nectarine, spinach, raisin, paprika, cabbage, pear, rice, legume and gherkin) comparing the response of reference standards prepared in solvent with the response of matrix-matched standards. Most of the pesticides showed an evident signal enhancement in the presence of matrix, and matrix-matched calibration using relative responses to an internal standard was required for the correct quantification of compounds. On the contrary, Nardelli *et al.* [20] performed the quantification of OC pesticides in fish feed by using standard solutions in solvent and in the matrix as calibration curves and no differences were observed between the results obtained using the different sets of standards. Other applications have been reported in the literature in which standard solutions in solvent were used for analysis of different matrices

such as cabbage [32], milk [25], mussel [21] and ginseng [56]. As occurs with other analyzers used in GC-MS, the triple quadrupole analyser, even working in the SRM mode, is affected by coextracted matrix components that may lead to an enhancement of the chromatographic signal or to a reduction of the analyte response in comparison to the signal in pure solvent, as these effects are normally a consequence of problems coming from the GC system. Thus, when coextracted matrix components compete with the target pesticides to access the active sites of the GC system and/or when they are protected from decomposition in the hot injector, a matrix-induced response enhancement is observed. Conversely, accumulation of non-volatile coextracted matrix components in the GC system helps to generate new active sites, and matrix-induced response diminishment occurs [45].

Sensitivity is an important parameter to measure the potential of a method in PRA. LOD and LOQ are usually calculated for this purpose, although their estimation is a controversial issue, due to the different ways of calculation. This makes it troublesome to perform a realistic comparison of the values reported in the literature. In order to compare the sensitivity of the applications reviewed, the lowest concentration level validated was used as an indicator of the sensitivity of GC-MS/MS methods. Most of the publications (***Table 1***) used a lowest level validated in the range of 5–10 μg kg^{-1} [8,10,17,24,27,37–39,43,44,46,48–52,54,57,59]. The use of a large injection volume resulted in an increase in the sensitivity, and lowered the method validation concentration down to 1 μg kg^{-1}. Thus, Leandro *et al.* [9], Belmonte Valles *et al.* [16] and Sapozhnikova and Lehotay [58] validated their procedure at the lowest level of 1 μg kg^{-1} in baby foods, fruits and vegetables, or in catfish muscle, using large volume injections of 8, 2 and 5 μL, respectively.

3. GC-MS/MS applications in the analysis of environmental samples

The extensive use of pesticides in agriculture and their industrial applications in the last decades, together with the persistence of some of these compounds, has led to their wide presence in the different compartments of the environment. Consequently, there is a need to know the concentration of these contaminants in the aquatic environment, although they normally are found at the μg L^{-1} level or below. To this aim, strict regulations and environmental monitoring programs have to be adopted to accurately determine the concentration levels of pesticides. GC-QqQ MS/MS is an attractive technique with strong potential in the determination of low levels of pesticides in environmental samples, as occurs in food analysis.

The number of papers published until now related to environmental applications of GC-QqQ MS/MS in pesticide residue analysis is not as large as for food (***Table 2***). Only eighteen publications have been found and among them eleven developed analytical methodology for the determination of multiclass pesticides [65–75]. Only two articles deal with around hundred target analytes: Barco-Bonilla *et al.* [67] included 139 analytes in waste water samples analysis and Martínez Vidal *et al.* [69] included 98 pesticides in soil analysis. It is noteworthy that the first publication dealing with the use of GC-QqQ MS/MS for pesticides together with other organic contaminants in environmental samples dated 2007 [65], which illustrates the novelty and the recent use of this technique.

Table 2. Pesticide residue analysis of environmental samples by GC-(QqQ) MS/MS

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg^{-1})	Number SRM transitions	Confirmation criteria	Comments
2007, E. Pitarch *et al.*[65]	Deionized water	2 ground, 3 surface and 2 waste waters	25 multiclass pesticides	SPE (C_{18})	(EI)MS/MS (NCI)MS	25 ng L^{-1}	2	Relative intensity ratios (Decision 2002/657/EC)	Standards in solvent
2010, A. Penetra *et al.*[66]	Drinking, ground and surface water	Drinking, ground and surface water (55 in total)	9 multiclass pesticides	SPE (OASIS HLB)	(EI)MS/MS	25 ng L^{-1}	2	Relative abundances of the 2 selected SRM (tolerance of ±15%)	Standards in solvent
2010, N. Barco-Bonilla *et al.*[67]	Waste water	5 waste waters	139 multiclass pesticides	SPE (C_{18}) for aqueous phase PLE for suspended particulate matter	(EI)MS/MS	100 ng L^{-1}	2–3	None	Study of matrix effect Matrix-matched standards
2010, E. Pitarch *et al.*[68]	Waste water	41 waste waters	26 multiclass pesticides	SPE (C_{18})	(EI)MS/MS	—	2	Relative intensity ratios (Decision 2002/657/EC)	Based on 2007, Pitarch *et al.*
2010, J. L. Martínez Vidal *et al.*[69]	Soil	26 agricultural soils	98 multiclass pesticides	PLE (ethyl acetate–MeOH)	(EI)MS/MS	5 ng g^{-1}	≥ 2	FIT >700 for SRM spectra	PTV injection (10 μL) Study of matrix effect Matrix-matched standards

Table 2. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2010, A. Rashid *et al.*[76]	Soil	6 soils	19 OC pesticides	Acetate buffering QuEChERS method with LLP clean-up (acetonitrile, H_2O, hexane)	(EI)MS/MS	1 ng g^{-1}	2	Acquisition of 2 SRM transitions	Matrix-matched standards
2011, M. L. Feo *et al.*[25]	Water and sediment	—	12 pyrethroid pesticides	Water: UAE (chloroform) Sediment: solvent extraction with hexane–dichloromethane and SPE clean-up (Florisil)	(NCI)MS/MS	0.25 ng L^{-1}(water) 5 ng g^{-1}(sediment)	2	Acquisition of 2 SRM transitions	PTV injection (3 μL) Standards in solvent
2011, F. J. Camino-Sánchez *et al.*[70]	Marine sediment	9 marine sediments	55 multiclass pesticides	PLE (MeOH) and SBSE	(EI)MS/MS	10–30 ng g^{-1}	2	Relative intensity ratios (tolerance of ±25%)	Matrix-matched standards

Table 2. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2011, J. Sánchez-Avila *et al.*[21]	Water and marine sediment	5 WWTP, 5 sea and 5 river waters, 5 sediments	12 OC pesticides	Water: SPE (Oasis HLB) Sediment: solvent extraction and SPE clean-up (Florisil)	(EI)MS/MS	10 ng L^{-1}(river and sea waters) 250 ng L^{-1}(WWTP effluents) 100 ng g^{-1}(sediment)	2	Relative intensity ratios (Decision 2002/657/EC)	Matrix-matched standards
2011, C. Coscollà *et al.*[71]	Airborne particulate matter	38 airborne particulate matters	40 multiclass pesticides	MAE (ethyl acetate) and GPC clean-up (methylene chloride)	(EI)MS/MS	10 ng mL^{-1}	2	Acquisition of 2 SRM transitions	Study of matrix effect Matrix-matched standards
2011, Cervera *et al.*[80]	Mineral water	2 WWTP, 2 SWTP and 1 surface water	8 OC pesticides	HS-SPME (CAR/PDMS)	(EI)MS/MS (EI)MS	100 ng L^{-1}	2	Relative intensity ratios (Decision 2002/657/EC)	Statistical optimization of extraction variables Calibration extracted with same procedure
2011, A. Garrido Frenich *et al.*[72]	Drinking water	41 drinking waters	77 multiclass pesticides	SPME (PDMS-DVB) and HF-LPME clean-up (octanol–dihexyl ether)	(EI)MS/MS	50 ng L^{-1}	≥ 2	Acquisition of 2 SRM transitions	Study of matrix effect Standards in solvent

Table 2. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2012, F. J. Camino-Sanchez *et al.*[73]	River water	—	50 multiclass pesticides	SBSE	(EI)MS/MS	0.14–10 ng L^{-1}	2	Relative intensity ratios (tolerance of ±25%)	Matrix-matched standards
2012, Devos *et al.*[78]	Mineral water	2 harbor, 1 tap, 1 mineral and 7 waste waters	Tributyltin	SBSE	(EI)MS/MS	1 ng L^{-1}	4	Relative intensity ratios	Two-dimensional GC approach. Calibration extracted with same procedure
2012, Chary *et al.*[77]	River water and WWTP effluent waters	River water and WWTP	25 OC pesticides	SBSE	(EI)MS/MS	10 ng L^{-1}	2	Acquisition of 2 SRM transitions	Matrix-matched standards
2012, Cavaliere *et al.*[79]	Drinking water	Mineral and tap water	5 carbamate pesticides	SPME (PDMS/DVB)	(EI)MS/MS	80 ng L^{-1}	2	Acquisition of 2 SRM transitions (Decision 2002/657/EC)	Statistical optimization of extraction variables Calibration extracted with same procedure
2013, Camino-Sánchez *et al.*[74]	Drinking water	500 drinking waters	45 multiclass pesticides	SBSE	(EI)MS/MS	0.25–10 ng L^{-1}	2	Relative intensity ratios (tolerance of ±25%)	Calibration extracted with same procedure

Table 2. (Continued)

Reference	Validated matrix	Applied samples	Analytes	Sample preparation	Analysis mode	Lowest level validated (μg kg–1)	Number SRM transitions	Confirmation criteria	Comments
2013, Robles-Molina *et al.*[75]	Waste water	33 waste waters	40 multiclass pesticides	LLE (hexane)	(EI)MS/MS	15 ng L^{-1}	3	Relative intensity ratios (Decision 2002/657/EC)	Matrix-matched standards

[a] **OC, organochlorine; WWTP, Waste Water Treatment Plant; SWTP, Solid Waste Treatment Plant; EI, Electron Ionization; NCI, Negative Chemical Ionization; SRM, Selected Reaction Monitoring; GPC, Gel Permeation Chromatography; HF-LPME, Hollow Fiber Liquid Phase Microextraction; LLP, Liquid–Liquid Partition; MAE, Microwave Assisted Extraction; PLE, Pressurized Liquid Extraction; PTV, Programmable Temperature Vaporizing; SBSE, Stir-Bar Sorption Extraction; SPE, Solid Phase Extraction; SPME, Solid Phase Microextraction; UAE, Ultrasound Assisted Extraction; LLE, Liquid–Liquid extraction; PDMS-DVB, Polydimethylsiloxane-Divinylbenzene.**

Depending on the complexity of the matrix, different techniques have been used to extract the analytes. For example, soil generally requires the use of stronger techniques capable of extracting potentially bound residues. Only two applications dealt with soil analysis: Martínez Vidal *et al.* [69] who used a pressurized liquid extraction (PLE) technique with a mixture of ethyl acetate and methanol for investigation of multiclass pesticides, and Rashid *et al.* [76] who developed a methodology based on acetate buffering QuEChERS with a posterior liquid–liquid partition (LLP) clean-up for the determination of OC pesticides. In the case of sediments, analytical methodologies are based on extraction with different solvents followed by an additional clean-up or concentration step using SPE [21,25] or stir-bar sorption extraction (SBSE) [70]. Airborne particulate matter has been another environmental matrix analyzed by GC-QqQ MS/MS [71], using microwave-assisted extraction (MAE) followed by GPC as a clean-up step. As shown in ***Table 2***, water has been the most common matrix studied in the environmental field. SPE using different sorbents has been the technique of choice by most authors [21,65–68]. Some exceptions applied to pyrethroids pesticides that were extracted using ultrasound-assisted emulsification extraction (UAEE) with chloroform [25], and SBSE which was used for the analysis of pesticides in river water [73,77] or in drinking water [74,78]. Garrido Frenich *et al.* [72] compared both SPME and hollow fiber liquid phase microextraction (HF-LPME) for the extraction of pesticides in drinking water, concluding that SPME and GC-MS/MS offered the best compromise in terms of quality, speed and reliability.

After extraction (and occasionally clean-up), GC-amenable pesticides described in ***Table 2*** were determined by GC-QqQ MS/MS in the SRM mode. In all the publications, at least two transitions were acquired and the most used was the EI mode. Feo *et al.* [25] concluded that the best selectivity and sensitivity for the

determination of pyrethroids in water and sediment was obtained by using GC-MS/MS in the NCI mode. Pitarch *et al.* [65] developed the first GC-MS/MS methodology for priority organic pollutants in water, including several pesticides. Although the EI mode was used for the general method, a supplementary methodology based on GC-(NCI) MS using the selected ion recording (SIR) mode was proposed for quantification and confirmation of OC pesticides as it allowed notable sensitivity improvement for these compounds. As regards confirmation identity, it is based on the presence of at least two SRM transitions [25,71,72,76,77,79] and R_t agreement, although several authors also took into account the experimental relative intensity ratio of the sample and the theoretical ratio of the reference standard, using the maximum deviations established in the European Commission Decision 2002/657/EC [21,65,68,75,79,80], or based on other defined tolerances [66,70,73,74,78].

Quantification for water samples has been mostly based on calibration in solvent [25,65,66,68,72]. For more complex matrices, matrix-matched calibration provided better results, as in the case of soil samples [69,76], marine sediments [21,70] or even airborne particulate matter [71].

The GC-MS/MS methods applied in the analysis of environmental samples presented excellent sensitivity. In the case of soils and sediments, the lowest level validated was as low as 1 ng g^{-1} [76] or 5 ng g^{-1} [25,69]. The purity of the air was evaluated by analyzing the airborne particulate matter performing the validation at the lowest level of 10 ng mL^{-1} [71]. As regards water samples, the lowest level validated is reported to be 0.14 ng L^{-1} for a variety of priority organic pollutants [73]. As expected, the lowest level validated in waste water was much higher, as a consequence of the higher matrix complexity [67,75,77].

4. Trends and perspectives

After its first use for PRA around 10 years ago, GC-QqQ MS/MS has been consolidated in most laboratories. This technique has the degree of robustness required to be widely applied at present, and the improvements offered as regards method sensitivity and selectivity are widely recognized. The determination of pesticide residues by GC-MS is commonly based on the use of relatively long capillary columns (25–30 m) with internal diameters of 0.25–0.32 mm by using typically low polarity stationary phases (from 100 % methyl silicone to 5 % phenyl methyl silicone in most cases) leading to chromatograms of tenths of minutes. Fast GC coupled to MS has been shown to be an interesting alternative that, through different instrumental approaches, allows increased sample throughput by reducing the analysis time [81,82]. In this way, low-pressure GC-QqQ MS/MS has been applied to pesticide residue analysis, with an important increase in sensitivity, shorter run time, higher sample loading and increased ruggedness [81,83]. Another approach is based on the use of narrower columns with internal diameters of 0.1 mm combined with fast column temperature programming, resulting in an increase in sensitivity, a reduction in the analysis time and peak width and thus an increase in resolution, thereby making it feasible to determine even more than one hundred pesticides in analysis times lower than 10 minutes [82,84].

The use of APCI as an ionization source for GC-MS methods is a major advance that will greatly improve pesticide residue determination (and other GC amenable compounds) due to its soft ionization behaviour in comparison with that obtained by EI. Portolés *et al.* [18,19] have demonstrated the capabilities of this soft ionization source for producing spectra with much lower fragmentation than

that obtained by EI, where the molecular ion is commonly absent (or with low abundance). In these cases, when using EI it is necessary to select a fragment ion as a precursor ion in the MS/MS method and, consequently, not only the sensitivity but also the specificity of the method can be affected. With APCI, M^+ or $[M+H]^+$ is the base peak of the spectra in most cases. Under these conditions, precursor-ion selection would no longer require a compromise between selectivity and sensitivity, allowing more specific MS/MS experiments. This approach has not yet been exploited in pesticide residues analysis but it will surely be a major advance in this field in the near future.

A combination of GC-MS/MS and LC-MS/MS, both with a triple quadrupole analyzer, is one of the most current powerful approaches in PRA. They are complementary techniques that allow the determination of pesticides and metabolites within the whole range of physico-chemical properties, such as volatility, polarity and thermal stability. The combined use of both techniques allows the monitoring of hundreds of compounds that are GC or LC amenable. The present trend in multiresidue analysis is the application of generic sample preparation leading to sample extracts that are analyzed by both LC-MS/MS and GC-MS/MS, this being nowadays one of the most "universal" approach in PRA.

Acknowledgements

This work forms part of the project P1 1B2010-23 (Universitat Jaume I-Fundació Bancaixa). The authors acknowledge the financial support from the Generalitat Valenciana, as a research group of excellence PROMETEO/2009/054. M.I. Cervera is very grateful to the Ministry of Economy and Competitivity for her predoctoral grant.

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3. Artículo científico 2

Anal Bioanal Chem (2010) 397:2873–2891
DOI 10.1007/s00216-010-3597-8

ORIGINAL PAPER

Multi-residue determination of 130 multiclass pesticides in fruits and vegetables by gas chromatography coupled to triple quadrupole tandem mass spectrometry

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Received: 11 January 2010 /Revised: 17 February 2010 /Accepted: 18 February 2010 /Published online: 17 March 2010

Abstract A multi-residue method has been developed and validated for the simultaneous quantification and confirmation of around 130 multiclass pesticides in orange, nectarine and spinach samples by GC-MS/MS with a triple quadrupole analyzer. Compounds have been selected from different chemical families including insecticides, herbicides, fungicides and acaricides. Three isotopically labeled standards have been used as surrogates in order to improve accurate quantitation. Samples were extracted by using accelerated solvent extraction (ASE) with ethyl acetate. In the case of spinach, an additional clean-up step by gel permeation chromatography was applied. Determination was performed by GC-MS/MS in electron ionization mode acquiring two MS/MS transitions for each analyte. The intensity ratio between quantitation transition (*Q*) and identification transition (*q*) was used as confirmatory parameter (*Q*/*q* ratio). Accuracy and precision were evaluated by means of recovery experiments in orange, nectarine, and spinach samples spiked at two concentration levels (0.01 and 0.05 mg/kg). Recoveries were, in most cases, between 70% and 120% and RSD were below 20%. The limits of quantification objective for which the method was satisfactorily validated in the three samples matrices were for most pesticides 0.01 mg/kg. Matrix effects over the GC-MS/MS determination were tested by comparison of reference standards in pure solvent with matrix-matched standards of each matrix. Data obtained showed enhancement of signal for the majority of analytes in the three matrices investigated. Consequently, in order to reduce the systematic error due to this effect, quantification was performed using matrix-matched standard calibration curves. The matrix effect study was extended to other food matrices such as raisin, paprika, cabbage, pear, rice, legume, and gherkin, showing in all cases a similar signal enhancement effect.

Keywords Pesticides · Gas chromatography tandem mass spectrometry · Triple quadrupole · Fruits and vegetables · Matrix effect · Accelerated solvent extraction · Multi-residue analysis

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Analytical and Bioanalytical Chemistry, 397, 2873-2891, ***2010***

Multi-residue determination of 130 multiclass pesticides in fruits and vegetables by gas chromatography coupled to triple quadrupole tandem mass spectrometry

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Abstract

A multi-residue method has been developed and validated for the simultaneous quantification and confirmation of around 130 multiclass pesticides in orange, nectarine and spinach samples by GC-MS/MS with a triple quadrupole analyzer. Compounds have been selected from different chemical families including insecticides, herbicides, fungicides and acaricides. Three isotopically labeled standards have been used as surrogates in order to improve accurate quantitation. Samples were extracted by using accelerated solvent extraction (ASE) with ethyl acetate. In the case of spinach, an additional clean-up step by gel permeation chromatography was applied. Determination was performed by GC-MS/MS in electron ionization mode acquiring two MS/MS transitions for each

analyte. The intensity ratio between quantitation transition (*Q*) and identification transition (*q*) was used as confirmatory parameter (*Q/q* ratio). Accuracy and precision were evaluated by means of recovery experiments in orange, nectarine, and spinach samples spiked at two concentration levels (0.01 and 0.05 mg/kg). Recoveries were, in most cases, between 70 % and 120 % and RSD were below 20 %. The limits of quantification objective for which the method was satisfactorily validated in the three samples matrices were for most pesticides 0.01 mg/kg. Matrix effects over the GC-MS/MS determination were tested by comparison of reference standards in pure solvent with matrix-matched standards of each matrix. Data obtained showed enhancement of signal for the majority of analytes in the three matrices investigated. Consequently, in order to reduce the systematic error due to this effect, quantification was performed using matrix-matched standard calibration curves. The matrix effect study was extended to other food matrices such as mango, raisin, paprika, cabbage, pear, rice, legume, and gherkin, showing in all cases a similar signal enhancement effect.

Keywords

Pesticides; Gas chromatography tandem mass spectrometry; Triple quadrupole; Fruits and vegetables; Matrix effect; Accelerated solvent extraction; Multi-residue analysis

INTRODUCTION

Pesticides are used to protect crops before and after harvest from infestation by pests and plant diseases. A consequence of their use may be the presence of pesticide residues in treated products, fruits, vegetables, grains, and other commodities. Even after being washed, stored, processed, and prepared, some residues may remain in both fresh products and processed foods. The European Commission has set harmonized Maximum Residue Levels (MRL) in the Regulation (EC) No 396/2005 [1], in order to avoid that different Member States gave different MRL values for the same pesticide in the same crop, a situation which gave rise to questions from consumers, farmers, and traders [2, 3].

Nowadays, the control of pesticide residues in food commodities has become a requirement for compliance with the legislation, ensuring safety of the population and international and national trade. Therefore, multi-residual methodologies capable to determine a large number of pesticides simultaneously with satisfactory sensitivity and selectivity are highly required. However, the different physicochemical properties presented by the different pesticide chemical classes increases the difficulty when developing a unique analytical method for multi-residue pesticide determination in food commodities.

Typically, the determination of pesticides in complex matrices, such as fruits and vegetables, involves a sample treatment using different techniques as Soxhlet extraction [4], solid-phase extraction (SPE) [5, 6], supercritical fluid extraction (SFE) [7], microwave-assisted extraction (MAE) [8], matrix solid-phase dispersion (MSPD) [9], and accelerated solvent extraction (ASE) [10–12]. Some of the procedures reported for fruits and vegetables require the application of additional clean-up steps to remove interferences (such as chlorophyll or fat) and

also to improve detection limits. Gel permeation chromatography (GPC) and SPE have been commonly applied for this purpose [13].

The QuEChERS (quick, easy, cheap, effective, rugged, and safe) method developed in 2005 by Lehotay *et al.* [14] could be referenced as an example of a sample preparation technique (extraction and clean-up) applied for the multi-residue determination of pesticides in food and agricultural products. The key of this approach is the development of a rapid extraction procedure called dispersive solid-phase extraction which quickly removes water and non-target compounds with magnesium sulfate and a primary–secondary amine sorbent. Several advantages have been reported for this method compared to traditional sample preparation methods of pesticide residue analysis, like high recoveries for a wide volatility range of pesticides, accurate results, quick treatment, reduced use of solvent and reactives, and, in addition, being robust and reliable. In combination with gas chromatography/mass spectrometry, with ion trap analyzer, and with liquid chromatography/tandem mass spectrometry, with triple quadrupole analyzer, this approach has been successfully validated for a large number of pesticides in lettuce and orange [14]. This method was subjected to improvements, using buffering during the extraction to improve the recoveries of problematic pesticides (*e.g.*, folpet, dichlofluanid, chlorothalonil, and pymetrozine), without sacrificing recoveries of other pesticides in fruits and vegetables samples [15]. It has been applied in a collaborative study to determine multiple pesticides residues in fruits and vegetables for twenty representative pesticides in three matrices (grapes, lettuces, and oranges) with satisfactory results [16].

The determination of GC-amenable pesticides in food samples has been traditionally carried out by gas chromatography (GC) coupled to mass spectrometry (MS), due to the excellent resolution of capillary GC and satisfactory sensitivity and confirmation power of GC-MS based on electron ionization (EI) full scan mass spectra. Several applications of multi-residue GC-MS methods have been described in the literature in different food commodities including vegetables (potato, cabbage, carrot, cucumber, and beans), fruits (apple and orange), rice, baby food, and other products, some of them reaching more than 100 compounds [17–21]. Most of them use single quadrupole MS analyzer working in selected ion monitoring (SIM) mode with one target and some qualifier ions for quantitative analysis of pesticides. However, in recent years, the application of tandem mass spectrometry (MS/MS) has emerged as a more valuable approach, which allows higher selectivity and sensitivity, minimizing or even removing some chromatographic interference.

The use of tandem mass spectrometry (MS/MS) with triple quadrupole (QqQ) analyzer takes advantage of adequate precursor and product ions selection and offers the possibility of applying selected reaction monitoring (SRM), one of the most selective and sensitive approaches for simultaneous quantification and confirmation. In this way, matrix interferences are minimized, even eliminated, improving the selectivity and the sensitivity, reaching very low detection limits, due to the lower chemical noise in the chromatograms. In addition, acquiring two SRM transitions and evaluating their *Q/q* ratio (quantification transition (*Q*), confirmation transition (*q*)) leads to a reliable confirmation of the compound detected in sample [22, 23].

Several authors have reported the application of GC-MS/MS using QqQ analyzer for the determination of pesticide residues in different food commodities, such as meat [24–26], cereals and dry animal feed [27, 28], eggs [29], and vegetables and fruits [30–35].

In this paper, a wide-scope multi-residue method has been developed based on GC-MS/MS with QqQ analyzer for the determination of a large number of pesticides in fruits and vegetables. The procedure has been applied for the screening, quantification, and confirmation of around 130 pesticides in three matrices (orange, nectarine, and spinach). Sample treatment is based on the standard operative procedures already applied in the Chemistry Laboratory of Public Health Agency of Barcelona (ASPB) and consists of an efficient ASE with ethyl acetate, an interesting alternative to the use of acetonitrile, which is especially needed at present due to the difficulties to get commercial acetonitrile available at low prices.

EXPERIMENTAL

Reagents

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 were stored in a freezer at –20 °C. Working pesticide standard mixtures were prepared by dilution of stock solutions in hexane (for GC-MS/MS optimization) or in ethyl acetate (for sample fortification and for matrix effect study).

Three isotopically labeled compounds, purchased from Dr. Ehrenstorfer, were used as surrogates: *p,p'*-DDE D_8 (100 μg/mL), hexachlorobenzene (HCB) $^{13}C_6$ (100 μg/mL), and terbutylazine D_5 (100 μg/mL). Individual stock solutions of 10 μg/mL were prepared by volume dilution in acetone. A mixture solution of labeled standards (2 μg/mL) was prepared by volume dilution of individual stock solutions in ethyl acetate. Further dilutions of this mixture were prepared in ethyl acetate.

In order to simplify chromatographic determination during optimization, analytes were divided in two groups. Two matrix-matched calibration curves containing the two pesticides mixtures were prepared from standards diluted in blank extracts for every matrix, orange, nectarine, and spinach, in order to perform sample quantification. The preparation was performed differently, for orange and nectarine, and for spinach. For the first group, 5 mL of sample extract was evaporated to dryness under a gentle nitrogen stream. Then, it was redissolved with 100 μL of the isotopically labeled compounds solution of 500 μg/L and 150 μL of the pesticide mixture at adequate concentration. For spinach, 250 μL of sample extract was evaporated to dryness under a nitrogen stream, and it was redissolved with 100 μL of the internal standard mixture of 625 μg/L and 150 μL of the pesticide mixture at adequate concentration.

Acetone (pesticide residue analysis quality), ethyl acetate, hexane (ultra trace quality) were purchased from Scharlab (Barcelona, Spain) and cyclohexane (for GC, Suprasolv) were purchased from Merck (Barcelona, Spain). Inert diatomaceous earth (high purity quality) Hydromatrix and anhydrous sodium sulfate were purchased from Varian (Middelburg, the Netherlands) and from Scharlab, respectively.

Apparatus

Accelerated solvent extraction (ASE) was performed using a Dionex (Sunnyvale, USA) ASE 200 system equipped with solvent controller that allowed automated delivery of up to four solvents. The volume of the extraction cell used was 33 mL and the bottom was covered with two cellulose filters (19.8 mm I.D). Ethyl acetate was selected as extraction solvent and the extraction temperature and pressure were set at 70 °C and 10.34 MPa (1500 psi), respectively. The pre-heating and static times were set at 2 and 3 min, respectively. The contact solvent time was 5 min, with a flush volume of 60 % and executing two cycles.

Gel permeation chromatography (GPC) clean-up step was performed with a GPC system Agilent 1100 (Palo Alto, USA) equipped with a fraction collector, adapted to inject large sample volumes and with two connected Envirogel GPC clean-up columns from Waters (Milford, MA, USA). Both columns were packed with high-performance, fully-porous, highly cross-linked, styrene divinylbenzene copolymer particles: 15 mm × 19 mm (pre-column) and 300 mm x 19 mm, respectively.

GC instrumentation

A GC system (Agilent 6890N, Palo Alto, USA) equipped with an autosampler (Agilent 7683) was coupled to a triple quadrupole (QqQ) mass spectrometer Quattro Micro GC (Waters, Boston, USA), operating in EI mode. The GC separation was performed using a fused silica HP-5MS capillary column with a length of 30 m, an internal diameter of 0.25 mm and a film thickness of 0.25 μm (J&W Scientific, Folson, CA, USA). The oven was programmed as

follows: 70 °C (1.5 min); 25 °C/min to 180 °C (3 min); 5 °C/min to 300 °C (5.1 min). Splitless injections of 1 μL of the sample extracts were carried out with an injector temperature of 240 °C and a splitless time of 1 min. Helium 99.999 % (Carburos metálicos, Valencia, Spain) was used as a carrier gas at a flow of 1 mL/min. The interface temperature was set to 260 °C.

The ionization mode selected was EI (with a solvent delay of 4 min), setting the source temperature at 250 °C. The MS/MS procedure was designed as SRM mode using argon 99.995 % (Carburos metálicos, Valencia, Spain) as the collision gas at a pressure of 2.5×10^{-3} mbar in the collision cell. A dwell time per channel between 0.01 and 0.05 s was chosen, depending on the number of transitions recorded in each window and on the peak width of each compound, in order to get a minimum of 16 points per peak.

Heptacosa (perfluorotri-n-butylamine), used for the daily mass calibration, was injected using a syringe in the reference reservoir for this purpose. The Quanlynx application manager was used to process the data obtained from calibration standards and from fruit and vegetable sample extracts.

Sample preparation

Orange, nectarine, and spinach samples were purchased directly from a local market, in the city of Barcelona (Spain). Samples were chopped, homogenized and then stored in a freezer at –20 °C until analysis.

The extraction of samples was performed as follows: 7 g of diatomaceous earth was added to 10 g of triturated sample and then homogenized in a mortar.

The content was transferred to a 33-mL extraction cell; a volume of 0.5 mL of the isotopically labeled internal standard mixture (1 μg/mL) was added, and then it was subjected to the ASE procedure with ethyl acetate as described before. The ethyl acetate extract (around 50 mL) was concentrated to approximately 35 mL in TurboVap at 35 °C under a nitrogen stream. Then, approximately 2 g of anhydrous sodium sulfate was added to eliminate the existing water. At this point, the need of applying clean-up step has to be considered according to sample type. In the case of orange and nectarine, this step was not required. The organic extract obtained from ASE was collected into a volumetric flask and the final volume was adjusted to 50 mL with ethyl acetate. An aliquot of 10 mL was evaporated to dryness in TurboVap and the residue was redissolved with 0.5 mL of ethyl acetate and directly injected into the GC-MS/MS system.

For spinach samples, a GPC clean-up step was necessary. For this purpose, the organic extract obtained from ASE, was evaporated to approximately 1 mL in TurboVap. Then, volume was adjusted to 2 mL with ethyl acetate, and filtered through a 0.45 μm, 25 mm Millex filter. A 1 mL aliquot of the filtered extract was injected into the GPC system and eluted with cyclohexane:ethyl acetate (1:1, v/v) at a flow rate of 5 mL/min (collect time 14.5–21.0 min). The total volume collected was evaporated to dryness in a TurboVap at 35 °C under a nitrogen stream. The residue was redissolved with 1 mL of ethyl acetate and injected into the GC-MS/MS system.

Validation study

The *linearity* of the method was studied by analyzing matrix-matched standards (five concentration levels, in duplicate) ranging by one side from 12 to 120 μg/L (which corresponded to 0.003–0.03 mg/kg in orange and nectarine and to 0.0024–0.024 mg/kg in spinach) and by the other side from 60 to 600 μg/L (which corresponded to 0.015–0.15 mg/kg in orange and nectarine and 0.012–0.12 mg/kg in spinach). Linearity was assumed when regression coefficient was >0.99 with residuals lower than 30 %.

The *accuracy* was estimated by means of recovery experiments, analyzing orange, nectarine and spinach samples (n = 5) spiked at two concentrations levels (0.01 mg/kg and 0.05 mg/kg).

Spiked samples were prepared by adding the adequate volume of standard mixtures over the triturated sample (10 g) and left to stand for 1 h. Then, they were subjected to extraction procedure as described in the **Sample preparation** section.

According to the Regulation (EC) 396/2005 [1], MRL values (or the lower limit of analytical determination) for pesticides selected are equal or higher than 0.01 mg/kg in orange, nectarine, and spinach. So, validating the method to 0.01 mg/kg should be appropriate for regulatory purposes.

Precision was determined from the above-mentioned recovery experiments, carried out at two fortification levels. It was expressed as repeatability in terms of relative standard deviation (RSD; n = 5) at each fortification level.

Selectivity of the method was estimated considering the absence of interfering peaks at the retention time of each compound and based on the acquisition of two MS/MS transitions for each analyte by selecting adequate precursor and product ions.

The *limit of quantification* (LOQ) objective was established as the lowest concentration level validated with satisfactory values of recovery (70–120 %) and precision (RSD ≤ 20 %), i.e., 0.01 mg/kg for most of analyte matrix combinations tested.

The *limit of detection* (LOD) was estimated as the analyte concentration that produced a peak signal of three times the background noise in the chromatogram of the sample spiked at the lowest level studied. The LOD was obtained using a software option for estimating the S/N ratio and referring/recounting this value to a S/N value of three.

As *confirmation criteria* of positives in samples, the *Q/q* ratio was considered, defined as the ratio between the intensity of the quantification transition (*Q*) and the intensity of the confirmation transition (*q*). The *Q/q* reference value for each compound in each sample matrix was calculated as the mean value obtained from matrix-matched standards at different concentration levels in the range of 60–600 µg/L. For the reliable confirmation of positive findings, a maximum ratio tolerance ±20 % (when *Q/q* ratio value is lower than 2), ±25 % (*Q/q* ratio between 2 and 5), ±30 % (*Q/q* ratio between 5 and 10) or ±50 % (*Q/q* ratio higher than 10) were accepted, in the line of the European Union Decision 2002/657/EC [23]. Obviously, agreement in the retention time between reference standard and sample was also required to give a detection as positive.

RESULTS AND DISCUSSION

The analytical procedures presented in this work were based on the methodology already established as standard operating procedures in the Chemistry Laboratory of ASPB (Spain) for the determination of pesticides in fruits and vegetables. These procedures have been satisfactorily applied in this laboratory but using GC-MS with single quadrupole analyzer for the measurement. Our purpose was to improve those methodologies by changing the analytical determination using GC-MS/MS with QqQ analyzer, in order to improve sensitivity and selectivity, taking advantage of the possibility of applying SRM acquiring two MS/MS transitions for each compound.

GC-MS/MS optimization

Optimization of the MS/MS method was performed for all pesticides using hexane standard solutions injected in the EI ionization mode. After obtaining the full scan spectra for each compound, the base peak of the spectrum was selected as precursor ion. Once the precursor ion was selected, different values of collision energy (between 5 and 40 eV) were tested to study the fragmentation. The final purpose was to develop a SRM method with two MS/MS transitions (with the exceptions of surrogates with only one transition), normally the most sensitive ones, for each compound in order to have a reliable confirmation of the pesticide detected in samples.

Table 1 shows the precursor and the product ions corresponding to the quantitative and confirmative transitions monitored. Optimum values of collision energy for most compounds were found to be between 10 and 30 eV. The dwell

time parameter was modified between 0.01 and 0.05 s in order to obtain a good chromatographic peak (with at least 16 points/peak) still maintaining satisfactory sensitivity for each compound.

The *Q/q* intensity ratios are also shown in ***Table 1*** for each matrix studied. Average *Q/q* ratios were calculated as the mean values obtained after injection of matrix-matched standards at four concentration levels (60, 150, 300, and 600 μg/L), obtaining RSD typically below 15 %. As *Q/q* ratio values, similar to retention times, might suffer slight variation along the time, they might be corrected with the matrix-matched standard calibration included in every sample analysis batch, if necessary.

Sample preparation optimization

Sample extraction was performed with ASE using ethyl acetate as extraction solvent. In the case of the more complex matrices, such as spinach, a purification step by GPC was required. As indicated above, these sample preparation procedures were already being applied in the Chemistry Laboratory of ASPB. Consequently, they were not really subjected to a complete optimization study in the present paper, as before introduction in the routine work, the ASE procedure was already optimized by ASPB on the basis of the commercial information and application notes, and testing different times (pre-heat, static, and heat). Ethyl acetate was chosen as extraction solvent because of its low cost and low toxicity. Moreover, ethyl acetate avoided problems of miscibility with subsequent solvent mixtures. ASE presented the advantages of

higher efficiency, low toxicity of extraction solvent and short extraction time, as well as the simplicity of an automated extraction.

In order to purify extracts, GPC clean-up was considered highly recommendable for a wide range of matrices. A mixture of cyclohexane:ethyl acetate was selected as elution solvent. Different proportions of this mixture were tested, and finally cyclohexane:ethyl acetate (1:1, v/v) was selected as an adequate elution solvent. The extract collect time was set from 14.5 to 21 min, as a compromise between clean-up efficiency and sensitivity.

Matrix effect

Matrix effects for orange, nectarine, and spinach samples were evaluated. The study was performed by comparing the response of reference standards prepared in pure solvent with the response of matrix-matched standards (prepared as described in the "Reagents" section). The ratio between response in matrix and response in pure solvent was taken as absolute matrix effect. Moreover, due to the fact that labeled internal standards may correct signal suppressions or enhancements resulting from matrix interference, the ratio between relative responses of standard in matrix and standard in solvent was also studied. This ratio was taken as relative matrix effect. In both cases, a ratio value of 0.8-1.2 was established as acceptable; this means that no severe matrix effects affected in this case the response of the analytes after application of the overall analytical procedure.

Table 1. Experimental conditions of the optimized GC-MS/MS method

Rt (min)	Window (min)	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (s)	Collision energy (eV)	Orange *Q/q* ratio[a]	Nectarine *Q/q* ratio[a]	Spinach *Q/q* ratio[a]
5.2	4.0–5.7	Dichlorvos	185	93	0.02	10	1.64 (11)	1.41 (11)	1.31 (2)
			109	79	0.02	10			
6.3		Mevinphos	127	109	0.01	10	1.65 (8)	1.53 (4)	1.69 (3)
			192	127	0.01	10			
6.4		Acephate	136	94	0.01	10	13.4 (15)	9.68 (5)	No response
			136	112	0.01	10			
6.7	6.4–7.1	Methacrifos	208	180	0.05	10	1.35 (14)	1.49 (9)	3.07 (13)
			240	180	0.05	10			
7.0		Pentachlorobenzene	250	142	0.05	30	1.70 (6)	1.66 (6)	1.40 (3)
			248	142	0.05	30			
7.3	6.9–8.3	Heptenophos	124	89	0.01	10	13.2 (19)	14.8 (11)	15.26 (9)
			109	79	0.01	10			
7.6		Omethoate	156	110	0.01	10	1.39 (7)	1.11 (13)	1.20 (7)
			110	79	0.01	10			
7.6		Tecnazene	178	143	0.01	10	1.25 (9)	1.23 (3)	1.19 (7)
			213	142	0.01	20			
7.8		Diphenylamine	168	167	0.01	10	107 (17)	114 (16)	108 (15)
			169	143	0.01	10			
7.8		Ethoprophos	158	97	0.01	10	1.79 (5)	2.25 (16)	2.21 (6)
			158	114	0.01	10			
8.0		Chlorpropham	127	65	0.01	20	16.5 (11)	9.82 (7)	17.7 (3)
			153	90	0.01	20			
8.2		Trifluralin	306	264	0.01	10	7.54 (15)	6.76 (12)	7.31 (13)
			264	160	0.01	20			
8.5	8.1–8.7	Phorate	121	65	0.05	5	2.07 (8)	2.53 (11)	2.91 (3)
			260	75	0.05	10			
8.6		α-HCH	217	181	0.05	10	1.08 (2)	1.13 (6)	1.04 (4)
			219	183	0.05	10			
8.8	8.4–9.3	HCB	284	249	0.02	20	2.48 (4)	2.61 (9)	1.08 (12)
			284	214	0.02	20			
8.8		HCB- $^{13}C_6$	292	257	0.02	20	–	–	–
9.0		Dimethoate	93	63	0.02	10	1.89 (3)	1.89 (11)	2.02 (9)
			125	79	0.02	10			
9.1		Simazine	201	173	0.02	10	1.14 (13)	1.21 (3)	1.02 (8)
			186	91	0.02	10			
9.2		Atrazine	200	122	0.02	10	1.54 (12)	1.56 (10)	1.44 (13)
			200	132	0.02	10			
9.4	8.8–10.2	γ-HCH	217	181	0.01	10	1.08 (11)	1.02 (6)	1.08 (6)
			219	183	0.01	10			
9.5		β-HCH	217	181	0.01	10	1.04 (3)	1.07 (6)	1.09 (8)
			219	183	0.01	10			
9.6		Terbutylazine D_6	234	178	0.01	10	–	–	–
9.6		Terbufos	231	129	0.01	20	1.32 (4)	1.44 (12)	1.89 (3)
			231	175	0.01	10			
9.6		Quintozene	265	237	0.01	10	1.99 (14)	1.85 (18)	1.00 (4)
			237	119	0.01	20			
9.6		Terbutylazine	214	132	0.01	10	1.36 (4)	1.30 (16)	1.32 (13)
			229	173	0.01	10			
9.7		Fonofos	137	109	0.01	10	2.62 (13)	2.8 (8)	3.14 (13)
			246	137	0.01	5			
9.7		Propyzamide	173	145	0.01	10	1.96 (14)	1.65 (16)	1.66 (3)
			173	109	0.01	20			
9.9		Pyrimethanil	198	118	0.01	30	1.80 (10)	1.74 (4)	2.47 (6)
			198	156	0.01	20			

Table 1. (Continued)

Rt (min)	Window (min)	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (s)	Collision energy (eV)	Orange *Q/q* ratio[a]	Nectarine *Q/q* ratio[a]	Spinach *Q/q* ratio[a]
9.9		Diazinon	304	179	0.01	10	5.56 (11)	5.74 (16)	5.72 (10)
			276	179	0.01	10			
10.0	9.5–11.5	Disulfoton	274	88	0.01	20	4.02 (6)	2.88 (10)	2.92 (13)
			186	115	0.01	5			
10.2		Tefluthrin	177	137	0.01	10	7.22 (9)	5.04 (4)	2.09 (10)
			197	141	0.01	20			
10.2		δ-HCH	217	181	0.01	10	1.04 (3)	1.04 (8)	1.05 (8)
			219	183	0.01	10			
10.4		Chlorothalonil	264	133	0.01	20	1.66 (3)	1.63 (5)	1.72 (9)
			266	168	0.01	30			
10.4		Etrimfos	181	153	0.01	10	2.32 (2)	1.98 (10)	2.31 (14)
			277	125	0.01	10			
10.7		Endosulfan ether	272	237	0.01	10	1.09 (5)	1.07 (1)	1.11 (3)
			239	204	0.01	10			
10.7		Pirimicarb	238	166	0.01	10	2.24 (5)	1.15(4)	1.52 (9)
			166	96	0.01	10			
11.1		Phosphamidon	127	109	0.01	10	2.86 (5)	3.19 (7)	3.94 (6)
			264	127	0.01	10			
11.2		Metribuzin	198	82	0.01	20	5.41 (12)	5.29 (16)	7.15 (18)
			198	111	0.01	10			
11.3	10.8–11.8	Chlorpyriphos methyl	288	93	0.01	10	3.53 (14)	3.94 (14)	4.48 (6)
			197	169	0.01	30			
11.3		Vinclozolin	285	212	0.01	10	1.04 (13)	1.2 (12)	1.01 (8)
			212	172	0.01	10			
11.4		Parathion methyl	263	109	0.01	10	8.71 (16)	7.82 (6)	9.49 (13)
			233	124	0.01	10			
11.5		Tolclofos methyl	265	250	0.01	10	3.17 (15)	3.13 (14)	2.86 (8)
			265	93	0.01	20			
11.5		Heptachlor	272	237	0.01	10	1.77 (10)	1.60 (1)	1.70 (2)
			274	239	0.01	10			
11.6		Alachlor	188	160	0.01	10	2.20 (16)	1.93 (16)	1.87 (6)
			188	131	0.01	20			
11.5		Carbaryl	144	115	0.01	10	6.73 (10)	8.75 (6)	2.48 (9)
			115	89	0.01	20			
11.8		Metalaxyl	206	132	0.01	20	1.79 (15)	1.85 (9)	1.86 (13)
			206	117	0.01	30			
11.8		Fenchlorphos	285	240	0.01	20	2.98 (12)	3.25 (9)	3.06 (11)
			285	164	0.01	30			
12.2	11.7–13.5	Fenitrothion	260	125	0.01	20	5.62 (10)	6.10 (17)	3.57 (7)
			260	79	0.01	10			
12.3		Methiocarb	168	91	0.01	30	1.27 (12)	1.18 (9)	1.68 (8)
			168	109	0.01	30			
12.3		Pirimiphos methyl	290	233	0.01	10	1.67 (6)	1.5 (8)	1.64 (6)
			290	151	0.01	10			
12.5		Dichlofluanid	224	123	0.01	10	1.86 (10)	2.08 (3)	2.09 (5)
			167	124	0.01	10			
12.6		Aldrin	261	191	0.01	30	1.61 (5)	1.47 (5)	1.43 (12)
			263	193	0.01	20			
12.6		Malathion	127	99	0.01	10	12.6 (5)	15.3 (4)	11.4 (12)
			173	99	0.01	5			
12.7		Metholachlor	238	162	0.01	20	2.91 (14)	2.34 (7)	1.98 (10)
			162	132	0.01	10			

Table 1. (Continued)

Rt (min)	Window (min)	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (s)	Collision energy (eV)	Orange *Q/q* ratio[a]	Nectarine *Q/q* ratio[a]	Spinach *Q/q* ratio[a]
12.9		Fenthion	278	245	0.01	20	6.35 (17)	6.77 (17)	6.50 (2)
			278	108	0.01	10			
12.9		Chlorpyriphos ethyl	199	171	0.01	10	1.01 (6)	1.55 (2)	2.68 (6)
			316	260	0.01	10			
12.9		Parathion ethyl	291	109	0.01	10	70.2 (15)	77.2 (19)	37.0 (19)
			155	124	0.01	10			
13.0		4,4'-Dichlorobenzophenone	250	139	0.01	35	1.32 (16)	1.55 (14)	1.25 (12)
			250	111	0.01	20			
13.5	13.0–14.2	Isodrin	193	157	0.02	20	1.62 (6)	1.66 (4)	1.53 (3)
			195	123	0.02	30			
13.7		Pirimiphos ethyl	304	168	0.02	10	1.53 (3)	1.26 (3)	1.31 (5)
			318	166	0.02	10			
13.8		Cyprodinil	224	207	0.02	10	7.12 (11)	3.81 (12)	3.89 (7)
			225	208	0.02	10			
13.9	13.3–14.3	Heptachlor epoxide	353	263	0.01	10	1.85 (16)	1.44 (16)	2.10(18)
			353	282	0.01	20			
14.0		Oxychlordane	185	121	0.01	20	1.98 (12)	1.94 (18)	1.66 (13)
			235	141	0.01	20			
14.0		Pendimethalin	252	161	0.01	10	0.94 (6)	1.09 (10)	1.16 (5)
			252	191	0.01	10			
14.1		Penconazole	248	157	0.01	20	1.53 (6)	1.71 (7)	1.64 (13)
			248	192	0.01	10			
14.2		Tolyfluanid	137	91	0.01	20	1.04 (8)	1.88 (16)	2.50 (5)
			238	137	0.01	10			
14.2		Chlozolinate	259	188	0.01	10	2.69 (11)	2.94 (16)	1.82 (19)
			188	153	0.01	10			
14.3	13.8–15.1	Chlorfenvinphos	267	159	0.01	10	2.78 (13)	3.15 (16)	2.75 (7)
			323	267	0.01	20			
14.3		Isofenphos	213	121	0.01	20	2.22 (6)	2.27 (7)	2.33 (5)
			255	121	0.01	20			
14.4		Quinalphos	157	129	0.01	10	1.12 (11)	1.07 (7)	1.05 (8)
			157	102	0.01	20			
14.5		Folpet	260	130	0.01	20	1.24 (6)	1.66 (17)	1.18 (14)
			262	130	0.01	10			
14.5		Captan	149	79	0.01	10	1.22 (18)	2.02 (18)	2.22 (9)
			149	105	0.01	10			
14.6		Procymidone	283	96	0.01	10	4.22 (8)	4.58 (11)	5.15 (11)
			283	255	0.01	10			
14.7		*trans*-Chlordane	373	266	0.01	20	1.85 (12)	2.24 (3)	1.82 (11)
			373	264	0.01	20			
14.8		Triflumizole	206	179	0.01	20	6.88 (13)	14.0 (20)	No response
			206	144	0.01	30			
14.9		Methidathion	145	85	0.01	5	14.5 (4)	14.8 (13)	12.1 (12)
			125	79	0.01	5			
15.1	14.4–16.0	Endosulfan I	239	204	0.02	20	1.07 (9)	1.38 (14)	1.18 (6)
			272	237	0.02	10			
15.2		Tetrachlorvinphos	329	109	0.02	20	1.27 (7)	1.14 (16)	8.67 (1)
			331	127	0.02	20			
15.6		Chlorfenson	111	75	0.02	10	1.28 (7)	1.30 (4)	1.47 (5)
			175	111	0.02	10			

Table 1. (Continued)

Rt (min)	Window (min)	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (s)	Collision energy (eV)	Orange *Q/q* ratio[a]	Nectarine *Q/q* ratio[a]	Spinach *Q/q* ratio[a]
16.0	15.2–17.6	Profenofos	339	269	0.01	10	8.02 (20)	9.16 (16)	6.40 (14)
			208	99	0.01	20			
16.0		*p,p'*-DDE D_8	324	254	0.01	20	–	–	–
16.0		Dieldrin	263	193	0.01	30	1.30 (8)	1.23 (17)	1.31 (7)
			261	191	0.01	20			
16.1		*p,p'*-DDE	316	246	0.01	20	1.83 (4)	1.85 (12)	1.64 (4)
			318	246	0.01	20			
16.4		Myclobutanil	179	125	0.01	10	4.29 (18)	4.26 (2)	3.57 (11)
			179	90	0.01	20			
16.5		Buprofezin	105	77	0.01	20	18.8 (11)	18.9 (7)	23.0 (13)
			172	115	0.01	10			
16.7		Bupirimate	208	165	0.01	20	5.15 (13)	4.64 (14)	7.11 (10)
			273	193	0.01	10			
16.7		Endrin	263	193	0.01	30	1.10 (13)	1.02 (17)	1.22 (11)
			261	191	0.01	20			
17.1		Endosulfan II	193	123	0.01	30	1.21 (13)	1.07 (14)	2.26 (16)
			241	170	0.01	20			
17.5	17.2–19.8	*p,p'*-DDD	235	165	0.05	20	1.68 (10)	1.68 (8)	1.74 (2)
			237	165	0.05	20			
17.6		*p,p'*-DDT	235	165	0.05	30	1.77 (4)	1.46 (8)	1.93 (3)
			237	165	0.05	10			
17.7		Oxadixyl	163	132	0.05	10	3.24 (16)	2.76 (9)	2.40 (5)
			163	117	0.05	20			
17.8		Ethion	231	129	0.05	20	7.40 (4)	7.71 (5)	7.61 (13)
			231	175	0.05	20			
18.3	17.8–19.8	Triazophos	161	134	0.05	5	1.24 (8)	1.21 (15)	1.40 (7)
			257	162	0.05	10			
18.6		Endosulfan sulfate	274	239	0.05	20	1.04 (3)	1.14 (5)	1.07 (3)
			272	237	0.05	10			
18.7		Propiconazole I	173	145	0.05	10	1.04 (2)	1.04 (7)	1.02 (4)
			173	109	0.05	20			
18.9		Propiconazole II	173	145	0.05	10	1.11 (1)	1.08 (4)	1.06 (3)
			173	109	0.05	20			
19.3		Tebuconazole	125	89	0.05	10	2.62 (8)	3.02 (5)	1.73 (4)
			250	125	0.05	10			
20.4	19.8–21.3	Iprodione	314	245	0.01	20	2.08 (12)	1.74 (3)	1.22 (9)
			187	124	0.01	10			
20.5		Phosmet	160	77	0.01	20	1.48 (2)	1.33 (9)	1.63 (2)
			160	133	0.01	10			
20.6		Bromopropylate	183	155	0.01	10	1.16 (4)	2.08 (5)	3.47 (12)
			343	185	0.01	10			
20.8		Bifenthrin	181	166	0.01	10	1.16 (6)	1.04 (3)	1.01 (4)
			181	165	0.01	20			
20.9		Dicofol	251	139	0.01	10	1.44 (13)	1.60 (11)	No response
			251	111	0.01	30			
20.9		Methoxychlor	227	169	0.01	30	1.87 (5)	1.24 (5)	1.01 (2)
			227	141	0.01	20			
21.5	20.7–22.6	Tetradifon	356	229	0.02	20	1.22 (9)	1.22(3)	1.26 (16)
			356	159	0.02	10			
21.9		Phosalone	182	111	0.02	10	5.25 (8)	5.01 (9)	4.43 (6)
			367	182	0.02	10			
21.9		Azinphos methyl	160	77	0.02	20	5.96 (13)	7.61 (6)	8.88 (4)
			160	132	0.02	10			

Table 1. (Continued)

Rt (min)	Window (min)	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (s)	Collision energy (eV)	Orange *Q/q* ratio[a]	Nectarine *Q/q* ratio[a]	Spinach *Q/q* ratio[a]
22.0		Mirex	272	237	0.02	10	1.85 (5)	1.81(5)	1.82 (6)
			274	239	0.02	10			
22.1		Pyriproxyfen	136	96	0.02	20	3.80 (14)	3.84 (9)	3.94 (10)
			136	78	0.02	30			
22.4	22.0–23.6	λ-Cyhalothrin I	181	152	0.05	20	1.09 (8)	2.49 (11)	2.20 (6)
			208	181	0.05	10			
22.8		λ-Cyhalothrin II	181	152	0.05	20	1.06 (9)	2.53 (16)	2.05 (7)
			208	181	0.05	10			
22.8		Fenarimol	251	139	0.05	10	1.45 (5)	1.42 (6)	1.42 (6)
			219	107	0.05	10			
23.2		Pyrazophos	221	193	0.05	10	2.60 (11)	2.47 (5)	2.67 (3)
			221	149	0.05	10			
23.3		Acrinathrin	181	152	0.05	10	0.97 (10)	2.01 (13)	1.87 (2)
			208	181	0.05	20			
24.1	23.6–24.8	Permethrin I	183	153	0.05	10	1.76 (6)	2.32 (6)	1.95 (1)
			183	165	0.05	10			
24.3		Pyridaben	147	117	0.05	20	1.95 (12)	2.12 (6)	2.28 (7)
			147	132	0.05	10			
24.3		Permethrin II	183	153	0.05	10	1.69 (4)	2.23 (4)	1.98 (2)
			183	165	0.05	10			
24.6		Coumaphos	362	226	0.05	20	1.24 (5)	1.07 (3)	1.29 (5)
			226	163	0.05	10			
25.3	24.1–27.1	Cyfluthrin I	163	91	0.05	10	1.14 (5)	1.32 (10)	4.82 (7)
			163	127	0.05	20			
25.5		Cyfluthrin II	163	91	0.05	10	1.26 (5)	1.38 (4)	5.10 (6)
			163	127	0.05	20			
25.6		β-Cyfluthrin	163	91	0.05	10	1.13 (2)	1.31 (7)	5.10 (6)
			163	127	0.05	20			
25.7		Cyfluthrin III	163	91	0.05	10	1.19 (3)	1.34 (4)	5.71 (4)
			163	127	0.05	20			
25.9		Cypermethrin I	163	91	0.05	10	1.47 (7)	1.52 (11)	5.05 (6)
			163	127	0.05	10			
26.1		Cypermethrin II	163	91	0.05	10	1.12 (6)	1.51 (8)	5.19 (10)
			163	127	0.05	10			
26.2		Cypermethrin III	163	91	0.05	10	1.16 (9)	1.29 (6)	4.99 (3)
			163	127	0.05	10			
26.3		Cypermethrin IV	163	91	0.05	10	1.21 (6)	1.42 (6)	4.94 (5)
			163	127	0.05	10			
26.4		Etofenprox	163	106	0.05	10	5.37 (14)	9.33 (1)	4.60 (13)
			163	134	0.05	10			
27.6	27.0–35.0	Fenvalerate	181	152	0.05	10	1.61 (9)	1.91 (5)	1.76 (4)
			225	119	0.05	20			
28.0		Esfenvalerate	181	152	0.05	10	1.71 (8)	1.90 (3)	2.54 (8)
			225	91	0.05	20			
28.0		τ-Fluvalinate I	252	200	0.05	20	6.20 (7)	5.29 (14)	2.60 (9)
			250	200	0.05	20			
28.2		τ-Fluvalinate II	252	200	0.05	20	5.64 (13)	5.37 (13)	2.55 (8)
			250	200	0.05	20			
29.0		Deltamethrin	181	152	0.05	20	2.92 (2)	2.39 (14)	2.64 (4)
			253	93	0.05	10			
29.6		Azoxystrobin	344	329	0.05	10	79.1 (12)	77.4 (15)	44.5 (14)
			344	156	0.05	30			

[a]Average value calculated from matrix-matched standard calibration (four concentration levels) and RSD in parenthesis. For every compound, the first transition corresponds to quantification (*Q*) and the second transition to confirmation (*q*)

Concentration levels tested for matrix effects were 150, 300, and 600 μg/L obtaining the average absolute response or relative response of analytes at these three levels. In the case of oranges, nearly 70 % of pesticides suffered significant matrix effect, with response ratio out of the range 0.8–1.2. Most of them showed an evident signal enhancement in the presence of matrix. A similar behavior was observed for nectarine and spinach matrices, although the degree of signal enhancement was higher, especially in spinach. When using responses relative to the internal standards, a notable correction was observed in all matrices. In spite of this, a considerable number of pesticides still gave a response out of the 0.8–1.2 range, as ***Figure 1a*** illustrates for nectarine. The strong matrix effect could not be corrected with I.S. for spinach, as depicted in ***Figure 1b***, where relative matrix effect for spinach reveals that most pesticides suffered signal enhancement with responses out of the 0.8–1.2 range. In such a case, a high number of I.S. would be surely necessary to properly correct matrix effect for each analyte.

It can be concluded that for correct quantification of pesticides in orange, nectarine and spinach samples, matrix-matched standards calibration using relative responses as regards internal standards would have to be used.

In order to further study the applicability of developed procedures to other food matrices, matrix effect was also evaluated in other matrices such as mango, raisin, paprika, cabbage, pear, rice, legume, and gherkin. The study was performed at a single concentration level of 100 μg/L. Typically more than 80 % of pesticides investigated showed enhancement of signal in matrix when mango, raisin, paprika, pear, and rice were studied. In the rest of the matrices, the percentage of pesticides showing signal enhancement was lower (around 40–50 %). So, although the degree of signal enhancement may vary from one vegetable

matrix to another, it seems that in all matrices studied it would be necessary to use matrix-matched calibration using relative responses to internal standard for correct quantification of pesticides.

Validation results

Validation of the multi-residue method in orange, nectarine and spinach was carried out in terms of accuracy, precision, selectivity, limits of detection, and limits of quantification. Three labeled internal standards were added as surrogates to improve quantitation. The use of the different surrogates was established considering the chemical families studied and the retention times of the analytes. Thus, the surrogates used for insecticides were: HCB $^{13}C_6$ or *p,p'*-DDE D_8 for OCs; HCB $^{13}C_6$ or terbutylazine D_5 for OPs; terbutylazine D_5 for pyretroids and carbamates and HCB $^{13}C_6$ for the rest of insecticides. The surrogates used for herbicides were: terbutylazine D_5 for triazines and HCB $^{13}C_6$ for the rest of herbicides. The surrogate used for acaricides and fungicides was HCB $^{13}C_6$.

Linearity of the chromatographic method using matrix-matched standards was satisfactory in the range of concentrations between 12 and 600 μg/L (0.003–0.15 mg/kg in orange and nectarine and 0.0024–0.012 mg/kg in spinach) with correlation coefficients higher than 0.99 and residuals lower than ±30 %.

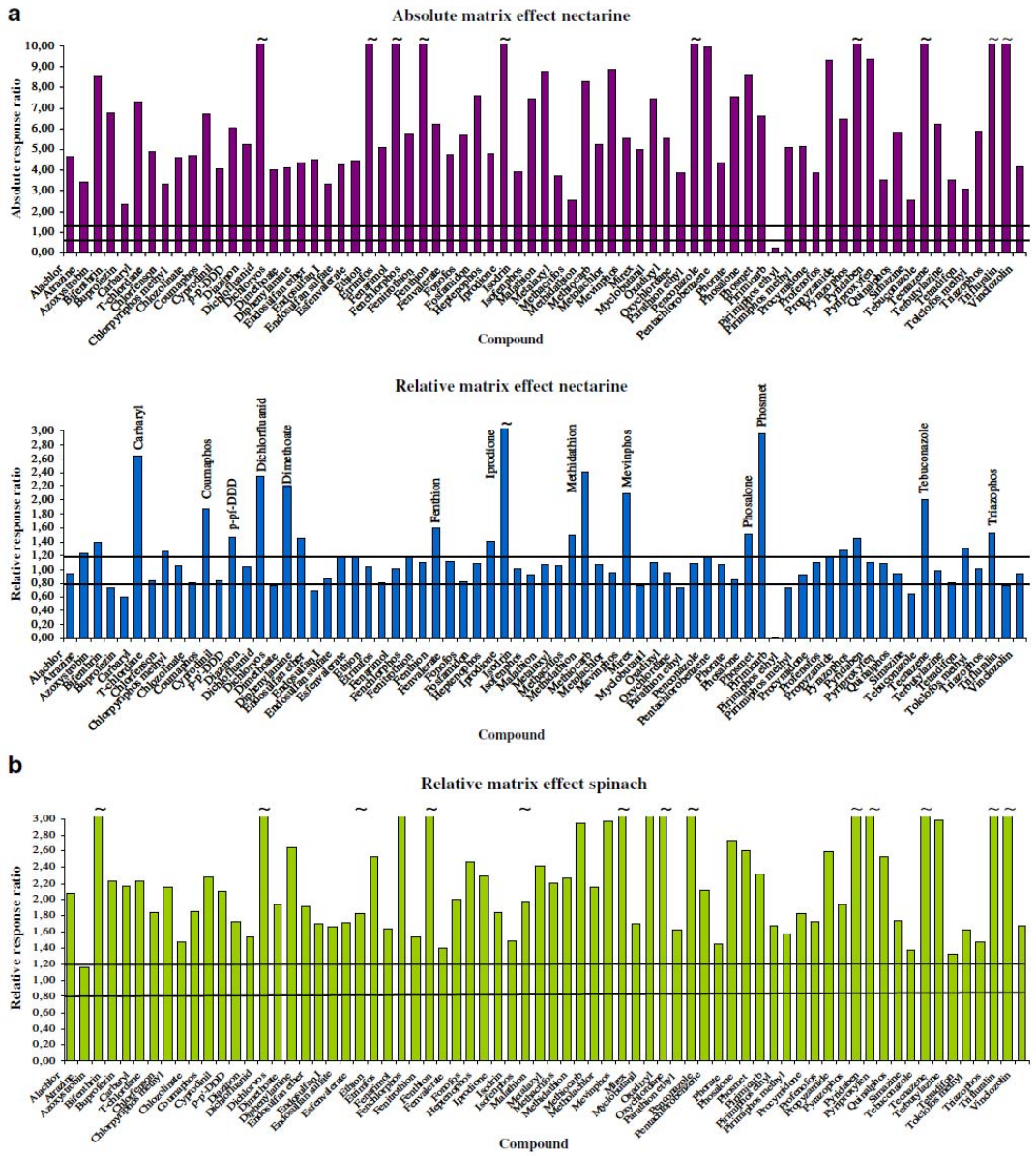

Figure 1. a. Absolute and relative matrix effect for nectarine samples. b. Relative matrix effect for spinach samples in the GC-MS/MS determination of selected pesticides

Accuracy and precision were estimated by means of recovery experiments (n = 5) at two concentration levels (0.01 mg/kg and 0.05 mg/kg) for each sample matrix studied. ***Table 2*** shows the results obtained for orange, nectarine, and spinach samples. As it can be seen, most compounds presented satisfactory recoveries in orange and nectarine with values between 70 % and 120 % at both spiked levels. Several exceptions were found with recoveries between 60 % and 70 %, especially at the lowest fortification level assayed, although normally with satisfactory RSD. Dicofol, heptachlor epoxide, and methoxychlor were poorly recovered at the lowest level in both matrices. Omethoate and pentachlorobenzene showed in general recoveries below 70 % in the three sample matrices tested. Captan was especially problematic in all samples due to the well-known difficulties associated to its determination [36]. The low recoveries for azinphos methyl at 0.05 mg/kg in both orange and nectarine, did not fit with those at the 0.01 mg/kg, and further experiments would be necessary to get a better knowledge about this fact. Apart from this exception, only four recovery values were slightly lower than 50 % and always corresponded at 0.01 mg/kg level (dicofol and methoxychlor in orange; methoxychlor and pentachlorobenzene in nectarine) but maintaining good precision (RSD ≤ 15 %).

Data of spinach reveal that it was the most difficult matrix among the three studied, and thus nine pesticides (acrinathrin, captan, λ-cyhalothrin I and II, disulfoton, τ-fluvalinate I and II, folpet and tefluthrin) could not be detected, probably due to their behavior during the GPC clean-up step. Improvement of these results might be achieved by further optimization of the GPC procedure. Additionally, acephate, dicofol, and triflumizole could not be determined in spinach samples as they did not show any response, even in matrix-matched standards, as shown in ***Table 1***. Some other compounds could not be determined

at the 0.01 mg/kg level in none of the matrices due to their low sensitivity, with heptachlor epoxide being an example of this behavior in the three sample matrices studied.

Precision was satisfactory as the majority of pesticides showed values of RSD lower than 20 %. The poorest RSD values were observed for dichlofluanid in spinach at 0.01 mg/kg and endrin and pyrimethanil in nectarine at 0.05 mg/kg. The lowest level validated, i.e., 0.01 mg/kg, could be established as the LOQ objective for most of the compounds investigated in orange, nectarine, and spinach samples, with the few exceptions where unsatisfactory data were obtained. LOD, estimated as the analyte concentration giving a peak of three times the background noise in the chromatograms corresponding at the LOQ level, were generally in the range of 0.0001 to 0.01 mg/kg. LOD values were obtained from the quantification transition (*Q*), i.e., the most sensitive one of the two transitions acquired.

In the procedure proposed, three internal standards have been used in combination with matrix-matched calibration in order to correct the demonstrated matrix effects over recoveries. This approach has been found satisfactory for most analyte/matrix combinations in view of the recoveries obtained. Obviously, the use of higher number of labeled internal standards should improve the recovery for some of the 130 compounds studied, especially for those with higher differences in chemical structure related to the internal standard used.

Table 2. Average recovery (%) and RSD (in parenthesis) after the application of the GC-MS/MS procedure to orange, nectarine, and spinach samples (n=5) at two concentration levels

Compounds	Orange			Nectarine			Spinach		
	Fortification levels (mg/kg)		LOD (mg/kg)	Fortification levels (mg/kg)		LOD (mg/kg)	Fortification levels (mg/kg)		LOD (mg/kg)
	0.01	0.05		0.01	0.05		0.01	0.05	
Acephate[1]	64(20)	70 (8)	0.002	74 (17)	109 (15)	0.004	–	–	–
Acrinathrin[2]	71 (8)	111 (17)	0.001	74 (12)	101 (23)	0.0003	–	–	–
Alachlor[1]	88 (17)	85 (5)	0.002	81 (18)	94 (9)	0.004	104 (10)	98 (15)	0.002
Aldrin[1]	51 (16)	95 (4)	0.005	85 (15)	103 (10)	0.003	73 (12)	114 (9)	0.0004
Atrazine[2]	70 (11)	91 (15)	0.004	72 (15)	78 (11)	0.002	107 (11)	79 (9)	0.004
Azinphos methyl[1]	71 (11)	36 (10)	0.003	108 (18)	22 (17)	0.002	–	107 (13)	0.02
Azoxystrobin[1]	80 (8)	87 (19)	0.002	109 (17)	93 (12)	0.001	104 (13)	85 (12)	0.004
Bifenthrin[2]	75 (4)	94 (18)	0.0001	71 (10)	99 (9)	0.0006	89 (12)	68 (10)	0.001
Bromopropylate[1]	90 (17)	89 (12)	0.0002	91 (13)	117 (10)	0.002	110 (1)	126 (19)	0.002
Bupirimate[1]	79 (10)	99 (38)	0.005	81 (10)	–	0.003	104 (19)	102 (15)	0.01
Buprofezin[1]	106 (9)	106 (20)	0.006	82 (7)	87 (22)	0.008	105 (19)	95 (6)	0.006
Captan[1]	–	100 (18)	–	–	59 (4)	–	–	–	–
Carbaryl[2]	78 (6)	99 (16)	0.0007	86 (15)	98 (12)	0.002	102 (10)	72 (15)	0.001
trans-Chlordane[1]	80 (8)	94 (13)	0.001	90 (17)	96 (7)	0.0005	100 (11)	85 (8)	0.001
Chlorfenson[1]	90 (8)	96 (8)	0.0001	93 (19)	101 (7)	0.0001	102 (7)	84 (10)	0.0005
Chlorfenvinphos[2]	70 (8)	81 (20)	0.007	70 (7)	105 (21)	0.002	99 (12)	76 (8)	0.01
Chlorothalonil[1]	100 (14)	86 (5)	0.001	117 (9)	94 (15)	0.003	75 (17)	90 (11)	0.005
Chlorpropham[1]	109 (12)	85 (4)	0.002	79 (7)	118 (12)	0.001	107 (13)	118 (12)	0.004
Chlorpyriphos ethyl[2]	90 (12)	100 (13)	0.001	89 (6)	117 (17)	0.002	110 (12)	72 (9)	0.0002
Chlorpyriphos methyl[2]	70 (15)	81 (14)	0.003	69 (15)	88 (12)	0.002	95 (11)	65 (3)	0.0004
Chlozolinate[1]	103 (20)	96 (16)	0.01	86 (15)	91 (7)	0.01	85 (11)	98 (24)	0.01
Coumaphos[2]	82 (9)	88 (20)	0.004	87 (6)	102 (13)	0.003	104 (22)	61 (8)	0.008
Cyfluthrin I[2]	78 (8)	100 (8)	0.002	84 (13)	110 (21)	0.001	62 (10)	72 (11)	0.004
Cyfluthrin II[2]	89 (8)	93 (10)	0.002	80 (12)	109 (20)	0.001	53 (15)	67 (3)	0.003
Cyfluthrin III[2]	86 (11)	90 (6)	0.002	76 (15)	101 (17)	0.0004	62 (14)	64 (2)	0.003
β-Cyfluthrin[2]	74 (10)	95 (6)	0.002	78 (5)	102 (17)	0.0006	84 (20)	68 (10)	0.003
λ-Cyhalothrin I[2]	85 (9)	114 (13)	0.002	82 (1)	100 (21)	0.001	–	–	–
λ-Cyhalothrin II[2]	83 (9)	109 (15)	0.002	102 (8)	104 (19)	0.001	–	–	–
Cypermethrin I[2]	106 (14)	92 (12)	0.001	105 (7)	94 (12)	0.001	105 (22)	63 (12)	0.008
Cypermethrin II[2]	98 (13)	93 (7)	0.003	79 (1)	105 (18)	0.002	107 (7)	59 (11)	0.01
Cypermethrin III[2]	73 (15)	92 (10)	0.001	73 (11)	–	0.003	98 (18)	59 (13)	0.01
Cypermethrin IV[2]	80 (15)	102 (11)	0.003	86 (4)	–	0.004	114 (7)	61 (14)	0.01
Cyprodinil[1]	76 (18)	93 (7)	0.005	90 (22)	108 (8)	0.008	92 (20)	90 (17)	0.01
p,p'-DDD[3]	65 (6)	73 (15)	0.0002	65 (5)	70 (3)	0.0001	102 (5)	54 (6)	0.0003
p,p'-DDE[3]	64 (2)	70 (7)	0.001	67 (6)	73 (4)	0.0002	109 (5)	58 (7)	0.0004
p,p'-DDT[3]	65 (2)	89 (8)	0.0003	60 (6)	74 (10)	0.0001	102 (3)	57 (16)	0.01
Deltamethrin[2]	90 (9)	78 (14)	0.002	82 (6)	124(24)	0.002	113 (13)	67 (11)	0.004
Diazinon[2]	63 (5)	89 (10)	0.001	57 (8)	91 (10)	0.0005	90 (9)	61 (6)	0.001
Dichlofluanid[1]	88 (16)	91 (4)	0.002	84 (19)	85 (7)	0.001	47 (31)	58 (4)	0.0004
4,4'-Dichlorbenzophenone[1]	69 (12)	111 (12)	0.01	82 (14)	–	0.009	90 (11)	71 (20)	0.002
Dichlorvos[1]	70 (18)	69 (6)	0.0003	57 (17)	83 (8)	0.0006	98 (17)	53 (9)	0.001
Dicofol[1]	48 (14)	103 (6)	0.01	55 (12)	69 (5)	0.01	-	-	–
Dieldrin[1]	87 (24)	114 (13)	0.005	67 (7)	106 (7)	0.002	92 (9)	124 (11)	0.004
Dimethoate[1]	110 (16)	76 (20)	0.001	105 (17)	81 (5)	0.003	102 (13)	80 (10)	0.01
Diphenylamine[1]	78 (5)	87 (16)	0.001	68 (16)	78 (7)	0.0003	87 (19)	71 (10)	0.001

Table 2. (Continued)

Compounds	Orange			Nectarine			Spinach		
	Fortification levels (mg/kg)		LOD (mg/kg)	Fortification levels (mg/kg)		LOD (mg/kg)	Fortification levels (mg/kg)		LOD (mg/kg)
	0.01	0.05		0.01	0.05		0.01	0.05	
Disulfoton[2]	87 (5)	111 (14)	0.01	75 (20)	92 (21)	0.009	-	-	–
Endosulfan I[1]	99 (18)	102 (16)	0.01	79 (20)	103 (7)	0.01	97 (16)	84 (15)	0.01
Endosulfan II[1]	85 (20)	86 (16)	0.01	–	89 (15)	0.02	110 (12)	88 (27)	0.006
Endosulfan ether[1]	79 (8)	84 (10)	0.005	79 (19)	87 (7)	0.005	72 (15)	75 (13)	0.004
Endosulfan sulfate[1]	105 (12)	107 (12)	0.002	97 (11)	103 (15)	0.001	112 (9)	80 (10)	0.0005
Endrin[1]	92 (15)	106 (13)	0.005	91 (20)	126 (33)	0.005	108 (11)	115 (17)	0.005
Esfenvalerate[2]	85 (8)	87 (19)	0.001	75 (5)	99 (13)	0.002	121 (15)	57 (9)	0.002
Ethion[2]	74 (9)	101 (20)	0.0004	77 (6)	96 (8)	0.0001	113 (10)	55 (9)	0.003
Ethoprophos[1]	94 (11)	85 (8)	0.001	82 (16)	115 (12)	0.001	89 (12)	99 (11)	0.003
Etofenprox[2]	64 (5)	89 (11)	0.002	79 (16)	105 (18)	0.001	111 (1)	54 (10)	0.005
Etrimfos[2]	66 (9)	84 (19)	0.001	63 (5)	90 (10)	0.001	93 (9)	65 (8)	0.002
Fenarimol[1]	102 (8)	102 (15)	0.001	102 (19)	76 (12)	0.0005	110 (17)	88 (9)	0.001
Fenchlorphos[2]	68 (6)	88 (16)	0.0009	63 (1)	87 (10)	0.002	98 (11)	67 (7)	0.0002
Fenitrothion[2]	81 (16)	97 (11)	0.004	87 (16)	94 (9)	0.009	97 (19)	83 (12)	0.001
Fenthion[2]	77 (2)	90 (14)	0.01	71 (10)	72 (12)	0.005	100 (14)	60 (12)	0.0007
Fenvalerate[2]	90 (6)	93 (13)	0.002	76 (4)	102 (13)	0.003	113 (9)	80 (10)	0.006
τ-Fluvalinate I[2]	74 (16)	77 (16)	0.003	72 (12)	123 (20)	0.002	–	–	–
τ-Fluvalinate II[2]	93 (17)	76 (16)	0.002	89 (17)	95 (16)	0.002	–	–	–
Folpet[1]	64 (13)	99 (8)	0.01	74 (17)	67 (11)	0.007	–	–	–
Fonofos[1]	72 (11)	88 (8)	0.001	83 (4)	86 (4)	0.0003	77 (14)	91 (10)	0.0004
HCB[1]	71 (5)	80 (4)	0.002	70 (7)	91 (10)	0.001	107 (3)	90 (9)	0.003
α-HCH[1]	83 (6)	83 (4)	0.001	81 (13)	104 (8)	0.0005	75 (7)	104 (10)	0.002
β-HCH[1]	85 (11)	87 (4)	0.001	78 (15)	108 (17)	0.002	78 (8)	118 (12)	0.001
δ-HCH[1]	95 (11)	83 (4)	0.002	86 (13)	118 (9)	0.004	105 (12)	123 (14)	0.005
γ-HCH[1]	88 (14)	90 (14)	0.002	105 (9)	119 (14)	0.003	90 (12)	127 (13)	0.002
Heptachlor[1]	86 (10)	88 (4)	0.002	79 (15)	100 (12)	0.001	73 (13)	104 (10)	0.0002
Heptachlor epoxide[1]	–	110 (26)	0.05	–	96 (11)	0.04	–	111 (11)	0.02
Heptenophos[1]	94 (6)	90 (5)	0.0002	85 (15)	101 (7)	0.003	109 (7)	96 (10)	0.001
Iprodione[1]	99 (20)	92 (17)	0.0004	–	–	–	101 (17)	78 (11)	0.004
Isodrin[1]	81 (8)	94 (12)	0.003	92 (9)	91 (6)	0.005	94 (12)	88 (6)	0.003
Isofenphos[2]	69 (7)	87 (13)	0.003	71 (11)	94 (8)	0.001	99 (14)	68 (7)	0.005
Malathion[2]	70 (5)	93 (19)	0.002	71 (4)	101 (8)	0.0007	110 (10)	65 (10)	0.001
Metalaxyl[1]	101 (17)	87 (8)	0.002	104 (15)	104 (14)	0.002	108 (12)	97 (15)	0.0001
Methacrifos[1]	70 (12)	81 (7)	0.0008	69 (5)	79 (4)	0.0006	111 (1)	72 (3)	0.001
Methidathion[2]	70 (5)	–	0.001	70 (9)	98 (12)	0.0005	108 (11)	65 (13)	0.001
Methiocarb[2]	71 (12)	101 (20)	0.006	90 (16)	99 (17)	0.009	104 (19)	67 (16)	0.004
Metholachlor[1]	81 (8)	86 (9)	0.0005	84 (16)	98 (9)	0.0008	108 (15)	88 (13)	0.0005
Methoxychlor[3]	44 (12)	84 (16)	0.0003	48 (15)	75 (11)	0.004	53 (20)	–	0.01
Metribuzin[2]	65 (18)	57 (23)	0.009	74 (20)	49(7)	0.007	115 (19)	72 (18)	0.003
Mevinphos[1]	87 (6)	93 (7)	0.0001	84 (17)	109 (10)	0.0006	106 (6)	94 (9)	0.0004
Mirex[1]	69 (9)	95 (4)	0.0003	72 (14)	100 (8)	0.0001	93 (14)	84 (10)	0.0003
Myclobutanil[1]	95 (14)	94 (9)	0.0004	100 (19)	98 (6)	0.001	105 (10)	86 (10)	0.0006
Omethoate[1]	61 (7)	56 (8)	0.001	74 (13)	–	0.005	–	68 (11)	0.01
Oxadixyl[1]	89 (4)	96 (4)	0.001	90 (16)	90 (7)	0.0003	106 (11)	89 (11)	0.001
Oxychlordane[3]	84 (19)	85 (10)	0.003	69 (2)	67 (12)	0.003	84 (17)	73 (10)	0.006
Parathion ethyl[2]	71 (10)	91 (11)	0.007	70 (10)	98 (13)	0.003	107 (9)	72 (13)	0.0004

Table 2. (Continued)

Compounds	Orange			Nectarine			Spinach		
	Fortification levels (mg/kg)		LOD (mg/kg)	Fortification levels (mg/kg)		LOD (mg/kg)	Fortification levels (mg/kg)		LOD (mg/kg)
	0.01	0.05		0.01	0.05		0.01	0.05	
Parathion methyl[2]	72 (13)	85 (17)	0.002	72 (15)	107 (20)	0.003	93 (16)	84 (11)	0.0002
Penconazole[1]	95 (11)	91 (9)	0.003	88 (19)	96 (7)	0.001	110 (10)	94 (11)	0.006
Pendimethalin[1]	86 (19)	83 (6)	0.01	95 (23)	110 (3)	0.009	125 (27)	121 (14)	0.0003
Pentachlorobenzene[1]	60 (8)	74 (9)	0.0005	45 (1)	70 (11)	0.003	63 (23)	60 (15)	0.01
Permethrin I[2]	81 (3)	99 (12)	0.002	89 (6)	103 (20)	0.002	111 (9)	61 (5)	0.004
Permethrin II[2]	86 (9)	97 (15)	0.002	80 (4)	100 (18)	0.002	123 (4)	66 (10)	0.003
Phorate[1]	81 (8)	94 (6)	0.0005	76 (18)	81 (5)	0.0004	69 (14)	60 (8)	0.002
Phosalone[2]	71 (8)	89 (17)	0.001	78 (9)	94 (12)	0.001	112 (14)	68 (7)	0.005
Phosmet[2]	76 (4)	89 (20)	0.0001	82 (5)	107 (13)	0.001	124 (27)	67 (10)	0.003
Phosphamidon[2]	75 (8)	83 (14)	0.001	80 (3)	98 (13)	0.001	113 (13)	70 (6)	0.0003
Pirimicarb[2]	73 (6)	102 (18)	0.001	69 (4)	–	0.001	107 (9)	60 (13)	0.0002
Pirimiphos methyl[2]	69 (6)	87 (13)	0.003	58 (11)	90 (9)	0.003	86 (8)	71 (5)	0.0003
Pirimiphos ethyl[2]	67 (6)	90 (9)	0.003	66 (3)	90 (9)	0.001	98 (15)	68 (7)	0.0002
Procymidone[1]	86 (14)	87 (13)	0.008	98 (19)	99 (8)	0.002	90 (8)	95 (11)	0.005
Profenofos[2]	76 (10)	87 (20)	0.008	77 (9)	93 (10)	0.002	110 (10)	63 (8)	0.008
Propiconazole I[1]	95 (14)	95 (5)	0.0004	96 (18)	115 (11)	0.002	102 (8)	125 (13)	0.01
Propiconazole II[1]	102 (12)	98 (7)	0.0003	93 (16)	118 (7)	0.001	112 (8)	128 (15)	0.001
Propyzamide[1]	65 (4)	87 (20)	0.001	67 (8)	99 (10)	0.0003	112 (8)	73 (7)	0.0005
Pyrazophos[2]	74 (10)	87 (14)	0.0006	73 (10)	91 (11)	0.0004	109 (16)	67 (9)	0.002
Pyridaben[1]	92 (20)	96 (10)	0.0002	80 (17)	103 (12)	0.0004	109 (15)	84 (10)	0.004
Pyrimethanil[1]	100 (11)	98 (15)	0.001	94 (20)	81 (34)	0.005	109 (13)	119 (6)	0.001
Pyriproxyfen[1]	111 (14)	105 (12)	0.003	90 (7)	104 (6)	0.005	108 (8)	91 (18)	0.01
Quinalphos[2]	78 (8)	98 (12)	0.007	73 (9)	94 (9)	0.003	110 (13)	70 (5)	0.003
Quintozene[1]	86 (17)	87 (7)	0.003	93 (16)	115 (16)	0.001	75 (14)	122 (7)	0.003
Simazine[2]	74 (17)	85 (19)	0.009	80 (19)	73 (15)	0.01	111 (14)	84 (5)	0.01
Tebuconazole[1]	107 (9)	96 (10)	0.0003	109 (15)	68 (17)	0.001	110 (2)	88 (11)	0.001
Tecnazene[1]	64 (2)	81 (4)	0.002	63 (12)	76 (5)	0.002	110 (12)	84 (5)	0.009
Tefluthrin[2]	69 (8)	94 (15)	0.002	66 (12)	93 (8)	0.003	–	–	–
Terbufos[1]	77 (10)	87 (4)	0.001	78 (14)	95 (8)	0.0003	84 (9)	88 (11)	0.001
Terbutylazine[2]	72 (1)	87 (22)	0.002	70 (7)	78 (6)	0.003	96 (12)	70 (9)	0.003
Tetrachlorvinphos[2]	65 (5)	111 (14)	0.005	70 (3)	104 (19)	0.002	105 (9)	95 (16)	0.004
Tetradifon[1]	107 (8)	94 (10)	0.001	103 (15)	105 (7)	0.01	104 (7)	100 (17)	0.01
Tolclofos methyl[1]	60 (6)	81 (14)	0.001	62 (8)	89 (11)	0.0007	83 (8)	70 (8)	0.0001
Tolyfluanid[1]	90 (16)	80 (6)	0.002	93 (19)	108 (11)	0.001	71 (10)	110 (15)	0.01
Triazophos[2]	74 (7)	88 (19)	0.0005	74 (8)	103 (12)	0.001	104 (14)	68 (9)	0.001
Triflumizole[1]	73 (17)	–	0.003	86 (18)	–	0.002	–	–	–
Trifluralin[1]	86 (5)	91 (10)	0.0003	83 (11)	90 (7)	0.0002	104 (11)	80 (10)	0.002
Vinclozolin[1]	102 (17)	97 (13)	0.004	95 (19)	97 (6)	0.005	100 (20)	96 (14)	0.0004

LOD limits of detection

The numbers in superscript indicate the I.S. used for each analyte: *1, HCB-* $^{13}C_6$ *; 2 Terbutylazine* D_6*; 3, p,p'-DDE* D_8

As an example, ***Figure 2*** shows GC-MS/MS chromatograms for several pesticides in orange, nectarine, and spinach samples fortified at 0.01 mg/kg. Pesticides have been chosen within a wide range of retention times (between 6 min and 29 min) to better illustrate the performance of the method. The selectivity of the method was satisfactory and came from the acquisition of two specific SRM transitions for each pesticide. GC-MS/MS chromatograms did not show the presence of interfering peaks at the analyte retention time for none of the pesticides investigated in this work.

As regards *Q/q* ratios (see ***Table 1***), they were, in general, rather similar in the matrices investigated for a given pesticide, with some exceptions, normally in spinach matrix (tefluthrin, metribuzin, carbaryl, fenitrothion, parathion ethyl, tolyfluanid, tetrachlorvinphos, bupirimate, bromopropylate, cyfluthrin, cypermethrin, fluvalinate and azoxystrobin). This would make necessary to use the *Q/q* ratios of standards in matrix for an adequate confirmation of positives in samples instead of standards, in solvent or in any other food matrix. In many pesticides, favorable *Q/q* ratios (around 1–2) were obtained, what indicates that confirmation transition had similar sensitivity to quantification transition, which would allow confirmation of positives at very low concentration levels. In a few compounds, confirmation would be problematic at low levels, due to unfavorable *Q/q* ratios (*e.g.*, diphenylamine, parathion ethyl, buprofezin, and azoxystrobin).

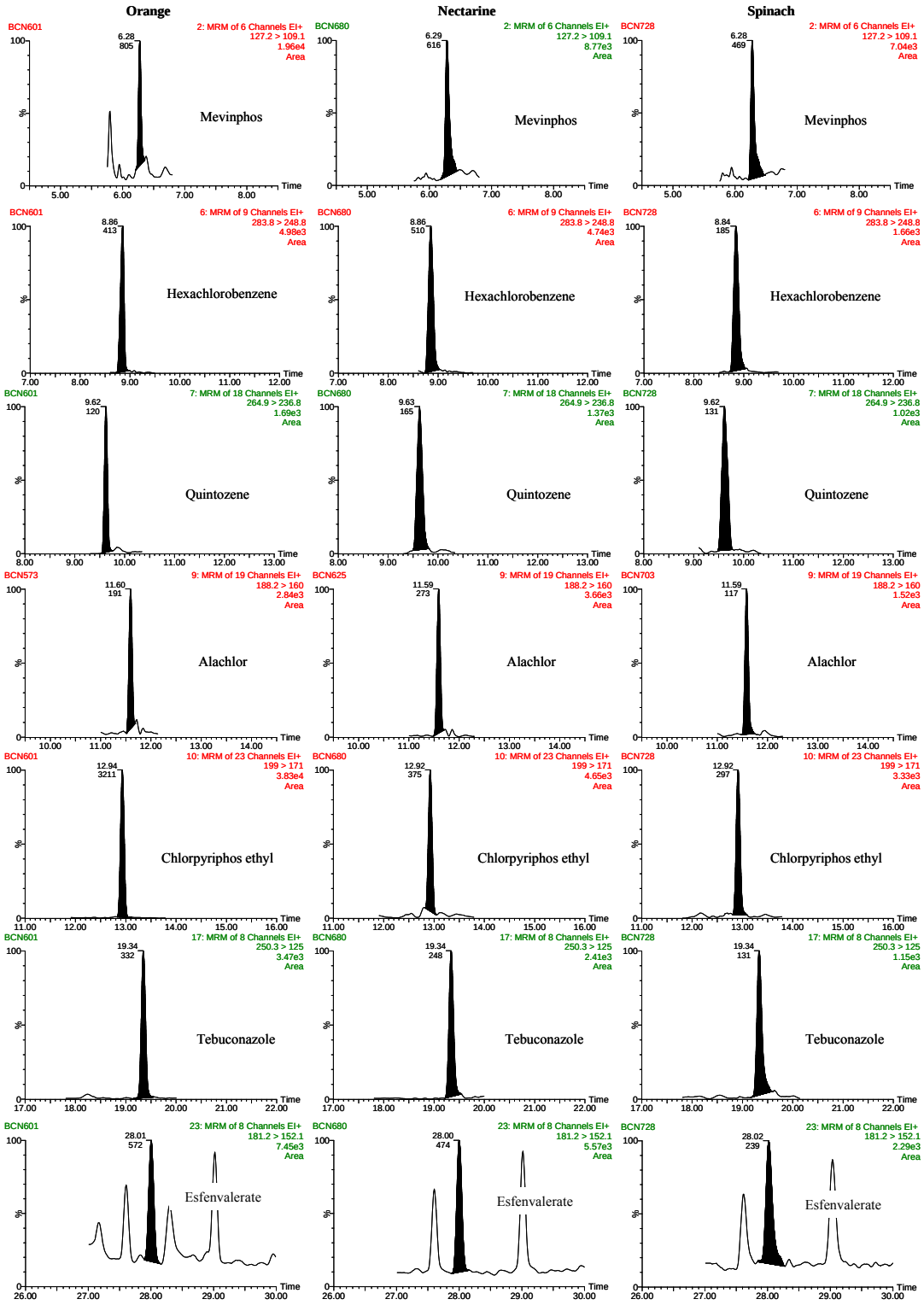

Figure 2. GC-MS/MS SRM chromatograms for selected pesticides (within a wide range of retention times) in orange, nectarine, and spinach samples fortified at 0.01 mg/kg. Only the quantification transition is shown

Application to real samples

In order to study the applicability of the methodology developed, several samples collected from a local market in Barcelona (Spain) were analyzed (six samples, two of each matrix).

The results obtained are shown in ***Table 3***. The OC insecticide mirex was detected in 50 % of the samples analyzed but at concentrations below 0.01 mg/kg. Persistent OC insecticides, like DDT and its metabolites DDD and DDE, or endosulfan sulfate were detected in some samples but at very low levels, very close to the LODs. Only three positive findings could be quantified, as they were above 0.01 mg/kg: chlorpyrifos in orange 2 (0.016 mg/kg) and, deltamethrin and phosmet in nectarine 1 (0.021 and 0.015 mg/kg, respectively). In these cases, the concentration was lower than the MRL established for three insecticides in the sample matrices analyzed.

Table 3. Pesticides found in orange, nectarine and spinach samples after application of the overall procedure (concentrations expressed in mg/kg)

Compounds	Orange 1	Orange 2	Nectarine 1	Nectarine 2	Spinach 1	Spinach 2
Chlorpyriphos ethyl	–	0.016	d	–	–	–
p,p'-DDD	d	–	–	–	d	–
p-p'-DDE	–	d	–	–	–	d
p,p'-DDT	d	–	–	–	–	
Deltamethrin	–	–	0.021	–	–	–
Endosulfan sulfate	d	–	–	–	–	–
Malathion	–	–	d	–	–	–
Mirex	–	d	–	d	–	d
Phosmet	–	–	0.015	–	–	–

d detected

As regards confirmation of positive findings, all pesticides detected were confirmed by the use of the two transitions monitored and the compliance of the *Q/q* intensity ratios. The acquisition of two transitions allows the simultaneous quantification and confirmation of pesticides in only one injection, as an alternative approach to the proposed elsewhere [30, 31] where one injection with only one transition is used as a screening method and a second injection, of only potentially positive samples, is required for confirmation and quantification purposes. Anyway, in the case of exceeding MRLs, a second independent analysis would be required to confirm the presence of the pesticide in the sample as well as its concentration to be above the MRL. All *Q/q* ratios were within the range of the tolerance accepted [23] around the experimental *Q/q* value obtained from reference standards in matrix injected in the same analysis sequence. ***Figure 3*** shows GC-MS/MS chromatograms corresponding to the positive findings detected in one of the nectarine samples. A reliable identification of analytes in this sample was feasible by means of the experimental *Q/q* intensity ratios, even at concentrations lower than 0.01 mg/kg.

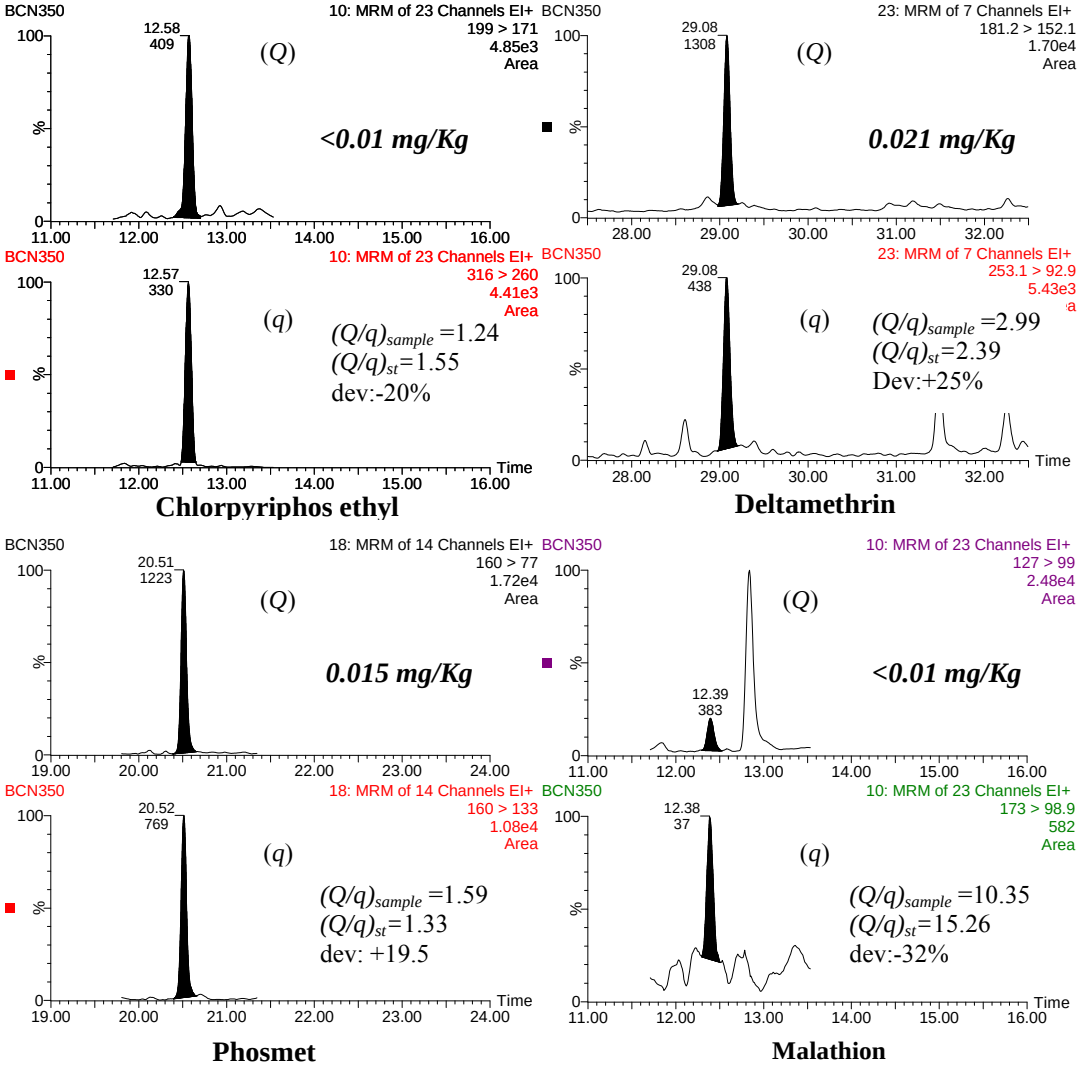

Figure 3. GC-MS/MS SRM chromatograms for pesticide detected in a nectarine sample (nectarine 1, Table 3). (Q) Quantification transition (q) confirmative transition.

CONCLUSIONS

A multi-residue method has been developed and validated for the simultaneous quantification and confirmation of around 130 pesticides in fruits and vegetables, selecting orange, nectarine, and spinach as matrices under study. The potential of GC-MS/MS with triple quadrupole analyzer has shown to be a

key tool for the quantitative determination of this high number of pesticides. The selection of two SRM transitions, one for quantification and one for confirmation, gives excellent selectivity and sensitivity and the possibility of safe identification, using *Q/q* intensity ratio as a confirmatory parameter.

Extraction of samples was made by ASE using ethyl acetate as solvent. The overall multi-residual method has been fully validated at 0.01 and 0.05 mg/kg in the three types of samples, obtaining satisfactory accuracy and precision in most cases. The methodology developed in this work was applied to the analysis of market samples, where some pesticides were detected and identified at low concentration levels, even below 0.01 mg/kg.

The study of matrix effect in orange, nectarine, and spinach samples showed an evident enhancement of signal produced by matrix components for the majority of pesticides investigated, especially in spinach. A similar behavior was observed for other food matrices investigated (mango, raisin, paprika, cabbage, pear, rice, legume and gherkin). The use of labeled I.S. helped to minimize matrix effects for some pesticide/matrix combinations, although did not always assure appropriate correction. Therefore, matrix-matched standard calibration was required in order to perform a correct quantification in samples.

Acknowledgments

The authors acknowledge the financial support of Generalitat Valenciana, as research group of excellence PROMETEO/2009/054.

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4. Discusión de resultados

Por lo que respecta a la información recogida en el **Artículo científico 1** (*review*) es inevitable destacar las indudables ventajas que ha aportado la cromatografía de gases acoplada a la espectrometría de masas en tándem con analizador de triple cuadrupolo en la determinación de residuos de plaguicidas durante los últimos años. En él se discuten de modo crítico las aportaciones de GC-MS/MS QqQ en este campo de trabajo, y se recogen los artículos publicados, desde sus primeros usos (año 2003 en muestras alimentarias y 2007 para el caso de muestras medioambientales) hasta la actualidad. Cabe mencionar que el primer artículo publicado sobre aplicación de GC-MS/MS QqQ en el campo ambiental corresponde a nuestro propio grupo de investigación (Pitarch, 2007).

Tras las primeras aplicaciones del analizador de QqQ acoplado a la cromatografía de gases, la técnica ha ido continuamente mejorando a la vista de los muchos métodos instrumentales desarrollados y reportados hasta el momento, tal como se muestra en la ***Tabla 1*** y ***Tabla 2*** del **Artículo científico 1**. Resulta evidente esta evolución a través del tiempo, tanto por la frecuencia del uso del QqQ como por otros aspectos del proceso analítico. Se han producido mejoras en las técnicas de preparación de muestras y se han aplicado etapas posteriores de purificación para eliminar las impurezas, minimizando posibles problemas en la cuantificación y en la correcta identificación de los analitos.

Otro aspecto que ha ido evolucionando es el relativo a los sistemas de inyección. Los sistemas *split/splitless,* comúnmente utilizados en cromatografía de gases, son en ocasiones sustituidos por otros sistemas como PTV, que permiten la inyección de disolventes menos recomendados para GC debido al elevado

volumen de expansión que generan dentro del inyector durante la vaporización. Así, con el uso de PTV se pueden evitar las etapas previas de cambio de disolvente a uno más adecuado para *split/splitless*, agilizando el proceso de extracción de la muestra (Sapozhnikova, 2013).

Algunos de los trabajos publicados recientemente (referencias 18. y 19., *Tabla 1*, Artículo científico 1), son pioneros en cuanto al uso de una nueva fuente de ionización a presión atmosférica para GC. APCI es una interesante alternativa a la ionización electrónica, más comúnmente utilizada, tal como se discute en este *review*. En estos trabajos, se exponen las ventajas derivadas de trabajar con una ionización más suave, especialmente en cuanto a la posibilidad de usar transiciones escogiendo como ion precursor el ion molecular o la molécula protonada, $M^{+\cdot}$ o $[M+H]^+$. Este ion presenta generalmente una elevada abundancia en APCI, en contraste con EI donde muchas veces el ion molecular está ausente debido a la extensa fragmentación que tiene lugar en esta fuente. Esto se traduce en una importante mejora de la sensibilidad y especificidad del método al usar sistemas GC-(APCI) MS/MS (Portolés, 2012a; Portolés, 2012b).

A modo de conclusión global, cabe destacar que todos los artículos citados en este *review* (Artículo científico 1) resaltan la elevada selectividad y sensibilidad conseguidas con este tipo de analizadores, al poder desarrollar métodos SRM con transiciones específicas para cada analito, lo que posibilita la identificación y la adecuada cuantificación a muy bajos niveles de concentración, en un único análisis.

El segundo artículo presentado en este capítulo (**Artículo científico 2**), corresponde a un trabajo de desarrollo y optimización de un método para la determinación multiresidual de 130 plaguicidas haciendo uso de GC-MS/MS QqQ. En el método desarrollado se seleccionan dos transiciones SRM para cada uno de los analitos estudiados y se lleva a cabo una validación cuantitativa para todos ellos en tres matrices de alimentos vegetales (naranja, nectarina y espinaca), a dos niveles de concentración, utilizando ASE como técnica de extracción. Como parte del trabajo, se llevó a cabo un estudio detallado del efecto matriz para las muestras mencionadas anteriormente con el fin de asegurar la correcta cuantificación de los analitos. Adicionalmente, se estudió el efecto matriz para otras matrices como mango, pasas, pimentón, col, pera, arroz, legumbres y pepinillo, mostrando sus diferentes comportamientos.

En primer lugar, se estudiaron y optimizaron las transiciones adecuadas para cada analito, usando un patrón de referencia que contenía todos los plaguicidas seleccionados (130 en total). Mediante análisis en modo de adquisición *scan*, se obtuvieron los espectros completos de cada compuesto y se seleccionaron un par de iones, preferiblemente el pico base y otro ion intenso (si era posible, el ion molecular), susceptibles de ser iones precursores. A continuación, en una segunda inyección del patrón de referencia, esta vez en modo de adquisición de barrido de iones producto (*product ion scan*), se probaron diferentes energías de colisión con los iones precursores seleccionados. Este proceso permitió seleccionar el ion producto más intenso y/o más selectivo que daba lugar a una transición lo más intensa y selectiva posible para cada uno de los analitos estudiados.

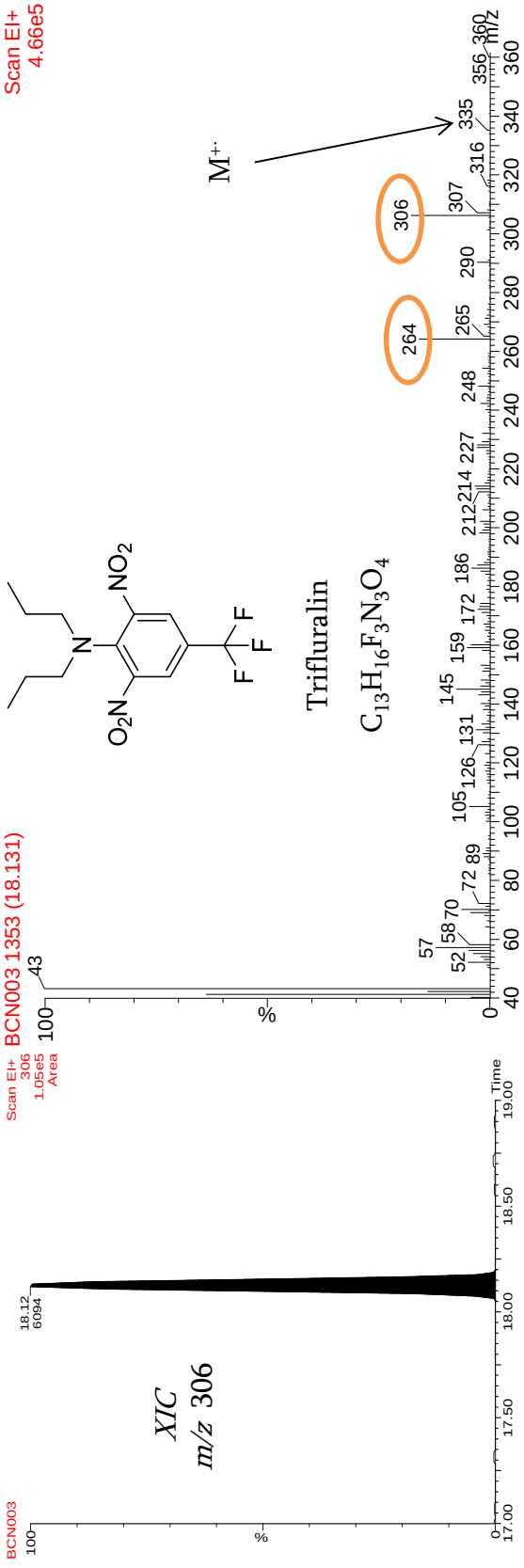

Figura 1. Cromatograma extraído del ion *m/z* 306 y espectro de masas obtenidos mediante GC-QqQ MS adquiridos en modo *scan* para la trifluralina (patrón solvente 2 mg/L), con la selección de los iones *m/z* 306 y 264 como iones precursores

A modo de ejemplo, se muestra la optimización de las transiciones adecuadas para un herbicida incluido en el método multiresidual, la trifluralina.

La **Figura 1** muestra el cromatograma y el espectro de masas de la triflurina obtenidos tras ser analizada en modo *scan* con GC-QqQ MS. Tras observar el espectro de masas, obtenido mediante ionización electrónica, se aprecia claramente la ausencia del ion molecular $M^{+\cdot}$, por lo que otros dos iones mayoritarios, el *m/z* 306 y el *m/z* 264 con intensidades similares, son susceptibles de ser los iones precursores. El pico base en este caso, el *m/z* 43 no fue elegido como ion precursor por ser un ion demasiado pequeño para obtener iones producto aceptables. Con los iones mencionados se analizó de nuevo, mediante modo de adquisición *product ion scan,* con diferentes energías de colisión (10, 20 y 30 eV).

En la **Figura 2** se pueden observar los espectros de masas de los iones producto obtenidos para el ion precursor *m/z* 264, a las diferentes energías de colisión.

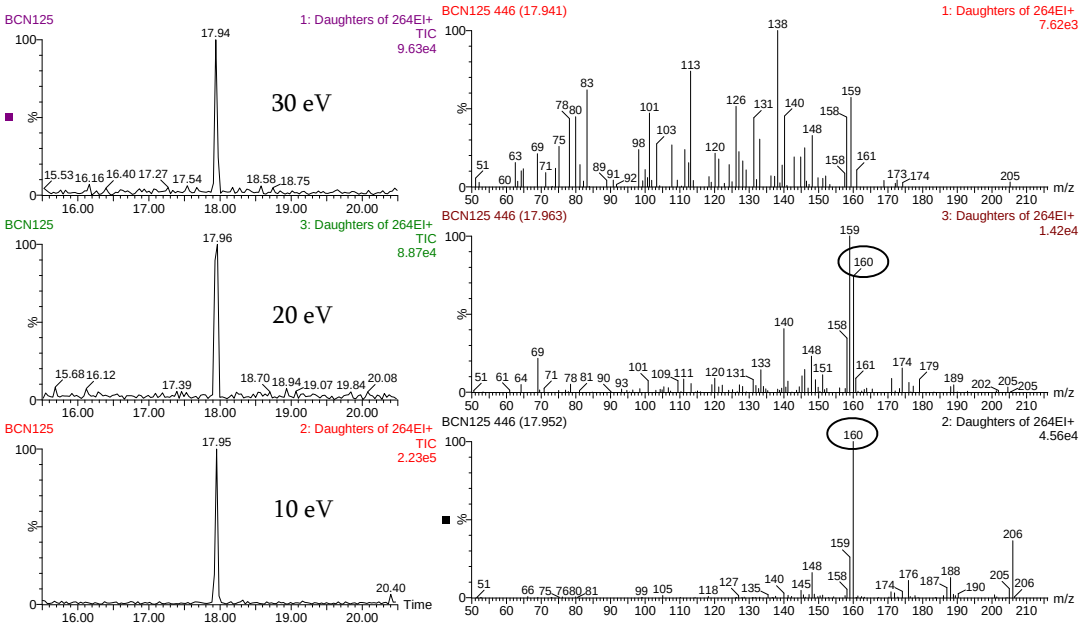

Figura 2. Cromatogramas y espectros de masas obtenidos por GC-QqQ MS en modo *product ion scan* para el ion *m/z* 264 a sus respectivas energías de colisión

A la vista de los resultados, se escogió el ion *m/z* 160 como ion producto del *m/z* 264, con las opciones de 10 y 20 eV. Finalmente, se seleccionó una energía de colisión de 20 eV por ser la transición 264>160 ligeramente más intensa a esta energía. Siguiendo los mismos pasos, se optimizó una segunda transición, en este caso con el ion precursor *m/z* 306, obteniendo *m/z* 264 como ion producto, con una energía de colisión de 10 eV. Ambas transiciones fueron las elegidas para la trifluralina, seleccionando la más intensa con fines de cuantificación y la menos intensa con fines de confirmación, tal como se muestra en la **Figura 3.**

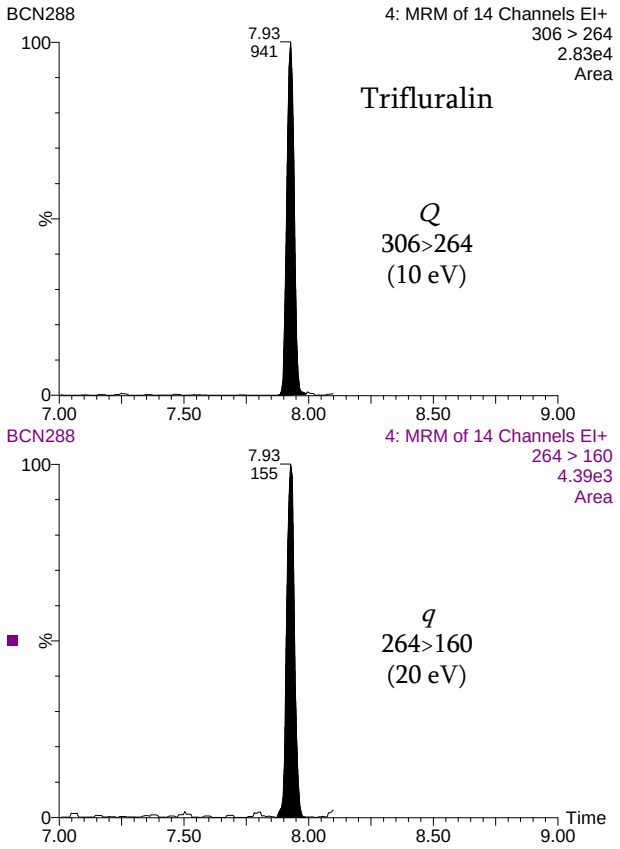

Figura 3. Cromatograma obtenido mediante GC-MS/MS QqQ mostrando las 2 transiciones SRM optimizadas de un patrón en solvente de la trifluralina (60 µg/L)

Aplicando esta misma metodología de trabajo para los 130 compuestos objeto de estudio, se creó un único método de análisis para todos, trabajando en modo SRM, con la transición más intensa (en la mayoría de los casos) para la cuantificación (*Q*) y la otra para confirmación (*q*). Al tratarse de un método con un número tan elevado de compuestos, para cada una de las ventanas de adquisición se optimizó minuciosamente el *dwell time*, de modo que cada pico cromatográfico presentara un mínimo de 16 puntos por pico, con buena forma y manteniendo una sensibilidad aceptable. Todas las transiciones seleccionadas,

junto con las condiciones de MS empleadas, se muestran en la *Tabla 1* del Artículo científico 2.

Una vez diseñado el método de GC-MS/MS QqQ para los 130 compuestos seleccionados se procedió a realizar la etapa de tratamiento de muestra. La técnica elegida fue una extracción acelerada con disolventes (ASE), que se realizó por completo en el laboratorio de Química de la Agencia de Salud Pública de Barcelona (ASPB), en el marco de una investigación colaborativa. En dicho laboratorio se disponía del material y equipos necesarios para realizar la etapa de tratamiento de muestra.

El método aplicado consistió en la utilización de un sistema ASE, el cual trabaja a elevada temperatura y presión. En cuanto al disolvente de extracción, se prefirió el acetato de etilo frente al acetonitrilo, pues, además de ser más barato, presenta menor toxicidad y evita problemas de miscibilidad en posteriores mezclas con otros disolventes. El método de extracción utilizado ya había sido optimizado por el laboratorio de la ASPB y aplicado en sus métodos de rutina por lo que se siguieron los mismos procedimientos normalizados de trabajo. La investigación se centró, por tanto, en el uso de GC-MS/MS con analizador de triple cuadrupolo, en un momento en el que apenas había antecedentes sobre el uso de este analizador en la determinación multiresidual de plaguicidas.

Las matrices que se seleccionaron inicialmente para llevar a cabo este proyecto fueron la naranja, como matriz representativa de productos con un elevado contenido en ácido; nectarina y espinaca, con un elevado contenido en agua (aunque ésta última también con elevado contenido en clorofila); y el aguacate, con un elevado contenido en aceite (European Commission (2007) SANCO/3131/2007). Para naranja y nectarina, la extracción de los analitos

mediante la técnica ASE fue adecuada, obteniendo aparentemente unos extractos limpios y sin apenas coloración. En cambio, para espinaca y aguacate fue necesaria una etapa adicional de purificación al obtenerse unos extractos con un elevado contenido en clorofila (con un color verde muy intenso) y en aceite, respectivamente. Para ello, se utilizó la técnica de cromatografía de exclusión por tamaños (GPC). A pesar del *clean-up* mediante GPC, el extracto del aguacate todavía contenía demasiado aceite, lo que provocaba graves problemas de contaminación durante la etapa de inyección (en jeringa, en *liner*…), además de una pobre reproducibilidad de los resultados. Por ello, considerando que la finalidad de nuestro estudio era demostrar las posibilidades y mejoras obtenidas por el uso de GC-MS/MS en general, y no el desarrollo de un método específico para la matriz del aguacate, en particular, el aguacate se eliminó del método a partir de este punto.

Por lo general, con el método de extracción aplicado se había logrado reducir la manipulación de muestra y la posibilidad de extraer un elevado número de muestras cada día. El sistema ASE utilizado (ASE® 200) dispone de 24 posiciones para las muestras y, de este modo, permite realizar la extracción automatizada de todas ellas al dejar trabajando al equipo durante la noche y tener al día siguiente los extractos preparados para proseguir con las etapas posteriores. Con el método utilizado, descrito en detalle en el apartado de "*Sample preparation"* del **Artículo científico 2**, se consigue una pre-concentración de 4 veces para las matrices en las que únicamente se realiza la extracción (naranja y nectarina), y de 5 veces para la espinaca, en la que se aplica además la etapa posterior de *clean-up* por GPC.

Tras aplicar el proceso de extracción con ASE (más purificación, en el caso de espinaca), se realizó una evaluación del efecto matriz sobre la respuesta obtenida por GC-MS/MS QqQ de las matrices objeto de estudio, tal como se explica en el **Artículo científico 2**. En esta parte del estudio, también se incluyeron otras matrices que fueron consideradas de interés, como mango, pasas, pimentón, col, pera, arroz, legumbres y pepinillo. El efecto matriz (absoluto) se estimó como el cociente entre la respuesta absoluta obtenida para un compuesto en un patrón preparado en matriz y la respuesta absoluta del patrón preparado en solvente, ambos a la misma concentración. Si en lugar de utilizar la respuesta absoluta se tiene en cuenta la respuesta relativa (corregida con el patrón interno) se le denomina efecto matriz relativo. Se consideró que para valores entre 0.8 y 1.2, donde las respuestas eran relativamente semejantes, no existía un destacado efecto matriz y que, por tanto, un calibrado en solvente no llevaría a errores relevantes en su cuantificación.

El comportamiento en cuanto al efecto matriz para los tres tipos de muestras seleccionadas naranja, nectarina y espinaca, se ilustra en la ***Figura 1*** (***a*** y ***b***) del **Artículo científico 2**. Adicionalmente, en la **Figura 4** se puede observar dicho efecto (absoluto) para el caso del mango, pasas, pimentón, col, pera y arroz. Para la mayor parte de los compuestos estudiados se obtienen valores superiores a 1.2, como resultado de la exaltación de la respuesta cromatográfica en presencia de matriz. Por el contrario, pocos plaguicidas presentaban valores inferiores a 0.8 (límite inferior). Este es un hecho frecuente, ya que el efecto matriz sufrido en GC es normalmente por exaltación de la señal (Schenck, 2000; Kwon, 2012; Rahman, 2013). Esto es causado por un aumento de la señal inducida, ya que las superficies activas del sistema, especialmente el inyector, y en menor medida la columna o el detector, provocan una retención o una degradación de los analitos. En un patrón

en solvente, hay más sitios activos disponibles para los plaguicidas que cuando se inyecta en matriz ya que aparte del solvente también hay componentes de la matriz que actúan bloqueando los lugares activos. Entonces, la eficiencia de inyección para los analitos a nivel de trazas es mayor en presencia de componentes de la matriz que en disoluciones con solo solvente, lo que lleva a obtener una respuesta mayor y una concentración erróneamente (por exceso) si no se corrige adecuadamente este efecto matriz.

Algunos analitos presentan mayor tendencia hacia esta exaltación de la señal debido a sus propiedades fisicoquímicas (por ejemplo, aquellos compuestos que presentan un valor del efecto matriz calculado superior a 4, ver **Figura 4**). Esto le ocurre a compuestos como *coumaphos*, *phosmet*, *iprodione*, *tolyfluanid*, *dichlofluanid*, *phosphamidon* o *mevinphos*, en dos o incluso en tres de las matrices estudiadas. Estos plaguicidas tienen en común grupos fosfato o amino (ver **Figura 5**), los cuales tienen tendencia a presentar un mayor efecto matriz (Schenck, 2000).

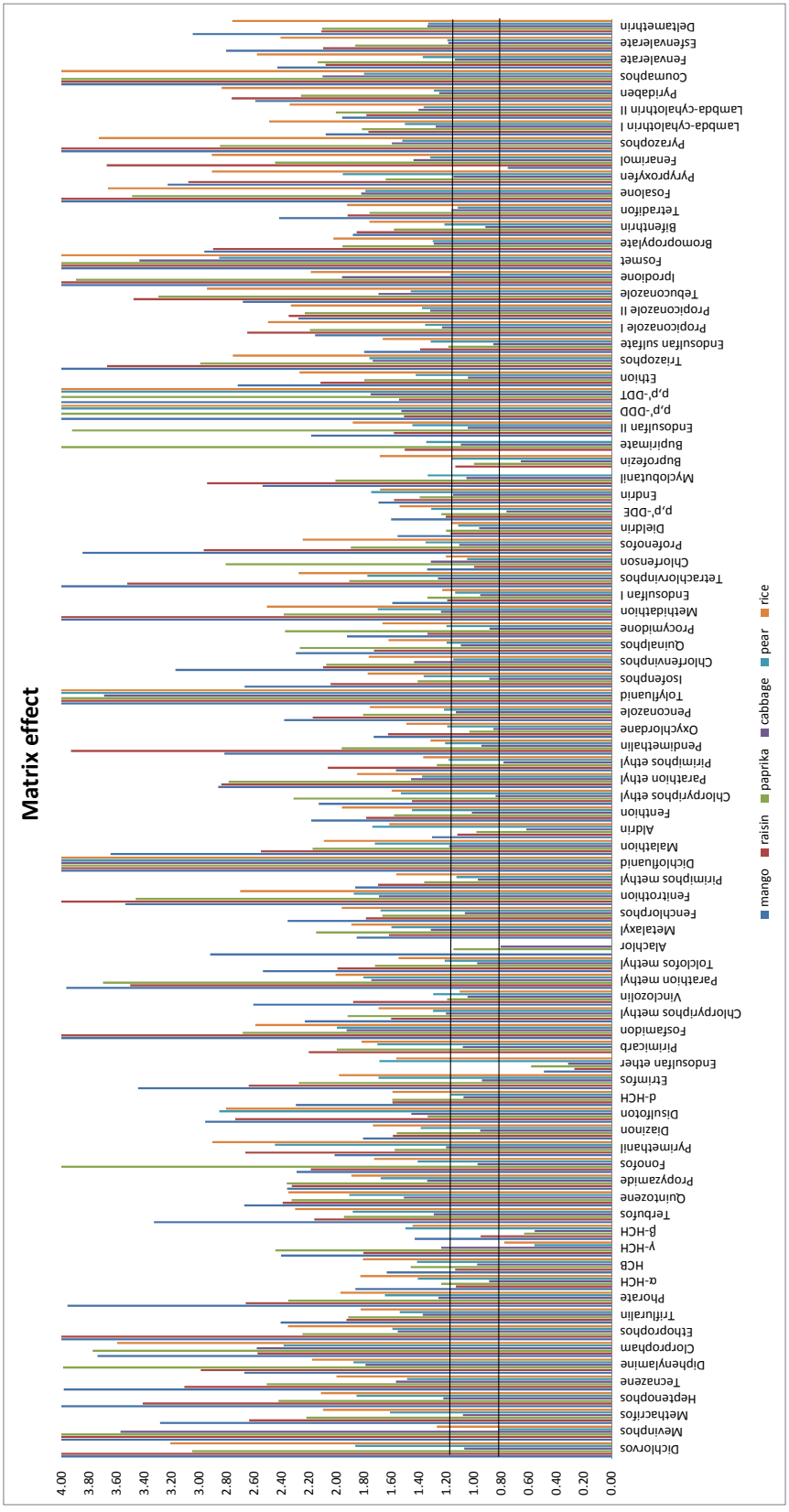

Figura 4. Efecto matriz calculado (respuesta patrón estándar en matriz/respuesta patrón estándar en solvente) en la determinación por GC-MS/MS de plaguicidas seleccionados en mango, pasas, pimentón, col, pera y arroz

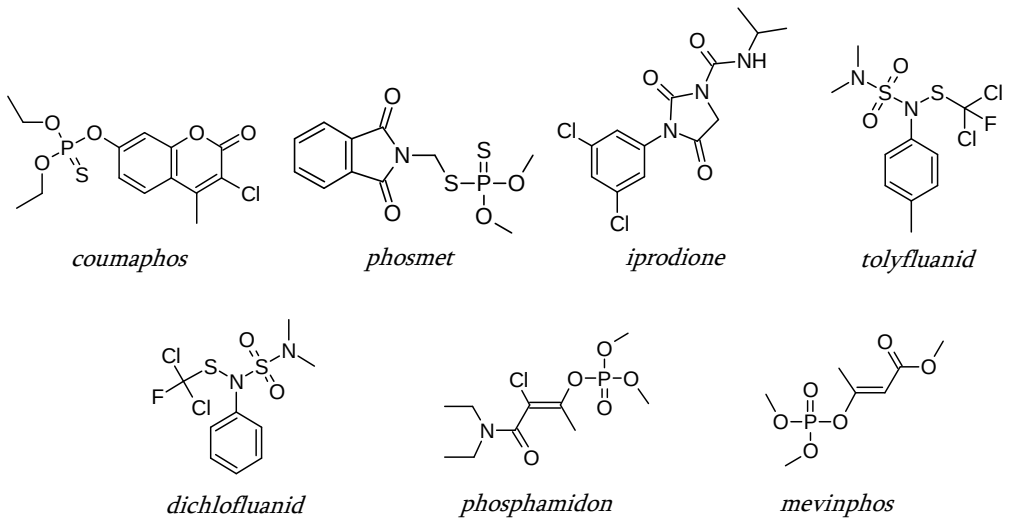

Figura 5. Estructuras de plaguicidas estudiados que presentan un elevado efecto matriz

Una de las opciones para reducir el efecto matriz consiste en disminuir la cantidad de muestra que entra en el sistema de GC diluyendo el extracto resultante. A modo de ejemplo, se muestra en la **Figura 6** el estudio realizado en muestras de legumbres y de pepinillo, en las que al diluir el extracto al 50 % con acetato de etilo, el efecto matriz calculado (absoluto) presentaba valores más cercanos a los límites establecidos como aceptables (0.8-1.2). El inconveniente que presenta esta aproximación es el poder alcanzar los límites de detección requeridos, al solo introducir parte del extracto final de la muestra.

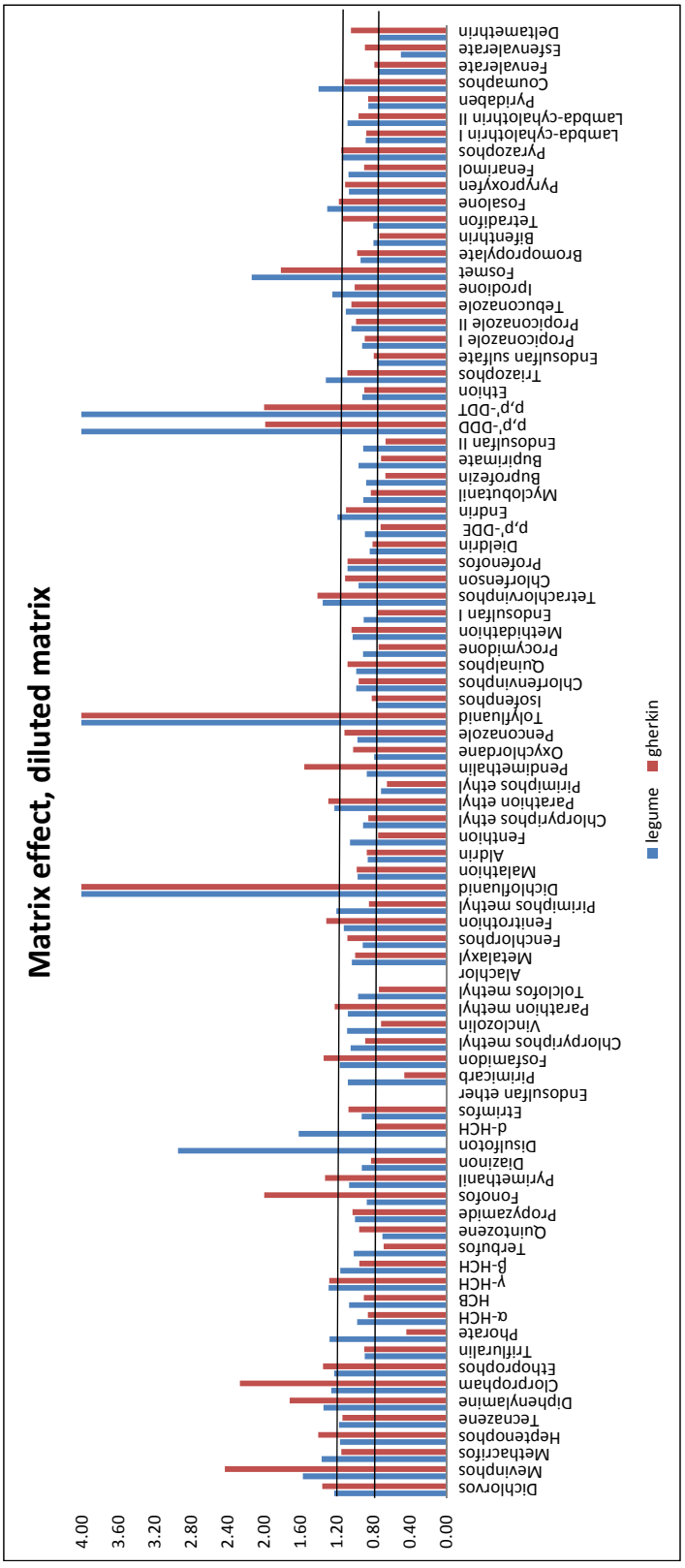

Figura 6. Efecto matriz calculado (respuesta patrón de referencia en matriz/respuesta patrón de referencia en solvente) en la determinación por GC-MS/MS de plaguicidas seleccionados en extractos de legumbres y pepinillo diluida (/2)

Otra opción disponible para corregir el efecto matriz es utilizar para la cuantificación un calibrado en matriz, para cada tipo de muestra. De este modo, en el método desarrollado se tomó una muestra ecológica de cada matriz como blanco y se llevó a cabo el mismo procedimiento de extracción. Los extractos resultantes fueron fortificados con la cantidad adecuada de patrón conteniendo los 130 plaguicidas para crear una curva de calibración preparada en la misma matriz a estudio y llevar a cabo la cuantificación corrigiendo, de este modo, el efecto matriz

Aparte del efecto matriz, que es un factor relevante que puede afectar a la cuantificación, los resultados pueden verse también afectados por las variaciones sufridas a lo largo del proceso de extracción, así como en la etapa de inyección y también durante el análisis, por la posible variabilidad instrumental. El uso de patrones internos mejora en gran medida la correcta cuantificación de los analitos al corregir dichas variaciones. La opción ideal para corregir las desviaciones y/o errores sufridos a lo largo de todo el proceso analítico seria utilizar como patrón interno el propio analito marcado isotópicamente, ya que en principio se vería afectado por las mismas circunstancias que el analito, al tener una estructura química semejante. Sin embargo, en este trabajo resultaría inviable y extremadamente caro el uso de tantos patrones marcados (130 compuestos) y supondría además un trabajo adicional en cuanto a su preparación y a la optimización de las transiciones. Por ello, se decidió seleccionar únicamente 3 patrones internos (IS), HCB $^{13}C_6$, *p,p'*-DDE D_8 y *terbutylazine* D_6, para corregir las variaciones sufridas.

Para seleccionar cuál de los tres IS era más adecuado para corregir la señal de cada analito se llevaron a cabo los cálculos de las recuperaciones y las

desviaciones (RSD) de los extractos procedentes de la validación de la naranja (n=5) a nivel de 0.05 mg/kg. Se usaron las áreas relativas con respecto a cada uno de los tres IS y también con áreas absolutas (sin corrección con IS) para realizar el cálculo, cuantificando con calibrado en matriz.

En la **Figura 7** se muestra, a modo de ejemplo, el comportamiento de 20 plaguicidas seleccionados incluyendo una representación de los principales grupos: organoclorados (OC), organofosforados (OP), carbamato, piretroides, triazinas y otros. Se evaluaron tanto las recuperaciones obtenidas, como las RSD en %, de las 5 réplicas de los experimentos de validación, ya que ambos son parámetros importantes a tener en cuenta.

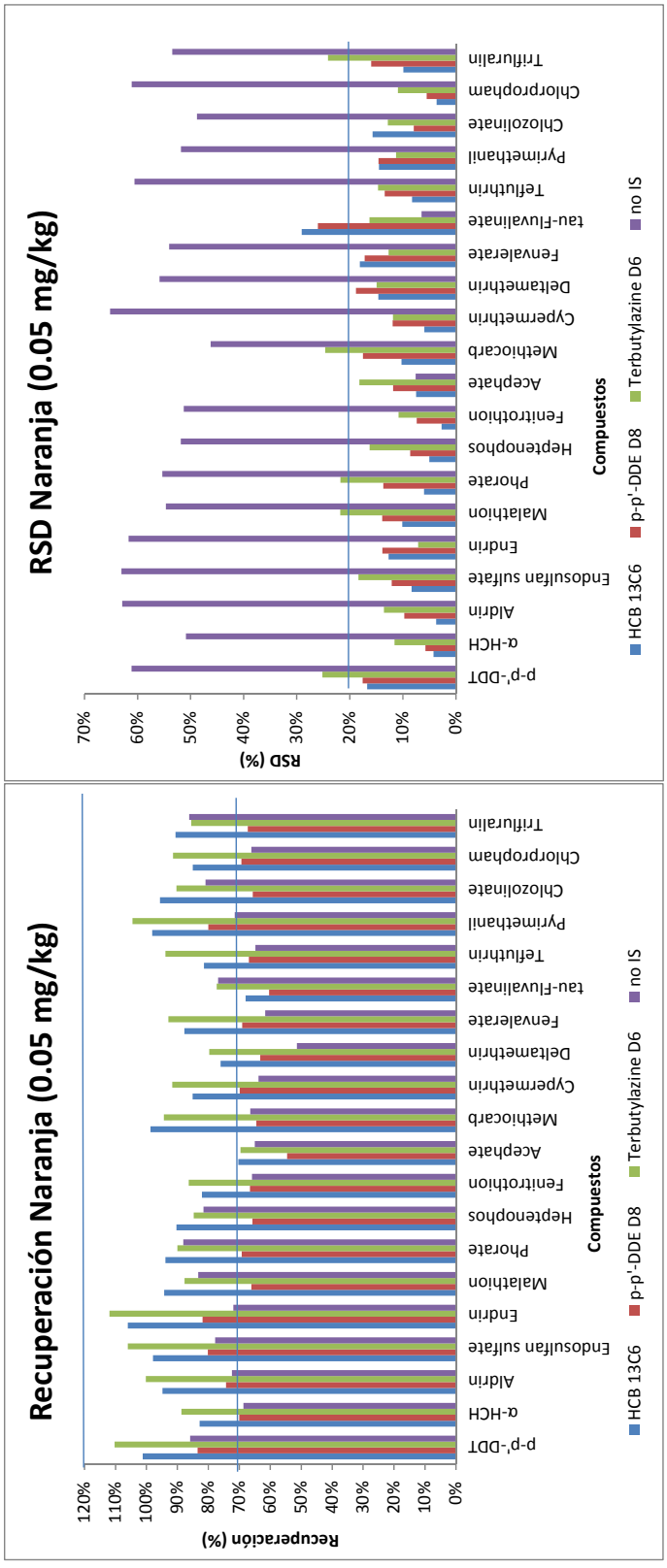

Figura 7. Recuperación y RSD de las muestras fortificadas de la validación realizada a nivel de fortificación de 0.05 mg/kg alto para la matriz de naranja en su determinación por GC-MS/MS de unos plaguicidas elegidos como representativos para la elección del patrón interno para la cuantificación.

Se puede observar que, en general, con los 3 IS se producía una cierta corrección de la señal, al mejorar las recuperaciones obtenidas sin IS. Con el fin de escoger el IS adecuado para cada grupo de plaguicidas, se observó la respuesta global de ellos, en función del que proporcionaba mejores resultados en la recuperación así como en la RSD. De este modo, para *p,p'*-DDT, α-HCH, *aldrin, endosulfan sulfate* y *endrin* (todos los plaguicidas OC), con HCB $^{13}C_6$ y *p,p'*-DDE D_8 se obtuvieron las mejores recuperaciones, entre 70 y 100 %, mientras que con el IS *terbutylazine* D_6 fueron ligeramente superiores al 100 %. En cuanto a las RSDs, los mejores resultados se obtuvieron con el IS HCB $^{13}C_6$ y *p,p'*-DDE D_8. De este modo, para los OC se seleccionaron los dos IS mencionados, escogiendo como el más adecuado para cada uno de ellos, el más próximo en tiempo de retención. Para los siguientes 5 plaguicidas mostrados en la **Figura 7** (compuestos OP: *malathion, phorate, heptenofos, fenitrotion* y *acephate*) pareció ser la *terbutylazine* D_6 la que mejor corrigió junto con HCB $^{13}C_6$, mientras que con *p,p'*-DDE D_8 se obtuvieron recuperaciones muy pobres. Por proximidad en tiempo de retención, se seleccionó uno u otro IS. *Methiocarb*, único representante de los carbamatos en esta selección a estudio, presentó mejor recuperación y menor desviación con HCB $^{13}C_6$ por lo que no hubo ninguna duda en su elección. En cuanto a los piretroides *cypermethrin, deltamethrin, fenvalerate*, *tau-fluvalinate y tefluthrin*, en general las recuperaciones fueron mejores con la *terbutylazine* D_6, por lo que se seleccionó dicho IS. La *terbutylazine* D_6 corregía satisfactoriamente a las triazinas, seguramente por ser de la misma familia (no se muestra en el gráfico) y éste fue, por tanto, el IS seleccionado para esta familia de compuestos. Por último, para el grupo de los otros tipos de compuestos (en representación, *pyrimethanil*, *chlozolinate, chlorpropham* y *trifluralin*) el HCB $^{13}C_6$ pareció ofrecer mejor corrección en general, por lo que fue el seleccionado para ellos.

Tras este estudio detallado del efecto matriz y de la correcta elección del IS, se llevó a cabo la validación de las matrices objeto de estudio a dos niveles de concentración, 0.01 y 0.05 mg/kg, utilizando calibrado en matriz y obteniendo recuperaciones en la mayoría de los casos entre 70 y 120 %, con desviaciones menores de 20 %, tal como se muestra en la *Tabla 2* del Artículo científico 2.

Como conclusión general del presente capítulo, se puede indicar que la espectrometría de masas en tándem con analizador de triple cuadrupolo es una de las aproximaciones que ofrece mayor sensibilidad y selectividad para la cuantificación y confirmación simultánea de residuos de plaguicidas de diversas familias químicas. La adquisición de, al menos, dos transiciones por compuesto en modo SRM permite minimizar las interferencias de la matriz, debido a la excelente selectividad de la técnica GC-MS/MS con triple cuadrupolo. Del mismo modo, su excelente sensibilidad permite alcanzar límites de detección muy bajos, debido a la baja señal del ruido en el cromatograma. Además, la adquisición de dos transiciones y la evaluación de su *Q/q ratio* ofrece una confirmación altamente fiable de la identidad de los compuestos detectados en las muestras.

CAPÍTULO 3

APLICACIÓN DE LA ESPECTROMETRÍA DE MASAS CON ANALIZADOR DE TIEMPO DE VUELO Y CUADRUPOLO-TIEMPO DE VUELO EN ANÁLISIS DE RESIDUOS DE PLAGUICIDAS

1. Introducción

El uso del analizador de tiempo de vuelo (*time-of-flight*, TOF) en espectrometría de masas ha ampliado el concepto de análisis, que típicamente ha estado dirigido hacia la búsqueda de analitos preestablecidos, atendiendo a sus características físico-químicas al aplicar un método instrumental específico, caracterizado por una elevada selectividad y sensibilidad. Este tipo de aproximación se conoce como análisis dirigido o *target* y tiene como propósito investigar únicamente unos compuestos previamente seleccionados. Un ejemplo representativo del análisis *target* son los métodos GC-MS/MS multiresiduos basados en el uso de analizadores de triple cuadrupolo, donde únicamente se adquieren las transiciones SRM optimizadas para cada compuesto. Con esta metodología el número de compuestos analizados es limitado, existiendo pocas referencias de métodos GC-MS/MS QqQ *target* que excedan los 100-200 compuestos. No obstante, la excelente selectividad y sensibilidad que se alcanza

con esta técnica para los compuestos seleccionados es indudable (Walorcyzk, 2008; Banerjee, 2012). El uso del analizador TOF ha propiciado un cambio en la filosofía de trabajo, ya que permite, gracias a la adquisición completa del espectro de masas, trabajar en modo de análisis no dirigido (*non-target*), adicionalmente a la aproximación *target*. En este sentido toman especial protagonismo los softwares de tratamiento de datos para realizar la búsqueda de compuestos una vez se han adquirido los datos. Es por ello que el campo de análisis se puede ampliar tanto como se desee y en cualquier momento tras la adquisición de toda la información espectral.

Según las características del analizador de masas de tiempo de vuelo, se puede distinguir entre analizadores de elevada resolución, HR TOF MS, o aquellos que presentan una elevada velocidad de adquisición, HS TOF MS, característica que hace ideales a estos últimos para ser acoplados a GCxGC o GC ultrarrápida.

Tradicionalmente los HR TOF MS desarrollados para ser acoplados a un sistema de GC con fuente de ionización de EI o CI conseguían un poder de resolución medio-alto, de entre 5000-7000 FWHM (*full width at half maximum*). Las nuevas fuentes de ionización a presión atmosférica (APCI) han permitido los acoplamientos de sistemas de GC con analizadores de TOF MS de resoluciones más elevadas, llegando incluso a alcanzar dicho acoplamiento los 50000 FWHM. Estos instrumentos permiten resolver (completa o parcialmente) los componentes de la matriz con la misma masa nominal que los analitos, reduciendo en gran medida las interferencias producidas por el ruido de fondo y ruido químico, mejorando así la identificación. Otra de las ventajas que ofrecen los HR TOF MS es la posibilidad de realizar medidas con una elevada exactitud de masa, ya que se puede trabajar con errores de masa inferiores a 5 ppm (incluso con nueva

instrumentación, 1 ppm), lo cual permite determinar de modo más fiable la composición elemental de cada *m/z* y facilitar la elucidación de compuestos desconocidos.

Especialmente interesante es la peculiaridad que tienen los analizadores TOF MS de adquirir el espectro completo de masas, con una elevada sensibilidad en modo *scan,* notablemente superior a otros instrumentos de barrido. Así, gracias a su diseño ortogonal, la eficiencia de adquisición del espectro de masas completo en estos instrumentos es mayor si se compara con otros analizadores como el cuadrupolo (aprox. 100 veces). Este hecho permite disponer de la información espectral, incluso a niveles de ultra-traza, de cualquier compuesto y como consecuencia la posibilidad de identificarlo gracias a la librería teórica de espectros de masas (en caso de adquisición mediante modo EI) (Godfrey, 2012; Cajka, 2007).

El conjunto de todas las características mencionadas, es decir, elevado poder de resolución, posibilidad de medida de masa exacta, y elevada sensibilidad en modo *scan,* confieren al TOF MS un elevado potencial para el análisis de residuos de plaguicidas con la posibilidad de trabajar usando distintas aproximaciones, dependiendo de cuál sea el objetivo de los análisis: *target*, *post-target* o *non-target* (Hernández, 2011).

El modo de trabajo *target* es la aproximación típica de los métodos analíticos convencionales, con el objetivo de analizar compuestos previamente seleccionados. En el caso del TOF MS, en modo *target* se crean métodos de tratamiento de datos en los que se busca la presencia de iones característicos (o fragmentos) medidos en masa exacta a su tiempo de retención, para poder llevar a cabo la detección del compuesto objeto de estudio y su identificación fiable. Para

ello, es necesaria la inyección de patrones de referencia con el fin de conocer el Rt y los fragmentos característicos que se generan en la fuente de ionización usada. En el caso de la ionización mediante EI, la fragmentación suele ser abundante, con lo cual es habitual la presencia de numerosos iones fragmento en el espectro de masas. Para calcular la masa exacta (teórica) de cada uno de los fragmentos es necesario determinar previamente la composición elemental de cada uno de ellos, lo cual resulta ser una tarea complicada, aun disponiendo del patrón de referencia. La existencia de programas que simulan la fragmentación ayuda a determinar las posibles composiciones elementales y proponer estructuras de los fragmentos presentes en el espectro de masas de un determinado compuesto, escogiendo el más adecuado tras evaluar su error de masa y las posibles rutas de fragmentación. Con esta aproximación *target*, la cuantificación de los analitos es también posible al tener la oportunidad de analizar en la misma secuencia un calibrado externo (bien en solvente, bien en matriz) para los compuestos seleccionados y reportar, por tanto, valores de concentración.

Tras el análisis dirigido a una serie de compuestos, y dado que la adquisición del espectro de masas es completa, la lista de analitos a estudio puede ser ampliada (modo *post-target)* tanto como se desee aun cuando no se disponga de patrones de referencia. Es por ello, que el campo de aplicación es ilimitado, y se puede detectar cualquier analito que haya sido eluido de la columna, ionizado por la fuente y adquirida su información espectral. Así, la localización de candidatos se puede realizar por la búsqueda de sus iones característicos, bien $M^{+\cdot}$ o $[M+H]^+$ con APCI, o $M^{+\cdot}$ y/o iones fragmento con EI; medidos en masa exacta (Mol, 2010).

Otra aproximación completamente diferente que se puede aplicar a los datos obtenidos por TOF MS es el modo *non-target*, que se ve, en parte facilitado gracias a las librerías teóricas existentes para la búsqueda de desconocidos. Así, tras la adquisición inicial en modo *full scan*, se aplica un software de deconvolución (*ChromaLynx XS*) que ofrece un pico cromatográfico, como consecuencia de la detección de varios iones a un mismo tiempo de retención, y muestra su espectro de masas completo medido en masa exacta. De forma automatizada, por comparación con la librería espectral teórica, se genera una lista de posibles candidatos con los errores de masa para cada uno de los supuestos fragmentos y un porcentaje de analogía (*match*) con el espectro de masas teórico. La decisión de tomar un candidato como positivo no es una labor sencilla ya que junto con los compuestos exógenos a las muestras aparecen también los compuestos endógenos de la matriz que dificultan la identificación de los contaminantes de interés. Hasta el momento, este método *non-target* sólo se puede realizar usando búsquedas en librerías en aquellos análisis llevados a cabo mediante ionización por EI ya que son las únicas librerías comerciales y normalizadas que existen actualmente.

Cabe destacar que cuando se utiliza como modo de ionización EI (el más habitual en GC-MS), existe la ventaja de poder comparar el espectro de masas obtenido experimentalmente con el de la librería comercial teórica, aunque para facilitar la tarea cuando se trabaja con HR TOF MS se preferiría la existencia de una librería teórica con masas exactas de cada uno de los fragmentos presentes. Por el contrario, si la ionización se lleva a cabo mediante una fuente de ionización suave, como APCI, los espectros de masas suelen presentar $M^{+\cdot}$ o $[M+H]^+$ como iones más abundantes, siendo sencillo calcular su masa exacta y realizar así la detección del analito basándose en la búsqueda de dicho ion. En el caso del

analizador híbrido QTOF, con fuente APCI, es posible la adquisición de forma simultánea del espectro de masas mediante dos funciones: baja energía (4 eV) y alta energía de colisión (rampa 10-40 eV). Esto ofrece la posibilidad de identificar los analitos de forma absolutamente fiable, al combinar información sobre su ion (cuasi) molecular (función de baja energía) con la de sus iones fragmento (función de alta energía), en ambos casos medidos en masa exacta.

Cuando se trabaja con TOF MS es fundamental disponer de conocimientos básicos de espectrometría de masas, así como conocer los modelos isotópicos y las reglas de fragmentación que, junto con ayuda de softwares específicos, permiten establecer las posibles composiciones elementales de los iones (fragmento) y llevar a cabo la creación de métodos. Estos conocimientos posibilitan además la reducción a simple vista de la lista, por ejemplo, de los candidatos a positivos ofrecida con una aproximación *non-target*. Esto es aún más importante cuando no se dispone del espectro de masas en la librería teórica de EI, cuando no existe patrón de referencia o en casos en los que debe interpretarse correctamente el espectro de masas obtenido mediante APCI en la función de alta energía. Sin duda, una buena base de conocimientos sobre MS ayuda, y mucho, en el proceso de elucidación e identificación de un compuesto desconocido (Godfrey, 2012).

Tras mencionar brevemente las ventajas del analizador TOF MS, también hay que indicar algún inconveniente, que afortunadamente se ha ido mejorando en diseños instrumentales recientes, como su limitado rango lineal dinámico, que no suele exceder de 3-4 órdenes, mientras que con otros instrumentos en modo *scan* se pueden obtener hasta 5 o 6 órdenes de magnitud de rango lineal (Cajka, 2007).

En este capítulo se presentan dos metodologías analíticas desarrolladas en la **Tesis**, basadas en cromatografía de gases acoplada a TOF MS, para la determinación de residuos de plaguicidas en matrices vegetales. Una de ellas mediante ionización por EI usando analizador de tiempo de vuelo (método GC-(EI)TOF MS; **Artículo científico 3**) y la otra mediante ionización por APCI usando analizador híbrido cuadrupolo-tiempo de vuelo (método GC-(APCI)QTOF MS; **Artículo científico 4**).

En el **Artículo científico 3** se ha desarrollado y validado un método *target* para llevar a cabo la detección, identificación y cuantificación de 55 plaguicidas mediante GC-TOF MS en modo EI en muestras de manzanas, tomates, zanahorias y naranjas. El método se ha aplicado a muestras reales llevando a cabo la cuantificación de los compuestos detectados, así como un posterior análisis *non-target* para determinar la presencia de otros compuestos que no habían sido previamente seleccionados en el método.

En el **Artículo científico 4** se ha aplicado un método de *screening* para 130 plaguicidas en muestras de frutas y verduras mediante GC-(APCI)QTOF MS. De entre todos los plaguicidas investigados, solo 15 resultaron detectados e identificados. Para estos compuestos encontrados en las muestras, se realizó una validación cuantitativa con el fin de comprobar la idoneidad del analizador QTOF MS para llevar a cabo un análisis cuantitativo. Tras comprobar la validez del método en términos cuantitativos, se procedió a la cuantificación de los compuestos identificados. En una segunda etapa, se extendió el *screening* a más de 400 plaguicidas, basándose en la búsqueda de su ion molecular o molécula protonada en la función de baja energía. En aquellos casos en los que se encontraron posibles positivos, se llevó a cabo un estudio detallado de su

fragmentación gracias a la información obtenida en la función de alta energía de colisión, con el fin de realizar la correcta identificación de los mismos.

2. Artículo científico 3

Journal of Chromatography A, 1244 (2012) 168–177

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Application of gas chromatography time-of-flight mass spectrometry for target and non-target analysis of pesticide residues in fruits and vegetables

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ARTICLE INFO

Article history:
Received 14 December 2011
Received in revised form 25 April 2012
Accepted 26 April 2012
Available online 4 May 2012

Keywords:
Fruits and vegetables
Pesticides
QuEChERS
GC–TOF MS
Target and non-target
Quantitative analysis

ABSTRACT

In this work, the capability of gas chromatography coupled to time-of-flight mass spectrometry (GC–TOF MS) for quantitative analysis of pesticide residues has been evaluated. A multiclass method for rapid screening of pesticides (insecticides, acaricides, herbicides and fungicides) in fruit and vegetable matrices has been developed and validated, including detection, identification and quantification of the analytes. To this aim, several food matrices were selected: high water content (apples, tomatoes and carrots), high acid content (oranges) and high oil content (olives) samples. The well known QuEChERS procedure was applied for extraction of pesticides, and matrix-matched calibration using relative responses versus internal standard was used for quantification. The sample extracts were analyzed by GC–TOF MS. Up to five ions using narrow window (0.02 Da)-extracted ion chromatograms at the expected retention time were monitored using a target processing method. The most abundant ion was used for quantification while the remaining ones were used for confirmation of the analyte identity. Method validation was carried out for 55 analytes in the five sample matrices tested at three concentrations (0.01, 0.05 and 0.5 mg/kg). Most recoveries were between 70% and 120% with relative standard deviations (RSDs) lower than 20% at 0.05 and 0.5 mg/kg. At 0.01 mg/kg, roughly half of the pesticides could be satisfactorily validated due to sensitivity limitations of GC–TOF MS, which probably affected the ion ratios used for confirmation of identity. In the case of olive samples, results were not satisfactory due to the high complexity of the matrix. An advantage of TOF MS is the possibility to perform a non-target investigation in the samples by application of a deconvolution software, without any additional injection being required. Accurate-mass full-spectrum acquisition in TOF MS provides useful information for analytes identification, and has made feasible in this work the discovery of non-target imazalil, fluoranthene and pyrene in some of the samples analyzed.

Journal of Chromatography A, 1244, 168-177, ***2012***

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Abstract

In this work, the capability of gas chromatography coupled to time-of-flight mass spectrometry (GC–TOF MS) for quantitative analysis of pesticide residues has been evaluated. A multiclass method for rapid screening of pesticides (insecticides, acaricides, herbicides and fungicides) in fruit and vegetable matrices has been developed and validated, including detection, identification and quantification of the analytes. To this aim, several food matrices were selected: high water content (apples, tomatoes and carrots), high acid content (oranges) and high oil content (olives) samples. The well-known QuEChERS procedure was applied for extraction of pesticides, and matrix-matched calibration using relative responses versus internal standard was used for quantification. The sample extracts were analyzed by GC–TOF MS. Up to five ions using narrow window (0.02 Da)-extracted ion chromatograms at the expected retention time were monitored using a target processing method. The most abundant ion was used for

quantification while the remaining ones were used for confirmation of the analyte identity. Method validation was carried out for 55 analytes in the five sample matrices tested at three concentrations (0.01, 0.05 and 0.5 mg/kg). Most recoveries were between 70 % and 120 % with relative standard deviations (RSDs) lower than 20 % at 0.05 and 0.5 mg/kg. At 0.01 mg/kg, roughly half of the pesticides could be satisfactorily validated due to sensitivity limitations of GC–TOF MS, which probably affected the ion ratios used for confirmation of identity. In the case of olive samples, results were not satisfactory due to the high complexity of the matrix. An advantage of TOF MS is the possibility to perform a non-target investigation in the samples by application of a deconvolution software, without any additional injection being required. Accurate-mass full-spectrum acquisition in TOF MS provides useful information for analytes identification, and has made feasible in this work the discovery of non-target imazalil, fluoranthene and pyrene in some of the samples analyzed.

Highlights

► QuEChERS is highly appropriate for sample treatment in multi-residue pesticide methods for fruits and vegetables. ► GC–TOF MS allows the quantification and reliable identification of pesticide residues. ► Accurate-mass full-spectrum acquisition in TOF MS allows investigation of non-target compounds. ► Using different internal standard *m/z* ions, depending on the analyte ion monitored, notably improves quantification.

Keywords

Fruits and vegetables; Pesticides; QuEChERS; GC-TOF MS; Target and non-target; Quantitative analysis

1. Introduction

The importance of food quality control is widely recognized nowadays to assure the compliance of regulation of these products and guarantee consumer health. The presence of pesticide residues in food is a matter of concern. For this reason, strict legislation exists at the EU level that establishes maximum residue levels (MRL), i.e. the upper legal concentration allowed for a pesticide residue in or on food or feed [1].

Keeping in mind the large number of pesticides applied worldwide, multiresidue methods are commonly used for monitoring pesticide residues in food. Both gas chromatography (GC) and liquid chromatography (LC) have been widely applied coupled with mass spectrometry (MS) using different analyzers. As regards GC–MS, single quadrupole [2-5], ion trap (ITD) [6-8] or triple quadrupole (QqQ) [3,9-13], have been frequently used. ITD and QqQ analyzers are normally applied under tandem mass spectrometry (MS/MS) mode, offering notable advantages in sensitivity and selectivity. The information acquired in target MS/MS method is analyte-specific (*e.g.* characteristic ions/transitions monitored). Therefore, other pesticides that might be present in the samples would not be detected if they are not included in the scope of the method.

The recent progress in instrumentation has increased the use of time-of-flight (TOF) mass analyzers coupled to GC for analyzing pesticides in food [14-18]. The main advantage of TOF MS comes from the full spectrum acquisition, with better sensitivity than conventional scanning instruments (*e.g.* quadrupole) [19]. There are two commercially available approaches for the time-of-flight analyzers using gas chromatography: high-speed (HS) and high-resolution (HR). HS instruments allow acquiring at 100–500 spectra/s but only

provide unit resolution. HS-TOF instruments are suitable for detection of very narrow chromatographic peaks generated by fast and ultra-fast GC or by GC × GC and most applications reported are focused on quantification. On the other hand, HR instruments have normally 5000–10000 FWHM (full width at half-maximum) resolution and moderate scan speed (up to 20 Hz). HR-TOF has the possibility of resolving matrix components yielding ions with the same nominal mass as that of the target analyte, reducing background interferences and improving the analyte identification [20].

Accurate-mass full-spectrum data available in HR-TOF MS enable to obtain extracted ion chromatograms using narrow mass windows (nw-XICs). Reducing the mass window can notably improve the signal-to-noise due to exclusion of a large proportion of the chemical background and quasi-isobaric interferences. The potential of GC–TOF MS has been mainly explored in the qualitative field pursuing the detection and identification of GC-amenable organic contaminants [19,21-24]. Up to five *m/z* ions of each target analyte are monitored, using as confirmation of identity criteria the presence of at least two *m/z* ions and the accomplishment of the intensity ratio within established tolerances [25]. Thus, wide-scope screening has been developed and validated from a qualitative point of view for around 150 organic micropollutants in water [24]. The elucidation of non-target compounds is also possible after MS data acquisition, without the need of reinjecting the sample, making use of powerful deconvolution software [19,21-23,26,27]. Although there is wide consensus on the great qualitative potential of HR-TOF MS however its low dynamic range compared with conventional MS instrumentation limits its quantitative applications and also affects mass accuracy, which can be deteriorated at certain concentration levels. The analog-to-digital converter

(ADC) detector offers linear dynamic range of four orders of magnitude but, at low analyte signal intensities, noise becomes a limiting factor. The time-to-digital converter (TDC) detector, on the contrary, is suitable for detection of weak signals, which is the case of analytes at ultra-traces levels, but it may present problems of saturation at high concentrations. New generations of HR-TOF MS typically use TDC for data acquisition, and allow dynamic range to be extended (DRE). The new DRE option overcomes the problems of saturation and makes quantification easier in HR-TOF MS instruments [15,19,20]. Even with some limitations, mainly as regards sensitivity, quantitative applications have been reported using GC–TOF MS in the food safety field [14-18,28] but it has not been implemented for routine monitoring analysis yet, where GC–MS/MS remains the instrument of choice.

To take full advantage of the capabilities of GC–TOF MS for screening a large number of pesticides, a generic procedure with a wide scope is required. To this aim the QuEChERS method [12,17,18,28-36], a rapid extraction procedure based on the use of acetonitrile as extractant, has been widely applied in food residue analysis. After the original method, developed in 2003 [29], several modifications have improved the scope of the method, like the use of acetate buffering during the extraction step (AOAC Official Method 2007.1) [30,32] or citrate buffering (CEN Standard Method EN 15662) [33,34]. The QuEChERS method has been tested for hundreds of pesticides using GC–MS and LC–MS for measurement, obtaining satisfactory results. Therefore, it seems appropriate to be used in combination with GC–TOF MS for a wide-scope screening [17,18].

In this paper, a multi-residue method based on QuEChERS extraction and GC–TOF MS analysis has been developed for target and non-target analysis of

pesticides in fruits and vegetables. The potential of GC–TOF for quantitative analysis has been investigated for 55 target analytes in different food commodities (orange, apple, carrot, tomato and olive). The developed target methodology has been applied to the analysis of several samples containing incurred analytes. Additionally, taking advantage of the use of GC–TOF MS, the screening has been extended to non-target pesticides in the samples under study.

2. Experimental

2.1. Reagents

Individual reference standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany) with a purity >93–99 %. Stock standard solutions (around 500 mg/L) were prepared in acetone and were stored in a freezer at –20 °C. Eight mixtures of pesticide standards (individual concentration of each pesticide around 50 mg/L) were prepared by volume dilution of stock individual solutions in acetone. Two working standard solutions containing all analytes at 5 mg/L were prepared by combining the eight standard mixtures and diluting in hexane or in acetonitrile. Further dilutions were prepared in hexane (for preparing matrix-matched calibration curves) or in acetonitrile (for sample spiking purposes).

Triphenyl phosphate (TPP), purchased from Dr. Ehrenstorfer with a purity 99.5 %, was used as internal standard. Stock standard solution (around 500 mg/L) was prepared by dissolving reference standard in acetone. Then, a solution at 5 mg/L was prepared by volume dilution in toluene.

Acetone (pesticide residue analysis quality), hexane (ultra trace quality), acetonitrile (reagent grade), toluene (for GC residue analysis) and glacial acetic acid were purchased from Scharlab (Barcelona, Spain). Anhydrous magnesium sulfate (extra pure) and anhydrous sodium acetate (reagent grade) were purchased from Scharlab. The QuEChERS commercial products, 2 mL micro-centrifuge tubes for d-SPE containing 50 mg primary secondary amine (PSA) and 150 mg anhydrous $MgSO_4$ or containing additionally 50 mg C_{18} were purchased from Teknokroma (Barcelona, Spain).

2.2. GC–TOF instrumentation

The GC instrumentation used consisted in an Agilent 6890N GC system (Palo Alto, CA, USA), equipped with an Agilent 7683 autosampler, coupled to a TOF mass spectrometer, GCT, 1.0 GHz TDC (Waters Corporation, Manchester, UK), operating in electron ionization (EI) mode. The GC separation was performed using a fused silica HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) J&W Scientific (Folson, CA, USA). The oven temperature was programmed as follows: 90 °C (hold 1 min); 5 °C/min to 300 °C (hold 2 min). The cycle time was 45 min. The temperature program was designed to get an optimum chromatographic separation between analytes and matrix components. Additionally, this improved chromatographic separation is expected to allow better non-target detection of unknown compounds, avoiding coelutions. Splitless injections of 1 μL of sample extracts were carried out with an injector temperature of 300 °C and with a splitless time of 1 min. Helium 99.999 % (Praxair, Valencia, Spain) was used as carrier gas at a constant flow of 1 mL/min.

The interface and ion source temperatures were set to 260 °C and 250 °C, respectively. A solvent delay of 4 min was used to prevent damage in the ion source filament. TOF MS was operated at a scan time of 0.95 s in the mass range *m/z* 50–650 and using a multi-channel plate voltage of 2850 V. As the GC–TOF instrument used in this work did not have the dynamic range enhancement (DRE) mode available, for high analyte concentrations (i.e. highest validation level), the scan time was reduced to 0.65 s to avoid problems of detector saturation, as a consequence of the low dynamic range of TOF MS. For sample analysis, a scan time of 0.95 s was selected. If under these conditions a positive finding led to detector saturation, a second injection of the sample extract at 0.65 s would be required to obtain a suitable quantification. TOF MS resolution was about 6700 (FWHM) at *m/z* 264. Mass spectrometric grade PFTBA (perfluorotri-n-butylamine), used for the daily mass calibration/verification as well as for lock mass, was injected via syringe (~1 μL) in the reference reservoir at 30 °C. The *m/z* monitored was 218.9856. The application manager TargetLynx, a module of Masslynx 4.0 software, was used to process data obtained for target compounds in samples extracts. The application manager Chromalynx was used to investigate the presence of non-target (unknown) compounds in sample extracts. Library searching was performed using the commercial NIST library.

2.3. Samples

Sample matrices used in this work were chosen to cover different commodity groups as classified in Annex 1 of SANCO/10684/2009 [25]. Apples, tomatoes and carrots were selected as high water content products; oranges were

chosen due to their high acid content; and finally, olives were taken as high oil content products.

For each commodity, a blank sample (for validation purposes) was acquired from ecological agriculture. In addition, samples of four different varieties were obtained from local markets and/or particular crops from several areas of Spain.

2.4. Analytical procedure

The extraction procedure was carried out following the modified acetate-buffered version of the QuEChERS method [30]. The samples were chopped and homogenized in Homogeniser Thermomix TM30 (Vorwerk, Madrid, Spain) at room temperature during 2 min. 15 g of chopped and homogenized sample were weighed in a 50-mL Falcon conical tube and 15 mL of 1 % acetic acid (HAc) in acetonitrile (MeCN) (v/v) were added. After shaking for 30 s, 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous NaAc were added and immediately shaken vigorously for 1 min. The tubes were centrifuged at 3000 rpm for 2 min and 1 mL of the upper layer of the extract was transferred to the dispersive-SPE tubes containing 50 mg of PSA and 150 mg of anhydrous $MgSO_4$ (for orange and olive samples, SPE-tubes also contained 50 mg of C_{18}). The extracts were vortexed for 30 s and then 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 50 μL of the internal standard TPP at 5 mg/L. This extract was evaporated to approximately 300 μL under a gentle nitrogen stream at 50 °C. The extracts were

adjusted to a final volume of 500 μL with toluene prior to injection into GC–TOF MS.

For analyte quantification, matrix-matched calibration curves were prepared for every matrix as follows: 500 μL of acetonitrile sample blank extract was transferred into an evaporation tube containing 1 mL of toluene. The mixture was evaporated to approximately 300 μL under a gentle nitrogen stream at 50 °C. Then, 50 μL of 5 mg/L TPP and 50 μL of hexanic pesticide standard solution of adequate concentration were added, adjusting the final volume to 500 μL with toluene.

2.5. Validation study

Linearity of the method was studied by analyzing matrix-matched standards in duplicate at concentrations ranging from 5 to 1000 μg/L. Linearity was assumed when regression coefficient, *r*, was higher than 0.99 with residuals lower than 20 %.

Accuracy was estimated by means of recovery experiments, analyzing orange, apple, carrot, tomato and olive samples spiked at three concentrations (0.01, 0.05 and 0.5 mg/kg). Experiments were performed by sextuplicate at each concentration. The spiking of samples was made by adding the appropriate volume of the mixed pesticide standard solution in acetonitrile to 15 g of homogenized fresh sample before extraction with acetonitrile.

Based on SANCO/825/00 guideline [37], recoveries were considered satisfactory in the range of 70–120 % at 0.05 and 0.5 mg/kg spiked concentrations, and from 60 to 120 % at 0.01 mg/kg.

Intraday precision was estimated from recovery experiments (n = 6). It was expressed as repeatability of the method in terms of relative standard deviation (RSD). RSD values below 20 %, at 0.05 and 0.5 mg/kg spiked concentrations, and below 30 % at 0.01 mg/kg were considered satisfactory [37].

Selectivity, considered as the ability of the method to discriminate between the analyte peak and other chromatographic peaks, was tested by determining every analyte in the presence of the rest of compounds included in the screening. It was based on the monitoring of characteristics *m/z* ions, measured at accurate mass in the EI spectrum for each compound.

The limit of quantification (LOQ) objective was established as the lowest concentration that was validated with satisfactory recovery and precision in spiked samples.

The confirmation of identity criterion of positive findings in samples was the presence of, at least, two *m/z* ions in the spectrum of the chromatographic peak at the expected retention time, measured at accurate mass in the respective narrow window-extracted ion chromatograms, nw-XIC (0.02 Da). The ion intensity ratio was evaluated in order to know whether it fitted within the tolerances established by SANCO/10684/2009 guideline [25].

3. Results and discussion

3.1. QuEChERS extraction and GC–TOF MS analysis

In this work, we have applied the QuEChERS AOAC Official Method 2007.01 [30], without any additional optimization. This version uses strong acetate buffering at pH 4.8 and gives better recoveries for some problematic pesticides than other QuEChERS versions [35]. In this method, the acetonitrile extract is directly injected in a PTV injector, which seems more adequate than split/splitless due to the large expansion volume of MeCN during vaporization [30,38]. In our work, a split/splitless injector was used, as the PTV was unavailable in our GC–TOF instrument. So, it was necessary to perform a solvent exchange before GC-injection. Toluene was chosen due to the advantages reported [38]: miscibility with MeCN, high response for some polar-GC amenable pesticides and higher boiling point with the possibility of increasing the initial temperature in the GC oven.

TPP was used as internal standard (IS) in order to improve quantification by compensating the variations of the system. TPP was chosen on the basis of its use in most QuEChERS procedures, as it gives sharp peaks and intense signal [29].

Once pesticides were extracted from fruits and vegetables, their determination was performed by GC–TOF MS, taking the advantage of the accurate mass full-spectrum acquisition. First, an identification and quantification of target analytes was performed in the method validation. Later, the method was applied to different fruit and vegetable samples where the target analytes were determined. A non-target analysis was also carried out, without the need of

reanalyzing samples, in order to detect the presence of other compounds not included in the target method.

A total of 55 target analytes were selected, including organophosphate, organochlorine and pyrethroid insecticides, as well as several herbicides, fungicides and acaricides. The detection of target analytes in the samples was carried out by obtaining a minimum of two (up to five, when possible) nw-XICs, with a mass window of 0.02 Da, at selected *m/z* analyte ions. The selected *m/z* ions were optimized in a previous work performed at our laboratory [24]. Quantification ion (*Q*) was used for quantification purposes (showed in ***Table 1***), while the rest of ions (*qi*) were used for confirmatory of identity analysis (see Ref. [24] for more detailed information on the confirmatory ions selected).

Initially, the base peak of TPP spectrum, *m/z* 326.0708, was selected to calculate relative responses for each analyte. Unexpectedly, a reduction in the intensity of all spectra was observed along the sample sequence, which led to unsatisfactory IS correction. This reduction was more noticeable for low *m/z* ions and was mainly produced by the matrix. The voltage applied into the beam stearing, a half plate lens located between the ion chamber and the focus lenses, was optimized every day in order to get an adequate PFTBA spectrum (intensity and ion ratios). During a sequence, and mainly due to the effect of the matrix, this lens gets contaminated. Consequently, the optimized voltage at the beginning of the sequence did not remain sufficient to maintain a satisfactory sensitivity, especially at low *m/z* values. To improve the IS correction, the following strategy was applied: three *m/z* ions of TPP were selected (m/z_1 170.0732, m/z_2 233.0368 and m/z_3 326.0708) in order to calculate relative responses for analytes depending

on the characteristic monitored ions. TPP m/z_1 was used for analyte ions < m/z 190, m/z_2 for those between m/z 190 and 250, and m/z_3 for those ions > m/z 250. ***Table 1*** shows the TPP m/z ion selected for each analyte.

In order to perform non-target analysis, a deconvolution package ChromaLynx Application Manager was used to automatically process the MS data acquired [21].

3.2. Method validation

Validation of the multi-residue method was carried out using orange, apple, carrot, tomato and olive in terms of linearity, accuracy, precision, selectivity and LOQ. Matrix-matched calibration curves using relative areas versus IS were used for quantification in spiked and non-spiked samples.

Linearity was tested in the general range of concentrations from 5 to 1000 μg/L. For more accurate quantification, the calibration set was split into the three ranges, adjusted to the concentration present in the spiked samples: 5–100 μg/L (for the lowest concentration, where 10 μg/L corresponds to 0.01 mg/kg in sample), 10–250 μg/L (for intermediate concentration, where 50 μg/L corresponds to 0.05 mg/kg), and 100–1000 μg/L (for the highest concentration, where 500 μg/L corresponds to 0.5 mg/kg). Correlation coefficients were higher than 0.99 and randomly distributed residuals were lower than 20 %.

Table 1. List of compounds studied, retention time (RT), quantification m/z ion (Q), its elemental composition and TPP m/z ion selected.

RT (min)	Compound	TPP ion (*m/z*)	*Ion (Q)*	*m/z*
7.27	Dichlorvos	170.0732	$C_2H_6O_3P$	109.0055
16.37	Chlorpropham	170.0732	C_6H_6NCl	127.0189
17.05	Trifluralin	326.0708	$C_{11}H_{11}N_3O_4F_3$	306.0702
17.18	Phorate	170.0732	$C_4H_{10}O_2P$	121.0418
17.23	α-HCH	170.0732	$C_6H_4Cl_3$	180.9379
17.49	HCB	326.0708	$C_6\ ^{35}Cl_5{}^{37}Cl$	283.8102
18.47	Atrazine	233.0368	$C_7H_{11}ClN_5$	200.0703
18.53	β-HCH + lindane	170.0732	$C_6H_4Cl_3$	180.9379
19.03	Terbuthylazine	233.0368	$C_8H_{13}ClN_5$	214.0859
19.16	Propyzamide	170.0732	$C_7H_3OCl_2$	172.9561
19.60	Diazinon	170.0732	$C_7H_9N_2O$	137.0715
19.85	Chlorothalonil	326.0708	$C_8\ ^{35}Cl_3\ ^{35}ClN_2$	265.8786
20.58	Pirimicarb	170.0732	$C_8H_{12}N_3O$	166.098
21.05	Metribuzin	233.0368	$C_8H_{12}N_3OS$	198.0701
21.27	Chlorpyriphos methyl	326.0708	$C_7H_7Cl_2NO_3PS$	285.9261
21.27	Parathion methyl	170.0732	$C_2H_6O_2PS$	124.9826
21.36	Heptachlor	326.0708	$C_5\ ^{35}Cl_5\ ^{37}Cl$	271.8102
21.60	Alachlor	170.0732	$C_{11}H_{14}N$	160.1126
22.38	Fenitrothion	170.0732	$C_2H_6O_2PS$	124.9826
22.53	Pirimiphos methyl	326.0708	$C_{10}H_{17}N_3O_3PS$	290.0728
22.67	Aldrin	326.0708	$C_7H_2\ ^{35}Cl_4{}^{37}Cl$	262.8570
22.89	Malathion	170.0732	$C_6H_7O_3$	127.0395
22.98	Metholachlor	170.0732	$C_{11}H_{16}N$	162.1283
23.12	Fenthion	326.0708	$C_{10}H_{15}O_3PS_2$	278.0200
23.21	Chlorpyriphos ethyl	233.0368	$C_5H_2Cl_3NO$	196.9202
23.24	Parathion ethyl	326.0708	$C_{10}H_{14}NO_5PS$	291.0330
23.75	Isodrin	233.0368	$C_7H_4Cl_3$	192.9379
24.15	Cyprodinil	233.0368	$C_{14}H_{14}N_3$	224.1188
24.41	Pendimethalin	326.0708	$C_{11}H_{14}N_3O_4$	252.0984
24.79	Chlorfenvinphos	326.0708	$C_8H_6Cl_2O_4P$	266.9381
24.85	Quinalphos	170.0732	$C_8H_6N_2O$	146.0480
25.13	*trans*-Chlordane	326.0708	$C_{10}H_6{}^{35}Cl_6{}^{37}Cl$	372.8260
25.31	Methidathion	170.0732	$C_4H_5N_2O_2S$	145.0072
25.55	*a*-Endosulfan	170.0732	$C_8H_4Cl_2$	169.9690
26.51	Dieldrin	326.0708	$C_7H_2{}^{35}Cl_4{}^{37}Cl$	262.8570
26.62	*p,p'*-DDE	233.0368	$C_{14}H_8Cl_2$	246.0003
27.07	Buprofezin	170.0732	C_7H_7N	105.0578
27.29	Endrin	326.0708	$C_7H_2{}^{35}Cl_4{}^{37}Cl$	262.8570
27.66	β-Endosulfan	170.0732	$C_8H_4Cl_2$	169.9690
28.16	*p,p'*-DDD	233.0368	$C_{13}H_9Cl_2$	235.0081
28.35	Oxadixyl	170.0732	$C_{10}H_{13}NO$	163.0997
28.42	Ethion	233.0368	$C_5H_{12}O_2PS_3$	230.9737
29.24	Endosulfan sulfate	326.0708	$C_5{}^{35}Cl_5{}^{37}Cl$	271.8102
29.40	Propiconazole I	170.0732	$C_7H_3OCl_2$	172.9555
29.47	*p,p'*-DDT	233.0368	$C_{13}H_9Cl_2$	235.0081
29.63	Propiconazole II	170.0732	$C_7H_3OCl_2$	172.9555
30.37	TPP (IS)	-	$C_{18}H_{15}O_4P$	326.0708
31.21	Phosmet	170.0732	$C_9H_6NO_2$	160.0399
31.59	Bifenthrin	170.0732	$C_{13}H_{10}$	166.0783
31.61	Methoxychlor	233.0368	$C_{15}H_{15}O_2$	227.1072
32.25	Tetradifon	170.0732	C_6H_4ClOS	158.9665
32.89	Pyriproxyfen	170.0732	$C_8H_{10}NO$	136.0762
33.54	Fenarimol	170.0732	C_7H_4OCl	138.9951
38.34	Fenvalerate I	170.0732	C_7H_6Cl	125.0158
38.74	Fenvalerate II	170.0732	C_7H_6Cl	125.0158

Table 2 shows the validation results for oranges, apples, carrots and tomatoes. Data on olives are not shown, because they were not satisfactory for most pesticides. The matrix interferences caused by high concentrations of co-extractives in olives meant that TPP could not correct these deviations. Despite the use of additional clean-up, the high oil content in olives caused the contamination of the syringe, inlet, column and MS ion source making necessary extra maintenance [36,39].

As it can be seen in ***Table 2***, at the medium and high concentrations (0.05 and 0.5 mg/kg), most compounds presented satisfactory recoveries, ranging from 70 to 120 %. A few exceptions were observed with values slightly lower (between 60 and 70 %): hexachlorobenzene (HCB) (orange); malathion and chlorfenvinphos (apple); *p*,*p*'-DDD (tomato). Recoveries lower than 60 % were only found for chlorpropham (orange), and chlorpyriphos ethyl and parathion ethyl (apple). However, in two out of these three cases precision was satisfactory, with RSD below 20 %. Finally, only three recoveries were slightly higher than 120 %, with the highest value of 129 % corresponding to phorate in tomato at 0.5 mg/kg.

Table 2. Average recovery (%) and RSD (%, in parenthesis) for the GC-TOF MS method applied to orange, apple, carrot and tomato samples (n=6) at three spiked levels (0.01, 0.05 and 0.5 mg/Kg). Limit of quantification (LOQ) objective[a] in mg/Kg.

Compound	Family	Orange			LOQ	Apple			LOQ	Carrot			LOQ	Tomato			LOQ
		0.01	0.05	0.5		0.01	0.05	0.5		0.01	0.05	0.5		0.01	0.05	0.5	
Alachlor	HERB chloroacetanilide	-[e]	81 (11)	102 (15)	0.05	-	81 (10)	83 (8)	0.05	-	114 (9)	89 (5)	0.05	-	78 (10)	93 (6)	0.05
Aldrin	INS OC cyclodiene	**44**[f] (28)	70 (6)	72 (7)	0.05	77 (19)	102 (10)	86 (8)	0.01	88 (11)	88 (8)	91 (4)	0.01	79 (14)	70 (6)	92 (2)	0.01
Atrazine	HERB chlorotriazine	*72 (8)*[g]	92 (5)	73 (6)	0.05	-	-	86 (4)	0.5	*88 (11)*	104 (5)	98 (3)	0.05	106 (7)	84 (3)	91 (6)	0.01
Bifenthrin	INS pyretroid ester	71 (15)	81 (4)	77 (8)	0.01	87 (5)	80 (7)	87 (5)	0.01	-	105 (9)	88 (4)	0.05	**130** (20)	80 (13)	105 (3)	0.05
Buprofezin	INS chitin synthesis inhibitors	-	101 (17)	70 (11)	0.05	-	100 (19)	87 (4)	0.05	-	106 (10)	90 (5)	0.05	-	84 (12)	83 (7)	0.05
trans-Chlordane	INS OC cyclodiene	-	70 (6)	75 (5)	0.05	-	93 (8)	93 (7)	0.05	-	98 (7)	93 (5)	0.05	-	72 (8)	83 (1)	0.05
Chlorfenvinphos	INS OP organophosphate	-	78 (17)	81 (10)	0.05	-	**60** (14)	86 (6)	0.5	-	106 (6)	91 (7)	0.05	-	82 (7)	94 (7)	0.05
Chlorothalonil	FUNG aromatic	-	-	70 (13)	0.5	-	-	97 (8)	0.5	-	72 (10)	**125** (6)	0.05	-	-	-	-
Chlorpropham	HERB carbanalite	**53** (17)	**42 (24)**	96 (12)	0.5	-	-	74 (11)	0.5	75 (30)	96 (13)	100 (7)	0.01	81 (20)	77 (6)	93 (8)	0.01
Chlorpyrifos ethyl	INS OP pyridine organophosphate	74 **(37)**	76 (14)	84 (4)	0.05	[b]	**53** (11)	85 (5)	0.5	92 (16)	98 (5)	98 (4)	0.01	108 (9)	81 (5)	83 (2)	0.01
Chlorpyrifos methyl	INS OP pyridine organophosphate	78 (14)	77 (6)	71 (7)	0.01	104 (18)	82 (7)	78 (6)	0.01	92 (13)	101 (8)	89 (3)	0.01	95 (6)	76 (4)	93 (5)	0.01
Cyprodinil	FUNG anilinopyrimidine	88 (9)	84 (5)	86 (5)	0.01	73 (11)	72 (3)	91 (6)	0.01	101 (8)	104 (5)	95 (6)	0.01	[b]	[b]	[b]	-
p,p'-DDE	INS OC	72 (19)	72 (5)	83 (6)	0.01	85 (9)	85 (3)	91 (4)	0.01	89 (9)	94 (5)	86 (5)	0.01	91 (3)	73 (3)	90 (5)	0.01
p,p'-DDD	INS OC	77 (5)	84 (4)	91 (4)	0.01	75 (16)	87 (6)	93 (7)	0.01	93 (9)	99 (6)	79 (3)	0.01	103 (3)	**63** (3)	**66** (5)	0.01
p,p'-DDT	INS OC	*80 (8)*	83 (5)	76 (6)	0.05	69 (12)	90 (12)	92 (8)	0.01	89 (14)	96 (6)	90 (4)	0.01	105 (8)	79 (4)	78 (5)	0.01
Diazinon	INS OP pyrimidine organothiophosphate	85 (18)	82 (18)	73 (10)	0.01	76 (20)	80 (8)	81 (10)	0.01	*89 (19)*	101 (7)	93 (4)	0.05	101 (18)	78 (7)	79 (3)	0.01
Dichlorvos	INS OP organophosphate	81 (11)	74 (10)	79 **(25)**	0.01	69 (27)	96 (9)	86 (17)	0.01	61 (12)	85 (10)	98 (17)	0.01	96 (7)	73 (8)	100 (4)	0.01
Dieldrin	INS OC cyclodiene	**56 (36)**	100 (10)	97 (8)	0.05	-	97 (10)	96 (7)	0.05	92 (12)	93 (6)	94 (6)	0.01	91 (14)	70 (6)	99 (2)	0.01
α-Endosulfan	INS OC cyclodiene	-	91 (13)	73 (6)	0.05	-	71 **(34)**	93 (11)	0.5	93 (22)	99 (7)	93 (7)	0.01	94 (20)	76 (9)	96 (5)	0.01
β-Endosulfan	INS OC cyclodiene	-	82 (14)	77 (15)	0.05	-	75 **(41)**	94 (7)	0.5	89 (21)	110 (7)	102 (11)	0.01	65 (24)	82 (7)	94 (2)	0.01
Endosulfan sulfate	INS OC cyclodiene	70 (28)	99 (15)	94 (9)	0.01	-	91 (12)	94 (5)	0.05	105 (16)	102 (6)	93 (5)	0.01	91 (21)	71 (2)	96 (4)	0.01
Endrin	INS OC cyclodiene	-	79 (9)	82 (10)	0.05	-	91 (13)	92 (5)	0.05	-	103 (10)	94 (5)	0.05	-	74 (5)	103 (4)	0.05
Ethion	INS OP aliphatic organothiophosphate	-	80 (9)	75 (10)	0.05	-	70 (8)	89 (5)	0.05	96 (16)	104 (6)	90 (3)	0.01	120 (10)	85 (3)	91 (6)	0.01
Fenarimol	FUNG pyrimidine	*82 (24)*	96 (10)	105 (7)	0.05	-	100 (7)	106 (2)	0.05	*109 (4)*	101 (8)	107 (5)	0.05	*98 (3)*	90 (3)	113 (6)	0.05
Fenitrothion	INS OP phenyl organothiophosphate	-	78 (17)	105 (13)	0.01	-	-	70 (5)	0.5	110 (10)	110 (8)	82 (2)	0.01	99 (18)	84 (5)	78 (7)	0.01
Fenthion	INS OP phenyl organothiophosphate	80 (24)	82 (9)	79 (5)	0.01	70 (9)	70 (9)	81 (5)	0.01	96 (8)	96 (7)	83 (3)	0.01	108 (8)	77 (3)	80 (4)	0.01
Fenvalerate I	INS pyretroid ester	-	118 (17)	104 (9)	0.05	-	103 (19)	92 (10)	0.05	-	112 (12)	99 (11)	0.05	-	90 (8)	95 (3)	0.05
Fenvalerate II	INS pyretroid ester	-	80 **(28)**	116 (11)	0.5	-	100 (20)	80 (4)	0.05	-	78 (12)	85 (6)	0.05	-	88 (13)	87 (8)	0.05
HCB	FUNG aromatic	**52** (22)	**63** (8)	74 (10)	0.5	111 (21)	103 (12)	79 (11)	0.01	78 (9)	84 (9)	90 (7)	0.01	81 (11)	70 (9)	96 (4)	0.01
α-HCH	INS OC	-	78 (13)	70 (18)	0.05	80 (23)	87 (15)	87 (14)	0.01	85 (18)	100 (11)	102 (10)	0.01	103 (24)	74 (9)	**127** (4)	0.01

Table 2. (Continued)

Compound	Family	Orange			LOQ	Apple			LOQ	Carrot			LOQ	Tomato			LOQ
		0.01	0.05	0.5		0.01	0.05	0.5		0.01	0.05	0.5		0.01	0.05	0.5	
θ-HCH + lindane	INS OC	**52** (30)	83 (15)	79 (12)	0.05	77 (25)	78 (8)	100 (9)	0.01	72 (17)	94 (5)	104 (10)	0.01	88 (22)	77 (5)	[c]	0.01
Heptachlor	INS OC cyclodiene	**48** (25)	98 (8)	70 (11)	0.05	102 (25)	102 (7)	83 (8)	0.01	71 (16)	97 (12)	88 (5)	0.01	79 (22)	70 (6)	90 (2)	0.01
Isodrin	INS OC cyclodiene	-	70 (13)	86 (6)	0.05	73 (11)	70 (18)	90 (6)	0.01	85 (26)	91 (8)	97 (4)	0.01	75 (30)	74 (7)	81 (3)	0.01
Malathion	INS OP aliphatic organothiophosphate	-	77 (10)	105 (7)	0.05	-	**68 (25)**	85 (6)	0.5	79 (30)	105 (8)	91 (4)	0.01	85 (24)	88 (5)	86 (6)	0.01
Methidathion	INS OP thiadiazole organothiophosphate	-	79 (12)	89 (9)	0.05	-	-	78 (7)	0.5	-	98 (15)	86 (4)	0.05	-	82 (4)	88 (9)	0.05
Metholachlor	HERB chloroacetanilide	90 (16)	81 (9)	70 (10)	0.01	85 (9)	71 (6)	83 (7)	0.01	99 (9)	102 (8)	93 (3)	0.01	105 (6)	86 (3)	84 (3)	0.01
Methoxychlor	INS OC	-	89 (5)	91 (9)	0.05	-	87 (8)	86 (7)	0.05	76 (12)	99 (8)	87 (4)	0.01	96 (16)	83 (3)	84 (7)	0.01
Metribuzin	HERB triazinone	*86 (11)*	88 (16)	79 (12)	0.05	-	84 (6)	90 (4)	0.05	*89 (2)*	70 (13)	99 (3)	0.05	-	93 (5)	99 (6)	0.05
Oxadixyl	FUNG anilide	-	91 (20)	85 (6)	0.05	*68 (14)*	89 (9)	88 (6)	0.05	*109 (18)*	120 (12)	93 (5)	0.05	101 (20)	90 (3)	90 (2)	0.01
Parathion ethyl	INS OP phenyl organothiophosphate	-	83 (20)	70 (11)	0.05	-	**47** (16)	78 (7)	0.5	-	95 (8)	89 (8)	0.05	-	80 (2)	93 (3)	0.05
Parathion methyl	INS OP phenyl organothiophosphate	74 (18)	86 (10)	96 (5)	0.01	70 (23)	75 (12)	81 (6)	0.01	90 (14)	96 (6)	85 (4)	0.01	100 (11)	82 (5)	83 (4)	0.01
Pendimethalin	HERB dinitroaniline	-	103 (15)	70 (12)	0.05	-	99 (17)	95 (5)	0.05	-	106 (10)	86 (5)	0.05	112 (9)	78 (4)	112 (3)	0.01
Phorate	INS OP aliphatic organothiophosphate	-	98 (16)	78 (20)	0.05	-	95 (10)	84 (11)	0.05	-	102 (14)	101 (7)	0.05	-	80 (9)	**129** (6)	0.05
Phosmet	INS OP phtalimide	-	76 (20)	95 (14)	0.05	-	-	70 (12)	0.5	101 (12)	111 (8)	90 (5)	0.01	103 (17)	88 (2)	81 (17)	0.01
Pirimicarb	INS dimethylcarbamate	101 (17)	85 (9)	70 (14)	0.01	96 (11)	94 (10)	84 (8)	0.01	[d]	113 (7)	92 (3)	0.05	[d]	87 (4)	78 (4)	0.05
Pirimiphos methyl	INS OP pyrimidine organothiophosphate	62 **(39)**	76 (6)	71 (11)	0.05	69 **(37)**	85 (7)	86 (6)	0.05	99 (11)	99 (7)	92 (4)	0.01	108 (8)	78 (4)	79 (2)	0.01
Propiconazole I	FUNG conazole	*96 (15)*	83 (12)	92 (6)	0.05	-	84 (15)	90 (4)	0.05	98 (17)	109 (6)	96 (3)	0.01	108 (7)	95 (4)	94 (4)	0.01
Propiconazole II	FUNG conazole	*91 (12)*	85 (11)	90 (6)	0.05	-	102 (6)	92 (3)	0.05	102 (13)	107 (6)	97 (2)	0.01	108 (11)	88 (3)	101 (3)	0.01
Propyzamide	HERB amide	79 (5)	77 (13)	70 (8)	0.01	-	-	88 (7)	0.5	96 (7)	100 (6)	96 (4)	0.01	101 (7)	81 (5)	83 (5)	0.01
Pyriproxyfen	INS juvenile hormone mimics	*80 (23)*	84 (15)	120 (10)	0.05	-	93 (6)	92 (6)	0.05	-	117 (9)	99 (2)	0.05	-	86 (3)	88 (5)	0.05
Quinalphos	INS OP quinoxaline organothiophosphate	-	78 (11)	88 (6)	0.05	-	77 (12)	82 (4)	0.05	-	103 (8)	101 (2)	0.05	-	91 (3)	88 (4)	0.05
Terbuthylazine	HERB chlorotriazine	76 (18)	79 (16)	71 (6)	0.01	69 (21)	91 (10)	93 (6)	0.01	90 (7)	103 (7)	96 (3)	0.01	100 (9)	83 (4)	85 (6)	0.01
Tetradifon	ACAR bridged diphenyl	78 (14)	89 (4)	116 (13)	0.01	69 (19)	94 (6)	98 (4)	0.01	104 (7)	102 (4)	102 (5)	0.01	96 (16)	86 (4)	90 (3)	0.01
Trifluralin	HERB dinitroaniline	**55 (32)**	94 (8)	85 (19)	0.05	*78 (12)*	104 (13)	74 (9)	0.05	91 (14)	101 (11)	86 (8)	0.01	93 (22)	72 (5)	101 (4)	0.01

INS, insecticide; HERB, herbicide; FUNG, fungicide; ACAR, acaricide; OP, organophosphate; OC, organochloride.

[a] LOQ objective is the lowest level that was validated in spiked samples with satisfactory accuracy and precision.

[b] Data not available due to the poor calibration.

[c] Data not available due to the detector saturation.

[d] Data not available due to the presence of an interfering peak.

[e] –, not detected.

[f] Bolded values out of the acceptance interval.

[g] Italic, in these cases (always at 0.01 mg/kg) the compound could be quantified, but *Q/q* ratio was out of tolerance due to the poor sensitivity for the confirmatory transitions.

At the lowest spiked concentration (0.01 mg/kg), around 50 % of target analytes were satisfactorily validated. A notable number of analytes (54 % in orange, 60 % in apple, 38 % in carrot and 32 % in tomato) could not be detected, due to the lack of sensitivity. Recoveries lower than 60 % were found in orange for chlorpropham, trifluralin, HCB, β-HCH + lindane, heptachlor, aldrin and dieldrin.

A few compounds were particularly problematic, including chlorothalonil, probably due to its degradation during sample preparation or in the hot inlet during GC-injection as reported by some authors [32,40]; the pyrethroid fenvalerate showed poor sensitivity [31]; and β-HCH and lindane were very close in retention time, making difficult their individual determination, so the results for these two compounds were expressed as the sum of both responses.

Intraday precision was satisfactory for most of pesticides at 0.05 and 0.5 mg/kg, with RSDs below 20 %. Only in three cases, RSDs were higher than 25 % (fenvalerate in orange and α- and β-endosulfan in apple, all at 0.05 mg/kg). At the lowest concentration assayed (0.01 mg/kg), only in five cases RSD were higher than 30 % (trifluralin, pirimiphos methyl, chlorpyrifos ethyl and dieldrin in orange; pirimiphos methyl in apple) surely because of the poor sensitivity.

The LOQ objective was 0.01 mg/kg, and was achieved for around 50 % of the compounds investigated. Obviously, the statistical LOQ estimated for a signal-to-noise ratio of 10 from the chromatograms at the lowest spiked concentration was substantially lower than 0.01 mg/kg for the majority of pesticides.

Ion intensity ratios were evaluated for all compounds in every matrix, updating the reference values in each sequence of analysis. Values of reference

corresponded to the matrix-matched calibration standard at the same spiked concentration. Maximum tolerances, established by SANCO/10684/2009 guideline, were: ±10 % when *Q*/*q* intensity ratio was lower than 2, ±15 % for *Q*/*q* between 2 and 5, ±20 % for *Q*/*q* 5 and 10 and ±50 % for *Q*/*q* ratio higher than 10 [25]. In the validation experiments, most analytes had ion ratios within the acceptance intervals. However, some exceptions were observed, indicating that accomplishment of the ion ratios within the maximum deviations admitted (between 10 % and 50 % depending on the relative signals) is a problematic issue, especially at low analyte concentrations. Thus, at 0.01 mg/kg several analytes could be quantified with satisfactory recovery, but the *Q*/*q* ratio was out of the tolerance as a consequence of the poor sensitivity for the confirmatory transition. These compounds are highlighted in ***Table 2***. Further investigations are being made in our group on this relevant matter, as the non-accomplishment of the ion ratio can lead to report an actual positive as negative.

As an illustrative example, ***Figure 1*** shows the nw-XICs obtained (mass window of 0.02 Da) for tetradifon in all matrices studied at the lowest concentration validated. Three ions were monitored in this case. The narrow mass window used allowed a notable improvement in selectivity, also decreasing the background noise of the chromatogram.

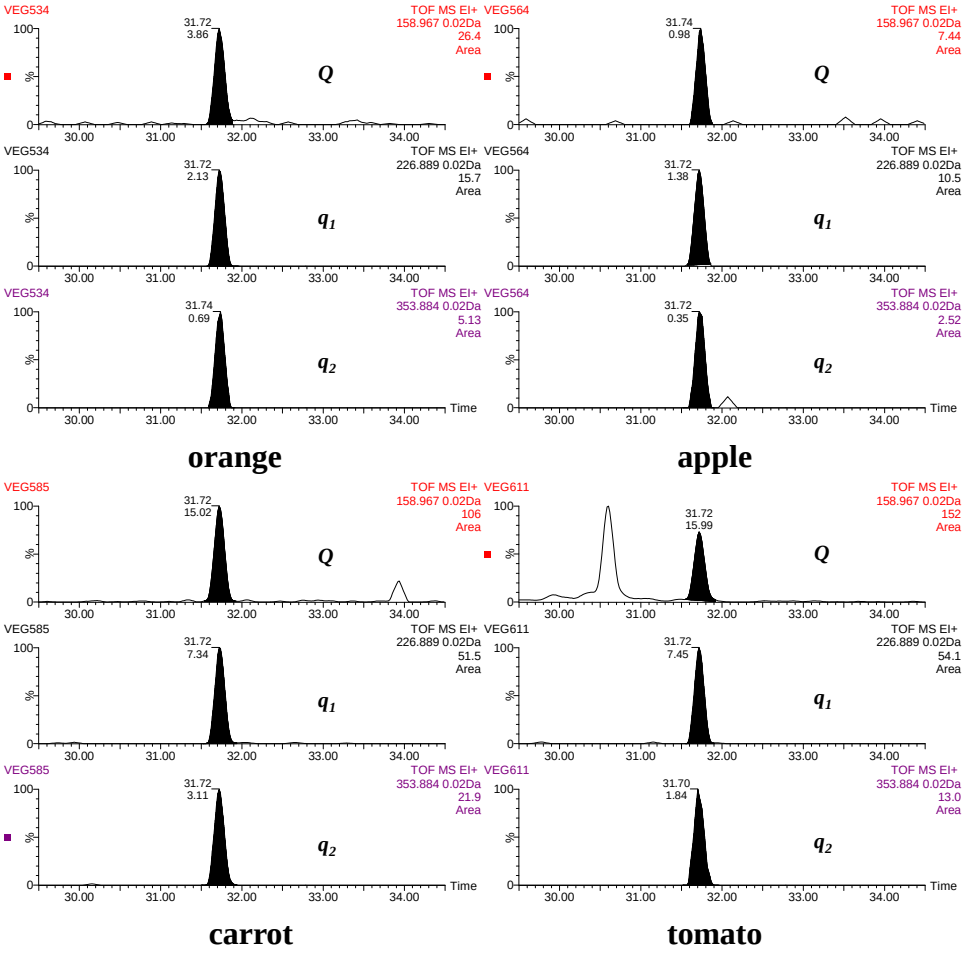

Figure 1. GC–TOF MS narrow window-extracted ion chromatogram (mass window of 0.02 Da) at different m/z (Q: 158.9665, q_1: 226.8886, q_2: 353.8843) ions for tetradifon in orange, apple, carrot and tomato spiked at 0.01 mg/kg.

3.3. Real sample analysis

3.3.1. Target analysis

The GC–TOF MS procedure was applied to 16 samples (four different varieties of the four sample matrices validated: orange, apple, carrot and tomato) collected from several areas of Spain. The results obtained are shown in ***Table 3***.

Table 3. Target pesticides found in samples after application of the overall procedure. Characteristic ion monitored (m/z) and experimental mass errors (mDa) are shown

Compound detected	Oranges		Apples			Carrots	Tomatoes
	Navelina	Clemenules	Royal gala	Golden	Fuji	Mantesa	Hanging
Terbuthylazine		0.02 mg/kg					
m/z 1: 214.0859 (*Q*)		1.8 mDa					
m/z 2: 216.0831 (q_1)		7.5 mDa					
m/z 3: 229.1094 (q_2)		3.8 mDa					
m/z 4: 173.0468 (q_3)		nd					
m/z 5: 138.0708 (q_4)		nd					
Chlorpyrifos ethyl	0.11 mg/kg	0.16 mg/kg	*<LOQ*	*<LOQ*			
m/z 1: 196.9202 (*Q*)	2.4 mDa	1.3 mDa	2.8 mDa	3.2 mDa			
m/z 2: 198.9172 (q_1)	2.1 mDa	1.4 mDa	4.1 mDa	4.6 mDa			
m/z 3: 257.8948 (q_2)	1.8 mDa	0.9 mDa	0.8 mDa	0.3 mDa			
m/z 4: 285.9261 (q_3)	0.9 mDa	0.9 mDa	-1.8 mDa	-0.8 mDa			
m/z 5: 315.9545 (q_4)	1.3 mDa	0 mDa	-0.5 mDa	-2.8 mDa			
Cyprodinil				0.03 mg/kg			
m/z 1: 224.1188 (*Q*)				2.2 mDa			
m/z 2: 225.1266 (q_1)				3.1 mDa			
m/z 3: 210.1031 (q_2)				0.7 mDa			
p-p'-DDE						0.02 mg/kg	
m/z 1: 246.0003 (*Q*)						1 mDa	
m/z 2: 247.9975 (q_1)						2.7 mDa	
m/z 3: 317.9352 (q_2)						-2.8 mDa	
m/z 4: 315.9380 (q_3)						-0.8 mDa	
m/z 5: 176.0626 (q_4)						nd	
Bifenthrin					0.03 mg/kg		
m/z 1: 166.0783 (*Q*)					1 mDa		
m/z 2: 165.0704 (q_1)					0.3 mDa		
m/z 3: 181.1017 (q_2)					nd		
Pyriproxyfen							0.05 mg/kg
m/z 1: 130.0762 (*Q*)							2.2 mDa
m/z 2: 186.0681 (q_1)							4.1 mDa
m/z 3: 226.0994 (q_2)							3.4 mDa

nd, not detected; *Q*, quantification ion; q_i, confirmation ions; *<LOQ*, concentrations lower than limit of quantification.

Regarding oranges, one of the samples analyzed was found to contain chlorpyriphos ethyl (0.1 mg/kg) and other showed the presence of terbuthylazine (0.02 mg/kg) and chlorpyriphos ethyl (0.16 mg/kg) (***Figure 2***). In the case of apples, cyprodinil (0.03 mg/kg) and chlorpyriphos ethyl (<LOQ) were also found in one and two samples, respectively. In carrots, unexpectedly *p*,*p*'-DDE was detected, although at low concentration (0.02 mg/kg) in one of the samples. As already known, *p*,*p*'-DDE is the DDT metabolite and is more frequently found in the environment (*e.g.* soil). Finally, one tomato sample was positive to pyriproxyfen (0.05 mg/kg). All positive findings were below the MRL established for each crop and were consistent with authorizations of use with the crop they appeared. Identification of these analytes in the samples was confirmed by means of the presence of at least two *m/z* ions at the expected retention time with mass errors normally below 3 mDa with few exceptions. The high number of co-extractives in QuEChERS extracts might lead to matrix-induced mass shifts. However, in this work the mass errors observed in samples were similar to those normally obtained with our instrument in other matrices, as water or more diluted food extracts. New GC–TOF generations provide better sensitivity and improved mass accuracy, leading to lower mass errors, compared with the GC–TOF used in this work. The measured ion intensity ratios were also evaluated. Ion ratios were in good accordance with those of calibration standards within the tolerances established [25]. However, as observed in the validation study, a few cases exceeded the maximum tolerance, but a clear evidence existed that they were positive findings from the rest of parameters evaluated (retention time, several ions present in the samples and accurate masses). This fact illustrates that maximum tolerances established in the current guidelines for *Q*/*q* ratio are a controversial issue, and may require revision, as we previously suggested for

organic contaminants in water [24]. Concentrations found in samples were generally higher than the LOQ objective, except for chlorpyriphos ethyl in apple.

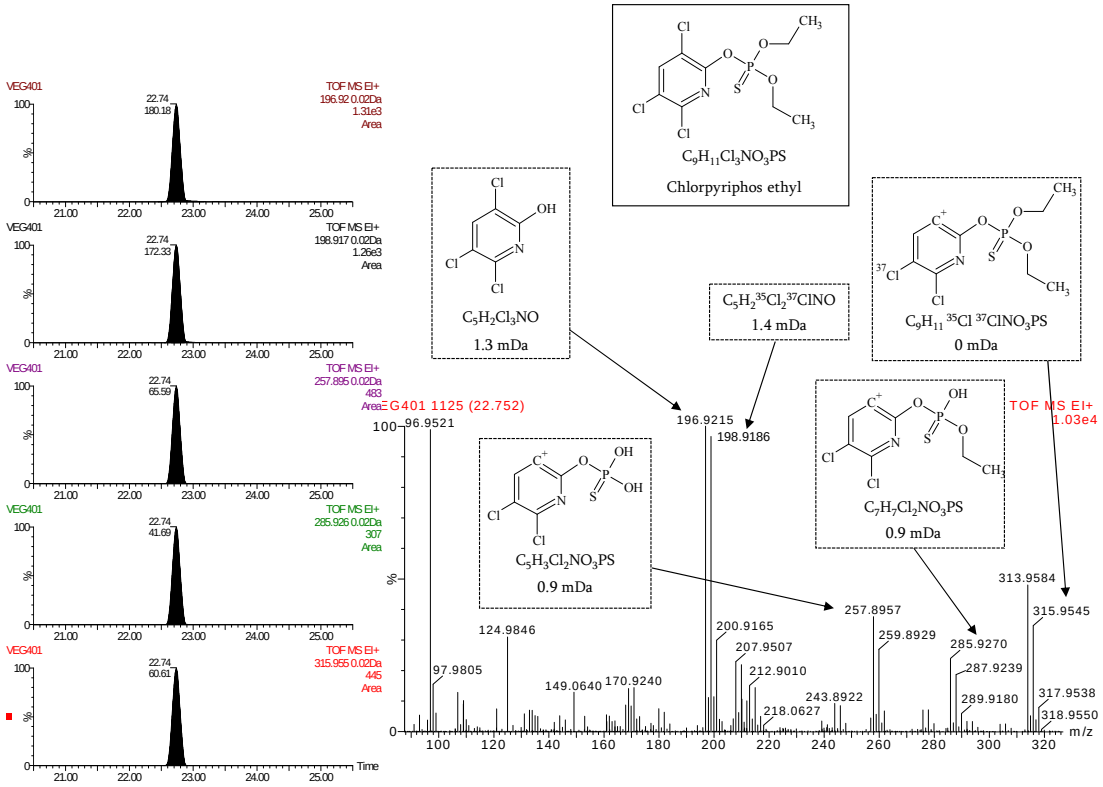

Figure 2. GC–TOF MS narrow window-extracted ion chromatogram (mass window 0.02 Da) showing the detection of target chlorpyriphos ethyl in orange. Experimental EI accurate mass spectrum and chemical structures proposed for the most abundant fragment ions together with experimental mass errors (in mDa).

3.3.2. Non-target analysis

One advantage of GC–TOF MS is the possibility of investigating the presence of non-target compounds, others than those included in the initial target list of the method. This searching can be made in a post-target way, i.e. by obtaining XICs at certain characteristic/abundant ions of the additional pesticides investigated, or also in a non-target way, without any kind of selection of the

compounds to be searched. Obviously, the non-target analytes would include not only pesticides but also other GC–MS amenable compounds that might be present in the samples, which could include organic pollutants or simply a common constituent of the sample. In this work, the non-target analysis allowed the detection of other compounds not included in the validated method. It was carried out by applying the Chromalynx Application Manager, which allowed the automated detection of sample components and their subsequent identification from the full-acquisition accurate-mass data obtained.

In this way, the post-harvest fungicide imazalil was detected in one orange and three apple samples. ***Figure 3*** shows the residue of imazalil in an apple sample, detected and identified in a non-target way. Accurate mass confirmation automatically performed for four representative ions and the library forward match (>700 used as criterion) led to the confirmation of the identity of imazalil with mass errors below 1.8 mDa for all ions. In addition, the structures proposed for at least four fragments ions observed in the EI spectrum were compatible with the chemical structure of imazalil. The injection of reference standards allowed the presence of imazalil in the samples to be confirmed.

Two PAHs, fluoranthene and pyrene, were also found. Both compounds presented the same spectra, so standard solutions of these compounds were necessary for their discrimination from retention times. Fluoranthene was only detected in one carrot sample, whereas pyrene was detected in most of samples. ***Figure 4*** shows the detection of pyrene finding in carrot. Accurate mass confirmation automatically performed for representative ions and library forward match (>700) suggested that the candidate compound detected was pyrene, with mass errors below 1.9 mDa for the ions shown. The structures proposed for at

least three fragments ions observed in the EI spectrum were supported with the chemical structure of this compound. Retention time information obtained by injection of the reference standard provided further supporting evidence for the confirmatory of identity.

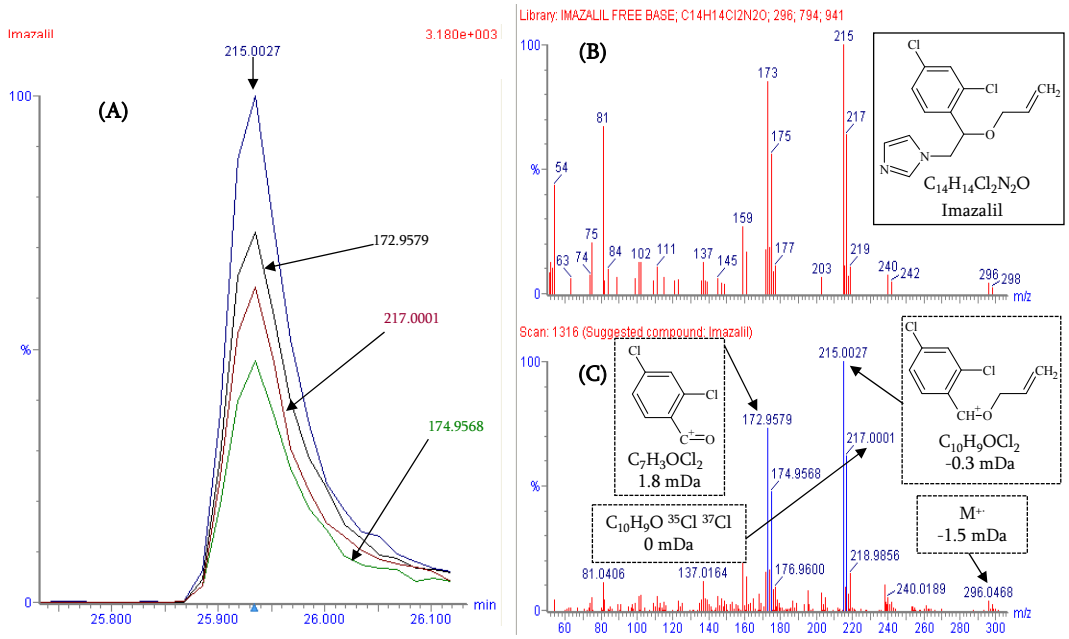

Figure 3. Identification of non-target imazalil in apple. (A) Extracted ion chromatograms for four imazalil ions used for deconvolution. (B) Library mass spectrum of imazalil at nominal masses (match 839). (C) Deconvoluted accurate mass spectrum of imazalil from the sample and chemical structures proposed for representative EI fragment ions together with mass errors.

Trans-limonene oxide was also identified in orange samples. This compound is present as a racemic mixture of cis and trans-limonene oxide, with a strong smell of orange-lemon. Other components detected in orange were sesquiterpenes (α-farnesene, α-humulene or copaene) and the flavor enhancer, maltol. The natural pesticide, falcarinol, was also detected in some carrot samples.

When required, the unequivocal confirmation of these compounds could be carried out by injecting reference standards.

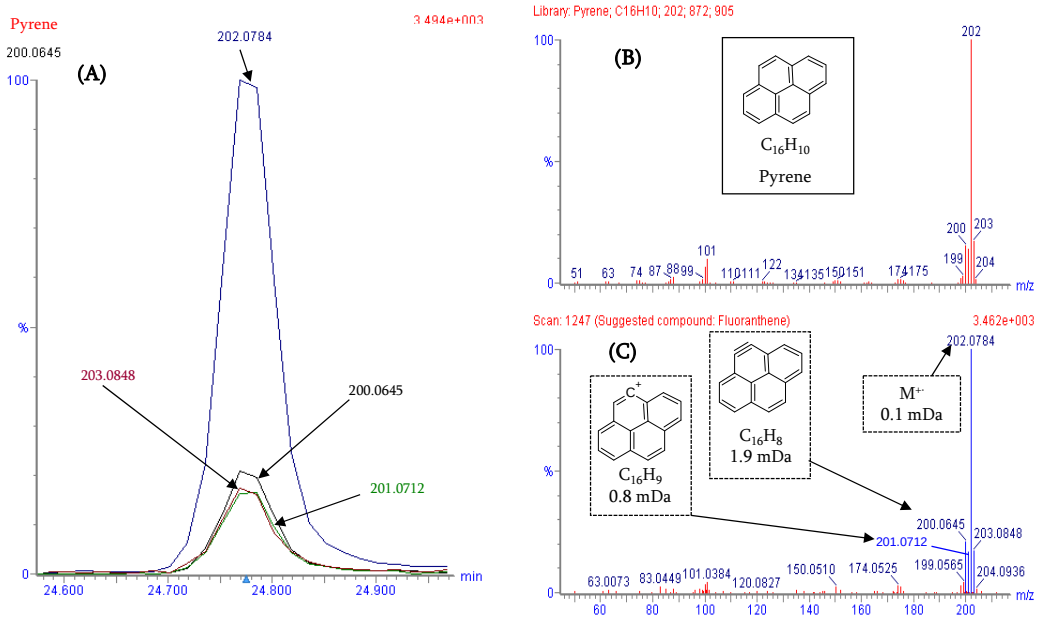

Figure 4. Identification of non-target pyrene in carrot. (A) Extracted ion chromatograms for four pyrene ions used for deconvolution. (B) Library mass spectrum of pyrene at nominal masses (match 872). (C) Deconvoluted accurate mass spectrum of pyrene from the sample and chemical structures proposed for abundant EI fragment ions together with mass errors.

4. Conclusions

In this work, a multi-residue method has been developed for a total of 55 pesticides and metabolites in representative fruit and vegetable matrices. The use of QuEChERS in combination with GC–TOF MS allowed reliable analysis in orange, apple, carrot and tomato. Validation of the method for olives was hampered by the greater complexity of the matrix, even after dispersive SPE clean-up using C_{18} sorbent. In the case of olives, further clean-up, or an

alternative extraction and clean-up is required to improved detectability of analytes by GC–TOF MS.

For these four matrices, recoveries and precision were acceptable at 0.05 mg/kg and 0.5 mg/kg. At 0.01 mg/kg spiked concentration, satisfactory data were obtained for approximately 50 % of the compounds, mainly due to insufficient sensitivity of our GC–TOF instrument. Particularly problematic was the accomplishment of the ion ratios due to the poor signal of the confirmatory ions in several analyte/matrix combinations.

The potential of GC–TOF MS has been proved both in target and non-target analysis. Target identification requires the presence of at least two *m/z* ions, measured at their accurate mass using narrow window-extracted ion chromatograms at the expected retention time. TPP was used as internal standard to minimize deviations in responses and to improve quantification. It is noteworthy that appropriate correction required to use different TPP *m/z* ions depending on the analyte *m/z* ion used for quantification. Matrix-matched standard calibration was applied in order to perform a correct quantification in orange, apple, carrot and tomato samples. Full-spectrum accurate-mass data acquired in GC–TOF MS has also allowed a non-target research of the samples analyzed. The analysis of samples from different origin and varieties has revealed the presence of several target analytes, included terbuthylazine, chlorpyrifos ethyl, cyprodinil, bifenthrin and pyriproxyfen, all at concentrations below 0.2 mg/kg, together with other non-target compounds such as imazalil, fluoranthene or pyrene. New GC–TOF instruments provide improved sensitivity and dynamic linear range compared to the instrument employed in this work. Hopefully, we will see many qualitative and quantitative applications in the field

of pesticide residue analysis in the near future based on GC–TOF MS. Continuous software developments will also facilitate the non-target analysis, which may become an interesting approach in GC–MS due to the commercial availability of standardized spectra libraries.

Acknowledgments

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of the time-of-flight mass spectrometer. This work forms a part of the projects P1 1B2010-23 (Universitat Jaume I-Fundació Bancaixa) and CTQ2009-12347 (Ministerio de Ciencia e Innovación). The authors acknowledge the financial support of Generalitat Valenciana, as research group of excellence PROMETEO/2009/054. M.I. Cervera is very grateful to Ministerio de Ciencia e Innovación for her predoctoral grant.

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3. Artículo científico 4

Anal Bioanal Chem (2014) 406:6843–6855
DOI 10.1007/s00216-014-7853-1

RESEARCH PAPER

Screening and quantification of pesticide residues in fruits and vegetables making use of gas chromatography–quadrupole time-of-flight mass spectrometry with atmospheric pressure chemical ionization

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Received: 3 March 2014 / Revised: 17 April 2014 / Accepted: 23 April 2014 / Published online: 15 May 2014

Abstract An atmospheric pressure chemical ionization source has been used to enhance the potential of gas chromatography coupled with quadrupole time-of-flight (QTOF) mass spectrometry (MS) for screening and quantification purposes in pesticide residue analysis. A screening method developed in our laboratory for around 130 pesticides has been applied to fruit and vegetable samples, including strawberries, oranges, apples, carrots, lettuces, courgettes, red peppers, and tomatoes. Samples were analyzed together with quality control samples (at 0.05 mg/kg) for each matrix and for matrix-matched calibration standards. The screening strategy consisted in first rapid searching and detection, and then a refined identification step using the QTOF capabilities (MS^E and accurate mass). Identification was based on the presence of one characteristic *m/z* ion (*Q*) obtained with the low collision energy function and at least one fragment ion (*q*) obtained with the high collision energy function, both with mass errors of less than 5 ppm, and an ion intensity ratio (*q*/*Q*) within the tolerances permitted. Following this strategy, 15 of 130 pesticides were identified in the samples. Afterwards, the quantitation capabilities were tested by performing a quantitative validation for those pesticides detected in the samples. To this aim, five matrices were selected (orange, apple, tomato, lettuce, and carrot) and spiked at two concentrations (0.01 and 0.1 mg/kg), and quantification was done using matrix-matched calibration standards (relative responses versus triphenyl phosphate used as an internal standard). Acceptable average recoveries and relative standard deviations were obtained for many but not all pesticide–matrix combinations. These figures allowed us to perform a retrospective quantification of positives found in the screening without the need for additional analysis. Taking advantage of the accurate-mass full-spectrum data provided by QTOF MS, we searched for a higher number of compounds (up to 416 pesticides) in a second stage by performing extra data processing without any new sample injection. Several more pesticides were detected, confirmed, and/or tentatively identified when the reference standard was unavailable, illustrating in this way the potential of gas chromatography–QTOF MS to detect pesticides in addition to the ones targeted in quantitative analysis of pesticides in food matrices.

Keywords Fruits and vegetables · Pesticides · QuEChERS · Gas chromatography · Quadrupole time-of-flight mass spectrometry · Screening · Quantitative validation

Published in the topical collection *Advanced Food Analysis* with guest editors Michel W.F. Nielen, Jana Hajslova, and Rudolf Krska.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-014-7853-1) contains supplementary material, which is available to authorized users.

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Springer

Analytical and Bioanalytical Chemistry, 406, 6843-6855, ***2014***

Screening and quantification of pesticide residues in fruits and vegetables making use of gas chromatography–quadrupole time-of-flight mass spectrometry with atmospheric pressure chemical ionization

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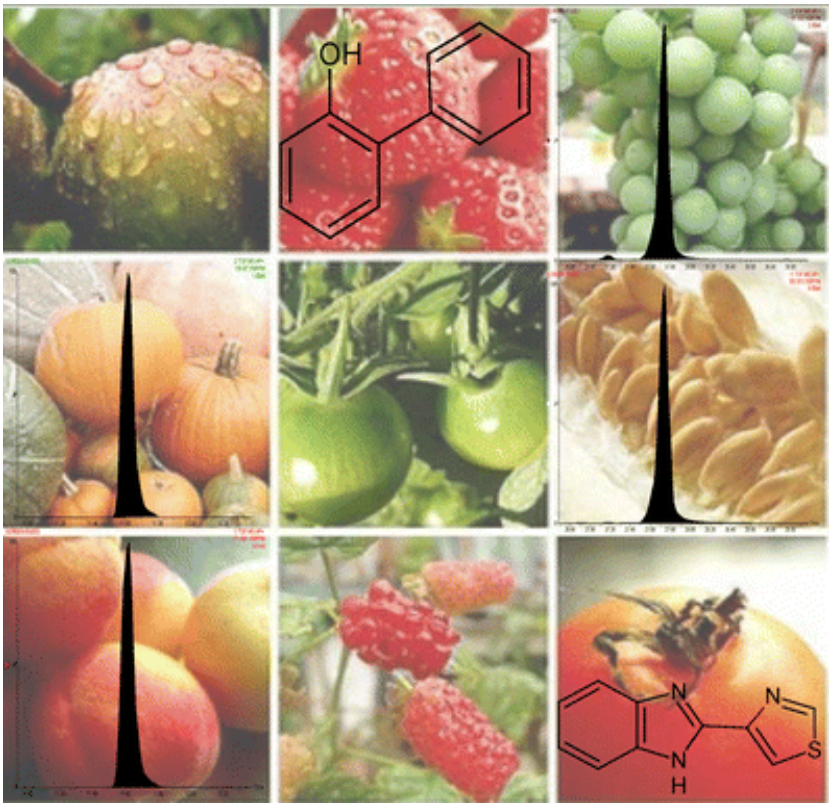
Graphical abstract



INTRODUCTION

The use of pesticides in food production has become common practice around the world to kill living things and manage pest problems. Toxicity and health risk associated with pesticides make necessary the control of their residues in food matrices. Although the detection of the pesticides present in samples is important, quantification is also necessary to accurately know their concentrations and verify if they are within the legal limits of residues established by the European Commission or other regulatory frameworks [1, 2].

Gas chromatography (GC) combined with mass spectrometry (MS) is one of the most important techniques for monitoring pesticide residues (volatile and non-polar/low-polarity compounds) together with liquid chromatography (LC) coupled with MS (non-volatile and medium-polarity/high-polarity compounds). GC–MS with single quadruple, triple quadrupole (QqQ), ion trap, and more recently, time-of-flight (TOF) analyzers has been applied in this field in recent decades. Different working modes have been used depending on the type of mass analyzer in pesticide residue analysis (PRA). Single quadrupole analyzers working under selected ion monitoring [3–5], and QqQ [6–9] and ion trap [10, 11] analyzers working under selected reaction monitoring in tandem MS methods have allowed simultaneous identification and quantification in most target methods developed until now. In the case of high-resolution (HR) TOF analyzers, the advantage of acquiring the accurate-mass full-spectrum has allowed researchers to work both in target and in non-target modes [12–14]. Full-scan nominal mass spectra from a single quadrupole analyzer have demonstrated their usefulness for non-target analysis in vegetables and fruits using DRS (deconvolution reporting software) [15,16]. However, TOF MS has offered new

possibilities in the food safety field and has facilitated the investigation of a large number of compounds, even if they were not included initially in the target lists of pesticides. This is due to the high resolving power and accurate mass measurements with better sensitivity, which drastically improves its potential for identification of the compounds detected.

Most GC–MS (/MS) applications are based on ionization modes operating under a vacuum, mainly using electron ionization (EI) [6, 12] but also chemical ionization [17, 18]. An alternative source, much less explored, is atmospheric pressure chemical ionization (APCI). Although its appearance dates from the 1970s [19, 20], it has hardly ever been applied up to now coupled with GC. Some authors have recently reported its use in GC–MS-based methods for PRA in fruit and vegetables, using QqQ [21–23] or quadrupole TOF (QTOF) [24] analyzers. GC–(APCI) TOF MS has also been used in other fields, for example, for impurity identification in pharmaceutical development [25], for profiling of phenolic compounds in oils [26], for metabolic fingerprinting and profiling [27], or coupled with comprehensive two-dimensional GC for flame retardants and plasticizers [28]. A few works have reported the use of QTOF MS to investigate substances in acrylic adhesives of food packaging materials [29], or polycyclic and nitro-polycyclic aromatic hydrocarbons in mosses [30], profiting from the APCI source.

EI is the preferred ionization technique in GC-based methods, and it is the most widely applied owing to its robustness and reproducibility, facilitating the identification of compounds thanks to the availability of standardized commercial spectra libraries. However, the use of APCI in GC has some advantages, as recently reported by Portolés *et al.* [22] in a comparative study of APCI and EI sources for analysis of pyrethroid insecticides. As the molecular ion (or

protonated molecule) is highly abundant under the soft ionization occurring in APCI, selectivity and sensitivity are notably enhanced when it is used as the precursor ion in tandem MS methods. The advantages of the APCI source together with the increasing use of HR TOF MS (accurate-mass full-spectrum data available) have made GC–(APCI) QTOF MS a promising technique for target screening of a large number of compounds in PRA [24]. To increase the amount of information obtained from each sample run, acquisition with a QTOF analyzer can be performed by alternating two scan events (MS^E mode): one at low collision energy and another at higher energy using a collision energy ramp. Mass data acquired at low collision energy, where the molecular ion is commonly highly abundant, provide the possibility of identifying a potential candidate through its accurate mass measurement. In addition, the final identification is supported by fragmentation information acquired in the second event. Another advantage is the accurate-mass full-spectrum acquired, which allows one to search for other compounds that had not been included in the target list, even without having the reference standard available, as the relevant information provided by MS^E mode makes feasible the tentative identification of the compound detected.

In recent years, different screening methods for PRA in food have been applied with the new instrumentation available in GC–MS. Broad screening methods have been described with QqQ analyzers, for example, in cucumber by performing the screening in a first injection and the confirmation and quantification in a second injection [31], or the screening, identification, and quantification applied to different crops between 2007 and 2010 and analyzing up to 528 samples [32]. Some articles have reported the use of TOF analyzers in PRA. HR TOF MS has been used, for example, in analysis of fresh celery, rape, scallion, and spinach in a rapid screening and identification method for pesticides [33],

and high-speed TOF MS has been used for analysis of lettuces, oranges, potatoes, strawberries, and tomatoes, achieving a high sample throughput with an analysis time of less than 10 min [34]. High-speed TOF MS is usually the choice when comprehensive two-dimensional GC is applied for screening of pesticide residues [35, 36]. Hayward *et al.* [37] compared five different MS systems for their application to targeted and non-targeted screening of pesticides in ginseng and spinach matrices. As a complementary approach, screening methods have been reported for polar pesticides in food [38, 39] and water [40–42] making use of LC–MS.

An efficient and useful screening analysis should be capable of investigating a notable number of pesticides in a large number of samples. To this aim, the screening method should be previously validated, a laborious task that, however, is required to provide evidence of the applicability and robustness of the method applied. The screening detection limit, an important parameter of the method, must be determined as well. However, until now, only a few works have reported screening validation in the field of pesticides from a qualitative point of view [39, 43–46].

In this work, we applied a previously developed and validated screening method (paper in preparation) for around 130 pesticides, based on GC–(APCI) QTOF MS, to a notable number of fruit and vegetable samples in order to test its applicability to different sample matrices. After detection and identification of several pesticides in the samples, a quantitative validation of the compounds detected was performed to evaluate the possibilities for quantitative analysis. Afterwards, taking advantage of the accurate-mass full-spectrum data under MS^E mode provided by QTOF MS, we extended the screening to 416 pesticides, in

most cases without having the reference standard available. This allowed the detection of several more pesticides in the samples in a retrospective way, without the need for additional analysis.

EXPERIMENTAL

Reagents

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 were stored in a freezer at -20 °C. Eighteen mixtures of pesticide standards (around 50 μg/mL) were prepared by volume dilution of individual stock solutions in acetone. Two mixtures were prepared, each containing nine of the 18 solutions mentioned above, at around 5 μg/mL in acetone. Then, working standard solutions were prepared by mixing both previous standard mixtures: in acetone for spiking and in hexane for calibration purposes. Further dilutions for calibration were done using hexane.

Triphenyl phosphate (TPP), purchased from Dr. Ehrenstorfer, was used as an internal standard. A stock standard solution (around 500 μg/mL) was prepared by dissolving the reference standard in acetone. Then, further dilutions were prepared by volume dilution in toluene.

Acetone (PRA quality), hexane (ultratrace quality), acetonitrile (MeCN; for GC residue analysis), toluene (for GC residue analysis), formic acid (content greater than 98 %), and glacial acetic acid were purchased from Scharlab (Barcelona, Spain). Anhydrous $MgSO_4$ and anhydrous sodium acetate (NaAc)

were purchased from Scharlab. The QuEChERS commercial products, 2-mL microcentrifuge tubes for dispersive solid-phase extraction containing 50 mg primary–secondary amine (PSA), 150 mg anhydrous $MgSO_4$, and 150 mg C_{18}, were purchased from Teknokroma (Barcelona, Spain).

GC–(QTOF) MS instrumentation

The chromatographic determinations were performed using a 7890A GC system (Agilent, Palo Alto, CA, USA), equipped with an Agilent 7693 autosampler, coupled to a Xevo G2 QTOF MS system (Waters, Manchester, UK), operating with an APCI source. The GC separation was performed using a fused-silica DB-5 ms column (30 m; 0.25-mm inner diameter, 0.25-μm film thickness; J&W Scientific, Folson, CA, USA). The oven temperature was programmed as follows: 90 °C (1 min); 5 °C/min to 275 °C; 40 °C/min to 320 °C (2 min). The total run time was 40 min. Pulsed splitless (50 psi) injections of 1 μL of sample extracts were done with an injector temperature of 280 °C and with a splitless time of 1 min. Helium (99.999 %; Praxair, Valencia, Spain) was used as the carrier gas at a constant flow rate of 2 mL/min.

To promote the protonation ionization mechanism in the APCI source, an uncapped vial containing high performance LC grade water was placed in the designed holder in the APCI source door. The interface temperature was set to 310 °C and the source temperature was set to 150 °C using N_2 as the auxiliary gas at a flow rate of 150 L/h, the makeup gas flow rate was set at 300 mL/min, and the cone gas flow rate was set at 16 L/h. The APCI corona pin was set at 1.6 μA and the cone voltage was set at 20 V. The Xevo G2 QTOF MS system was operated

with a scan time of 0.4 s, acquiring the mass range *m*/*z* 50–650. The TOF MS resolution was approximately 18,000 full width at half maximum at *m*/*z* 614. For MS^E measurements, two alternating acquisition functions were used applying different collision energies: a low collision energy function selecting 4 eV, and a high collision energy function. In the latter case, a collision energy ramp (10–40 eV) rather than a fixed higher collision energy was used. Continuous internal calibration was performed using a background ion coming from the GC-column bleed as the lock mass ($[M+H]^+$ of octamethylcyclotetrasiloxane, *m*/*z* 297.0830).

MassLynx (Waters) was used to collect and process the data. The application manager ChromaLynx was used to investigate the presence of the pesticides studied. POSI±IVE was used to perform the quantitative validation, the *q*/*Q* evaluation, and to report the quantitative results for positively detected compounds.

Materials

A total of 34 fruit and vegetable samples were analyzed for screening. Different varieties of tomatoes, apples, carrots, oranges, lettuces, courgettes, red peppers, and strawberries were obtained from local markets from the province of Castellón (Spain). For each type of commodity, a blank sample (for validation and matrix-matched calibration purposes) was acquired from a local organic food store in Castellón (Spain).

According to Annex A of SANCO/12571/2013 [47], the matrices analyzed were chosen as being representative of a high-water-content commodity group

(apples, tomatoes, peppers, lettuces, carrots, and courgettes) and a high-acid-content and high-water-content commodity group (oranges and strawberries).

Sample treatment

The extraction procedure was performed following a modified acetate-buffered version of the QuEChERS method [48]. Additionally, formic acid was also used in this work to help stabilize pesticides that can be degraded in MeCN under the basic conditions of QuEChERS clean-up [49].

The procedure for the sample was as follows. First, 10 g of chopped and homogenized sample was weighed in a 50-mL Falcon conical tube, and 10 mL of 1 % glacial acetic acid in MeCN (v/v) was added. After the mixture had been shaken for 30 s, 4 g of anhydrous $MgSO_4$ and 1 g of anhydrous NaAc were added and the resulting mixture was vigorously shaken for 1 min. The tubes were centrifuged at 5,000 rpm for 3 min, and then two aliquots of the supernatant of 1 mL each were transferred into dispersive solid-phase extraction tubes containing 50 mg primary–secondary amine, 150 mg anhydrous $MgSO_4$, and 150 mg C_{18} for clean-up purposes. After clean-up, the two separate extracts were vortexed for 30 s and centrifuged at 3,000 rpm for 2 min. Then, 500 μL of each cleaned-up extract was transferred into the same conical graduated tube, containing 2 mL of toluene, 20 μL of 1.25 % formic acid in MeCN, and 50 μL of the internal standard TPP at 2.5 μg/mL. The mixture was evaporated to approximately 300 μL under a gentle nitrogen stream at 50 °C. Then, each extract was adjusted to a final volume of 500 μL with toluene prior to injection into the GC–MS system.

A quality control sample was also prepared for each sample matrix, with the corresponding blank sample being spiked with 1 mL of working standard solution in acetone containing the 130 target pesticides at 500 µg/L. This spiked sample was extracted with the same procedure as described before.

For quantification purposes, a matrix-matched calibration curve was prepared by applying the aforementioned sample treatment to blank samples. For this, 500 µL of the cleaned-up blank extract was transferred into an evaporation tube containing 1 mL of toluene and 20 µL of 1.25 % formic acid in MeCN. It was evaporated to dryness under a gentle nitrogen stream at 50 °C. Then, 25 µL of TPP at 2.5 µg/mL and 25 µL of working standard solution of adequate concentration were added together with 200 µL of toluene.

TPP was used as an internal standard. The relative response between the quantitative *m*/*z* ion (*Q*) of the compound obtained with the low collision energy function, and *m*/*z* 327.0786, corresponding to the TPP protonated molecule $[C_{18}H_{15}O_4P + H]^+$, was used for quantification.

For validation experiments, blank matrices were spiked by adding 100 µL for the lowest concentration (0.01 mg/kg) or 1 mL for the highest concentration (0.1 mg/kg) of the working standard solution at 1 mg/L.

Screening analysis

A qualitative screening method for around 130 pesticides previously developed and validated in our laboratory (paper in preparation) was applied in the present work to 34 fruit and vegetable samples, including several varieties of

apples, oranges, courgettes, strawberries, red peppers, carrots, lettuces, and tomatoes. In each sample batch, a quality control sample at 0.05 mg/kg for each matrix was analyzed in order to check the reliability of the method with respect to detection of the targeted compounds and to test for potential false negatives. As part of the working strategy, a matrix-matched calibration standard (from 10 to 250 μg/L) for each sample matrix, containing the 130 pesticides, was injected together with the samples to allow quantification of the positive findings in a retrospective way. In this way, new injections were not required in the quantification step, except for those samples which had to be diluted and reanalyzed to quantify them correctly.

Analysis was performed by GC–QTOF MS, and the data obtained were processed by performing a quick search for the target compounds. The search was performed using ChromaLynx in target mode looking for the most abundant ion (*Q*), commonly the protonated molecule obtained with the low collision energy function, which led to the most sensitive detection (narrow-window extracted ion chromatogram ±75 ppm). For the reliable identification of the compound, in addition to the presence of the *Q* ion, one fragment ion (*q*) obtained with the high collision energy function was also required, both with mass errors lower than 5 ppm, together with a retention time with a deviation within ±0.2 min. Verification was performed by the analyst to check if the positives were three or more times the signal-to-noise ratio to assess if they were truly a peak. The ratio between the intensity of the *q* ion and intensity of the *Q* ion (*q*/*Q*) was also tested (with POSI±IVE) to be within the tolerances established by the SANCO/12571/2013 guidelines [47].

Table S1. Pesticides found in the screening of 34 fruit and vegetable samples. Mass errors (in ppm) for the m/z ion at the LE function

Compounds	Courgette				Strawberry				Lettuce				Apple				Orange				Red pepper				Tomato						Carrot		
	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	S5	S6	S1	S2	S3	S4
2-Phenylphenol										2.2[a]			3.7[a]			4.1[a]		1.7													4.2[a]		
Diphenylamine								1.1[a]	2.9[a]	1.0[a]		0.6[a]	3.1	1.7[a]	2.4	2.7	0.2[a]			0.2[a]						5.0[a]	3.0[a]	4.6[a]	1.3[a]		5.2[a]		4.6[a]	2.4[a]
Terbuthylazine																	4.6[a]			3.0[a]													2.2[a]
Chlorothalonil																							3.2[b]		1.5	5.0[c]	5.0[b]		2.0[b]	1.6	5.0[b]			
Tefluthrin																																0.7[c]		
Chlorpyrifos methyl																				1.6														
Metalaxyl				0.4[a]				3.4[a]		1.7														1.4[a]										1.4
Malathion																				1.9[b]														
Chlorpyrifos									1.1[b]	0.5[c]		3.3[b]	2.1	3.0[b]	0.0[c]	1.5	0. 3[a]	3.5		5.0										5.0[a]				0.7
Cyprodinil					1.2				6.6[a]					2.0[a]																				
Fipronil																															2.6[a]	5.0		
Thiabendazole																		4.5																
Procymidone			2.8[a]																															
Imazalil															2.4[a]																			
Fenhexamid	2.0[c]	1.8	3.7[a]	3.2[a]	1.6[a]	3.7[a]	0.1	0.5[c]	1.0[a]			5.0		2.2[a]		1.8[a]		5.4[a]			1.0	3.2[a]	2.4	0.7[a]	0.9		0.0[a]			2.9[a]	4.3[a]			
Phosmet															1.0											0.1[a]								
Iprodione												1.6	5.1[a]		2.0										5.0	2.8								
Fenarimol														0.3[a]			5.1[a]	5.0[a]									0.4[a]	5.0[a]		2.2[a]	5.0[a]			
λ-Cyhalothrin													4.6	2.0	5.9[a]	0.4[b]									0.3	1.3[b]								
Cypermethrin I												1.7																						1.6
Cypermethrin II												0.8																						0.6
Cypermethrin III												0.8																						0.0
Cypermethrin IV												2.9																						0.8
Azoxystrobin																								1.0	1.9	1.9	2.4			0.5[b]		0.7	4.4[a]

Unless specified, positives were confirmed by the presence of the *Q m/z* ion at LE and at least one fragment ion (*q*) at HE, both with mass errors < 5ppm. *q/Q* ratio was also within maximum tolerance deviation admitted

[a] Only the *Q* ion at LE (mass error < 5 ppm) was observed.
[b] The fragment ion at HE presented mass error > 5 ppm.
[c] Both *Q* and *q* ions were observed, but *q/Q* ratio was out of tolerance.

Table 1. Compounds identified in the qualitative screening of fruits and vegetables for which a quantitative method has been developed.

Rt (min)	Compound	Quantitative ion (*Q*)			Confirmative ion (*q*)		*q/Q* ratio				
		m/z	Elemental composition		*m/z*	Elemental composition	Orange	Apple	Tomato	Letucce	Carrot
11,27	2-Phenylphenol	171,0810	$C_{12}H_{11}O$	$[M+H]^+$	153,0704	$C_{12}H_9$	0.228 (12) [b]	0.230 (6)	0.232 (1)	0.218 (9)	0.224 (8)
13,67	Diphenylamine	170,0970	$C_{12}H_{12}N$	$[M+H]^+$	93,0578	C_6H_7N	0.075 (14)	0.102 (9)	0.108 (9)	0.106 (13)	0.112 (11)
17,15	Chlorothalonil	264,8894	$C_8Cl_4N_2H$	$[M+H]^+$	229,9205	$C_8HN_2Cl_3$	0.316 (19)	0.156 (11)	0.160 (8)	0.649 (29)	0.169 (5)
18,92	Chlorpyrifos methyl	321,9028	$C_7H_8Cl_3NO_3PS$	$[M+H]^+$	124,9826	$C_2H_6O_2PS$	- [c]	0.382 (2)	0.388 (1)	0.386 (2)	0.376 (2)
19,62	Metalaxyl	280,1549	$C_{15}H_{22}NO_4$	$[M+H]^+$	220,1338	$C_{13}H_{18}NO_2$	0.200 (1)	0.127 (4)	0.143 (7)	0.136 (8)	0.134 (10)
20,83	Chlorpyrifos	349,9341	$C_9H_{12}Cl_3NO_3PS$	$[M+H]^+$	197,9280	$C_5H_3NOCl_3$	0.205 (12)	0.214 (7)	0.218 (9)	0.212 (5)	0.219 (2)
22,10	Cyprodinil	226,1344	$C_{14}H_{16}N_3$	$[M+H]^+$	93,0578	C_6H_7N	0.023 (14)	0.022 (6)	0.022 (19)	0.016 (22)	0.019 (18)
22,47	Fipronil	436,9465	$C_{12}H_5Cl_2F_6N_4OS$	$[M+H]^+$	367,9513	$C_{11}H_5N_4OF_3SCl_2$	0.185 (3)	0.183 (2)	0.185 (8)	0.180 (12)	0.188 (16)
22,70	Thiabendazole	202,0439	$C_{10}H_8N_3S$	$[M+H]^+$	175,0330	$C_9H_7N_2S$	0.119 (24)	0.296 (9)	0.280 (9)	0.274 (5)	0.292 (23)
27,33	Fenhexamid	302,0715	$C_{14}H_{18}Cl_2NO_2$	$[M+H]^+$	97,1017	C_7H_{13}	0.303 (7)	0.319 (20)	0.235 (6)	0.267 (6)	0.383 (19)
28,20	Triphenyl phosphate	327,0786	$C_{18}H_{16}O_4P$	$[M+H]^+$	-	-	-	-	-	-	-
29,05	Phosmet	318,0024	$C_{11}H_{13}NO_4PS_2$	$[M+H]^+$	160,0399	$C_9H_6NO_2$	1.112 (2)	1.138 (5)	1.151 (3)	1.189 (1)	1.216 (5)
29,10	Iprodione	330,0412	$C_{13}H_{14}Cl_2N_3O_3$	$[M+H]^+$	244,9885	$C_9H_7N_2O_2Cl_2$	0.267 (2)	0.253 (4)	0.240 (10)	0.249 (5)	0.249 (4)
31,48	λ-Cyhalothrin	450,1084	$C_{23}H_{20}ClF_3NO_3$	$[M+H]^+$	225,0294	$C_9H_9OF_3Cl$	0.784 (17)	0.585 (10)	0.771 (1)	0.817 (6)	0.854 (14)
34,60	Cypermethrin [a]	416,0820	$C_{22}H_{20}Cl_2NO_3$	$[M+H]^+$	127,0315	C_7H_8Cl	0.280 (4)	0.519 (1)	0.507 (14)	0.498 (13)	0.563 (3)
38,08	Azoxystrobin	404,1246	$C_{22}H_{18}N_3O_5$	$[M+H]^+$	372,0984	$C_{21}H_{14}N_3O_4$	0.239 (13)	0.182 (5)	0.199 (3)	0.193 (7)	0.197 (3)

Rt retention time

[a] Cypermethrin exhibited four peaks. Its quantification was performed as the sum of the four peaks, giving a single value of response.

[b] Average *q/Q* ratio and relative standard deviation (RSD) calculated from matrix-matched calibration standards

[c] Coelution of interferences

Quantitative validation

Quantitative validation was performed only for those compounds detected and fully identified in the previous screening of food samples (***Table S1***). The quantitative ion (*Q*), confirmative ion (*q*), and *q*/*Q* reference value (calculated as the average for matrix-matched calibration standards) are shown in ***Table 1***. Data analysis was done using POSI±IVE, taking advantage of reporting the mass errors and quantitative results, simultaneously.

Five of the eight matrices studied were selected to perform the validation in order to represent the commodity groups analyzed in this work. The matrices selected for quantitative validation were apple, tomato, lettuce, and carrot for the high-water-content group, and orange for the high-acid-content and high-water-content group [47]. Organic samples, expected to be free from pesticides, were analyzed in the same batch as the spiked samples used in validation, but with addition of TPP to check if they had some detectable concentrations of pesticides and quantify them properly.

The following parameters were evaluated in the method validation:

- Linearity was studied by analyzing matrix-matched calibration curves at concentrations ranging from 10 to 250 μg/L. Linearity was assumed when the regression coefficient was greater than 0.99 with residuals lower than 20 %.
- Accuracy was estimated by means of recovery experiments from 15 food samples (five different matrices in triplicate) spiked at two concentrations (0.01 and 0.1 mg/kg). Recoveries within the range 70–120 % were considered as satisfactory.
- Precision was determined from the recovery experiments described before and was expressed as the repeatability of the method in terms of the relative

standard deviation (RSD). RSDs below or equal to 20 % were considered as satisfactory.

- The limit of quantification (LOQ) was defined as the lowest concentration that was satisfactorily validated with acceptable recoveries and RSDs.

Extended pesticide screening

In a second stage of the work, a wider screening searching for up to 416 pesticides (***Table S2***) was applied by reprocessing the mass data from the 34 fruits and vegetables already analyzed. Searching for the additional 286 pesticides (over the 130 initially studied) was done by looking for the molecular ion and the protonated molecule ion obtained with the low collision energy function, the presence of which was assumed (on the basis of our experience with other pesticides) when using APCI. With the potential list of detected candidates, a deeper study of their identity was done by evaluating the experimental spectra as well as the compatibility of the plausible structures of fragment ions observed with the high collision energy function. Also, a bibliographic search was performed on the fragment ions reported for the candidates using atmospheric pressure ionization sources, both electrospray ionization (ESI) and APCI. Information reported in the literature, although mostly related to LC–MS instrumentation, was helpful to explain the fragmentation of the protonated molecule in the collision cell. When the standards were available in the laboratory, confirmation was done by comparison of retention times and mass spectra.

Table S2. List of compounds searched in the widened screening method

Compounds			
Acephate	Boscalid	Chlorsulfuron	Dichlobenil
Acequinocyl	Bromocyclen	Chlorthal dimethyl	Dichlofenthion
Acetamiprid	Bromofenvinphos	Chlorthion	Dichlofluanid
Acetochlor	Bromophos	Chlorthiophos	Dichloran
Aclonifen	Bromophos ethyl	Chlozolinate	4,4'-Dichlorobenzophenone
Acrinathrin	Bromopropilate	Cinidon-ethyl	Dichlorprop
Alachlor	Bromuconazole	Clodinafop propargyl	Dichlorvos
Aldicarb	Bupimirate	Clomazone	Diclobutrazol
Aldicarb sulfone	Buprofezin	Coumaphos	Diclofop methyl
Aldrin	Butachlor	Crotoxyphos	Dicofol
Allethrin	Butamifos	Crufomate	Dicrotophos
Ametryn	Butralin	Cyanazine	Dieldrin
Amidithion	Cadusafos	Cyanofenphos	Dienochlor
Ancymidol	Captafol	Cyanophos	Difenoconazole
Anilazine	Captan	Cycloxydim	Diflufenican
Anthraquinone	Carbaryl	Cyfluthrin	Dimefox
Aramite	Carbendazim	λ-Cyhalothrin	Dimethachlor
Atrazine	Carbetamide	Cymoxanil	Dimethenamide
Atrazine desethyl	Carbofuran	Cypermethrin	Dimethipine
Atrazine desisopropyl	*trans*-Chlordane	Cyphenothrin	Dimethoate
Azaconazole	Chlordecone	Cyproconazole	Dimethomorph
Azamethiphos	Chlorethoxyfos	Cyprodinil	Dimoxystrobin
Azinphos ethyl	Chlorfenapyr	Cyprofuram	Diniconazole
Azinphos methyl	Chlorfenprop methyl	*p,p'*-DDE	Dinitramine
Azoxystrobin	Chlorfenson	*p,p'*-DDD	Dinobuton
Benalaxyl	Chlorfenvinphos	*p,p'*-DDT	Dinocap
Benfluralin	Chloridazon	DEF	Dinoseb
Benoxacor	Chlormephos	Deltamethrin	Dinoterb
Bentazone	Chloroneb	Demeton	Dioxathion
Benzoylprop ethyl	Chloropropylate	Demeton-S-methyl	Diphenylamine
Bifenox	Chlorothalonil	Dialifos	Disulfoton
Bifenthrin	Chlorotoluron	Diazinon	Disulfoton sulfone
Binapacryl	Chlorpropham	4,4-Dibromobenzophenone	Ditalimfos
Bitertanol	Chlorpyrifos	Dicamba	Diuron
Bixafen	Chlorpyrifos methyl	Dicapthon	DNOC

Table S2. (Continuation)

Compounds			
Edifenphos	Fenson	β-HCH	Mecarbam
α-endosulfan	Fensulfothion	γ-HCH	Mecoprop
β-endosulfan	Fensulfothion sulfone	δ-HCH	Mepanipyrim
Endosulfan ether	Fenthion	Heptachlor	Mephosfolan
Endosulfan sulfate	Fenvalerate	Heptachlor epoxide A	Merphos
Endrin	Fipronil	Heptachlor epoxide B	Metalaxyl
EPN	Flamprop isopropyl	Heptenophos	Metamitron
Epoxyconazol	Flamprop methyl	Hexachlorobutadiene	Metazachlor
Etaconazole	Fluazifop-p-butyl	Hexaconazole	Metconazole
Ethalfluralin	Flubenzimine	Hexythiazox	Methabenzthiazuron
Ethiofencarb	Fluchloralin	Imazalil	Methacriphos
Ethion	Flucythrinate	Imibenconazole	Methamidophos
Ethiprole	Fludioxonil	Indoxacarb	Methidathion
Ethofumesat	Flufenoxuron	Iodofenphos	Methiocarb
Ethoprophos	Flumethrine	Ioxynil	Methiocarb sulfone
Ethoxyquin	Flumetralin	Ioxynil octanoate	Methomyl
Etofenprox	Fluopicolide	Iprobenfos	Methoprene
Etoxazole	Fluopyram	Iprodione	Methoxychlor
Etridiazole	Fluorodifen	Iprovalicarb	2,4-D-Methylester
Etrimfos	Fluotrimazole	Isazophos	2,4,5-T-Methylester
Famophos	Fluquinconazole	Isobenzan	Metolachlor
Famoxadone	Flurenol butyl	Isocarbofos	Metrafenon
Famphur	Flurochloridone	Isodrin	Metribuzin
Fenamidone	Flurprimidol	Isofenphos	Mevinphos
Fenamiphos	Flurtamone	Isofenphos methyl	Mirex
Fenarimol	Flusilazole	Isomethiozin	Molinate
Fenbuconazole	Flutriafol	Isopropalin	Monocrotophos
Fenchlorazole ethyl	τ-Fluvalinate	Isoxaben	Monuron
Fenchlorphos	Folpet	Isoxadifen ethyl	Myclobutanil
Fenfluthrine	Fonofos	Isoxathion	Nitralin
Fenhexamid	Formothion	Ketoendrin delta	Nitrofen
Fenitrothion	Fosalone	Kresoxim methyl	Omethoate
Fenobucarb	Furathiocarb	Lactofen	Oxadixyl
Fenoxaprop ethyl	Furfural	Lenacil	Oxyfluorfen
Fenoxycarb	Halfenprox	Leptophos	Paclobutrazol
Fenpropathrin	Haloxyfop methyl	Lufenuron	Paraoxon ethyl
Fenpropimorph	HCB	Malaoxon	Paraoxon methyl
Fenpyroximate	α-HCH	Malathion	Parathion ethyl

Table S2. (Continuation)

Compounds		
Parathion methyl	Propiconazole	TEPP
Penconazole	Propisochlor	Terbacil
Pendimethalin	Propoxur	Terbufos
Pentachloranisol	Propyzamide	Terbumeton
Pentachloroaniline	Prosulfocarb	Terbumeton desethyl
Pentachlorobenzene	Prothioconazole-desthio	Terbuthylazine
Pentachlorophenol	Prothiophos	Terbuthylazine desethyl
Pentanochlor	Prothoat	Terbutryn
Permethrin	Pymetrozine	Tetrachlorvinphos
Perthane	Pyraclofos	Tetraconazole
Phenkapton	Pyraclostrobin	Tetradifon
Phenothrin	Pyraflufen ethyl	Tetramethrin
Phenthoate	Pyrazophos	Tetrasul
2-Phenylphenol	Pyridaben	Thiabendazole
Phorate	Pyridaphenthion	Thiometon
Phosalone	Pyrifenox	Thionazin
Phosfolane	Pyrimethanil	Thiophanate methyl
Phosmet	Pyrimitate	Tolclofos methyl
Phosphamidon	Pyriproxyfen	Tolyfluanid
Picloram methyl ester	Quinalphos	Transfluthrin
Picolinafen	Quinoxyfen	Triadimefon
Picoxystrobin	Quintiofos	Triadimenol
Pirazofos	Quintozene	Triallate
Pirimicarb	Quizalofop ethyl	Triamiphos
Pirimiphos ethyl	Resmethrin	Triazophos
Pirimiphos methyl	Sebuthylazin	Tribufos
Plifenate	Silafluofen	Trichlorfon
Prallethrin	Simazine	Trichloronat
Prochloraz	Spirodiclofen	Tridiphane
Procymidone	Spiromesifen	Trietazine
Profenofos	Sulfotep	Trifloxystrobin
Profluralin	Sulprofos	Triflumizole
Prometryn	Swep	Triflumuron
Propachlor	Tebuconazole	Trifluralin
Propanil	Tebupirimfos	Triticonazole
Propargite	Tecnazene	Vamidothion
Propazine	Teflubenzuron	Vinclozolin
Propetamphos	Tefluthrin	
Propham	Temephos	

RESULTS AND DISCUSSION

Screening analysis

The qualitative screening was firstly applied to 130 compounds for which the method had been previously validated (paper in preparation). To this aim, 34 fruit and vegetable sample extracts obtained by a QuEChERS method were analyzed by GC–(APCI) QTOF analysis in MS^E mode. The screening was performed in a three-step scheme. In the first step, the samples were processed by searching for the m/z Q analyte ion obtained with the low collision energy function (ChromaLynx XS, in target mode). As ***Table S1*** shows, 21 pesticides were detected since the ion observed at low collision energy had a mass error of less than 5 ppm, with a chromatographic peak at the reference retention time (±0.2 min). Next, in the second processing step (ChromaLynx XS, targeted-fragment-ion confirmation mode), the 21 candidates were examined to check if a fragment ion at high collision energy was also present at the same retention time with mass error of less than 5 ppm. Finally, the q/Q ratio was evaluated by comparing the experimental values with the reference ones obtained as the average from matrix-matched calibration standards. After this process had been performed, different situations could be observed. In most detections, all requirements were fulfilled; therefore, the presence of the compound was unequivocally confirmed in the samples. However, in several cases the Q ion (commonly the protonated molecule) was observed at low collision energy but no fragment ion was present at high collision energy, surely due to the lower abundance of the fragment ion. Particularly, this situation occurred at low concentrations (close to the screening detection limit), where the differences in abundance become more important. Another situation occurred for those

compounds where both ions were observed, but the fragment ion had a mass error higher than 5 ppm. Finally, in a few cases the *q*/*Q* ratio was out of the acceptable deviation tolerances (±30 %) [47]. Although evidence for the identity of the pesticide already existed in the last three cases, from a strict point of view the compound should, however, be taken as fully identified only when all requirements were fulfilled. Thus, identification of six pesticides (terbuthylazine, tefluthrin, malathion, procymidone, imazalil, and fenarimol) was considered as tentative, and they could not be finally identified, but 15 of the 21 candidates were satisfactorily confirmed (***Table S1***). The first rapid searching for one *m/z* ion (*Q*) allowed us to reduce the workload in terms of confirmation, as only those potential positives were examined in the next processing steps in order to confirm their identity.

Quantitative validation

After qualitative screening, a quantitative validation for the analytes found in the samples was performed, selecting apple, tomato, lettuce, carrot, and orange as representative matrices. TPP was used as an internal standard in order to properly quantify the compounds by compensating for the variations of the system.

For each spiking level, three replicates were analyzed in order to evaluate accuracy and precision. ***Table 2*** shows the recoveries and RSDs, as well as the LOQs (commonly 0.01 mg/kg) and the maximum residue levels (MRLs) established by the European Commission [1, 2] in order to illustrate whether the method developed was useful for MRL compliance.

Linearity was satisfactory in the concentration range from 10 to 250 μg/L, with correlation coefficients higher than 0.99 and residuals lower than 20 % in all matrices tested for all 15 compounds under study.

Recoveries were between 70 and 120 % for most analyte–matrix combinations, with some exceptions (***Table 2***), such as chlorothalonil, which could not be validated for orange, tomato and lettuce, since it needs particular conditions to minimize its degradation during extraction, as previously reported [6]. Metalaxyl was not validated in orange at the two spiking levels tested because of the lack of sensitivity observed in this matrix. Moreover, azoxystrobin in lettuce showed recoveries above 120 % at the two spiking levels, and fipronil in lettuce, carrot, and apple also gave recoveries above the range of acceptance at 0.01 mg/kg. Thiabendazole exhibited, in general, a poor peak shape, and this complicated the quantification, particularly in some matrices (*e.g.*, orange). ***Figure S1*** summarizes the results from validation experiments at both spiking levels. It can be seen that satisfactory recoveries were obtained for around 80-90 % of the analytes tested in orange, carrot, and apple for both levels, and for around 70-80 % in lettuce and tomato.

For more than 90 % of the analyte–matrix combinations, the RSDs were lower than 20 %, and for around 50 % they were lower than 10 % (***Table 2***).

Table 2. Average recovery (%) and RSD (%, in parenthesis) for the gas chromatography (GC)-(atmospheric pressure *chemical ionization) (APCI) quadrupole time-of-flight (QTOF) quantitative method applied to orange, tomato, lettuce, carrot and apple samples (n=3) at two spiking levels (0.01 and 0.1 mg/kg).*

Compound	Orange				Tomato				Lettuce				Carrot				Apple			
	0.01	**0.1**	**LOQ**	**MRL**	**0.01**	**0.1**	**LOQ**	**MRL**	**0.01**	**0.1**	**LOQ**	**MRL**	**0.01**	**0.1**	**LOQ**	**MRL**	**0.01**	**0.1**	**LOQ**	**MRL**
2-Phenylphenol	105 (20)	87 (11)	0.01	5	78 (5)	84 (20)	0.01	0.05	114 (16)	61 (7)	0.01	0.05	103 (10)	92 (12)	0.01	0.05	110 (12)	101 (20)	0.01	0.05
Azoxystrobin	118 (4)	97 (9)	0.01	15	87 (18)	101 (19)	0.01	3	153 (7)	158 (5)	-	15	120 (3)	94 (20)	0.01	1	94 (22)	112 (11)	0.01	0.05
Chlorothalonil	-	-	-	0.01	-	-	-	2	-	-	-	0.01	99 (4)	131 (1)	0.01	1	83 (9)	65 (2)	0.01	1
Chlorpyrifos	105 (7)	101 (3)	0.01	0.3	-	83 (20)	0.1	0.5	123 (1)	107 (5)	0.1	0.05	78 (3)	98 (10)	0.01	0.1	117 (7)	83 (7)	0.01	0.5
Chlorpyrifos methyl	-[a]	-[a]	-	0.5	-	94 (24)	0.1	0.5	120 (1)	97 (4)	0.01	0.05	86 (3)	96 (11)	0.01	0.05	98 (11)	84 (7)	0.01	0.5
Cypermethrin	124 (8)	72 (13)	0.1	2[b]	118 (19)	127 (20)	0.01	0.5[b]	104 (12)	103 (9)	0.01	2[b]	117 (7)	108 (21)	0.01	0.05[b]	92 (27)	98 (11)	0.01	1[a]
Cyprodinil	117 (10)	88 (4)	0.01	0.05	52 (30)	93 (13)	0.1	1	105 (10)	118 (7)	0.01	15	93 (2)	94 (11)	0.01	2	111 (8)	90 (3)	0.01	1
Diphenylamine	110 (3)	87 (11)	0.01	0.05	-	80 (21)	0.1	0.05	108 (10)	71 (6)	0.01	0.05	96 (3)	87 (10)	0.01	0.05	102 (14)	92 (13)	0.01	5
Fenhexamid	107 (4)	87 (1)	0.01	0.05	74 (16)	114 (13)	0.01	1	95 (5)	113 (2)	0.01	40	114 (6)	101 (16)	0.01	0.05	139 (1)	107 (6)	0.1	0.05
Fipronil	110 (20)	105 (18)	0.01	0.005	62 (17)	101 (12)	0.01	0.005	130 (11)	111 (2)	0.1	0.005	192 (3)	109 (20)	0.1	0.005	139 (1)	101 (2)	0.1	0.005
Iprodione	110 (4)	96 (8)	0.01	0.02	72 (5)	111 (6)	0.01	5	113 (9)	94 (4)	0.01	10	56 (14)	92 (12)	0.1	0.5	118 (4)	108 (4)	0.01	5
λ-Cyhalothrin	100 (2)	94 (10)	0.01	0.2	116 (12)	134 (15)	0.01	0.1	130 (7)	104 (11)	0.1	0.5	106 (5)	119 (12)	0.01	0.02	109 (20)	108 (7)	0.01	0.1
Metalaxyl	-	60 (8)	-	0.5	63 (14)	89 (13)	0.1	0.2	108 (15)	121 (5)	0.01	3	77 (6)	99 (17)	0.01	0.1	114 (4)	94 (7)	0.01	1
Phosmet	98 (9)	109 (5)	0.01	0.2	87 (37)	101 (29)	-	0.05	109 (11)	151 (7)	0.01	0.05	107 (5)	112 (15)	0.01	0.05	127 (1)	102 (3)	0.01	0.2
Thiabendazole	151 (2)	60 (17)	-	5	-	110 (27)	0.1	0.05	72 (11)	89 (7)	0.01	0.05	-	-	-	0.05	117 (8)	114 (5)	0.01	5

The limit of quantification (*LOQ*) and the maximum residue level (*MRL*) are given in milligrams per kilogram
[a] Coelution of interferences
[b] MRL including other mixtures of constituents isomers

The results obtained are promising and suggest that this technique might be used also for quantification soon. Although its potential for screening and identification is evident and has been demonstrated in recent years, its quantitative capability is less well known. The data shown in this work can be improved using other well-established methods, for example, using QuEChERS and GC–MS/MS with a QqQ analyzer [7], a combination that in the present state of the art is surely the choice in pesticide quantitative analysis. However, the possibility of combining large screening, reliable identification, and reasonable quantification with GC–(APCI) QTOF MS is very attractive and worth investigating in the next few years.

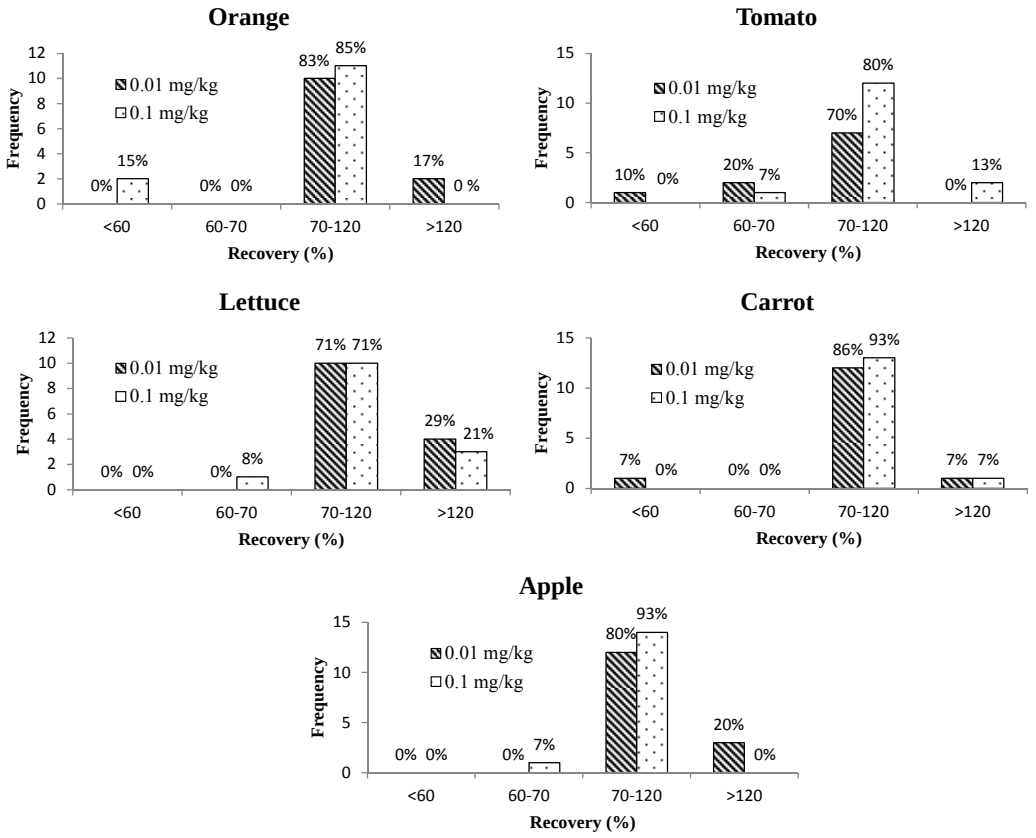


Figure S1. Histograms for recoveries experiments at 0.01 mg/kg and 0.1 mg/kg for the five validated matrices: orange, tomato, lettuce, carrot and apple.

Quantitative analysis of samples

The samples analyzed for qualitative screening could be quantified without the need for new injections (except for those samples which had to be diluted to quantify them properly) and analysis making use of the matrix-matched calibration standards already injected in every batch of samples. Besides, quality control samples for each matrix were also analyzed. Quantitative results for the samples analyzed are shown in ***Table 3***. Quantitative validation was performed for five of the eight matrices analyzed (orange, tomato, lettuce, carrot, and apple). For the remaining three matrices (courgette, strawberry, and red pepper), quantification was performed by means of matrix-matched calibration standards as well. In addition, quality control samples were analyzed together with the samples, and they gave acceptable recoveries for the compounds detected, with values ranging from 60 to 140 % as established in SANCO/12571/2013 for individual recoveries in routine multiresidue analysis [47].

Most of the residue concentrations found were below the corresponding MRL. However, this value was exceeded in a few cases: fenhexamid and cypermethrin in a lettuce sample; cypermethrin and metalaxyl in carrot. Thiabendazole was also found at high concentration in orange. Although validation in this matrix was not fully satisfactory at the low levels tested (0.01 and 0.1 mg/kg), we estimated a concentration of around 6 mg/kg, which is slightly above of its MRL (5 mg/kg). ***Figure 1*** shows illustrative examples of positives found slightly over the MRL in lettuce and carrot: the GC–(APCI) QTOF MS extracted ion chromatograms, the experimental low collision energy and high collision energy accurate mass spectra, the chemical structures proposed

for the most abundant fragment ions, and the experimental mass errors are shown.

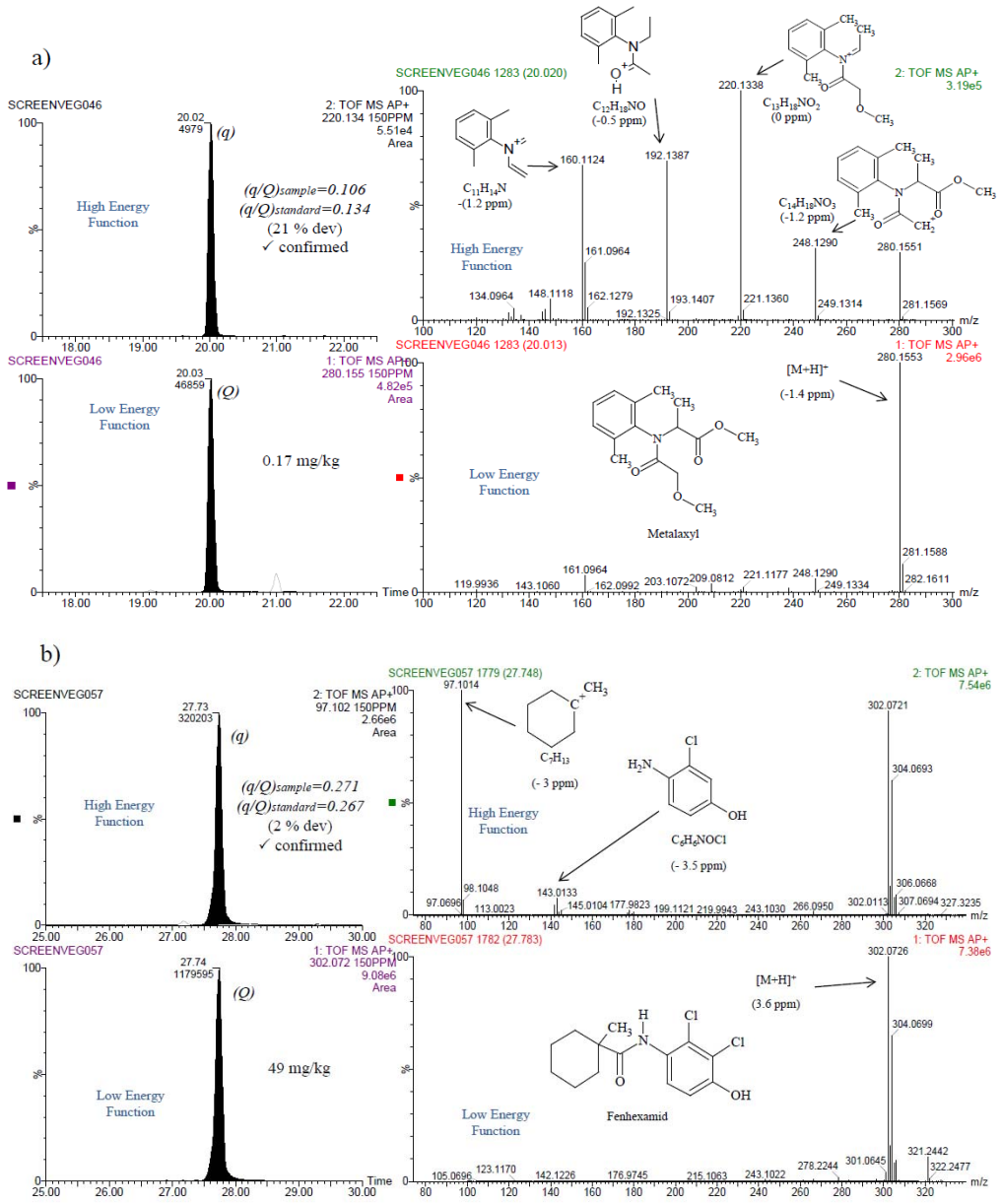


Figure 1. Gas chromatography (GC)-(atmospheric pressure chemical ionization) (APCI) quadrupole time-of-flight (TOF) mass spectrometry (MS) narrow-window extracted ion chromatogram (mass window 150 ppm) showing the detection of a) metalaxyl in one carrot sample (maximum residue level 0.1 mg/kg) and b) fenhexamid in lettuce (maximum residue level 40 mg/kg). Experimental APCI accurate-mass spectra for the high collision energy function and for the low collision energy function are also shown with the chemical structures proposed for the most abundant fragment ions together with the experimental mass errors (in parts per million)

Table 3. Pesticide concentrations (mg/kg) found in different fruits and vegetables samples (S) by GC–(APCI)QTOF MS

Compound	Courgette	Strawberry		Lettuce		Apple				Orange			Red pepper			Tomato				Carrot		
	S2	S1	S3	S1	S4	S1	S2	S3	S4	S1	S2	S4	S1	S3	S4	S1	S2	S3	S6	S1	S3	S4
2-Phenylphenol											3.6											
Diphenylamine						0.01		0.25	0.01													
Chlorothalonil																						
Chlorpyrifos methyl												*D*										
Metalaxyl				0.02																		0.17
Chlorpyrifos						0.05			0.02		0.02	0.07										0.01
Cyprodinil		0.15																				
Fipronil																					*D*	
Thiabendazole											6.0[a]											
Fenhexamid	*D*		0.11		49								0.02	0.04		0.03						
Phosmet								0.02														
Iprodione					0.01			0.03								0.09	0.17					
λ-cyhalothrin						0.02	0.02									0.01						
Cypermethrin					5.0																	0.07
Azoxystrobin															0.03	0.01	0.01	0.07			0.02	

D detected (concentration lower than the LOQ), *S* sample

[a]Estimated value, as validation at the low levels tested (0.01 and 0.1 mg/kg) was not fully satisfactory

Extended pesticide screening analysis

After the broader screening had been applied for 286 additional pesticides, 13 new compounds were detected in the fruits and vegetables analyzed. No additional sample injections were required, as only mass data reprocessing was applied at this stage. ***Table 4*** shows the new compounds (tentatively) identified after this extended search.

To confirm unequivocally the identity of the compounds detected, the use of reference standards was required. This was done for tetraconazole, flutriafol, fludioxonil, tebuconazole, etoxazole, and boscalid, for which both the retention times and the spectra of reference standards and positives in samples perfectly matched; besides, the *Q* and *q* mass errors were lower than 5 ppm.

When reference standards were unavailable, additional parameters were checked. When the protonated molecule was the diagnostic ion, data from methods reported in the literature using positive LC–ESI methods [50–52], which in most cases act as the APCI source, were consulted. This allowed us to test whether the fragment ions observed with the high collision energy function were in agreement with the fragmentation obtained in the collision cell for positive LC–ESI methods. Another step was to verify if the fragment ions had structures compatible with the candidate. MassFragment was useful to suggest probable structures of fragment ions coming from the protonated molecule and to evaluate whether they were in accordance with the compound being evaluated. Finally, the last parameter evaluated was the mass error for *Q* and *q* ions to verify whether it was below 5 ppm. When at least two of the three requirements were fulfilled, we considered the compound as tentatively identified. For myclobutanil, bupirimate, and acetamiprid, the protonated molecule observed was in fact

expected from information reported in the literature, and the fragment ions were compatible with their chemical structure with mass error below 5 ppm; therefore, these compounds were tentatively identified despite the reference standards being unavailable.

In those few cases where the molecular ion was the diagnostic ion, instead of the protonated molecule, the fragmentation behavior reported in the literature for EI sources or commercial EI libraries was used as a tool to help in interpretation. In the case of the insecticide methoprene in lettuce, the molecular ion $M^{+\cdot}$ had a mass error of 0.3 ppm, and coherent structures were found for some fragment ions (mass errors of less than 1.7 ppm); however, no references were found taking the molecular ion as the precursor ion in an EI source as this is absent in the theoretical spectra, and therefore, it was tentatively identified as two out of three requirements were fulfilled.

Another interesting example of tentative identification can be seen in ***Figure 2***, where the GC–(APCI) QTOF MS (narrow-window extracted ion chromatogram 150 ppm) is shown for the herbicide aclonifen in carrot. The experimental APCI accurate-mass spectra obtained with the high and low collision energy functions support its identification, with chemical structures compatible with aclonifen as found in the literature [52], all with mass errors lower than 3.7 ppm.

Table 4. Additional compounds detected in fruits and vegetables after widening the GC-QTOF screening up to 416 pesticides

Rt (min)	Compound	Matrix (number of positives/total number of samples analyzed)	Diagnostic ion	Reference standard	Fragmentation in agreement with literature	Compatible fragment ions	*Q* mass error (ppm)	*q* mass error (ppm)	Confirmed
21.81	Tetraconazole	Tomato (2/6)	$[M+H]^+$	Available	-	-	3.8	1.2	YES
23.75	Methoprene	Lettuce (1/4)	$M^{+\cdot}$	Unavailable	-	YES	0.3	1.7	Tentatively identified
24.29	Flutriafol	Red pepper (3/4)	$[M+H]^+$	Available	-	-	4.6	1.8	YES
24.81	Fludioxonil	Strawberry (1/4)	$M^{+\cdot}$	Available	-	-	2	1.5	YES
25.18	Myclobutanil	Tomato (1/6) Strawberry (1/4)	$[M+H]^+$	Unavailable	YES [51]	YES	4.1	3.9	Tentatively identified
25.4	Bupirimate	Tomato (2/6)	$[M+H]^+$	Unavailable	YES [52]	YES	5	2	Tentatively identified
26.55	Aclonifen	Carrot (1/4)	$[M+H]^+$	Unavailable	YES [53]	YES	1.1	3.7	Tentatively identified
27.13	Allethrin	Carrot (4/4)	$[M+H]^+$	Unavailable	-	YES	1	0.4	Tentatively identified
28.35	Tebuconazole	Tomate (1/6) Apple (1/4)	$[M+H]^+$	Available	-	-	2	4.8	YES
29.29	Acetamiprid	Lettuce (1/4)	$[M+H]^+$	Unavailable	YES [51]	YES	1.8	4.6	Tentatively identified
30.15	Etoxazole	Strawberry (1/4)	$[M+H]^+$	Available	-	-	1.7	2	YES
32.47	Isomethiozin	Orange (2/4)	$M^{+\cdot}$	Unavailable	-	YES	4.8	6.3	Tentatively identified
34.89	Boscalid	Lettuce (3/4) Strawberry (1/4)	$[M+H]^+$	Available	-	-	2.3	2.3	YES

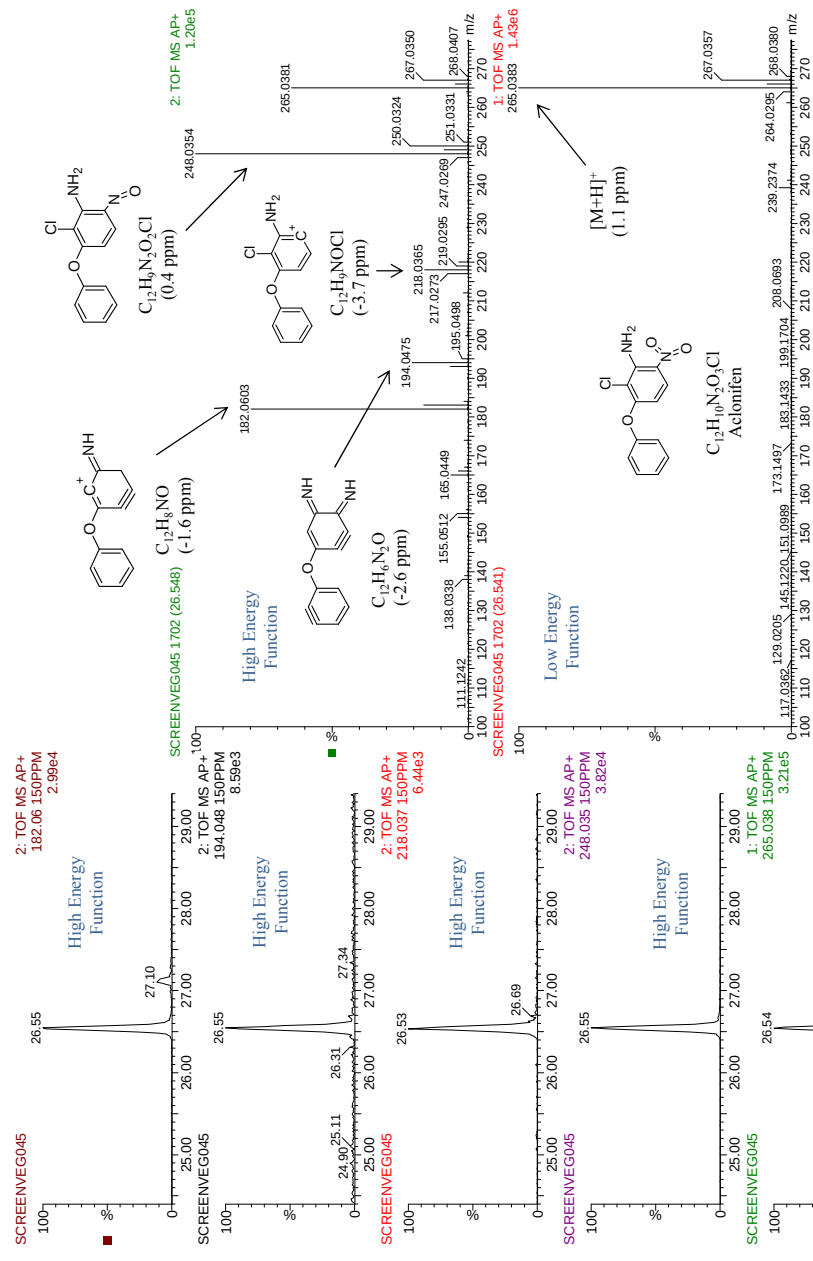


Figure 2. GC–(APCI) quadrupole TOF MS narrow-window extracted ion chromatogram (mass window 150 ppm) showing the detection of the herbicide aclonifen in a carrot sample. Experimental APCI accurate-mass spectra for the high collision energy function and for the low collision energy function are also shown with the chemical structures proposed for the most abundant fragment ions together with the experimental mass errors (in parts per million)

CONCLUSIONS

A wide-scope screening developed for 130 pesticides, based on the use of GC–(APCI) QTOF MS, has been applied to 34 fruit and vegetable samples from different food groups. After identification of several pesticides in these samples, the possibilities for quantitative analysis were evaluated. To this aim, a quantitative validation for 15 pesticides identified in the samples was performed, using five food matrices that were taken as a model. The potential of GC–(APCI) QTOF MS for screening purposes has been demonstrated, broadening the screening up to 416 pesticides in a subsequent step. This is facilitated by the common presence of the molecular ion (mostly the protonated molecule) in APCI spectra under the soft ionization conditions occurring in this source. Accurate-mass full-spectrum data provided by QTOF MS made possible the search for additional pesticides in a retrospective way without the need for new sample injections.

In this work, we have shown that GC–MS with an APCI source using a QTOF analyzer has great potential for investigation of GC-amenable pesticides, as it allows the detection and reliable identification of a large number of pesticides in compliance with the regulatory MRLs. In addition, the analytes can be searched for at any time after data acquisition, and tentative identifications are feasible owing to the useful information provided by QTOF MS under MS^E (accurate-mass molecular ion/protonated molecule, accurate-mass fragment ions, isotope pattern, compatibility of fragment ions with the chemical structure of the candidate, ion ratios). Nowadays, GC–(EI) MS with nominal-mass single MS or tandem MS instruments is the technique of choice for GC-amenable pesticides, but GC–(APCI) QTOF MS also has promising features for

quantification. In the present work, LOQs of 0.01 mg/kg were obtained for most analyte–matrix combinations tested. More work will be required to explore fully the quantitative applicability of GC–(APCI) QTOF MS on the basis of the results obtained in the first articles reported on this issue.

Acknowledgments

M.I. Cervera is very grateful to the Ministry of Economy and Competitiveness of Spain for her predoctoral grant. The authors acknowledge the financial support of Generalitat Valenciana (research group of excellence PROMETEO/2009/054; ISIC 2012/016).

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4. Discusión de resultados

En el **Artículo científico 3** se ha desarrollado y validado un método cuantitativo para la determinación de residuos de plaguicidas en matrices de frutas y verduras mediante cromatografía de gases acoplada a espectrometría de masas con analizador de tiempo de vuelo de alta resolución, GC-HR TOF MS, en modo de ionización electrónica. Además del enfoque *target* inicial, se llevó a cabo una aproximación *non-target* mediante aplicación, a los datos inicialmente adquiridos, de un software de deconvolución para la búsqueda de desconocidos.

Hasta el año de publicación del artículo (2012), no se habían reportado apenas trabajos sobre HR TOF MS para fines cuantitativos en el campo del análisis de residuos de plaguicidas; solo algunos autores lo hicieron aunque con la utilización de analizadores HS TOF MS (Patil, 2009; Koesukwiswat, 2010). HR TOF MS había sido, hasta ese momento, una excelente herramienta para análisis cualitativo (detección e identificación) de numerosos contaminantes orgánicos en matrices muy variadas, tanto en modo *target* como *non-target* (Hernández, 2011). Por ello, se pensó en explorar también la capacidad de cuantificación del acoplamiento GC-HR TOF MS aun cuando, *a priori*, su limitado rango lineal dificultaba este proceso.

Debido a la ligera deriva (pérdida paulatina de la señal) sufrida por el sistema de masas utilizado durante la realización de este trabajo (modelo GCT, Waters), la cuantificación de los analitos podía haberse visto afectada de modo negativo, lo que podría dar lugar a resultados no satisfactorios en cuanto a validación y aplicación de métodos cuantitativos. Así, se observó que durante secuencias largas de análisis, el equipo sufría esta variación en la respuesta

instrumental, que se podía apreciar fácilmente en el espectro de masas de la *perfluorotri-n-butylamine* (PFTBA), adquirida continuamente a lo largo de cada una de ellas. Este compuesto es utilizado para calibrar el equipo diariamente, y de forma constante durante el análisis, ajustando el eje de masas por comparación con la masa exacta adquirida de su pico base, *m/z* 218.9856, denominada *lock mass*. En la **Figura 1** se muestra su espectro de masas experimental en modo EI, siendo remarcados los 6 iones seleccionados para llevar a cabo el estudio de la variabilidad instrumental del sistema utilizado. Como se puede observar, los iones escogidos abarcan un amplio rango de masas del espectro, con el fin de tener una visión global del comportamiento de cada uno de ellos.

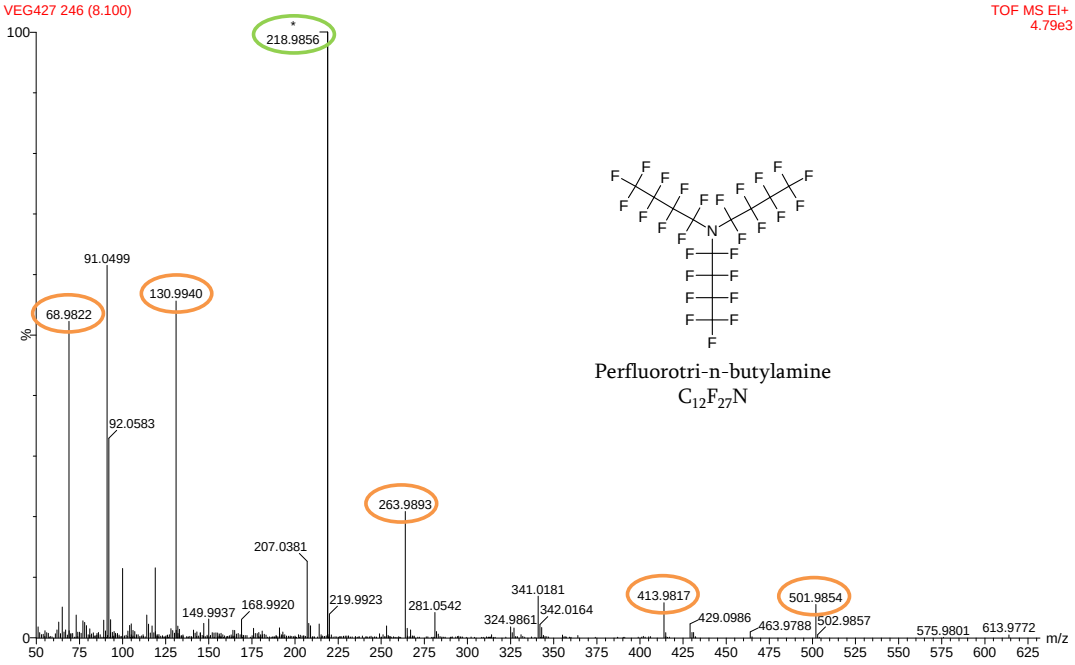


Figura 1. Espectro de masas experimental de la *perfluorotri-n-butylamine*, obtenido mediante GC-(EI)TOF MS, con 6 iones seleccionados para llevar a cabo el estudio de deriva de la respuesta instrumental

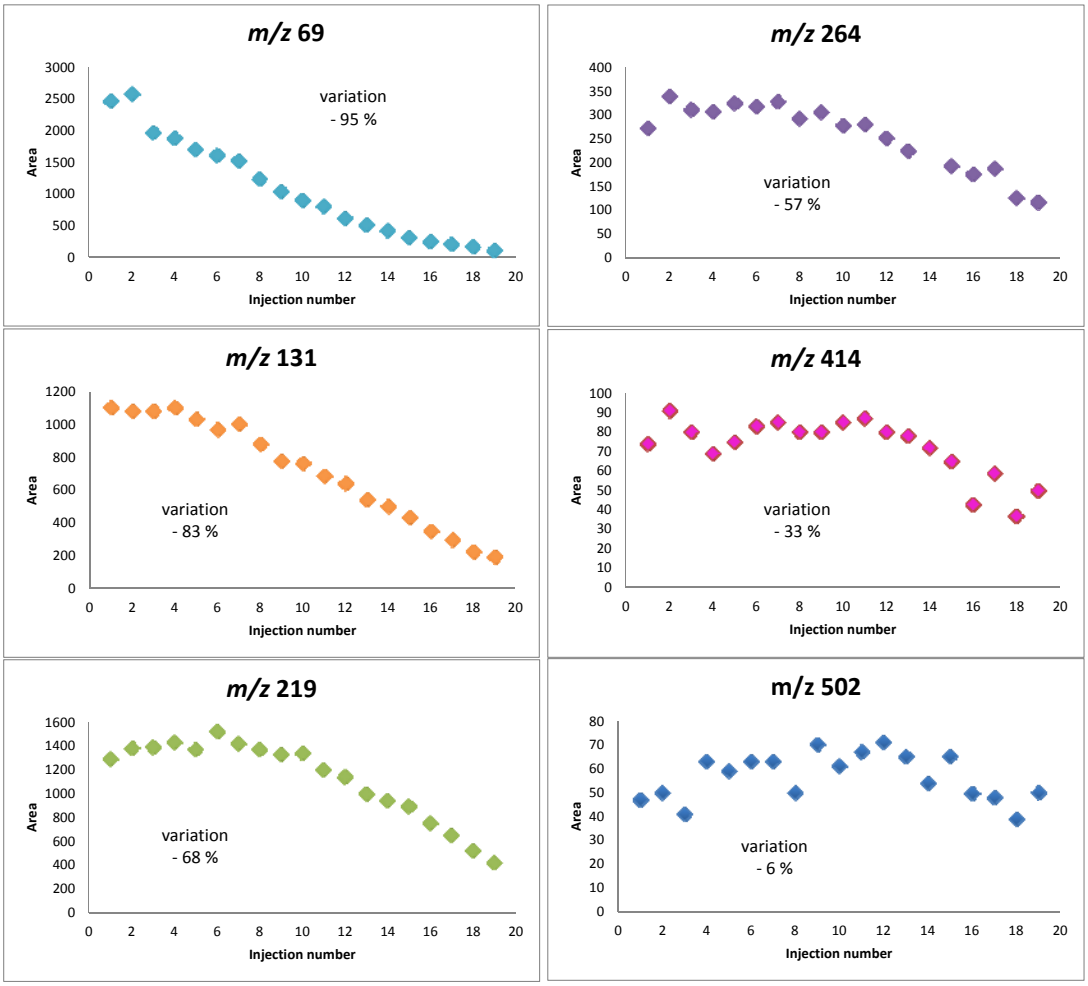


Figura 2. Gráficas de la variación sufrida por los iones seleccionados de la PFTBA durante una secuencia de 19 inyecciones consecutivas

La **Figura 2** muestra la variación en las respuestas de los iones seleccionados de la PFTBA a lo largo de las 19 inyecciones de una secuencia. Con el fin de tener una idea global de la variación sufrida por el sistema instrumental desde el inicio hasta el final de la secuencia, se ha calculado la diferencia entre el área final y la inicial, con respecto al área inicial (en porcentaje):

$$\%\ \text{variación} = [(A\ \text{final} - A\ \text{inicial})/A\ \text{inicial}] \cdot 100 \qquad \textbf{Ecuación 1}$$

Así pues, en cada gráfica se puede observar el comportamiento de cada ion, mostrando una notable deriva durante el transcurso de la secuencia. Existe un claro descenso de la señal desde la primera inyección hasta la última, aunque de forma mucho más pronunciada para las masas más pequeñas, como *m/z* 219 y 264, con valores de variación de más del 50 %, e incluso para *m/z* 69 y 131 con valores mayores del 80 %. Por el contrario, la variación sufrida por el ion *m/z* 502 es mínima, apenas del 6 %.

Es lógico pensar que la PFTBA sufra una ligera pérdida de señal, al ser un compuesto añadido al principio de la secuencia en un depósito diseñado para tal efecto, que entra continuamente en la fuente de ionización. Por ello es razonable que se vaya consumiendo, dando lugar a una cierta pérdida de señal con el tiempo, en secuencias muy largas. Pero esta situación debería dar lugar a una reducción de la señal por igual de todos los iones del compuesto. Sin embargo, esta pérdida de señal apenas se apreció para los iones de masas más altas; mientras que para las masas más pequeñas fue bastante elevada. Se pensó entonces que lo ocurrido con la PFTBA podría producirse también con los iones de los analitos, afectando a su correcta cuantificación y con ello a la validación del método, objetivo principal del trabajo. Con el fin de minimizar este posible problema, se consideró el uso de un patrón interno (IS) introducido como *surrogate*, es decir añadido a la muestra desde el principio del procedimiento analítico, como solución a dicha desviación. Además, al ser añadido desde el principio, también podría ayudar a corregir posibles pérdidas durante la etapa de extracción.

En la práctica habitual, la elección del ion del IS utilizado para llevar a cabo la corrección corresponde normalmente al pico base, el más intenso en modo

de EI. Por supuesto, siempre es necesario comprobar que no existan interferencias y que tenga una buena forma de pico, de modo que corrija adecuadamente. El patrón interno utilizado en este trabajo fue el *triphenyl phosphate* (TPP) y el ion seleccionado para la corrección fue el ion molecular, de *m/z* 326.0708 (ver **Figura 3**).

Los datos obtenidos hasta ese momento parecían indicar que las desviaciones sufridas eran diferentes dependiendo de la masa. Por ello, para mejorar la corrección con el IS se estudió la posibilidad de seleccionar otros iones de su espectro de masas de *m/z* semejantes a las de los analitos que tenían que corregir, de modo que ambos iones (IS y analito) variaran de forma similar. En la **Figura 3**, se observa el espectro de masas del TPP, medido en masa exacta, con tres de sus iones seleccionados, abarcando así un rango de masas más amplio.

Para cuatro plaguicidas seleccionados, *chlorpropham, p,p'-DDE, malathion* e *isodrin*, se estudió esta deriva instrumental y la corrección de sus respuestas mediante el IS, en una secuencia de 19 análisis consecutivos del mismo vial conteniendo dichos plaguicidas y el TPP. En la **Figura 4** se observa la variación sufrida, sin llevar a cabo ninguna corrección; corrigiendo los iones de cuantificación (*Q*) y confirmación (*q*) mediante el ion típico del TPP (*m/z* 326.0708); o bien corrigiendo las respuestas de los analitos con los diferentes iones seleccionados del TPP (*m/z* 326.0708, 233.0368 y 170.0732), en función de su proximidad con los iones *Q* y *q*.

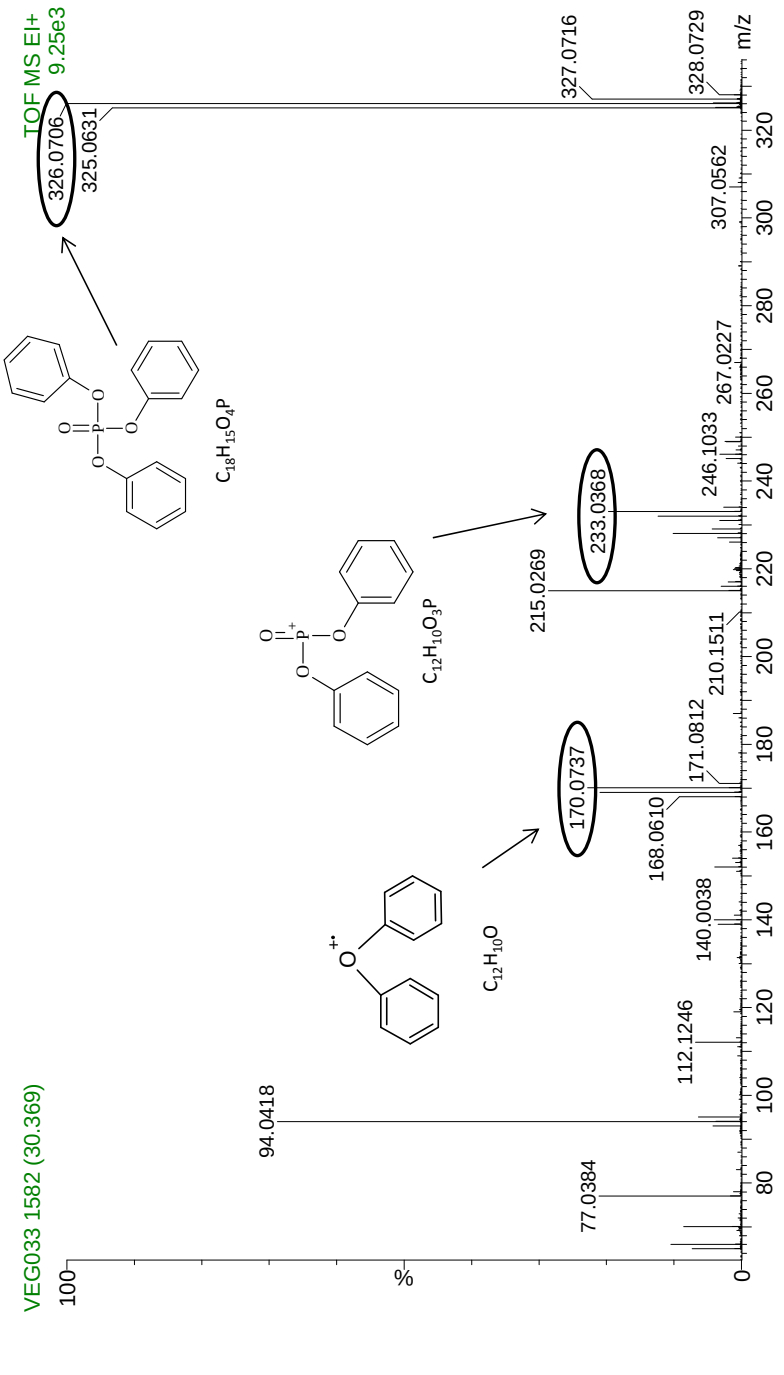


Figura 3. Espectro de masas experimental de EI medido en masa exacta para el *triphenyl phosphate* (500 µg/L) junto con la composición elemental y sus posibles estructuras

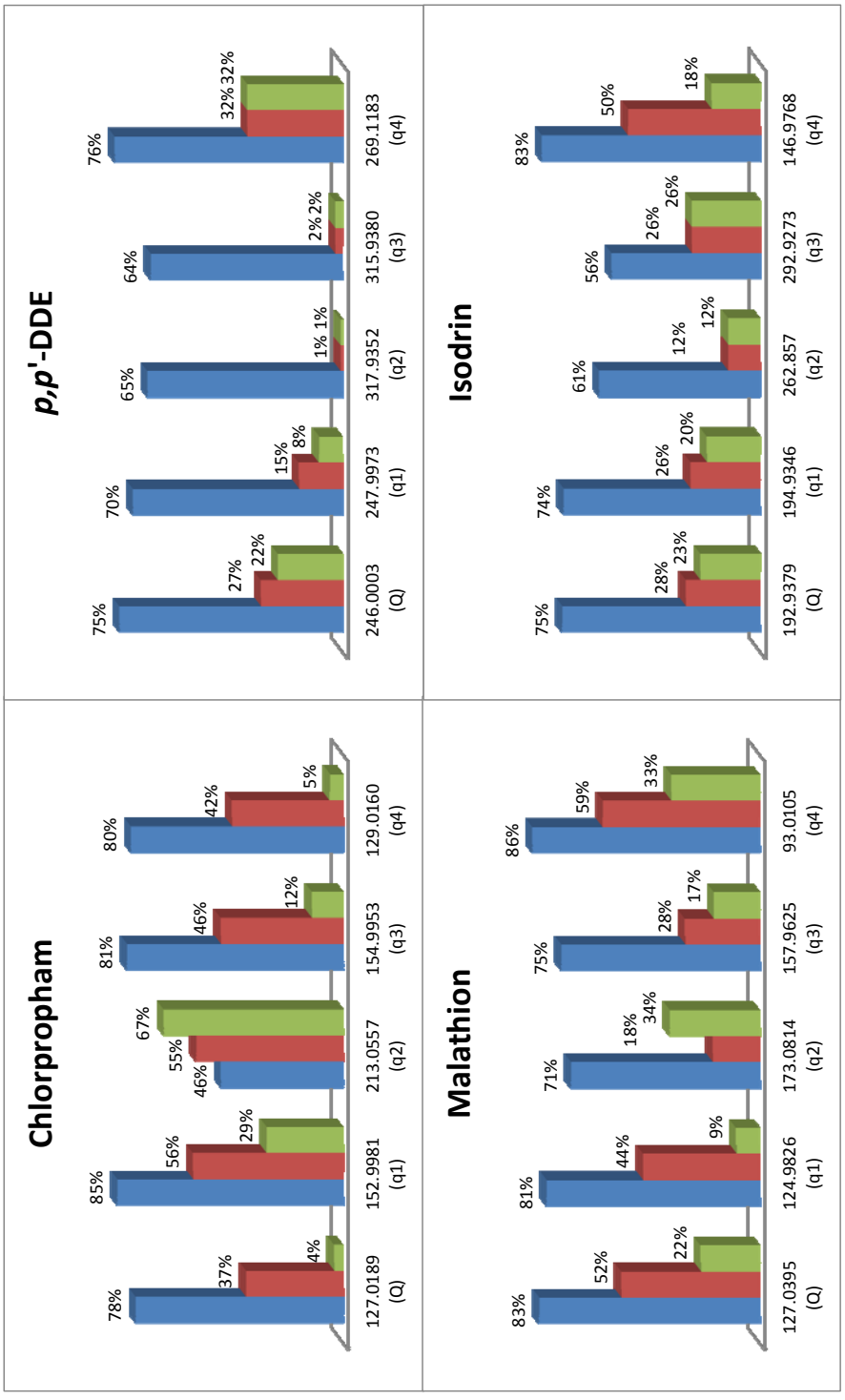


Figura 4. Graficas de las variaciones (en valor absoluto) de los iones característicos de 4 plaguicidas seleccionados para el área sin corregir (en azul), el área corregida con el *m/z* 326.0708, característico del TPP (rojo) ; y el área corregida con los iones *m/z* 170.0732 (ion característico de *m/z* <190), 233.0368 (ion característico *de m/z* entre190-250) o 326.0708 (ion característico de *m/z*> 250) del TPP, dependiendo del valor de su ion característico utilizado (verde)

Las variaciones representadas en las gráficas de la **Figura 4** se han calculado del mismo modo, con la **Ecuación 1** (aunque se muestra en valor absoluto), con los valores de las áreas iniciales y finales, con respecto a la inicial. En primer lugar, de color azul, se observa la variación de la señal sufrida por los diferentes iones, sin ningún tipo de corrección; de color rojo, las variaciones cuando son todos corregidos mediante el mismo ion característico del IS, independientemente de su masa; y por último, en color verde, la variación sufrida con la corrección con distintos iones del IS en función de su proximidad a la *m/z* del analito.

Se observa que todos los compuestos estudiados presentan una deriva considerable de la señal, traducida en una gran variación de las áreas desde el inicio hasta el final, lo que evidencia la necesidad de una corrección en sus respuestas.

En segundo lugar, se observa para los iones Q y q de cada plaguicida, que la corrección usando el ion característico del TPP (*m/z* 326.0708) resulta satisfactoria, con una minimización notable de la variación de la señal. Cabe destacar que para una gran mayoría de los iones de los plaguicidas estudiados, la desviación se vio reducida incluso a la mitad.

Por último, si para la corrección se selecciona el ion del TPP con un valor de *m/z* más cercano al de los iones Q y q, para muchos de los casos la variación se ve aún más reducida; llegando incluso a valores menores de 10 %, lo que asegura que la cuantificación no se verá tan afectada por la deriva instrumental. Esta mejora constata la necesidad de utilizar distintos iones del IS para la correcta corrección de la deriva instrumental, con el fin de llevar a cabo los análisis cuantitativos de forma satisfactoria.

En conclusión, para minimizar este problema instrumental se utilizaron diferentes iones del TPP para la corrección de la señal, dependiendo del *m/z* de los iones de cuantificación de los compuestos a estudiar, según el siguiente esquema:

- ✓ TPP *m/z* 170.0732, para aquellos compuestos cuyo ion de cuantificación es menor de *m/z* 190
- ✓ TPP *m/z* 233.0368, para aquellos compuestos cuyo Q se encuentra entre *m/z* 190 y 250
- ✓ TPP *m/z* 326.0708, para aquellos compuestos cuyo Q es mayor de *m/z* 250

Trabajando de este modo y llevando a cabo los estudios de validación, los resultados obtenidos para los compuestos seleccionados en las matrices a estudio (naranja, manzana, zanahoria y tomate) resultaron ser adecuados, tal como se muestra en la ***Tabla 2*** del **Artículo científico 3**. La única excepción fue para algunos compuestos en los que la baja sensibilidad condujo a problemas de confirmación y/o cuantificación, por lo que no quedaron debidamente validados. Sin embargo, el resto de compuestos presentaron resultados adecuados tras corregirse los errores de la deriva y cuantificar con calibrado en matriz, ya que de acuerdo con estudios anteriores (en el **Artículo científico 2**) era necesario este tipo de cuantificación para contrarrestar el indeseable efecto matriz.

En este trabajo, al utilizar un analizador TOF MS, con adquisición en modo *full scan*, no fue necesaria una etapa previa de optimización del método de adquisición de masas, por lo que la labor fue más sencilla que en trabajos realizados por QqQ donde se requería la optimización previa de las transiciones MS/MS. Por el contrario, para el desarrollo de metodología basada en este tipo de instrumentos, se requiere un minucioso y complejo tratamiento de datos. Así, en

la aproximación *target* (*TargetLynx*) se seleccionan hasta 5 iones (fragmentos) característicos del espectro de masas, medidos en masa exacta, de cada compuesto. Es imprescindible disponer de un patrón de referencia para observar su espectro de masas experimental y escoger la masa exacta de cada ion fragmento, aunque no siempre es fácil discernir a que composición elemental corresponde cada uno de ellos. Para ayudar en esta elección, se utilizaron varias aplicaciones incluidas en el software de *MassLynx*, como *MassFragment* o *Elemental composition.* La primera de ellas, permite conocer los fragmentos más probables procedentes de un determinado compuesto, con su correspondiente composición elemental, así como su posible estructura; información que se puede comparar con el espectro de masas adquirido, calculando sus errores de masa. La segunda aplicación ofrece posibles composiciones elementales para una masa exacta establecida, con los errores de masa de cada opción comparando con el espectro de masas experimental.

En el análisis en modo *target*, los parámetros necesarios para la correcta identificación y cuantificación de los compuestos fueron la presencia, como mínimo, de dos iones al tiempo de retención esperado junto con el cumplimiento de las *Q/q* ratios (en los límites establecidos).

Esta misma evaluación de los errores de masa y de las posibles estructuras de los fragmentos también se llevó a cabo en el análisis *non-target* tras la obtención mediante *ChromaLynx XS* de una lista de candidatos positivos, mostrando la composición elemental de cada uno de los iones fragmento así como su error de masa. En el caso de matrices complejas, como vegetales, la lista de candidatos suele ser muy extensa, ya que junto con los plaguicidas o compuestos orgánicos de interés, pueden aparecer también muchos componentes de la matriz.

Es necesario revisar la lista de compuestos presentes de forma manual y decidir qué compuestos son relevantes y eliminar los restantes, reduciendo en gran medida la lista inicial. Uno de los inconvenientes que presenta esta aproximación es su reducida sensibilidad en comparación con el método *target*. Esto se tradujo en que no todos los positivos detectados mediante la búsqueda dirigida aparecieron después incluidos en la lista creada de forma automatizada en el método *non-target*.

En la aproximación *non-target,* tras la aplicación del método de deconvolución a las muestras analizadas, uno de los positivos encontrados fue el fungicida *folpet* en una muestra de manzana. Los resultados obtenidos con el software *ChromaLynx XS* sugerían este compuesto, junto otros que presentaban un espectro de masas semejante, aunque *folpet* era el candidato con mayor porcentaje de similitud (*match*) con el espectro de masas teórico. Se estudiaron con mayor detalle los fragmentos obtenidos y se obtuvieron los errores de masa, con el fin de identificar con mayor fiabilidad este compuesto En la **Figura 5** se observa el espectro de masas para este fungicida detectado en la muestra, así como los errores de masa y las estructuras sugeridas, todas ellas compatibles con la estructura química del candidato. Con ello el compuesto quedó identificado de forma tentativa en la muestra de manzana. Tras la adquisición del patrón de referencia y por comparación con su Rt y espectro de masas, se pudo confirmar de forma inequívoca.

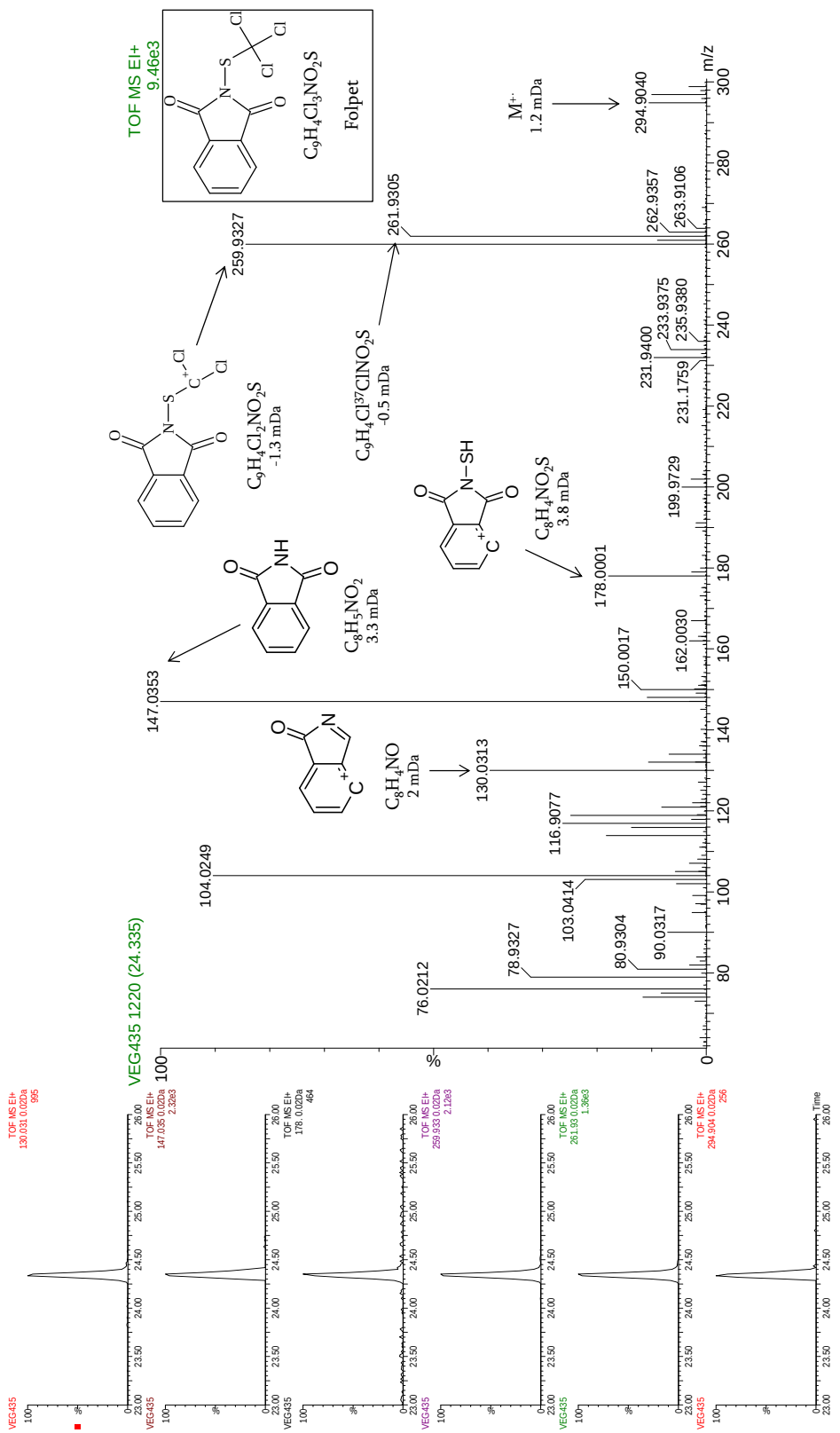


Figura 5. GC-TOF MS (nw-XIC 0.02 Da) ilustrando la detección del fungicida *folpet* en manzana. Espectro de masas experimental EI con las estructuras propuestas para los iones fragmento más abundantes junto con sus errores de masa experimentales.

En este trabajo, el uso de GC-TOF MS ha permitido llevar a cabo la detección, identificación y cuantificación de los 55 plaguicidas incluidos en el método *target* de forma satisfactoria. Además, se ha demostrado la ventaja que supone la adquisición completa del espectro de masas con medidas de masa exacta al aplicar un método automatizado de detección para compuestos no seleccionados (*non-target analysis*) que, de otro modo, hubieran pasado desapercibidos en un método *target*.

En el **Artículo científico 4** se describe la metodología de trabajo desarrollada para el análisis de residuos de plaguicidas en matrices vegetales incluyendo diferentes aproximaciones dependiendo de la información previa disponible y de los objetivos propuestos (*target* o *post-target*). La metodología desarrollada está basada en el uso de la cromatografía de gases acoplada a la espectrometría de masas con analizador cuadrupolo-tiempo de vuelo mediante la novedosa fuente de ionización química a presión atmosférica (GC-(APCI)QTOF MS).

La primera aproximación se basó en la realización de un análisis de tipo *screening* aplicado a 34 muestras de frutas y verduras. Junto con las muestras, en la secuencia de análisis se inyectaron también los calibrados en matriz respectivos, con la finalidad de poder realizar estudios cuantitativos, en caso necesario. También se utilizaron estos datos para actualizar en cada secuencia los tiempos de retención y los valores de *q/Q ratios*.

Al ser APCI una fuente de ionización suave, se obtiene para la mayoría de los compuestos un espectro de masas donde el ion molecular o la molécula

protonada son los mayoritarios en la función de baja energía de colisión (*low energy*, LE). Con el fin de favorecer la formación de la molécula protonada, se introdujo agua, como modificador, en la fuente de ionización. Fomentando la presencia de la molécula protonada, la sensibilidad mejora debido a la mayor abundancia del ion monitorizado (se minimiza la formación de $M^{+\cdot}$), y además el screening se puede dirigir hacia la búsqueda de $[M+H]^+$, que sería el más abundante en los compuestos investigados. Gracias al uso de un analizador híbrido QTOF, se puede obtener una segunda función, de alta energía (*high energy*, HE), aplicando una rampa de energía de colisión (10-40 eV) de modo simultáneo a la función de baja energía. En esta segunda función HE, se promueve la fragmentación y es habitual la presencia de iones fragmento en el espectro de masas.

Así, con estas dos funciones, se desarrolló el método de tratamiento de datos para procesar las muestras analizadas. Se llevó a cabo la búsqueda de una lista de compuestos *target*, de los cuales como previamente se disponía del patrón de referencia, ya se habían establecido los tiempos de retención, así como los iones de cuantificación y confirmación característicos para cada compuesto. Con el método creado se examinó la presencia de 130 plaguicidas, en base a la detección de un ion en la función de baja energía (típicamente la molécula protonada, *Q*) con error de masa < 5 ppm a un determinado tiempo de retención, junto con al menos un ion fragmento (*q*, típicamente en la función de alta energía) con un error < 5 ppm, al mismo tiempo de retención. Una vez se identificaron los candidatos, se evaluó su *q/Q ratio* para poder confirmar su identidad. Esta metodología se aplicó para el *screening* de 34 muestras reales, incluyendo matrices como fresa, lechuga, zanahoria, manzana, calabacín, pimiento rojo, naranja y tomate. Aquellos plaguicidas detectados que cumplieron

los parámetros confirmatorios pudieron ser correctamente identificados. A pesar de que este sistema ofrece muy buenas prestaciones, la falta de sensibilidad para ciertos compuestos presentes a muy bajos niveles pudo dar lugar a falsos negativos. Aunque parecía clara la presencia de ciertos compuestos en algunas muestras, sin embargo, no se pudieron reportar como positivos al no cumplir todos los requisitos confirmatorios, algunas veces debido a la ausencia del (menos abundante) ion fragmento, y en otras por el no cumplimiento del *ion ratio*.

Entre los compuestos detectados en el *screening* pero no confirmados, se presentaron tres situaciones diferentes que podrían estar relacionadas con problemas de sensibilidad o selectividad del ion de confirmación seleccionado y/o debido a que la concentración del compuesto detectado estaba cercana al límite de detección, tal como se muestra en la ***Tabla S1*** del **Artículo científico 4**. El primer caso (**Figura 6**) es cuando no se observa el ion fragmento en la función de alta energía pero sí el ion molecular o la molécula protonada en la de baja energía, al presentar una notable diferencia de sensibilidad entre ellos. Estos son los casos mostrados en la **Figura 6**, donde para *fenarimol*, *metalaxyl* y *procymidone* el ion de cuantificación aparece de forma clara, con buena relación *S/N*, pero el ion de confirmación no se ve o apenas se intuye, en la función de alta energía.

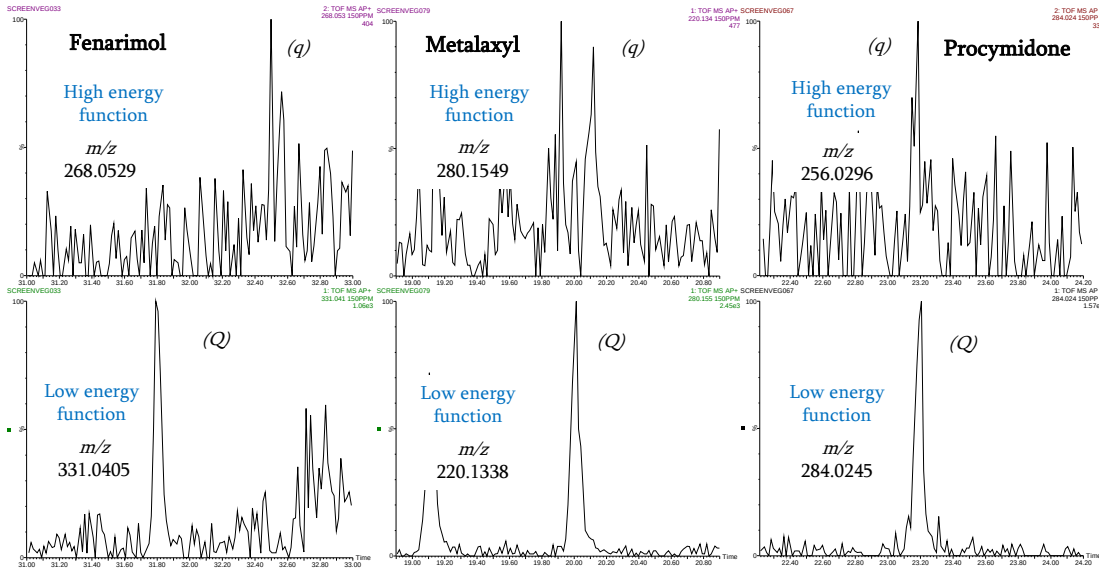


Figura 6. Cromatogramas obtenidos mediante GC-APCI (QTOF) MS (nw-XIC 150 ppm) mostrando posibles positivos detectados: *fenarimol* en naranja, *metalaxyl* en pimiento rojo y *procymidone* en calabacín

Otra situación que se puede encontrar, que también parece estar propiciada por la baja sensibilidad para algunos compuestos, es la presencia de iones fragmento pero con errores de masa superiores a 5 ppm, límite establecido para dar por satisfactoria la identificación. Esta situación se vería favorecida al aumentar el ruido de fondo del cromatograma, afectando a la hora de obtener un espectro de masas limpio, y con bajos errores de masa. Este el caso del insecticida piretroide *lambda-cyhalothrin*, mostrado en la **Figura 7**, con los picos cromatográficos extraídos en una ventana estrecha de masa (nw-XIC) de 150 ppm, en las funciones de alta y baja energía, junto con sus espectros de masas. El error de masa para la molécula protonada en la función de baja energía fue inferior a 5 ppm, pero en la función de alta energía el error del fragmento usado como ion de confirmación estuvo por encima del límite establecido.

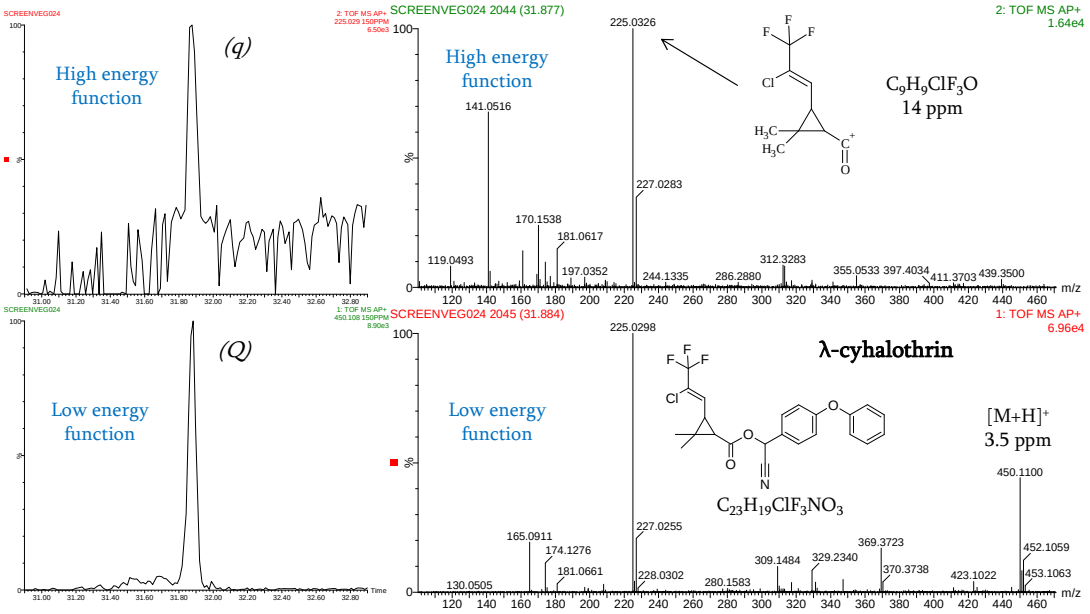


Figura 7. Cromatogramas y espectros de masas obtenidos mediante GC-APCI (QTOF) MS (nw-XIC 150 ppm) para un potencial positivo de *λ-cyhalothrin* en naranja

Por último, otra situación correspondió a aquellos casos en los que, tras calcular las relaciones iónicas *q/Q* de los compuestos detectados y compararlos con el valor obtenido con el patrón de referencia (preparado en la misma matriz), se observó que las desviaciones excedían los límites establecidos por la guía SANCO/12571/2013, usada en este trabajo para la confirmación de la identidad de los positivos.

Así, en la **Figura 8**, se muestra el ejemplo de un potencial candidato a positivo de *fenhexamid* encontrado en una muestra de calabacín. Para este candidato, se observó la presencia de los dos iones, *Q* y *q*, con errores de masa < 5 ppm; pero los valores de *q/Q* diferían un 32 % entre muestra y patrón preparado en matriz. La guía SANCO establece que para un análisis realizado con una técnica como QTOF MS, la tolerancia máxima permitida es de ± 30 %; por lo que,

aunque de forma leve, el valor de *q/Q del* positivo sobrepasaba los valores aceptables. Por ello, aplicando un criterio riguroso, no quedaría correctamente identificado.

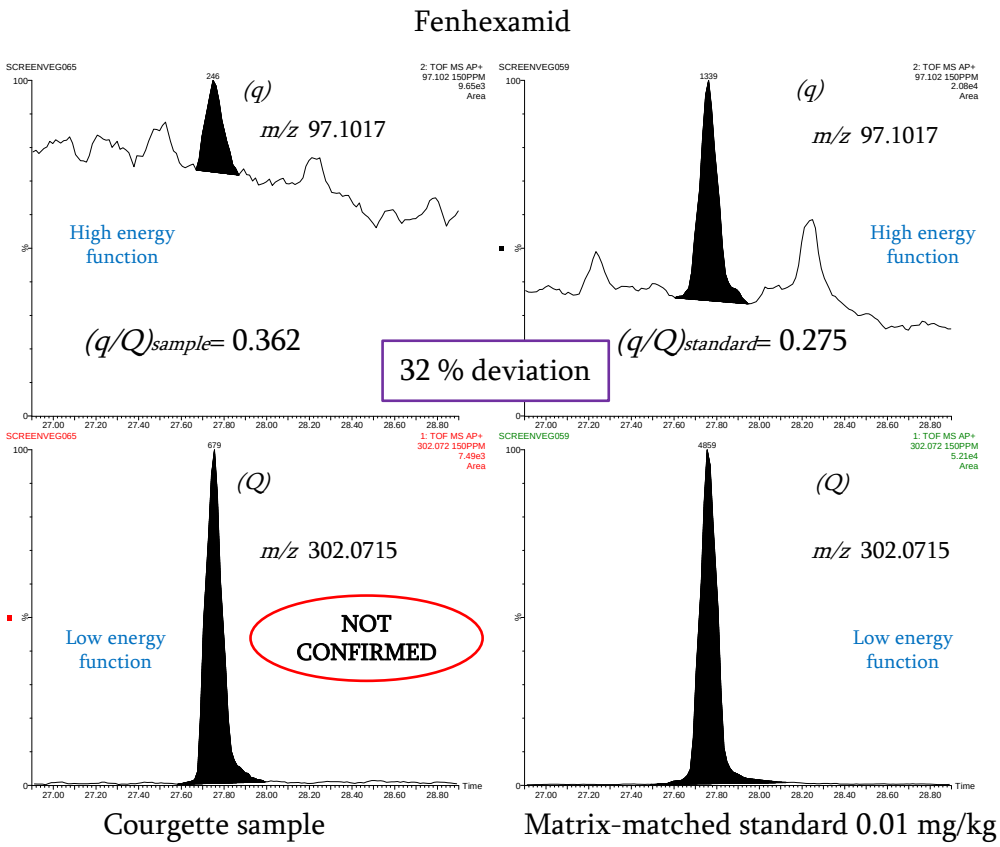


Figura 8. Cromatogramas obtenidos mediante GC-APCI (QTOF) MS (nw-XIC 150 ppm) para un positivo de *fenhexamid* en una muestra de calabacín. Comparación con el patrón de referencia preparado en la misma matriz

En las tres situaciones descritas anteriormente, de un modo estricto, no se deberían reportar las detecciones como positivas, a pesar de que, en ocasiones, parecía evidente la presencia del compuesto en las muestras analizadas.

Solo los compuestos adecuadamente confirmados, fueron seleccionados en una segunda fase con el fin de evaluar el potencial del QTOF para análisis cuantitativo. Para ello, se realizó una validación en 5 matrices características de los diferentes tipos de muestras analizadas (naranja, tomate, lechuga, zanahoria y manzana) a dos niveles de concentración, 0.01 y 0.1 mg/kg, por triplicado. Según se observa en la ***Tabla 2*** del **Artículo científico 4,** los resultados fueron satisfactorios para la mayoría de las combinaciones analito/matriz, alcanzando unos LOQs comúnmente por debajo de los MRL establecidos por la Unión Europea (Regulation (EC) No 396/2005). De forma gráfica, se pueden apreciar en la ***Figura S1*** del **Artículo científico 4**, las recuperaciones obtenidas para las 5 matrices, a los dos niveles de concentración. Tras quedar debidamente validado el método para los analitos detectados en las muestras, gracias a la inyección previa de calibrados en matriz con los 130 plaguicidas junto con las muestras, se pudo realizar una cuantificación de los positivos, sin la necesidad de volver a reinyectar las muestras.

En una última etapa, se llevó a cabo un *screening* ampliado hasta más de 400 plaguicidas haciendo uso de los datos de las muestras inicialmente adquiridos. Esta aproximación se pudo realizar gracias al uso de QTOF MS, por la completa adquisición de la información espectral, ya que aparte de realizar un *screening* previo y cuantificar los analitos en una única inyección, existe la posibilidad de buscar otros compuestos adicionales, con la creación de una lista de plaguicidas tan larga como se desee (análisis *post-target*). Para los nuevos compuestos investigados, se realizó la búsqueda tanto del ion molecular como de la molécula protonada, ya que al no disponer previamente del patrón no se conocían sus iones fragmento característicos, ni su Rt. En el caso de potenciales positivos, se realizaron estudios detallados de sus espectros de masas (baja y alta energía) y

gracias a las aplicaciones disponibles del software de tratamiento de datos se pudieron identificar tentativamente. La confirmación inequívoca se pudo efectuar en una fase posterior, adquiriendo el patrón de referencia, por comparación con su espectro de masas así como con su Rt. En la ***Tabla 4*** del **Artículo científico 4** se muestran los compuestos adicionales detectados con el *screening* ampliado.

Como ejemplo, en la **Figura 9** se observa un positivo (no confirmado) del fungicida *bupirimate*, que fue solo tentativamente identificado, ya que aunque no se disponía del patrón de referencia, cumplía una serie de requisitos como la presencia de la molécula protonada con un error < 5 ppm, así como distintos fragmentos con estructuras posibles también con errores de masa < 5 ppm.

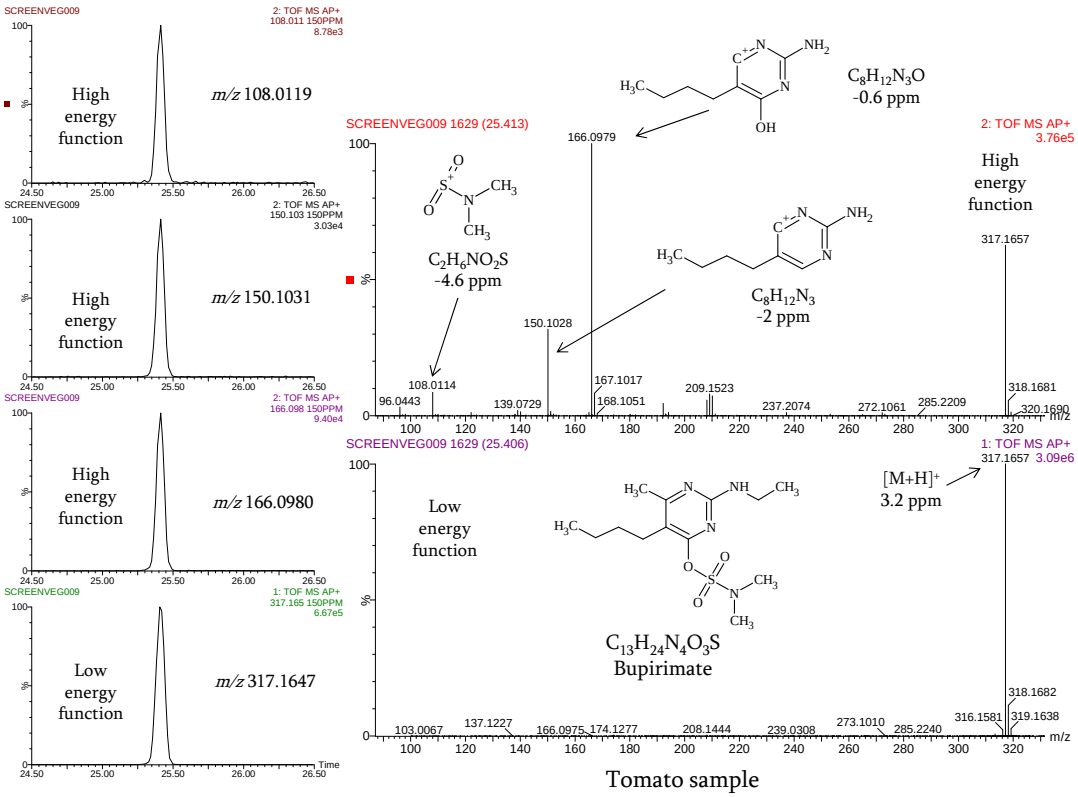


Figura 9. Cromatograma obtenido con GC-(APCI)QTOF MS (nw-XIC 150 ppm) relativo a la detección del fungicida *bupirimate* en una muestra de tomate

A modo de conclusión, cabe destacar con todas las aproximaciones que se han presentado relativas al uso de GC-(APCI)QTOF MS, que esta técnica es una excelente herramienta analítica en el campo del *screening* de plaguicidas, incluso cuando no se dispone del patrón de referencia. Evidentemente, el trabajo con patrones es más sencillo y rápido, y permite confirmar de forma inequívoca la presencia de los positivos detectados. Por otro lado, tras una validación cuantitativa del método para los positivos encontrados en el *screening*, sin necesidad de efectuar análisis adicionales de las muestras, se puede llegar a cuantificar los compuestos si en la secuencia de análisis se introdujeron calibrados en matriz para estos plaguicidas, así como una muestra control fortificada a 0.05 mg/kg.

Un aspecto interesante, común a ambos trabajos presentados, es el relativo a las tolerancias permitidas en las relaciones iónicas (*q/Q* o *Q/q*). Se trata de un tema controvertido al ser un parámetro confirmatorio por el cual algunos compuestos detectados pueden no reportarse, al no cumplirse el criterio de tolerancia máxima, llegando a reportar falsos negativos. Algunas de las guías que regulan estos parámetros se han ido actualizando con el paso del tiempo para las nuevas instrumentaciones, teniendo en cuenta los diversos modos de trabajo: MS/MS, MS^E, *scan*, SIM, etc. La tolerancia máxima admitida en las relaciones iónicas se recoge en la versión más actualizada de la guía SANCO (European Commission (2013) SANCO/12571/2013) donde con respecto a versiones anteriores, se han unificado las tolerancias permitidas a un único valor de ± 30 % para sistemas de QqQ MS, IT MS y sistemas híbridos. Este cambio de criterio se ha producido durante el periodo de realización de esta **Tesis**.

Las tolerancias de las relaciones iónicas usadas en los trabajos presentados en este **Capítulo 3** se han basado en la guía SANCO, la cual ha variado dependiendo de la fecha de su realización. Así en el **Artículo científico 3**, se consideró la guía SANCO/10684/2009 (European Commission (2009) SANCO/10684/2009) con las mismas tolerancias permitidas que en la posterior SANCO/12495/2011 (European Commission (2011) SANCO/12495/2011). En el **Artículo científico 4** se siguió la más reciente, la SANCO/12571/2013 (European Commission (2013) SANCO/12571/2013), con los cambios ya reflejados en cuanto a la mencionada unificación. Actualmente está también vigente la decisión de la comisión europea 657/2002/CE (Commission Decision 2002/657/EC), donde las tolerancias permitidas son diferentes a la guía SANCO actual, y, a su vez dependientes de la relación de intensidades.

En nuestra experiencia, la aplicación del criterio de *q/Q* ratio es un tema controvertido. Por ello, en el caso de que alguna *q/Q* ratio quedara fuera de los límites establecidos, sería necesario realizar una evaluación más detallada de los espectros de masas, teniendo en cuenta los modelos isotópicos, la exactitud de masa, estudiando la posibilidad de usar otros iones fragmentos, etc., con el fin de tomar la decisión final, ya que seguramente se trate de un positivo.

Tabla 1. Comparación (EI) TOF MS vs (APCI) QTOF MS

Analizador MS	Adquisición	Modo trabajo: *Target*	Modo trabajo: *Non-target/post target*
TOF (EI)	***Full scan***	- Monitorización hasta 5 iones (fragmento) característicos - nw-XIC: 0.02 Da - Confirmación: 2 iones y *Q/q* ratio al Rt adecuado - Detección, identificación y cuantificación	- Búsqueda desconocidos *(non-target)*, mediante software deconvolución - Ofrece lista automatizada de candidatos positivos por comparación librería teórica EI - Estudio detallado de cada positivo - Confirmación: comparación patrón de referencia (Rt y espectro de masas) - Detección e identificación
QTOF (APCI)	-***Low energy function*** -***High energy function*** **MS^E**	- nw-XIC: 150 ppm - Confirmación: 1 ion ($M^{+\cdot}$ o $[M+H]^+$) en LE y 1 fragmento en HE, error masa < 5 ppm al Rt adecuado - Detección, identificación y cuantificación	- Ampliación lista *screening* (*post target*) - Ofrece lista de candidatos positivos por búsqueda de $M^{+\cdot}$ o $[M+H]^+$ en LE - Estudio detallado de cada positivo - Confirmación: comparación patrón referencia (Rt y espectro de masas) -Detección e identificación

A modo de resumen del **Capítulo 3**, se incluye la **Tabla 1** en donde se ilustra el elevado potencial que tienen ambas metodologías desarrolladas para el análisis de residuos de plaguicidas en matrices vegetales. Además de permitir un análisis dirigido hacia una serie de compuestos *target*, existe la ventaja de la posterior detección de compuestos orgánicos, mediante aproximación *non-target* o *post-target* gracias a la adquisición completa de toda la información espectral de la muestra. Así, cuando existe un indicio de un candidato a positivo, se pueden realizar estudios más detallados sobre sus errores de masa, fragmentación, etc. y confirmar su identidad tras la adquisición del patrón de referencia.

CAPÍTULO 4

INVESTIGACIÓN DE COMPUESTOS VOLÁTILES ORGÁNICOS MEDIANTE CROMATOGRAFÍA DE GASES ACOPLADA A LA ESPECTROMETRÍA DE MASAS (TRIPLE CUADRUPOLO Y TIEMPO DE VUELO)

1. Introducción

La investigación sobre la presencia de compuestos orgánicos volátiles (VOCs) de diferentes familias químicas y en matrices muy distintas comporta el desarrollo de metodologías analíticas diseñadas de forma específica para su adecuada determinación. Estos compuestos presentan una elevada presión de vapor y una baja solubilidad en agua que los hacen tener comportamientos diferentes con respecto a otros compuestos, como los plaguicidas, ya estudiados en capítulos anteriores. Los VOCs engloban un elevado número de compuestos, incluyendo hidrocarburos aromáticos, parafínicos, olefínicos y pueden contener oxígeno, nitrógeno, azufre y/o haluros. Presentan un elevado interés en diferentes disciplinas, como la alimentación, en sabores y fragancias, así como en ciencias médicas, farmacéuticas, forenses y medioambientales. En el presente trabajo, se han estudiado los VOCs en dos tipos bien diferentes de matrices: aguas y bulbos de la flor del tulipán, como se comentará más adelante.

En cuanto a la metodología analítica, se requiere un método de extracción y/o pre-concentración que separe y retenga todos los analitos volátiles. La mayor volatilidad de los compuestos descarta la posibilidad de utilizar muchas técnicas de extracción comúnmente empleadas. Además, los VOCs en matrices medioambientales se encuentran en muy bajas concentraciones, con lo cual es necesaria una etapa de pre-concentración para conseguir la sensibilidad adecuada con la técnica utilizada para el análisis, comúnmente GC-MS.

Entre los diferentes procedimientos existentes, en el presente trabajo se han utilizado métodos de extracción basados en la técnica de espacio de cabeza o *headspace* (HS), que incluyen tanto el modo estático como dinámico. El objetivo es atrapar los analitos de la muestra que se encuentran expandidos en la fase gaseosa, y atraparlos en un sorbente donde quedan retenidos. Éstos son posteriormente desorbidos mediante elución en solvente o térmicamente (Demeestere, 2007; Chary, 2012). Una revisión de la bibliografía existente revela que tanto HS-SPME como la técnica de purga y trampa (P&T) son las más utilizadas para la extracción de compuestos volátiles, tanto en matrices acuosas (Canuti, 2009) como sólidas (Beltrán, 2006; Silva, 2009), porque proporcionan excelentes recuperaciones para un amplio rango de compuestos. Así P&T es una de las técnicas más frecuentes como método de pre-concentración en la determinación de VOCs en aguas, ya que presenta buena precisión y posibilidades de automatización. Por otra parte, SPME presenta ciertas ventajas en cuanto a la facilidad y rapidez de extracción, además de realizar la pre-concentración directa de los analitos para conseguir la sensibilidad deseada. También suele tener un menor coste por muestra ya que con esta modalidad de HS no se requiere la utilización de ningún disolvente orgánico para su elución, pues la desorción de la totalidad de los analitos retenidos se lleva a cabo térmicamente, directamente en

el puerto de inyección del cromatógrafo de gases (Beltrán, 2006; Chary, 2012; Demeestere, 2007).

En el **Artículo científico 5** se aborda la extracción de una matriz acuosa usando para ello la técnica de HS estática, en la cual la extracción se mantiene hasta que se alcanza el equilibrio de partición entre la fase acuosa, el espacio de cabeza y el absorbente. En este caso, se utiliza la técnica SPME en modo HS con posterior desorción térmica, directamente en el sistema de inyección del GC-MS. Por otro lado, en el **Artículo científico 6** se trata con una matriz poco usual y más delicada (bulbos de la flor del tulipán), ya que aparte de lograr la adecuada extracción de los analitos, se deben mantener las condiciones originales intactas. En este caso, se utiliza una técnica de HS dinámica, donde todos los analitos presentes en la matriz son exhaustivamente extraídos. Para esta matriz se ha utilizado la técnica de P&T, con los bulbos situados en el interior de un sistema hermético donde una corriente de N_2 arrastra los analitos hacia un sorbente, realizando una posterior elución de los analitos atrapados con un solvente.

En cuanto a la problemática de contaminantes orgánicos volátiles en aguas, la directiva de 2013 sobre política de aguas (Directive 2013/39/UE) regula algunos VOCs que se consideran prioritarios en lo relativo a contaminación de aguas superficiales, como benceno, cloroalcanos C_{10-13}, 1,2-dicloroetano, diclorometano, triclorobencenos y triclorometano. Los niveles máximos permitidos en aguas varían dependiendo de cada tipo de agua y cada analito, pero todos se encuentran en el rango de μg/L. Por ello, se necesita una técnica de detección (junto con la de extracción) que consiga alcanzar estos bajos niveles y que sobretodo permita cuantificar de forma precisa el contenido de estos contaminantes en las aguas. En el **Artículo científico 5**, con el propósito de analizar 23 VOCs en diversos tipos de

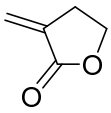
aguas, se ha elegido como técnica de extracción la SPME en modo HS, con la posterior desorción directamente al inyector del sistema GC-MS/MS QqQ, en modo EI.

La matriz de bulbos de tulipán fue seleccionada en el marco de un amplio proyecto de investigación cuyo objetivo era conocer la identidad de los compuestos volátiles que emiten estos bulbos bajo unas determinadas condiciones. Este trabajo se realizó en colaboración con el *Institute for Biodiversity and Ecosystem Dynamics* (IBED) de la *Universiteit van Amsterdam* (UvA) a raíz de una estancia realizada en este centro por parte de la doctoranda. El campo de trabajo de los tulipanes es de gran importancia en los Países Bajos, por sus repercusiones económicas y sociales. Son muchos los estudios que persiguen mejoras en la producción de bulbos y en el crecimiento correcto de las flores, así como en el control de plagas y enfermedades con métodos respetuosos con el medio ambiente.

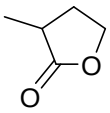
Se sabe que las plantas tienen diferentes mecanismos de defensa. Entre ellos, se encuentra la emisión de determinados compuestos químicos, que utilizan como respuesta a los daños sufridos en hojas o raíces, causados por insectos herbívoros o patógenos. Los compuestos que emiten las plantas pueden actuar como comunicación entre la planta y los insectos, las plantas de alrededor y ciertos patógenos, y pueden ser producidos por cualquier parte de la planta, desde la raíz hasta las hojas. Si la planta no está dañada puede también emitir estos compuestos pero con una menor intensidad. Los compuestos volátiles que pueden emitirse desde la parte floral y vegetativa de la planta pertenecen a tres clases principales de compuestos: terpenoides, fenilpropanoides/bencenoides y aldehídos C_6. Adicionalmente, existen otros compuestos volátiles orgánicos, considerados

fitohormonas, como el metil salicilato o el metil jasmonato que actúan frente al ataque de herbívoros o daños en la planta, como una importante señal en la comunicación, para optimizar la respuesta de defensa de la planta (Das, 2013).

Aunque en la bibliografía se encuentran numerosas referencias, con listas de compuestos presentes en diferentes tipos de plantas y flores (Kessler, 2013; Estell, 2013), sin embargo pocos investigadores han centrado sus estudios en los bulbos de tulipán. El **Artículo científico 6** se centra en los VOCs que emiten los bulbos de la flor del tulipán, justo en la época posterior a su recogida durante la etapa de almacenamiento (hasta su comercialización o siembra). El objetivo es conocer la identidad de los compuestos emitidos por los bulbos. Debido a la falta general de información sobre su identidad, es necesaria una técnica de análisis que suministre información espectral completa sobre las muestras para después realizar una búsqueda *non-target* de los compuestos presentes. Para ello, en este trabajo se ha hecho uso de GC-MS con analizador de TOF de alta resolución. Asimismo, y gracias a la información recogida en bibliografía (Lubbe, 2013; Aratchige, 2004), se han incluido también dos compuestos como posibles analitos *target* presentes en los bulbos de tulipán: el *tulipalin A (α-methylene-γ-butyrolactone)* y otro de semejante estructura, el *α-methyl-γ-butyrolactone*, cuyas estructuras químicas se muestran en la **Figura 1**.



Tulipalin A



α-methyl-γ-butyrolactone

Figura 1. Estructuras de los dos compuestos *target* seleccionados, por su posible presencia en los bulbos de tulipán

La estrategia de trabajo seguida en el **Artículo científico 6** consistió en aplicar en primer lugar, un método *target* para estos dos compuestos, para después realizar una búsqueda de desconocidos (*non-target*) que pudieran ser también emitidos por los bulbos de tulipán. Tras confirmar la identidad de ciertos compuestos presentes y con el propósito de realizar una correcta cuantificación, se repitieron los análisis de los mismos extractos mediante GC-MS/MS QqQ, una vez adquiridos los patrones de referencia de los compuestos descubiertos en las muestras.

2. Artículo científico 5

Analytica Chimica Acta 704 (2011) 87–97

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Determination of volatile organic compounds in water by head space-solid-phase microextraction gas chromatography coupled to tandem mass spectrometry with triple quadrupole analyzer

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ARTICLE INFO

Article history:
Received 3 May 2011
Received in revised form 22 July 2011
Accepted 5 August 2011
Available online 11 August 2011

Keywords:
Volatile organic compounds
Gas chromatography tandem mass spectrometry
Triple quadrupole
Environmental water
Wastewater
Headspace solid-phase microextraction

ABSTRACT

In the present work, a rapid method with little sample handling has been developed for determination of 23 selected volatile organic compounds in environmental and wastewater samples. The method is based on headspace solid-phase microextraction (SPME) followed by gas chromatography coupled to tandem mass spectrometry (GC–MS/MS) determination using triple quadrupole analyzer (QqQ) in electron ionization mode. The best conditions for extraction were optimised with a factorial design taking into account the interaction between different parameters and not only individual effects of variables. In the optimized procedure, 4 mL of water sample were extracted using a 10 mL vial and adding 0.4 g NaCl (final NaCl content of 10%). An SPME extraction with carboxen/polydimethylsiloxane 75 μm fiber for 30 min at 50 °C (with 5 min of previous equilibration time) with magnetic stirring was applied. Chromatographic determination was carried out by GC–MS/MS working in Selected Reaction Monitoring (SRM) mode. For most analytes, two MS/MS transitions were acquired, although for a few compounds it was difficult to obtain characteristic abundant fragments. In those cases, a pseudo selected reaction monitoring (pseudo-SRM) with three ions was used instead. The intensity ratio between quantitation (Q) and confirmation (q) signals was used as a confirmatory parameter.

The method was validated by means of recovery experiments ($n = 6$) spiking mineral water samples at three concentration levels (0.1, 5 and 50 $\mu g\,L^{-1}$). Recoveries between 70% and 120% were generally obtained with relative standard deviations (RSDs) lower than 20%.

The developed method was applied to surface water and wastewater from a wastewater treatment plant and from a municipal solid-waste treatment plant. Several compounds, like chloroform, benzene, trichloroethylene, toluene, tetrachloroethylene, dibromochloromethane, xylenes and bromoform were detected and confirmed in all the samples analyzed.

Analytica Chimica Acta, 704, 87-97, ***2011***

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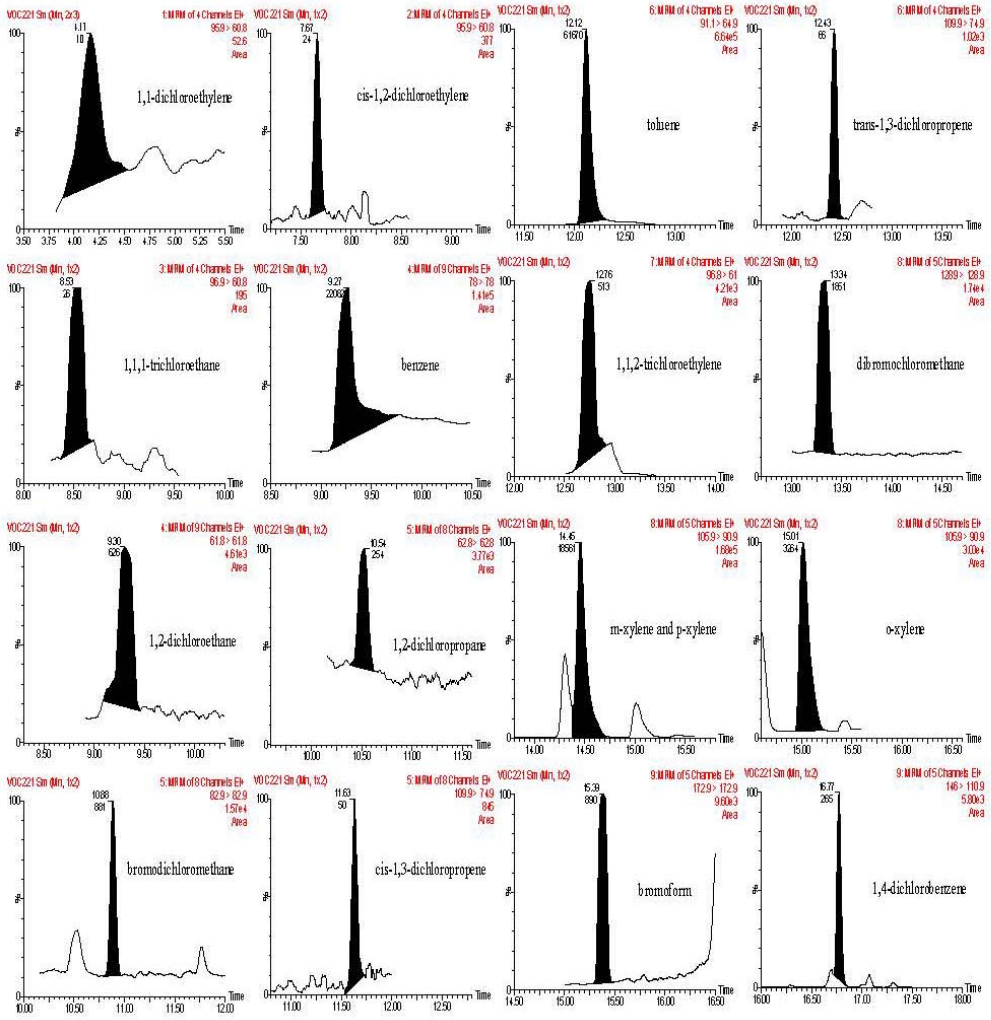
Graphical abstract



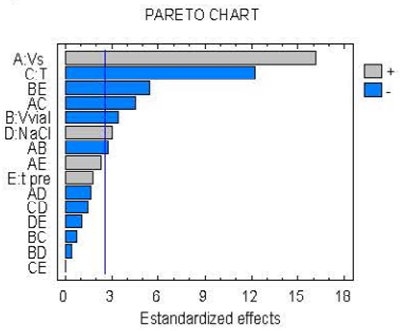
GC-MS/MS(SRM) chromatograms for all analytes validated at the fortification level 0.1 μg/L (only quantification transitions are shown)

1. SCREENING DESIGN

Experimental variables for screening design

essay	V_{sample}	V_{vial}	T[a]	% NaCl	$t_{pre-heating}$
1	-	+	+	-	+
2	+	-	+	-	+
3	-	-	-	+	-
4	-	+	-	+	+
5	+	-	-	+	+
6	0	0	0	0	0
7	0	0	0	0	0
8	+	+	-	+	-
9	0	0	0	0	0
10	+	+	+	-	-
11	-	-	+	-	-
12	+	-	-	-	-
13	0	0	0	0	0
14	0	0	0	0	0
15	+	+	-	-	+
16	-	+	+	+	-
17	-	+	-	-	-
18	-	-	-	-	+
19	+	-	+	+	-
20	0	0	0	0	0
21	-	-	+	+	+
22	+	+	+	+	+

-, minimum; 0, central point; + maximum



Parameters values:

Vsample→ 1-3 g
Vvial→ 10-20 mL
Temp.→ 35-65 ºC
Adit. NaCl→ 0-10 %
Tpre-heating→ 0-5 min

2. RESPONSE SURFACE DESIGN

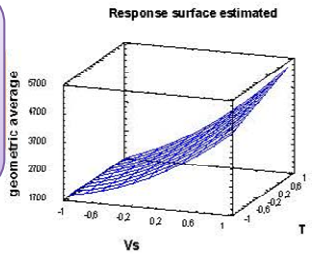
Experimental variables for response surface design

essay	V_{sample}	T[a]
1	-	-
2	+	+
3	0	0
4	0	-
5	+	0
6	0	0
7	0	+
8	0	0
9	+	-
10	0	0
11	-	+
12	-	0

-, minimum; 0, central point; + maximum

Parameters values:

Vsample→ 1-4 g
Temp.→ 20-50 ºC



Highlights

- Employing a statistical optimization improves results reducing experiments.
- Use of MS (QqQ) allows high sensitivity determination and improves identification capabilities.
- Using *Q*/*q* intensity ratios is a powerful tool to ensure compound identification.
- HS SPME GC–MS/MS method allows determination of VOCs in complex matrix water samples.

Keywords

Volatile organic compounds; Gas chromatography tandem mass spectrometry; Triple quadrupole; Environmental water; Wastewater; Headspace solid-phase microextraction

1. Introduction

Volatile organic compounds (VOCs) include a wide group of contaminants that have a high vapour pressure and low water solubility. According to the European Union [1], VOC is any organic compound having an initial boiling point less than or equal to 250 °C measured at a standard atmospheric pressure of 101.3 kPa. The most commonly known VOCs are chlorinated solvents like methylene chloride, chloroform, carbon tetrachloride, trichloroethylene, tetrachloroethylene, dibromochloromethane or 1,4-dichlorobenzene, among others, and fuel components, known as BTEX, like benzene, toluene or xylene. Some chlorinated solvents (trichloroethylene, tetrachloroethylene, methylene chloride, carbon tetrachloride and 1,2-dichloroethane) have proven to cause cancer at high doses in laboratory animals and are probable carcinogen for humans. Most chlorinated solvents have been banned because of their high toxicity, but some of these compounds are still widely used as solvents to produce polymers, waxes, resins, fats and lacquers, for producing dyes, for dry cleaning, as paint strippers and degreasers, as fire extinguishers, for paper coating, or as reagents in organic synthesis among other uses. Within the group of fuel components, benzene is a known carcinogen that was widely used in the past. Due to its proven toxicity, it was replaced by other solvents like toluene or xylene. Currently, it seems that benzene is still used in small amounts as industrial solvent and as precursor in the production of drugs, plastics, synthetic rubber, dyes, lubricants, detergents, explosives or pesticides. Long-term exposure to high levels of toluene or xylene may lead to liver and kidney damage too [2].

Due to their high volatility, VOCs can move easily through the environment and can eventually end up in groundwater and surface water. The

presence of VOCs in the aquatic environment has been widely reported [3-7]. The most effective way for determination of VOCs is by using gas chromatography, preferably GC–MS due to its higher confirmatory potential. Traditionally, purge and trap (P&T) has been used to determine VOCs in water [8,9], although other techniques have also been applied as static headspace [3, 10], direct aqueous injection [11] or solid-phase microextraction (SPME) [6,7,12-17]. Some authors have reported a comparison between P&T and SPME [18-20], indicating that they are similar in precision and accuracy, but SPME is somehow faster and simpler. Disadvantages as cost or the use of environmental unfriendly solvents are minimized, time of sample preparation is shorter and the design and operation of an SPME procedure is also simpler. Because of these advantages, SPME is an attractive and advantageous sample preparation method in environmental analysis. Using SPME, the extraction of volatile analytes from the sample can be done by direct immersion (DI) or by exposing the fiber to the headspace (HS) of the sample. Although DI-SPME is the more widely used technique for semivolatile compounds [21], HS-SPME seems to be more appropriate for volatile compounds, especially when they are dirty or complex matrices [13-17,22].

HS-SPME requires an optimization of the variables involved before its application, selecting the best fiber according to the nature of the analytes and then choosing the experimental conditions as amount of salt to be added, extraction temperature, sample volume, headspace volume, incubation or extraction time. These parameters have been traditionally optimised using univariate optimization approach (changing one variable at a time). However better results would be expected by applying a multivariate approach, as a

factorial design, which take into account not only individual effects but also interactions between variables [23-26].

As stated before the determination of VOCs fits well with gas chromatography (GC), which can easily be coupled with SPME. Once the analytes are concentrated in the stationary phase of the SPME fiber, it is introduced directly into the GC-injector and analytes are thermally desorbed [6,12,13,15-17,19]. The coupling of gas chromatography with a triple quadrupole mass spectrometer analyzer has demonstrated its strong potential for determination of organic pollutants in environmental and food samples [27-30]. With this analyzer, the use of tandem mass spectrometry (MS/MS) is feasible, selecting adequate precursor and product ions. Working in selected reaction monitoring (SRM) mode, typically acquiring two MS/MS transitions, gives the possibility of simultaneous confirmation and quantification with excellent selectivity and sensitivity, reaching very low detection limits. In addition, the use of the intensities ratio of the different transitions monitored can be used as confirmatory parameter [31].

In this work, a method based on HS-SPME has been developed for around 20 VOCs in water samples and using GC–MS/MS analysis with triple quadrupole for detection, quantification and reliable identification of the analytes present in the samples. Two MS/MS transitions have been acquired for each analyte. For some problematic compounds, it was difficult to find appropriate product ions and then, a "pseudo-SRM" approach has been applied, using the same ion as parent and product [32-35].

2. Experimental

2.1. Reagents

Acetone (pesticide residue analysis) was purchased from Scharlab (Barcelona, Spain) and HPLC-grade water was obtained by purifying demineralised water in a Milli-Q Gradient A10 (Millipore, Bedford, MA, USA). Sodium chloride was purchased from Scharlab.

A reference standard mixture (JMHW VOC Mix) was purchased from Supelco (Bellefonte, PA). The mixture contains 1000 mg L^{-1} of each of the following components in methanol: 1,1-dichloroethylene, methylene chloride, trans-1,2-dichloroethylene, cis-1,2-dichloroethylene, chloroform, 1,1,1-trichloroethane, carbon tetrachloride, benzene, 1,2-dichloroethane, trichloroethylene, 1,2-dichloropropane, bromodichloromethane, cis-1,3-dichloropropene, toluene, trans-1,3-dichloropropene, 1,1,2-trichloroethane, tetrachloroethylene, dibromochloromethane, m-xylene, p-xylene, o-xylene, bromoform and 1,4-dichlorobenzene. The commercial mixture was 10-times diluted with acetone to produce a 100 mg L^{-1} solution, which was stored at –18 °C. Further dilutions were made with water and solutions were stored at 4 °C. An isotopically labelled internal standard (ILIS), Benzene-D_6 (2000 mg L^{-1}), was purchased from Supelco and it was used as surrogate standard added to the sample before extraction. An individual stock solution of ILIS of 200 mg L^{-1} was prepared by dilution with acetone (stored at –18 °C) and further dilutions were made with water (stored at 4 °C).

Manual SPME holder used and SPME fibers have been purchased from Supelco.

2.2. GC instrumentation

Chromatographic determination was carried out using a GC system (Agilent 6890N, Palo Alto, USA) coupled to a triple quadrupole (QqQ) mass spectrometer Quattro Micro GC (Waters, Boston, USA). GC separation was performed using a BP624 capillary column with a length of 30 m, an internal diameter of 0.25 mm and a film thickness of 1.4 μm (SGE, Scharlab). The oven was programmed as follows: 30 °C (5 min); 10 °C min^{-1} to 125 °C; 30 °C min^{-1} to 220 °C (2.33 min). An adequate temperature to desorb the fiber was selected according to the manufacturer recommendations, establishing an injector temperature of 280 °C. Splitless time was adjusted to 5 min (maintaining the fiber in the injector for 10 min in split mode to ensure complete desorption) and an specific liner for SPME (Supelco) was used. Helium 99.999 % (Praxair, Valencia, Spain) was used as a carrier gas at a constant flow of 1 mL min^{-1}. The MS interface temperature was set to 230 °C.

The ionization mode selected was electron ionisation (EI) (solvent delay of 3.8 min) with the ionization source temperature set to 250 °C. For most compounds an MS/MS procedure was performed in Selected Reaction Monitoring (SRM) mode using argon 99.995 % (Praxair) as collision gas at a pressure of 2.5×10^{-4} kPa in the collision cell. For those compounds where two MS/MS transitions were not available, a pseudo Selected Reaction Monitoring (pseudo-SRM) was used selecting the precursor ion in first quadrupole, applying zero collision energy and isolating the same one as product ion in the third quadrupole. To ensure a reliable identification of analytes, two MS/MS transitions were selected, and when this was not feasible, three signals were acquired (one MS/MS and two pseudo-SRM, or three pseudo-SRM). A dwell time per channel

between 0.05 and 0.3 s was chosen, depending on the number of transitions recorded in each window and on the peak width of each compound, in order to get a minimum of 16 points per peak.

QuanLynx application manager was used to process the data obtained. PFTBA (perfluorotri-n-butylamine) was used for the daily mass calibration and it was injected using a syringe in the reference reservoir of the MS system for this purpose.

2.3. Headspace solid-phase microextraction procedure

A volume of 4 mL of water containing the surrogate at 20 μg L^{-1} was placed in a 10 mL vial adding 0.4 g of sodium chloride (10 % in sample) and it was sealed with a septum lined cap. In the case of water samples where lower analyte concentrations are expected, 10 mL of water in a 20 mL vial adding 1 g of NaCl was used and the final concentration of surrogate was 2 μg L^{-1}. The extraction was performed with a carboxen/polydimethylsiloxane (CAR/PDMS) 75 μm fiber purchased from Supelco and using magnetic stirring. The fiber was conditioned prior to the first use with the temperature and conditioning recommendations described by the manufacturer. Before extraction, the vial was pre-heated for 5 min at 50 °C; then, the fiber was placed into the headspace of the vial and extraction was carried out for 30 min at 50 °C. After the sorption process, the SPME fiber was immediately desorbed at 280 °C for 5 min on the GC injection port.

2.4. Validation study

Validation of the method was carried out at three levels of concentration (0.1, 5 and 50 μg L^{-1}). The following analytical characteristics were evaluated:

Linearity was studied by analyzing in duplicate water spiked at different concentrations, 0.05–1 μg L^{-1} (low concentration level), 1–25 μg L^{-1} (intermediate) and 10–100 μg L^{-1} (high level). Satisfactory linearity was assumed when regression coefficient was >0.99 with residuals lower than 20 %. *Accuracy* was estimated by means of recovery experiments (n = 6) using water spiked at three concentration levels (0.1, 5 and 50 μg L^{-1}). In order to ensure correct quantification, different calibration curves (four points each) were prepared and analyzed together with the recoveries experiments. For the high spiking level (50 μg L^{-1}) a calibration curve between 10 and 100 μg L^{-1} was used, for the intermediate level (5 μg L^{-1}), between 1 and 25 μg L^{-1}, and for low level (0.1 μg L^{-1}) between 0.05 and 1 μg L^{-1}. For the higher validation levels (50 and 5 μg L^{-1}) the ILIS was added to give a final concentration of 20 μg L^{-1}, and for the lowest validation level (0.1 μg L^{-1}) to give a final concentration of 2 μg L^{-1}. *Precision* was determined as repeatability of the method, expressed in terms of relative standard deviation, calculated from the same recovery experiments (n = 6) at the three fortification levels. *Selectivity* of the method was based on monitoring the appropriate MS/MS transitions for each analyte by selecting adequate precursor and product ions. Satisfactory selectivity was reached if no interfering peaks, higher than 30 % of LOQ signal, were present in the blank samples. *Limit of quantification* (LOQ) and *limit of detection* (LOD) were estimated as the analyte concentration that produced a peak signal of ten and three times, respectively, the background noise in the chromatogram at the

lowest fortification level tested. For identity confirmation we used the confirmation ratio (Q/q_i), i.e. the ratio between the intensity of the quantification (Q) and confirmation (q_i) transitions recorded for each compound. The theoretical value for each compound was obtained as an average of the standard solutions used for calibration (and updated in each analysis sequence). A maximum ratio tolerance of ±20 % was allowed when the theoretical Q/q_i ratio was <2; ±25 % for Q/q_i ratio between 2 and 5; ±30 % for Q/q_i ratio between 5 and 10; and ±50 % when the Q/q_i ratio was >10, according to the European Union Decision 2002/657/EC [36]. A good agreement in retention time between sample and standards was also required to confirm a positive identification in a sample. For quantification fitted curves of relative peak areas (ILIS used, Benzene-D_6) versus concentration (μg L^{-1}), were obtained after HS-SPME of spiked HPLC water.

3. Results and discussion

3.1. GC–MS/MS optimization

Optimization of the MS/MS method was performed using standard solutions prepared with HPLC-grade water extracted by SPME. Full scan spectrum was acquired for all compounds and then the most abundant ions (typically the base peak or other intense peaks) were selected as precursor ions. Once the precursor ion was selected, different values of collision energy (between 5 and 40 eV) were tested to perform the fragmentation. Again, the most abundant ions of the product ion spectra were selected as product ions with the final purpose of developing an SRM method with two transitions for each compound

in order to have a reliable confirmation in the samples. Six out of the 23 studied compounds showed one (or even none) adequate MS/MS transition. In these cases, a pseudo Selected Reaction Monitoring (pseudo-SRM) was used. This approach consists on selecting the precursor ion (characteristic of each compound) in the first quadrupole, and applying low collision energy, in such a way that the same ion is used as precursor and product ion to create a "pseudo" transition. After optimisation of the collision energy, the best results (in terms of sensitivity) were obtained with a zero value, as even with low collision energy, an important decrease in peak signal was observed. This approach was applied in order to determine simultaneously all compounds in one injection with the same acquisition mode. A few authors have been reported this mode of working, normally in LC–MS/MS methods, although using low collision energy instead of zero [32-35].

Table 1 shows the most relevant experimental conditions of the GC–MS/MS method for the 23 volatile organic compounds studied in this work. For 17 compounds, whose fragmentation was feasible, two transitions were used at their optimum collision energy, which value was between 5 and 30 eV. The most sensitive one was selected as quantification transition (*Q*) and rest as confirmation transitions (*qi*).

Table 1 also shows the molecular mass and the precursor and product ion for each compound, the molecular formula of the precursor ion and the fragment loss to get the product ion. Most of the losses corresponded to a chlorine atom as most analytes were chlorinated compounds.

The dwell time parameter showed in ***Table 1*** was also studied and optimized. Values selected ranged between 0.05 and 0.3 s in order to obtain good chromatographic peak shape still maintaining satisfactory sensitivity for each compound.

It has to be pointed out that even after optimisation m-xylene and p-xylene could not be separated neither chromatographically nor by MS, and they were considered as a group to simplify the quantification.

3.2. Solid-phase microextraction optimisation

The following parameters, which are typically considered in a SPME optimization, were studied: fiber type, extraction temperature, sample volume, headspace volume, incubation and extraction time, and amount of salt added.

First of all, the effect of the fiber type was studied, comparing two stationary phases: StableFlex divinylbenzene/carboxen/PDMS (DVB/CAR/PDMS) 50/30 μm and carboxen/polydimethylsiloxane (CAR/PDMS) 75 μm. A SPME generic procedure was applied using both fibers over 1 mL of HPLC-grade water fortified at 10 μg L^{-1} with the mixed standard solution, extracting at 35 °C for 30 min in a 10 mL vial with magnetic stirring. The results obtained for both fibers are shown in ***Figure 1***. Higher peak areas were obtained with the CAR/PDMS fiber for all analytes, except for 1,3-dichloropropene (cis and trans), xylenes (meta, para and ortho), bromoform and 1,4-dichlorobenzene (the last eluting compounds). Therefore, this fiber was considered the most suitable for this study and it was selected for further experiments.

Table 1. Experimental conditions of the optimized GC–MS/MS method.

Window (min)	t_R (min)	Compound	Molecular formula	Molecular mass (Da)	Precursor ion (Da)		Fragment loss	Product ion (Da)	Collision energy (eV)	Q/q_i[a]	Dwell (s)	Q/q_i ratio
3.8–6.8	4.12	1,1-Dichloroethylene	$C_2H_2Cl_2$	96	96	$[C_2H_2{}^{35}Cl_2]$	$[-{}^{35}Cl]$	61	10	*Q*	0.3	2.68
					98	$[C_2H_2{}^{35}Cl{}^{37}Cl]$	$[-{}^{35}Cl]$	63	10	*q*	0.3	
	5.17	Methylene chloride	CH_2Cl_2	84	84	$[CH_2{}^{35}Cl_2]$	$[-{}^{35}Cl]$	49	5	*Q*	0.3	3.45
					86	$[CH_2{}^{35}Cl{}^{37}Cl]$	$[-{}^{37}Cl]$	49	5	*q*	0.3	
	5.79	Trans-1,2-dichloroethylene	$C_2H_2Cl_2$	96	96	$[C_2H_2{}^{35}Cl_2]$	$[-{}^{35}Cl]$	61	10	*Q*	0.3	2.32
					98	$[C_2H_2{}^{35}Cl{}^{37}Cl]$	$[-{}^{35}Cl]$	63	10	*q*	0.3	
7.0–8.5	7.65	Cis-1,2-dichloroethylene	$C_2H_2Cl_2$	96	96	$[C_2H_2{}^{35}Cl_2]$	$[-{}^{35}Cl]$	61	10	*Q*	0.15	2.60
					98	$[C_2H_2{}^{35}Cl{}^{37}Cl]$	$[-{}^{35}Cl]$	63	10	*q*	0.15	
	8.07	Chloroform	$CHCl_3$	118	83	$[CH^{35}Cl_2]$	$[-{}^{35}Cl]$	48	30	*Q*	0.15	1.90
					83	$[CH^{35}Cl_2]$	$[-H^{35}Cl]$	47	30	*q*	0.15	
8.1–9.4	8.53	1,1,1-Trichloroethane	$C_2H_3Cl_3$	132	97	$[C_2H_3{}^{35}Cl_2]$	$[-H^{35}Cl]$	61	10	*Q*	0.25	3.18
					99	$[C_2H_3{}^{35}Cl{}^{37}Cl]$	$[-H^{35}Cl]$	63	10	q	0.25	
	8.92	Carbon tetrachloride	CCl_4	152	117	$[C^{35}Cl_3]$	$[-{}^{35}Cl]$	82	25	Q	0.25	1.33
					119	$[C^{35}Cl_2{}^{37}Cl]$	$[-{}^{35}Cl]$	84	25	*q*	0.25	
8.6–10.5	9.15	Benzene-d_6	C_6D_6	84	84	$[C_6D_6]$	$[-C_2D_2]$	*56*			0.05	
	9.21	Benzene	C_6H_6	78	78	$[C_6H_6]$	–	78	0	*Q*	0.05	1.86
					52	$[C_4H_4]$	–	52	0	*q1*	0.05	
					78	$[C_6H_6]$	$[-C_2H_2]$	52	10	*q2*	0.05	13.60
	9.30	1,2-Dichloroethane	$C_2H_4Cl_2$	98	62	$[C_2H_3{}^{35}Cl]$	–	62	0	*Q*	0.05	3.13
					64	$[C_2H_3{}^{37}Cl]$	–	64	0	*q1*	0.05	
					49	$[CH_2{}^{35}Cl]$	–	49	0	*q2*	0.05	5.68
	10.19	Trichloroethylene	C_2HCl_3	130	130	$[C_2H^{35}Cl_3]$	$[-{}^{35}Cl]$	95	10	*Q*	0.05	7.95
					95	$[C_2H^{35}Cl_2]$	$[-{}^{35}Cl]$	60	5	*q*	0.05	
10.2–12	10.51	1,2-Dichloropropane	$C_3H_6Cl_2$	112	63	$[C_2H_4{}^{35}Cl]$	–	63	0	*Q*	0.05	3.07
					65	$[C_2H_4{}^{37}Cl]$	–	65	0	*q2*	0.05	
					76	$[C_3H_5{}^{35}Cl]$	$[-{}^{35}Cl]$	41	5	*q1*	0.05	6.28

Table 1. Continuation

Window (min)	t_R (min)	Compound	Molecular formula	Molecular mass (Da)	Precursor ion (Da)		Fragment loss	Product ion (Da)	Collision energy (eV)	Q/q_i [a]	Dwell (s)	Q/q_i ratio
	10.88	Bromodichloromethane	$CHBrCl_2$	162	83	$[CH^{35}Cl_2]$	–	83	0	*Q*	0.05	1.54
					85	$[CH^{35}Cl^{37}Cl]$	–	85	0	*q1*	0.05	96.00
					47	$[C^{35}Cl]$	–	47	0	*q2*	0.05	
	11.63	Cis-1,3-dichloropropene	$C_3H_4Cl_2$	110	110	$[C_3H_4{}^{35}Cl_2]$	$[-{}^{35}Cl]$	75	5	*Q*	0.05	2.92
					112	$[C_3H_4{}^{35}Cl^{37}Cl]$	$[-{}^{35}Cl]$	77	5	*q*	0.05	
11.9–12.8	12.10	Toluene	C_7H_8	92	91	$[C_7H_7]$	$[-C_2H_2]$	65	10	*Q*	0.15	8.43
					92	$[C_7H_8]$	$[-C_2H_3]$	65	25	*q*	0.15	
	12.42	Trans-1,3-dichloropropene	$C_3H_4Cl_2$	110	110	$[C_3H_4{}^{35}Cl_2]$	$[-{}^{35}Cl]$	75	5	*Q*	0.15	3.06
					112	$[C_3H_4{}^{35}Cl^{37}Cl]$	$[-{}^{35}Cl]$	77	5	*q*	0.15	
12.5–13.4	12.74	1,1,2-Trichloroethane	$C_2H_3Cl_3$	132	97	$[C_2H_3{}^{35}Cl_2]$	$[-H^{35}Cl]$	61	10	*Q*	0.15	4.14
					99	$[C_2H_3{}^{35}Cl^{37}Cl]$	$[-H^{35}Cl]$	63	10	*q*	0.15	
	12.98	Tetrachloroethylene	C_2Cl_4	164	164	$[C_2{}^{35}Cl_4]$	$[-{}^{35}Cl]$	129	10	*Q*	0.15	1.07
					166	$[C_2{}^{35}Cl_3{}^{37}Cl]$	$[-{}^{35}Cl]$	131	10	*q*	0.15	
13.0–15.6	13.32	Dibromochloromethane	$CHBr_2Cl$	206	129	$[CH^{79}Br^{37}Cl]$	–	129	0	*Q*	0.1	2.06
					127	$[CH^{79}Br^{35}Cl]$	–	127	0	*q1*	0.1	
					81	$[^{81}Br]$	–	81	0	*q2*	0.1	5.81
	14.45	m-Xylene	C_8H_{10}	106	106	$[C_8H_{10}]$	$[-CH_3]$	91	5	*Q*	0.1	5.00
					91	$[C_7H_7]$	$[-C_2H_2]$	65	5	*q*	0.1	
	14.45	p-Xylene	C_8H_{10}	106	106	$[C_8H_{10}]$	$[-CH_3]$	91	5	*Q*	0.1	5.00
					91	$[C_7H_7]$	$[-C_2H_2]$	65	5	*q*	0.1	
	15.01	o-Xylene	C_8H_{10}	106	106	$[C_8H_{10}]$	$[-CH_3]$	91	5	*Q*	0.1	4.29
					91	$[C_7H_7]$	$[-C_2H_2]$	65	5	*q*	0.1	
15.0–17.5	15.39	Bromoform	$CHBr_3$	250	173	$[CH^{79}Br^{81}Br]$	–	173	0	*Q*	0.06	1.08
					175	$[CH^{81}Br_2]$	–	175	0	*q2*	0.06	
					171	$[CH^{79}Br_2]$	–	171	0	*q1*	0.06	2.30
	16.77	1,4-Dichlorobenzene	$C_6H_4Cl_2$	146	146	$[C_6H_4{}^{35}Cl_2]$	$[-{}^{35}Cl]$	111	10	*Q*	0.06	2.16
					111	$[C_6H_4{}^{35}Cl]$	$[-H^{35}Cl]$	75	5	*q*	0.06	

[a] *Q corresponds to quantification transition; qi corresponds to confirmation transitions.*

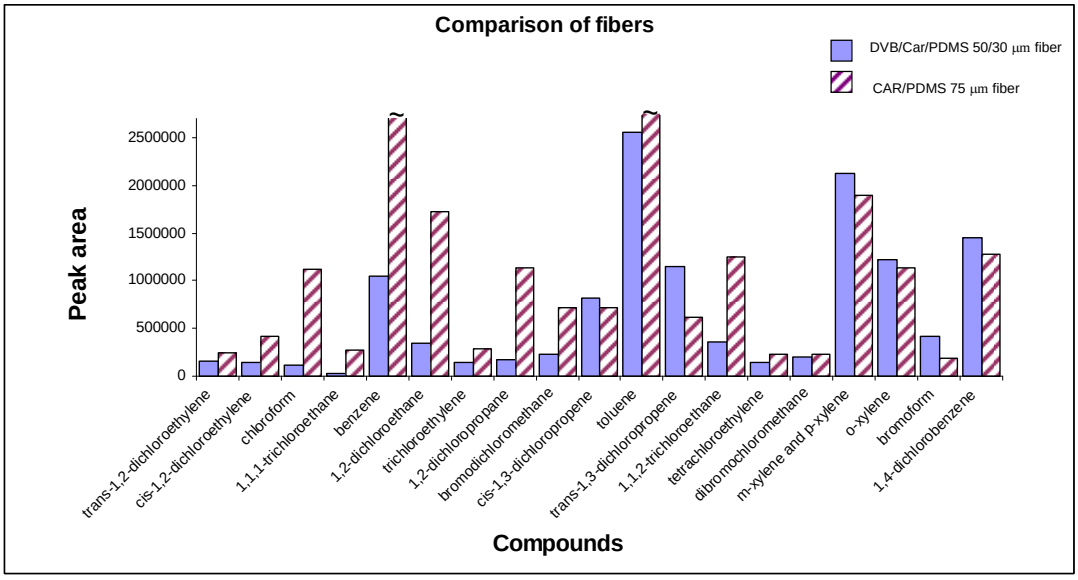


Figure 1. Comparison of SPME extraction efficiency using the two fibers selected for the study: divinylbenzene/carboxen/PDMS 50/30 μm, and carboxen/polydimethylsiloxane 75 μm (1 mL of HPLC-grade water spiked at 10 μg L^{-1}, extracted for 30 min at 35 °C).

Optimisation of the SPME parameters has been traditionally carried out following a univariate approach, studying each factor individually and maintaining the remaining variables constant. Recently, a multivariate experimental design has been used to optimise extraction procedures [23-26], which allows to appreciate the interactions between variables and take them into account to design an optimum extraction procedure.

In this work, a multivariate experimental design was applied to optimize the extraction procedure for the determination of VOCs in water. The statistical software StatGraphics was used for this purpose. The optimisation was made in two steps. First, a screening design was applied to detect significant variables, and then a surface response design was applied to estimate the optimum values for those variables. Five experimental factors (extraction temperature, sample

volume, headspace volume, incubation time and amount of salt) were studied in the first step, assigning a maximum and a minimum value for each variable and selecting a screening design consisting in a half fraction run in two blocks 2^{5-1} with three central points in each block. This led to 22 experiments divided into two blocks, described in ***Table 2A***, where the minimum, center and maximum values for each variable are also shown. The extraction time was fixed in all cases to 30 min. The experiments were performed using HPLC-grade water fortified with the standard solution at 10 μg L^{-1}. The fiber was desorbed into GC–MS and analyzed. The response function (desirability function) used to apply the optimization algorithm of the statistical software was the geometric mean of the response (ratio between the area of the compound and the area of the internal standard) of all compounds, similarly to other authors [25,26]. The effect of each factor and the interactions between them were studied by the resulting Pareto charts (***Figure 2A***), which show the standardized effect of the experimental factors. The vertical line in the figure defines 95 % of confidence level. If an effect exceeds this line, it is considered statistically significant. Sample volume (V_s) and extraction temperature (T) were the most significant experimental factors, followed by headspace volume (V_{vial}) and amount of salt (% NaCl). The interaction between sample volume and headspace volume, and between headspace volume and incubation time were also found to be significant. In a second step, the above mentioned factors were optimized by a response surface methodology. Sample volume and extraction temperature were selected as the most significant factors, fixing the other ones (headspace volume, incubation time and amount of salt) according to with the effect indicated in the Pareto chart, i.e. the minimum value if the effect was negative and the maximum value if the effect was positive. A response surface design with two variables at three levels

3^2 was selected; with 12 assays in one block including 3 central points (***Table 2B***). The values selected for sample volume were 1, 2.5 and 4 mL and for the extraction temperature were 20, 35 and 50 °C. When the experiments were performed and data analyzed using the statgraphics software, the Pareto chart (***Figure 2B***) clearly illustrated that both sample volume and extraction temperature showed a positive effect. ***Figure 2C*** shows the response surface estimated for these parameters. The maximum values assigned in the software for these experimental factors were selected as optimum values to ensure an efficient extraction.

Table 2. A) Experimental variables for screening design and values used in each experiment

Assay	V_{sample} (mL)	V_{vial} (mL)	T (ºC)	% NaCl	$t_{pre\text{-}heating}$ (min)
1	1	20	65	0	5
2	3	10	65	0	5
3	1	10	35	10	0
4	1	20	35	10	5
5	3	10	35	10	5
6	2	15	50	5	2.5
7	2	15	50	5	2.5
8	3	20	35	10	0
9	2	15	50	5	2.5
10	3	20	65	0	0
11	1	10	65	0	0
12	3	10	35	0	0
13	2	15	50	5	2.5
14	2	15	50	5	2.5
15	3	20	35	0	5
16	1	20	65	10	0
17	1	20	35	0	0
18	1	10	35	0	2.5
19	3	10	65	10	0
20	2	15	50	5	5
21	1	10	65	10	2.5
22	3	20	65	10	2.5

Table 2. B) Experimental variables for response surface design

Assay	V_{sample} (mL)	T (ºC)
1	1	20
2	4	50
3	2.5	35
4	2.5	20
5	4	35
6	2.5	35
7	2.5	50
8	2.5	35
9	4	20
10	2.5	35
11	1	50
12	1	35

During optimisation experiments, we found problems of contamination for methylene chloride and carbon tetrachloride in all experiments performed, as they were present even in the fiber blanks, probably, as a result of their presence in laboratory environment. Therefore, these two compounds could not be validated in this work.

Once the indicated parameters were optimised, it was necessary to study the effect of extraction time. Using mineral water spiked at 1 μg L^{-1}, different extraction times (5, 15, 30, 45, 60 and 120 min) were tested in duplicate. Experimental results were fitted to the equation (***Eq.*** (***1***)), proposed by Ai [37] and applied by us in a previous paper [38], which allows to estimate the theoretical equilibrium time (*t*):

$$n=n_0[1-e^{-at}] \qquad (1)$$

where *n* is the amount of analyte extracted at a given time (*t*) and n_0 is the amount extracted when equilibrium is reached.

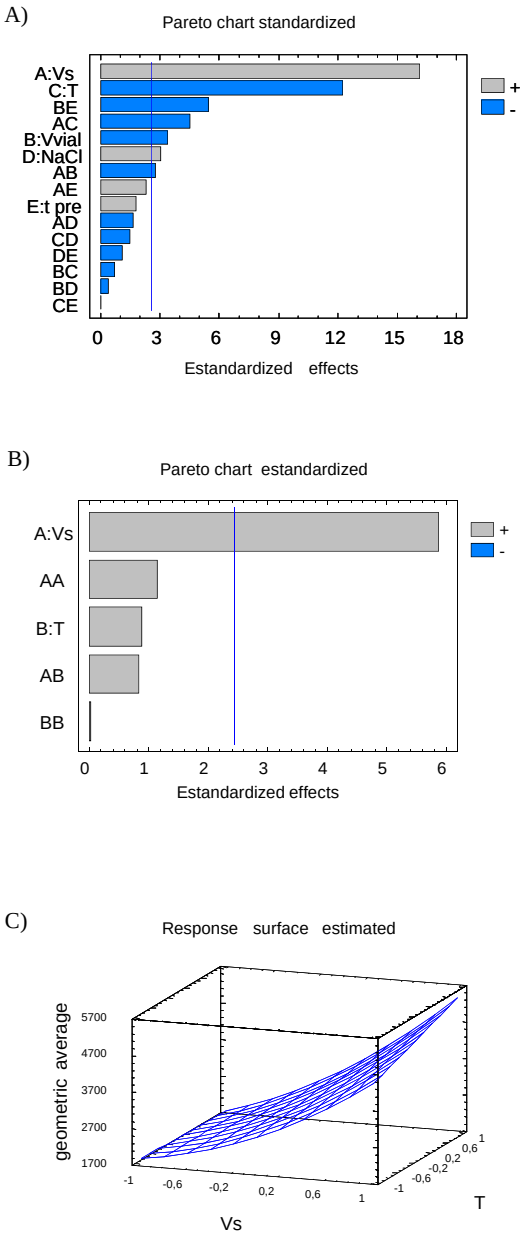


Figure 2. A) Standardized Pareto chart using as response the geometric mean for all studied compounds in a fractional factorial design (2^{5-1} experiments). B) Standardized Pareto chart using as response the geometric mean for all studied compounds in a surface response design (3^2 experiments). C) Diagram of surface response obtained for sample volume and temperature. + indicates a positive effect; –, indicates a negative effect.

The results obtained, considering the equilibrium time when amount extracted was 95% of total extracted amount at complete equilibrium, showed that most compounds reached the equilibrium at around 30 min (except five compounds, whose equilibrium times ranged between 45 and 80 min), and consequently we selected 30 min as extraction time for subsequent experiments. ***Figure 3*** shows data and theoretical fitted curves for some compounds, highlighting the behaviour of bromoform, which did not reach the equilibrium until approximately 80 min.

The optimum experimental conditions finally selected for HS-SPME are those shown in Section 2.3.

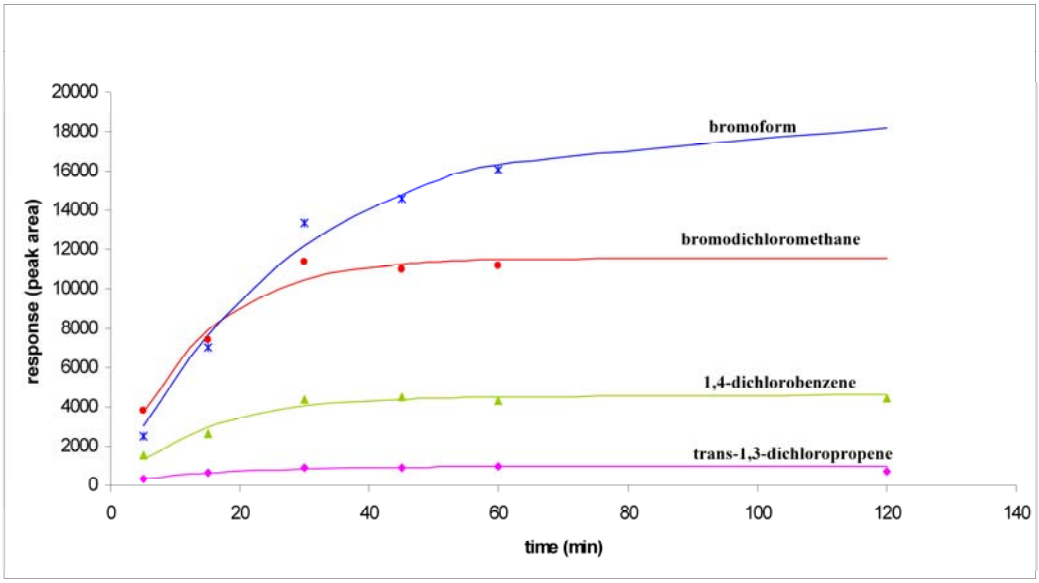


Figure 3. Effect of extraction time on extracted amount using SPME. Experimental data and theoretical (lines) curves for bromoform, bromodichloromethane, 1,4-dichlorobenzene and trans-1,3-dichloropropene (experimental conditions: 10 mL of mineral water spiked at 1 μg L^{-1}, extracted at 35 °C for different extraction times).

3.3. Method validation

Linearity was studied by extracting in duplicate standard solutions prepared in HPLC water (four levels from 10 to 100 μg L^{-1} and four levels from 1 to 25 μg L^{-1} maintaining the concentration of ILIS at 20 μg L^{-1}). For method application to cleaner samples, where lower analyte concentration are expected, linearity was also tested from 0.05 to 1 μg L^{-1} maintaining the ILIS at 2 μg L^{-1}, using in this case 10 mL of sample (in a 20 mL vial). All compounds studied showed good linearity results at all concentrations, ranges tested with correlation coefficients ≥0.99 and residuals lower than 20 %.

Accuracy and precision were estimated (n = 6) from mineral water spiked at three concentration levels (0.1, 5 and 50 μg L^{-1}). The procedure for higher levels (5 and 50 μg L^{-1}) was applied using 4 mL sample and 20 μg L^{-1} ILIS. In order to widen the applicability of the method to low contaminated water samples, additional recovery studies were carried out at 0.1 μg L^{-1}, using 10 mL of water and 2 μg L^{-1} of ILIS.

As it can be seen in ***Table 3***, most compounds present recoveries between 70 % and 120 %, with the only exception of trichloroethylene and tetrachloroethylene at the 5 μg L^{-1} level. Compounds as trans-1,2-dichloroethylene, chloroform, trichloroethylene and tetrachloroethylene could not be evaluated at the lowest level (0.1 μg L^{-1}), as no chromatographic peak was observed due to their low sensitivity. RSD in all cases was ≤20%, except for xylenes isomers at the lowest level validated.

Table 3. Average recovery (%) and RSD (in parenthesis) after the application of the method to mineral water samples (n=6) spiked at three concentration levels.

Compounds	Fortification level (μg L^{-1})			LOD (μg L^{-1})	LOQ (μg L^{-1})
	0,1	5	50		
1,1-Dichloroethylene	120 (8)	105 (7)	112 (16)	0.07	0.1
Methylene chloride	[a]	[a]	[a]	-	-
trans-1,2-Dichloroethylene	-	83 (12)	113 (7)	2	5
cis-1,2-Dichloroethylene	86 (6)	91 (1)	119 (9)	0.04	0.1
Chloroform	-	73 (18)	117 (20)	0.6	2
1,1,1-Trichloroethane	75 (17)	70 (8)	106 (6)	0.03	0.09
Carbon tetrachloride	[a]	[a]	[a]	-	-
Benzene	70 (5)	-	116 (7)	0.06	0.1
1,2-Dichloroethane	81 (13)	105 (9)	119 (4)	0.04	0.1
Trichloroethylene	-	57 (24)	111 (3)	0.2	0.6
1,2-Dichloropropane	73 (14)	91 (10)	112 (4)	0.05	0.1
Bromodichloromethane	110 (6)	103 (2)	120 (3)	0.03	0.1
cis-1,3-Dichloropropene	84 (8)	93 (3)	120 (4)	0.04	0.1
Toluene	83 (17)	101 (8)	84 (12)	0.004	0.01
trans-1,3-Dichloropropene	88 (12)	92 (4)	119 (4)	0.01	0.04
1,1,2-Trichloroethane	86 (14)	101 (3)	118 (3)	0.03	0.09
Tetrachloroethylene	-	67 (8)	80 (3)	0.6	2
Dibromochloromethane	70 (10)	99 (3)	108 (8)	0.02	0.06
m-Xylene + p-Xylene	96 (34)	82 (6)	95 (4)	0.005[b]	0.02[b]
o-Xylene	98 (34)	81 (7)	102 (4)	0.01	0.04
Bromoform	83 (10)	94 (3)	119 (6)	0.01	0.04
1,4-Dichlorobenzene	81 (17)	83 (7)	103 (5)	0.007	0.02

[a], not calculated due to high contamination found in the environment
[b], estimated corresponding to the sum of m-xylene and p-xylene.

Limits of quantification (LOQ) and limits of detection (LOD), calculated as described in Section 2, were estimated from the precision studies, and were mostly appropriate to ensure the determination of VOCs at the 0.1 μg L^{-1} level. ***Figure 4*** shows the GC–MS/MS chromatograms obtained for mineral water spiked at the 0.1 μg L^{-1} level, used to determine the LOD and LOQ values. This figure illustrates the method sensitivity for selected compounds.

3.4. Application to surface water and wastewater samples

Surface and wastewater samples (stored at −18 °C and defrosted before analyses) were collected from several localities of the Castellon province. The developed method (4 mL sample, 20 μg L^{-1} ILIS) was applied to several samples of water, including wastewater (influent and effluent) from an urban wastewater treatment plant, and from a municipal solid-waste-treatment plant (before and after treatment), and surface water. The objective was to show the applicability of the method developed rather than performing a detailed comparative study of VOCs pollution in the samples.

A calibration curve was obtained after SPME extraction prepared with HPLC water, ranging between 1 and 100 μg L^{-1} (20 μg L^{-1} ILIS). Some compounds were detected in several samples at concentrations below 1 μg L^{-1} (cis-1,2-dichloroethylene, bromodichloromethane, dibromochloromethane and bromoform). In these cases, repeated analyses were made with 10 mL sample in order to be able to accurately quantifying these compounds.

The results are shown in ***Table 4***. Some compounds such as chloroform, benzene, trichloroethylene, toluene, tetrachloroethylene, dibromochloromethane, xylenes and bromoform were detected in all samples, including surface water. Concentrations found for benzene, trichloroethylene, tetrachloroethylene, m-xylene and p-xylene were rather similar in all samples. It is noticeable that dichloroethylenes were not detected in surface water and the concentration for chloroform was much lower than in the wastewater samples analyzed. Low concentrations of bromodichloromethane, dibromochloromethane and bromoform were found in surface water, being similar to those of urban effluent wastewater.

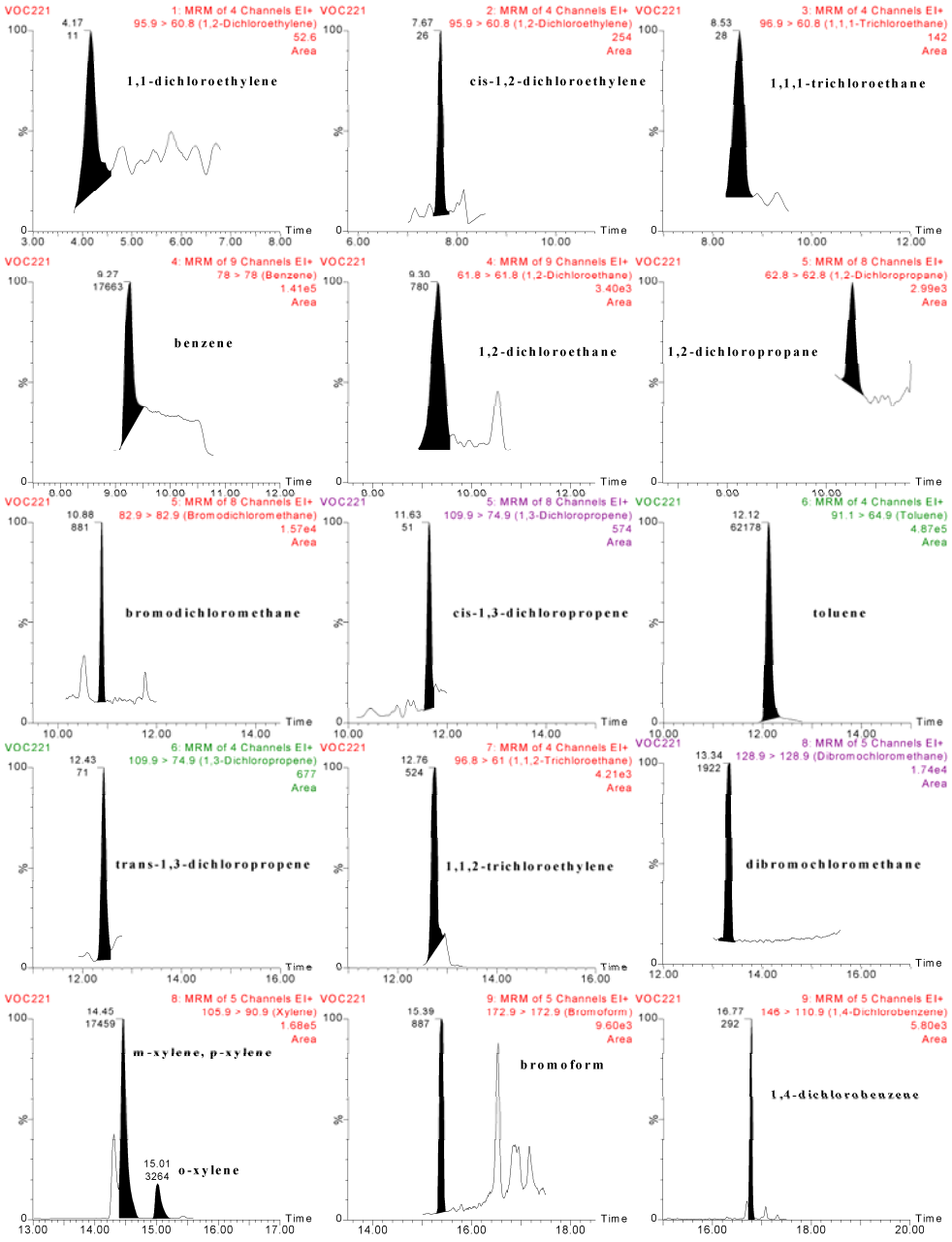


Figure 4. GC–MS/MS chromatograms (quantitative transitions are shown) obtained for a mineral water sample spiked at 0.1 μg L^{-1} extracted by HS-SPME, using the recommended procedure.

Table 4. Volatile organic compounds found in environmental water samples after application of the method (concentrations expressed in μg L^{-1}).

Compounds	IWW (wastewater treatment plant)	EWW (wastewater treatment plant)	Surface water	IWW (solid-waste treatment plant)	EWW (solid-waste treatment plant)
1,1-dichloroethylene	-	-	-	-	0.8
trans-1,2-dichloroethylene	2.0	-	-	-	41.3
cis-1,2-dichloroethylene	48.0	0.4	-	0.2	36.1
chloroform	96.0	67.7	3.1	174.5	177.8
benzene	32.0	34.3	45.8	23.9	53.3
trichlorethylene	49.3	39.9	41.8	41.4	54.9
bromodichloromethane	-	0.2	0.2	37.6	25.4
toluene	193.3	179.7	57.1	39.7	86.6
tetrachloroethylene	132.3	48.0	74.2	35.2	41.7
dibromochloromethane	0.3	0.2	0.1	1.2	8.1
m-,p-xylene	55.3	53.6	41.1	37.5	56.4
o-xylene	67.6	87.4	118.0	34.3	54.2
bromoform	0.4	0.2	0.3	0.4	5.1

According to the directive for Environmental Water Quality [39] the maximum permissible concentration for benzene is 50 μg L^{-1}. This value was not exceeded in any of the samples. For trichloroethylene and tetrachloroethylene maximum permissible concentration is not established, although an annual average of 10 μg L^{-1} is set up for both compounds.

Figure 5 shows the chromatograms obtained for a surface water, with all transitions monitored for the compounds detected in this sample.

In order to fully demonstrate the applicability of the proposed method and justify the use of the MS/MS detection, real samples have been analyzed both by GC–MS (Selected Ion Monitoring, SIM mode) and GC–MS/MS. Results obtained indicate that MS/MS leads to a notable increase in selectivity that, in some cases, results in a better sensitivity (in terms of higher signal-to-noise ratio). ***Figure 6*** shows selected chromatograms from real samples after HS-SPME and GC/MS (SIM) or GC–MS/MS (SRM). As it can be seen (***Figure 6A***), some of the studied compounds could be determined without remarkable differences by both modes. However, in most cases ***(Figure 6B***) the use of MS/MS improves the sensitivity with better signal-to-noise ratio. At low concentration levels, SIM mode even failed to measure the qualifier ions (***Figure 6C***). The improvement in selectivity is clearly illustrated in ***Figure 6D***, where an interfering compound affected the analyte chromatographic peak in SIM mode, while no interference was observed in GC–MS/MS. The analytical performance in SIM mode is limited by monitoring the isobaric background as well. The MS/MS process increases compound selectivity by monitoring either a product ion (SRM) or the unfragmented precursor ion. Even at low collision energies (set to 0 eV) the unspecific isobaric background is significantly reduced by the collision induced dissociation (CID)

process. The stable analyte precursor ion in these cases can hence be detected with increased S/N. These results support the use of GC–MS/MS even for those compounds, as studied VOCs, that seem less favourable for GC–MS/MS analysis due to their low molecular mass.

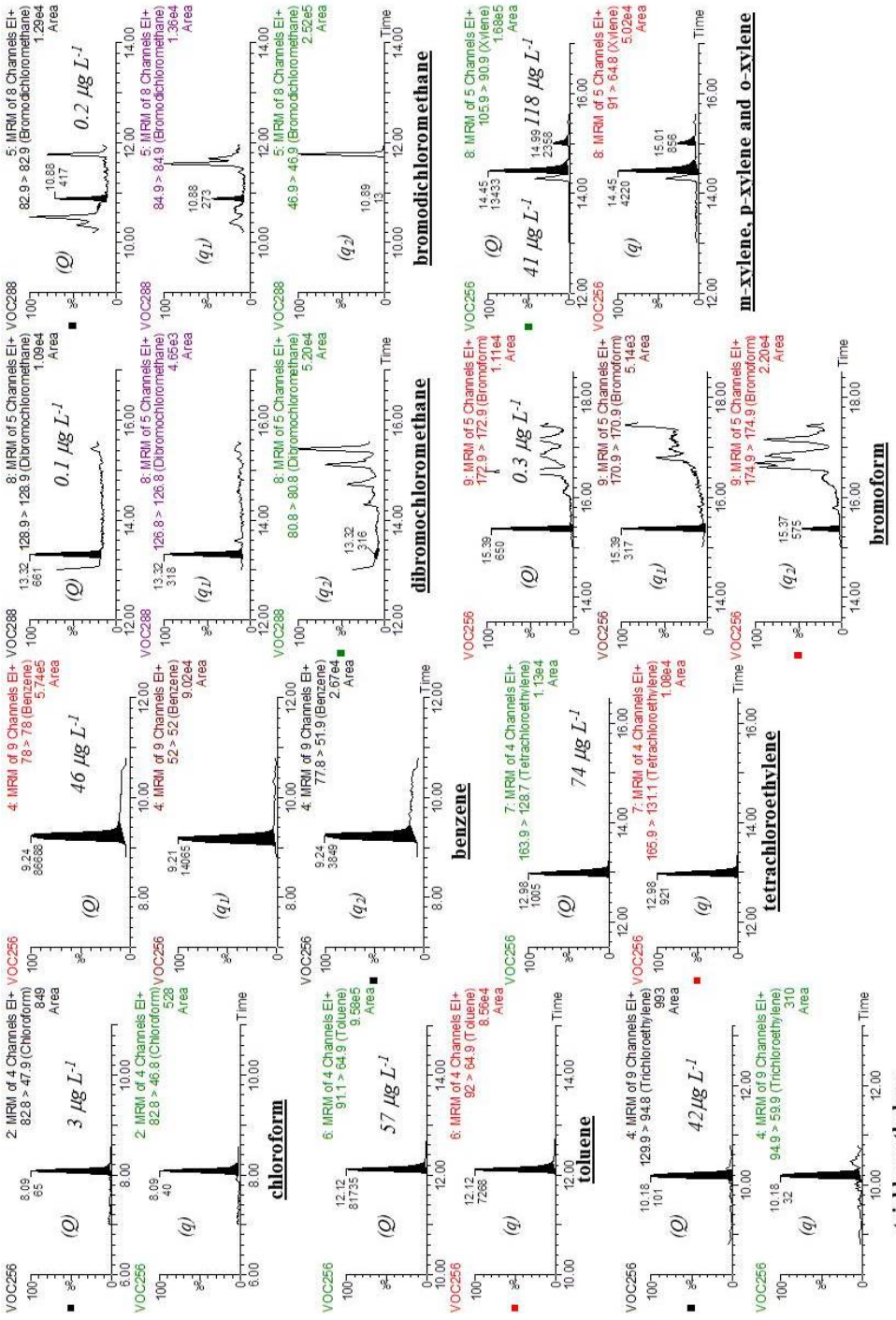


Figure 5. GC–MS/MS chromatograms for volatile organic compounds detected in surface water sample obtained after HS-SPME using the recommended procedure. (Q) Quantification transition, (q) confirmation transitions.

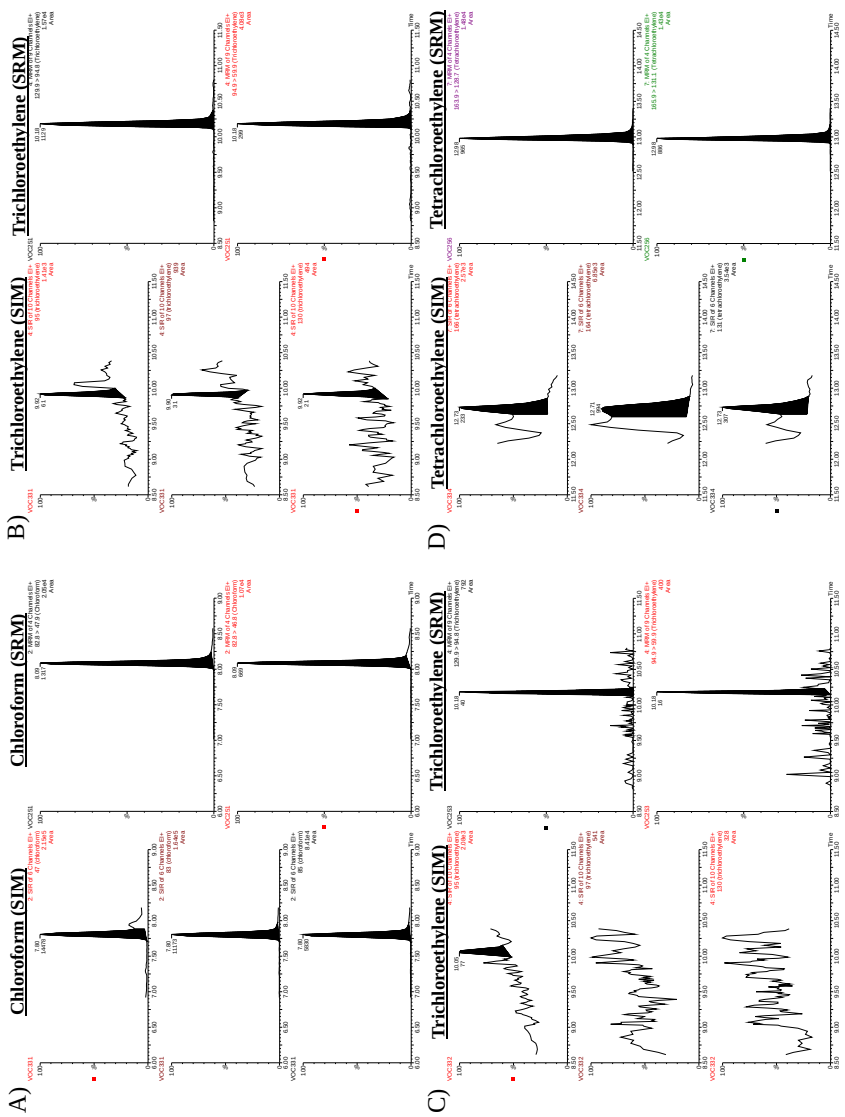


Figure 6. Comparison of GC–MS (SIM) and GC–MS/MS (SRM) chromatograms for volatile organic compounds detected in water. Three ions or two transitions are shown for each compound. (A) Chloroform detected in a IWW (wastewater treatment plant) sample. (B) Trichloroethylene detected in a IWW (wastewater treatment plant) sample. (C) Trichloroethylene detected in a EWW (wastewater treatment plant) sample. (D) Tetrachloroethylene detected in a surface water sample.

4. Conclusions

In this work, a method based on the use of HS-SPME has been designed, developed and validated for the determination of 23 VOCs in environmental water samples using GC–MS/MS with triple quadrupole analyzer.

Optimization of the SPME procedure applying a multivariate statistic design, allowed obtaining adequate information to decide the better extraction conditions, employing less time and effort than by optimising with the univariate form approach. In this way, it was also feasible to detect interactions between variables which can be significant for the study and that would not be detectable by univariate experimental design.

The solid-phase microextraction procedure developed based on headspace extraction overrode problems of most matrix interferences and made an easy, clean, rapid and solventless procedure to extract the compounds of interest with low limits of detection.

The advantageous use of gas chromatography coupled to mass spectrometry with a triple quadrupole analyzer, allowed choosing SRM transitions of each compound to carry out a correct quantification and confirmation, with the certainty of making a safe identification, using Q/q_i intensity ratio as a confirmatory parameter. Working in tandem MS, adequate MS/MS transitions were acquired, improving the sensitivity and selectivity of the method, as demonstrated in this article from the analysis of selected samples by both GC–MS (SIM) and GC–MS/MS (SRM) modes.

Optimum procedure was applied to 5 types of samples: wastewater (influent and effluent) from a water urban treatment plant, surface water and

wastewater from a municipal solid-waste-treatment plant (before and after treatment). Several positives were found and confirmed in the analyzed samples.

Acknowledgments

This work has been developed under financial support of Bancaixa (P1·1B2009-25). The authors acknowledge the financial support of Generalitat Valenciana, as research group of excellence PROMETEO/2009/054 and the financial support from Ministerio de Ciencia e Innovación (project CTQ2009-12347). M.I. Cervera is very grateful to Ministerio de Ciencia e Innovación for her predoctoral grant.

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2. Artículo científico 6

In process, ***2015***

Capturing chemical signals emitted by tulip bulbs before and after induction by herbivorous mites (Acari: Eriophyidae)

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Abstract

Gas chromatography (GC) coupled to time-of-flight mass spectrometry (TOF MS) and triple quadrupole (QqQ) tandem MS were the techniques applied in the investigation of volatile organic compounds (VOCs). The work was to investigate which VOCs are emitted by tulip bulbs that were either infested by the eriophyid mite, *Aceria tulipae*, or not; and that were subject to treatments with the plant hormone ethylene or a blocker of its receptor in the plant, 1-methylcyclopropene (1-MCP), as well as to appropriate control treatments. These treatments served to test the role of ethylene in mediating induction of the release of bulb volatiles. First, GC-TOF MS served as a powerful tool for qualitative/elucidative purposes, and it was applied to identify target analytes and also to discover non-target volatile compounds in samples from the headspace of

tulip bulbs. Second, GC-MS/MS QqQ was used to quantify initial target analytes and other potential candidates found in GC-TOF MS non-target analysis. An additional challenge of our work was to create a special set up to trap the volatile compounds emitted by tulip bulbs. The compounds α-methyl-γ-butyrolactone and α-methylene-γ-butyrolactone (tulipalin A) were the initial target analytes based on the literature. Sample extracts were analyzed by GC-TOF MS acquiring accurate-mass full-spectrum data. The presence of at least two accurate-mass *m/z* ions (narrow-mass window of 0.02 Da) at the expected retention time, together with the correspondence of their ion intensity ratios to those of reference standards, were the parameters required for unequivocal identification. Additionally, a non-target investigation was performed with the help of deconvolution software. The spectral library match together with the accurate mass measurements of up to five characteristic ions were evaluated to tentatively identify the non-target compounds. Confirmation was made by acquiring some of the reference standards of the candidates. In this way, R-limonene, 3-carene, benzaldehyde and ethyl-4-ethoxybenzoate were satisfactorily identified. Subsequently, some of the compounds previously identified and others that were suspected to be present or had an interesting biological meaning, were included in a target list, together with the two initial target analytes, in order to quantify them by GC-MS/MS QqQ analysis of extracts from the same samples. Quantification was carried out using calibration with standards in solvent with relative responses versus an internal standard response (pentachlorobenzene). Results obtained by GC-MS/MS QqQ allowed to quantify both initial target compounds in the different samples, with values ranging from 10 to 4500 ng extracted, during the whole third experiment. Additionally, ethyl-4-ethoxybenzoate, benzaldehyde, acetophenone and p-*tert*-butyl-phenol were also

quantified in the different extracts. From an ecological perspective, the volatile chemicals emanating from damage-free and *Aceria*-infested tulip bulbs are essential because they may convey information on bulb condition to other organisms in the food web. In a sequel to this study we will test these chemicals for the responses they elicit in predators of *Aceria tulipae*, which – if attracted – could then function as 'bodyguards' of the plant struggling with this herbivorous mite.

Keywords

Volatile compounds, purge and trap, tulip bulbs, GC-TOF MS, target and non-target analysis, GC-MS/MS QqQ

1. Introduction

Tulips are bulbous flowers from the plant family *Liliaceae*. They are widely cultivated in the Netherlands but have their origin in the Ottoman Empire. Bulbs and flowers are distributed all over the world and almost two thirds of the worldwide bulb production is produced in the Netherlands. Current research efforts in bulb production are focused on improving the control of pests with new and preferably environment-friendly methods. The use of predatory arthropods to control other arthropods feeding on tulip bulbs represents one important line of research.

Predatory arthropods may have to search at random and at large distances of their prey, but they are keen to use any information that brings them more close to their target. In addition to other sensory capacities they use their sense of smell to detect and locate prey. Generally, prey will try to hide and avoid releasing odours. But if they are herbivores, the plants on which they feed may betray their presence by releasing odours in response to being damaged and these herbivory-induced plant odours may then act as attractants to predatory arthropods. Clearly, these chemical signals benefit the plant by increasing predation pressure on herbivores and they are considered to be part of the plant's battery of defences, usually referred to as indirect plant defence (as opposed to plant defence directly aimed at the herbivores). This phenomenon of indirect plant-herbivore interaction has been demonstrated in several plant species and could be regarded as a cost-saving strategy by the plant because it is activated only in the case of an attack by an herbivore [1]. This is the mechanism which tulip bulbs use as defence. Aratchige *et al.* in 2004 [2] studied this mechanism in tulip bulbs infested by *Aceria tulipae*, an herbivorous mite. Tulip bulbs modify

their interior structure in response to attack by a herbivorous arthropod so tiny that it can move in between the bulb scales. The resulting changes in distance between the bulb scales are microscopic, yet sufficient to allow predatory arthropods (*Neoseiulus cucumeris*) to enter the interior space of the bulb. The predators are at least six times larger in diameter and they cannot enter inside unless, caused by the response to the attack by *A. tulipae*, bulbs increase the distance between bulb scales in such a way that they can enter into the bulb and clean-up the herbivorous mites. These crucial changes in bulb morphology enabling predator access are controlled by ethylene, a plant hormone released after herbivore attack. This plant hormone simultaneously induces the release of plant volatiles being those which attract predatory mites [2].

Tulip bulbs should be treated carefully, avoiding stress factors, because these can reduce bulb and flower quality and induce the bulb to release ethylene. The major effects of ethylene on tulip bulbs include gummosis, flower bud abortion, bulb splitting, increased respiration, poor rooting and early flowering. Sources of ethylene during bulb transport and storage include *Fusarium*-infected bulbs, internal combustion engines or ethylene-producing plant material. Endogenous ethylene production in healthy bulbs is very low but depends on temperature, bulb maturity and presence of wounds. In order to prevent such damage and to resist the ethylene effect, 1-methylcyclopropene (1-MCP) is applied as it has been proven to block the ethylene receptor in the plant and to be effective in extending shelf-life and postharvest quality [3].

The characteristics of volatile compounds emitted by plants necessitates an extraction method suitable for trapping them. Sample preparation techniques as solid-phase microextraction (SPME) [4], mainly in headspace mode (HS-SPME),

have been commonly applied for the extraction of volatile analytes from bulbs, flowers, leaves, stems, roots, seeds and even the whole plant [5-7]. Also static headspace [8] and purge-and-trap (P&T) [7,9-11] have been used in this field. They have the common feature of partitioning the analytes into a gas phase and so non-volatile high molecular weight compounds are eliminated, which prevents contamination of the separation column. These methods require a sorbent trap to collect the analytes. Tenax has been found as a suitable trapping sorbent as it gives the cleanest extracts with a very low background [7,12,13]. Less frequently, volatile compounds are extracted with solvents [14] or after steam distillation with a posterior solid-phase extraction (SPE) [15].

The above-mentioned extraction methods and characteristics of volatile analytes fit well with gas chromatography (GC) determination. GC analysis in plants have been generally coupled to mass spectrometry (MS) with single quadruple analyser [5,10,11,15], with ion trap detector (ITD) [6,7,9,14], and less frequently with triple quadrupole (QqQ) [16] or time-of-flight (TOF) analysers [8]. Using QqQ allows to work in tandem MS (MS/MS) mode, which leads to excellent sensitivity and selectivity, being a suitable technique for quantification purposes at very low concentrations.

Recent progress in instrumentation has increased the use of time-of-flight (TOF) analyzers in different fields of applied science. The strong analytical potential of TOF MS comes from the accurate-mass full-spectrum acquisition data with good sensitivity, which is very useful for detection and identification of analytes. Elucidation of unknowns may also be possible using TOF MS without the need of re-injecting the sample, due to adequate information provided on sample composition and the availability of powerful deconvolution software [17].

In this study, we selected initially two target compounds, because of their interest in the field under study: (1) α-methylene-γ-butyrolactone, mostly known as tulipalin A, is one of the most relevant volatile components present in the tulips [9,18,19], (2) α-methyl-γ-butyrolactone, was found to be present in tulip bulbs in an earlier study performed at the University of Amsterdam (UvA). With the aim of searching for these two compounds and also other ones emitted by tulip bulbs, an extraction method based on purge-and-trap, using Tenax as sorbent, was developed. Analysis was carried out by GC-TOF MS, processing data in a target and in a non-target way. After qualitative analysis by TOF MS, the extracts were subsequently analyzed by GC-MS/MS QqQ in order to quantify the compounds previously identified by TOF MS.

The aim of this work was to first identify target and non-target volatile compounds present in tulips bulbs, which are free of herbivory or damaged by a herbivorous mite; and additionally treated with ethylene, an ethylene blocker (1-MCP) or ambient air. Once qualitative analysis was carried out, the quantification of these compounds using reference standards was pursued. The volatiles emanating from these tulip bulbs then serve to test the response of predatory mites in a sequel to this article, in which the elucidation of the compounds will explain predator attraction to infested versus non-infested bulbs.

2. Materials and methods

2.1. Reagents

Reference standards of α-methyl-γ-butyrolactone, α-methylene-γ-butyrolactone (Tulipalin A), benzaldehyde, α-phellandrene, acetophenone, 1-nonanal, benzothiazole, p-*tert*-butyl-phenol, ethyl-2-ethoxybenzoate, ethyl-3-ethoxybenzoate and ethy-4-ethoxybenzoate were purchased from Sigma-Aldrich (the Netherlands and Spain). Stock standard solutions of them were prepared individually by dissolving reference standards in iso-octane and stored in the fridge at 4 °C.

Pentachlorobenzene, purchased from Riedel de Haën, was used as injection standard. A stock standard solution (around 500 μg/mL) was prepared by dissolving the reference standard in iso-octane. Then, a solution at 50 μg/mL concentration level was prepared by dilution with iso-octane.

A mixture of α-methyl-γ-butyrolactone and Tulipalin A was prepared at concentration levels of around 2 and 40 μg/mL, respectively, for the optimization of the sample treatment and to perform the GC-TOF MS analysis. For confirmation and quantification purposes in GC- MS/MS QqQ, a standard calibration curve was prepared in iso-octane from 5 to 1000 μg/L containing α-methyl-γ-butyrolactone, Tulipalin A, benzaldehyde, α-phellandrene, acetophenone, 1-nonanal, benzothiazole, p-*tert*-butyl-phenol, ethyl-2-ethoxybenzoate, ethyl-3-ethoxybenzoate, ethyl-4-ethoxybenzoate with the injection standard, pentachlorobenzene, at 2 μg/mL.

Iso-octane (2,2,4-trimethylpentane, glass distilled grade) was purchased by Rathburn Chemicals LTD (Walkerburn, Scotland) and diethyl ether (stab./BHT, AR) was purchased from Biosolve BV (Valkenswaard, the Netherlands).

Tenax® TA (60/80) was supplied by Grace Discovery Sciences (Albany, USA) and the glass wool, treated with DMCS was supplied by Varian (Middelburg, the Netherlands).

2.2. GC instrumentation

Three different chromatographic systems were used: GC-Q MS during the extraction optimization procedure period and, in addition, GC-TOF MS and GC-MS/MS QqQ during analysis of sample extracts.

To optimize the parameters used in the extraction method, the instrumentation used at the University of Amsterdam (UvA) was a ThermoQuest Trace GC 2000 gas chromatograph connected to a Finnigan Trace MS quadrupole mass spectrometer, operating in electron ionization (EI) mode. The separation was performed using a fused silica DB-5MS column (60 m; 0.25 mm internal diameter (i.d.), 0.25 μm f.t.) J&W Scientific (Folson, CA, USA) and a pre-column, 2 m x 0.53 mm i.d., DPTMDS deactivated retention gap. The oven temperature was programmed as follows: 65 °C (1 min); 70 °C/min to 100 °C (9 min); 3.5 °C/min to 180 °C; 30 °C/min to 330 °C (10.65 min) (total run 48 min). Cold on-column injections of 1 μL of sample extracts were carried out. Helium 99.999 % was used as carrier gas at a constant flow of 1 mL/min. The interface and ion source temperatures were set at 270 °C and 200 °C, respectively. Quadrupole MS

acquisition was performed in full scan mode, *m/z* 40-550. Xcalibur was used as software for controlling the analytical equipment.

Sample analysis was performed at University Jaume I (UJI) with an Agilent 6890N GC system (Palo Alto, CA, USA), equipped with an Agilent 7683 autosampler, coupled to a time-of-flight mass spectrometer, GCT (Waters Corporation, Manchester, UK), operating in EI mode. The GC separation was performed using a fused silica DB-5MS column (30 m; 0.25 mm i.d., 0.25 μm f.t.) J&W Scientific (Folson, CA, USA). The oven temperature was programmed as follows: 60 °C (3 min); 40 °C/min to 100 °C (2 min); 5 °C/min to 220 °C; 40 °C/min to 300 °C (2 min) (total run 33 min). Splitless injections of 1 μL of sample extracts were carried out with an injector temperature of 270 °C and with a splitless time of 1 min. Helium 99.999 % (Praxair, Valencia, Spain) was used as carrier gas at a constant flow of 1 mL/min. The interface and ion source temperatures were set at 280 °C and 250 °C, respectively. A solvent delay of 4 min was used to prevent damage in the ion source filament. TOF MS was operated at a scan time of 0.65 s in the mass range *m/z* 40-400 and using a multi-channel plate voltage of 2750 V. TOF MS resolution was about 6700 (FWHM) at *m/z* 264. PFTBA (perfluorotri-n-butylamine), used for the daily mass calibration as well as for lock mass, was injected via a syringe in the reference reservoir at 30 °C. The *m/z* monitored was 218.9856.

Quantitative analysis was also performed at UJI with an Agilent 6890N GC system (Palo Alto, CA, USA), equipped with an Agilent 7683 autosampler, coupled to a triple quadrupole mass spectrometer, Quattro Micro GC (Waters, Boston, USA), operating in EI mode. The GC separation was performed using a fused silica DB-5MS column (30 m; 0.25 mm i.d., 0.25 μm f.t.) J&W Scientific

(Folson, CA, USA). The oven temperature was programmed as follows: 60 °C; 20 °C/min to 100 °C (2 min); 5 °C/min to 180 °C; 40 °C/min to 300 °C (1 min) (total run 24 min). Splitless injections of 5 μL of sample extracts were carried out with an injector temperature of 270 °C. Helium 99.999 % (Praxair, Valencia, Spain) was used as carrier gas at an initial flow of 5 mL/min (0.75 min) and then at a constant flow of 1.5 mL/min. The interface and ion source temperatures were set at 280 °C and 250 °C, respectively. A solvent delay of 4 min was used to prevent damage in the ion source filament. The MS/MS procedure was designed as Selected Reaction Monitoring (SRM) mode using Argon 99.995 % (Carburos metálicos, Valencia, Spain) as collision gas at a pressure of 2.5 x 10^{-4} KPa in the collision cell. Dwell times per channel between 0.1 and 0.3 s were chosen, depending on the number of transitions recorded in each window and the peak width of each compound, in order to get a minimum of 16 points per peak. PFTBA, used for the daily mass calibration was injected using a syringe in the reference reservoir of the MS system for this purpose.

The application manager TargetLynx, a module of MassLynx software, was used to process data obtained for target compounds in sample extract analysis by GC-TOF MS and GC-MS/MS QqQ. The application manager ChromaLynx was used to investigate the presence of non-target (unknown) compounds in sample extract analysis by GC-TOF MS. Library searching was performed using the commercial NIST library.

2.3. Samples

Tulip bulbs (Cultivar *Yokohama*) were provided by Wageningen UR Bulb Research Centre in Lisse (the Netherlands). The bulbs were in the storage phase, after being harvested in July 2012. From October to November, every two weeks, one batch of tulip bulbs were provided to carry out headspace extractions (3 different batches in total). The tulip bulbs in each sample were either free of herbivorous arthropods, or they were infested with an herbivorous mite, *Aceria tulipae*. Each of three different treatments were applied to them 24 hours before extraction. These treatments were performed by maintaining the bulbs inside a big container with a solution at 10 μg/mL of ethylene (treatment 1), with a solution at 1 μg/mL of 1-MCP (treatment 2) and with ambient air (treatment 3), respectively. In addition, bulb samples that did not receive any of the three treatments were taken directly from a warehouse with non-infested bulbs and another with infested bulbs (control).

Each of the three batches of infested or non-infested tulip bulbs, treated with ethylene (coded as ETHYLENE), with 1-methylcyclopropene (coded as MCP), with ambient air (coded as BLANCO) or without any treatment (coded as CONTROL) were processed. Thus, the headspace of all these treatments and controls were extracted, plus additionally an empty jar (coded as EMPTY) that had no bulbs and served to monitor the extraction step. All extractions were carried out in duplicate.

2.4. Purge and trap method

The procedure used to perform the extraction of analytes from the tulip bulb samples was based on a 'purge-and-trap' approach developed at the UvA laboratory.

The extraction system consisted of a 750 mL volume glass jar with a special glass cover with two connections on top; the inlet was connected to a dry pure air gas supply and the outlet to the already activated Tenax trap, as shown in ***Figure 1***.

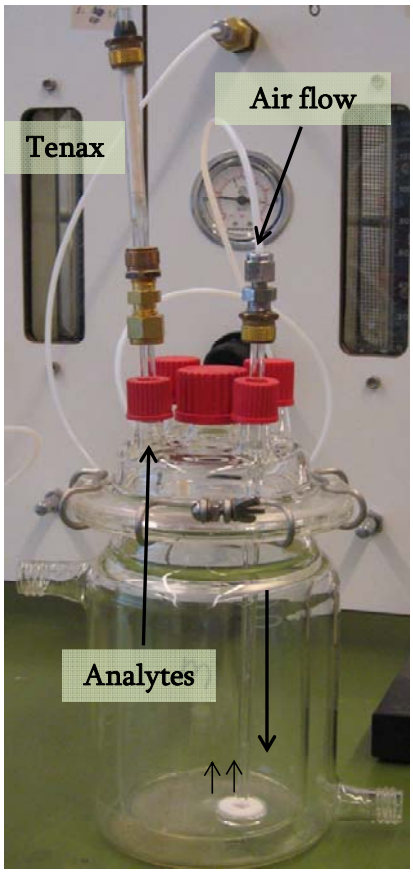

Figure 1. Schematic picture of the extraction assembly carried out in the sample extraction procedure indicating the air flow entrance which pushes the analytes from the gas phase present inside the system towards the exit with the Tenax trap set-up

First, Tenax traps were manually prepared by filling cylindrical glass tubes (12 x 0.5 cm) with 1 g of Tenax and putting two small pieces of glass wool at either side of the tube to enclose and compress the sorbent inside. The cleaning of each Tenax trap was carried out by passing through 3 x 10 mL of the elution solvent. After that, the traps were purged with pure N_2 in an oven used for this purpose with the following temperature program: 35 °C (15 min); 35 °C/min to 245 °C (39 min) (total run 60 min). Finally, the traps tubes were capped and stored in a desiccator until being used.

Ten tulip bulbs (roughly 250 g) were put inside the jar and this was hermetically closed. The extraction was carried out at 20 °C in darkness while an air stream was flowing through the jar to the Tenax trap at a speed of 150 mL/min (batch 1) or 10-20 mL/min (batches 2 and 3). After 24 hours, the Tenax trap was removed and replaced by another one. Then, this trap was eluted with 10 mL of iso-octane (batches 1 and 2) or with 3 mL of diethyl ether (batch 3). After 48 h, the second Tenax trap was removed and replaced by a third one. The elution for the second one was carried out in the same way. Finally, after 72 h, the third Tenax trap was removed and eluted as previously. Once the extracts were obtained, 0.5 mL of the final extract were transferred to a vial together with 20 µL of injection standard at 50 µg/mL to be injected into the GC system (batches 1 and 2). For the third experiment (batch 3), a pre-concentration step was additionally applied and the whole elution volume (3 mL) was evaporated by means of a gentle nitrogen stream by adding 0.5 mL of iso-octane and removing all the diethyl ether. The final volume was adjusted to 0.5 mL by weight and finally 20 µL of injection standard at 50 µg/mL were added, preceding chromatographic determination.

2.5. GC-TOF MS analysis

In the first stage of sample analysis, extracts were analysed by GC-TOF MS, in order to identify the presence of the two selected target compounds in the bulb samples. As ***Table 1*** shows, several *m/z* ions were monitored for each target compound, using TargetLynx application manager. The confirmation requirements of these compounds in the samples were the presence of at least two *m/z* ions at the expected retention time, measured at their accurate mass in the respective narrow window-eXtracted Ion Chromatograms, (nw-XIC) with mass window 0.02 Da. Also their *Q/q* ratio was evaluated by comparison with those of reference standards injected in the same sequence.

In addition to target analysis, deconvolution software (ChromaLynx application manager) was applied to the accurate-mass full-acquisition data obtained for the third batch in order to detect some positive candidates, not included initially in the list of target analytes, because they might be interesting in our research. Reference standards were subsequently acquired and injected for those compounds tentatively identified in order to confirm their identity by comparison of their Rt and mass spectra.

Table 1. List of target compounds for GC-TOF MS analysis with the monitoring ions selected and their elemental compositions

Rt (min)	Compound	Ion 1 (Q)	m/z 1	Ion 2 (q_1)	m/z 2	Ion 3 (q_2)	m/z 3	Ion 4 (q_2)	m/z 4	Ion 5 (q_2)	m/z 5
4.89	α-Methyl-γ-butyrolactone	$C_5H_8O_2$	100.0524	C_4H_8	56.0626	C_4H_7O	71.0497	C_3H_6	42.0471	C_4H_7	55.0548
5.28	Tulipalin A	$C_5H_6O_2$	98.0368	C_4H_4O	68.0262	C_3H_4	40.0313	-	-	-	-
16.38	Pentachlorobenzene (IS)	C_6HCl_5	142.9455	-	-	-	-	-	-	-	-

2.6. GC-MS/MS QqQ analysis

As several candidates for quantitative analysis were found in previous analyses by GC-TOF MS, the quantitative method was created after the putative compounds were confirmed against reference standards. The developed GC-MS/MS QqQ method was applied to the stored sample extracts (-20 °C), acquiring two characteristic SRM transitions per compound (***Table 2***). The confirmative parameters were the presence of two transitions at the expected Rt. Solvent standard calibration curves from 5 to 1000 µg/L were used for quantitative purposes, using the response of the analyte relative to the internal injection standard.

Table 2. List of target compounds for GC-MS/MS QqQ analysis with the SRM transitions selected

Window (min)	Rt (min)	Compound	SRM transition *Q*	Collision energy (eV)	SRM transition *q*	Collision energy (eV)	Dwell time
1 (4.0-5.0)	4.39	α-Methyl-γ-butyrolactone	100.1>56.1	5	56.1>41.1	5	0.1
	4.56	Benzaldehyde	105.8>104.9	10	104.9>77.0	10	0.15
	4.82	Tulipalin A	97.8>67.9	5	97.8>40.3	5	0.15
2 (5.0-5.8)	5.16	α-Phellandrene	92.8>77.0	10	92.8>91.0	10	0.3
3 (5.9-6.6)	6.22	Acetophenone	104.8>76.9	10	119.9>105.0	10	0.3
4 (6.6-7.5)	6.80	1-Nonanal	97.9>69.0	10	97.9>56.0	10	0.3
5 (9.0-13.0)	9.71	Benzothiazole	134.8>107.9	10	134.8>91.0	10	0.1
	11.17	p-*tert*-Butyl-phenol	134.9>107.0	10	149.9>135.1	10	0.1
6 (14.0-18.0)	15.22	Ethyl-2-ethoxybenzoate	119.8>63.9	20	119.8>91.9	10	0.1
	15.93	Ethyl-3-ethoxybenzoate	120.9>93.0	10	137.9>121.0	10	0.1
	16.98	Ethyl-4-ethoxybenzoate	121.1>93.1	10	121.1>65.0	10	0.1
7 (16.0-18.0)	16.95	Pentachlorobenzene (IS)	249.7>179.8	30	-	-	0.2

3. Results and Discussion

3.1. Sample extraction optimization

The extraction procedure was designed in order to trap the different volatile compounds that tulip bulbs emit depending on the treatment received before extraction. Purge and trap, a dynamic headspace method, was chosen to trap the volatile compounds into the sorbent performing a posterior solvent desorption. Taking into account previous experiments made in our laboratory, the assembly used (see ***Figure 1***) was the best option to maintain the original conditions of tulip bulbs without any further damage by stress or similar and in order to get the same compounds that they emit normally in the storage location. Darkness and temperature were also chosen to avoid degradation of volatile analytes, because tulipalin A is air, light and moisture sensitive.

The extraction of the three different batches of samples was performed once per two weeks, extracting them during the first week and optimizing one extraction parameter the following week. Thus, the final optimization was obtained in the last batch (batch 3), taking the best conditions for a more efficient extraction of the volatile compounds from the bulb samples.

The first batch was performed as it had been established in the laboratory, with an air flow set at 150 mL/min and after, the Tenax trap eluted with 10 mL of iso-octane, getting the extracts directly to be injected into the GC system. After the first batch was finished, the air flow was better optimized, testing the extraction procedure at two different flows: 10-20 and 150 mL/min. In order to select the best value, two Tenax traps were spiked on top of the glass tube with 1 mL of a mixture α-methyl-γ-butyrolactone and tulipalin A standards at 2 and 40

μg/mL, respectively, and leaving the solvent to dry. Both traps were installed in the extraction assembly in two different jars and also, in order to check the breakthrough, a second trap was coupled to the first one on-line for both selected flows during 24 hours. After this extraction time, the two traps were eluted with 10 mL of iso-octane and analysed in the GC-Q MS system. Results indicated that breakthrough was not yield for any novel assembly since no detectable peaks were seen in the chromatograms of both coupled Tenax traps. Higher areas of target analytes (2-3 times) were observed with the lowest flow selected and for this reason, 10-20 mL/min was selected as the optimum flow to use in the following experiments.

Thus, the samples from batch 2 were extracted with an air flow set at 10-20 mL/min and the Tenax trap eluted with 10 mL of iso-octane. Before carrying out batch 3, a further optimization in the elution step was made, i.e. the solvent and volume elution were then studied. To this aim, the study consisted in two Tenax traps which were spiked with 1 mL of the standards mixture and dried, as previously described. The two solvents selected for cartridge elution were iso-octane and diethyl ether. Once the spiking solvent was dried, the Tenax traps were eluted with 10 mL of iso-octane and 10 mL of diethyl ether, respectively. Each solvent was added in ten fractions of 1 mL, and collecting them individually for posterior analysis by GC-Q MS system. A solvent exchange was required for diethyl ether, adding 1 mL of iso-octane to each fraction obtained, evaporating it with a gentle nitrogen stream down to 1 mL as final volume and eliminating completely the diethyl ether from the extracts. Thereafter, fractions obtained for each solvent were analysed by GC-Q MS. The results showed that for iso-octane extracts, the peaks corresponding to target compounds were detected in the ten fractions. On the contrary, the target compounds were detected only in the first

three fractions eluted with diethyl ether. According to these data, it seemed that elution for the target analytes was more efficient with diethyl ether by using less volume to extract the same amount of analytes. Thus, the solvent selected for elution (in batch 3) was diethyl ether with an elution volume of 3 mL. Therefore, this solvent was not suitable to be injected into the available injection system and a solvent exchange was carried out at the same time as the pre-concentration step, improving the detection limits. Thus, 0.5 mL of iso-octane was added into the 3 mL of diethyl ether extract, evaporating by a gentle nitrogen stream down to a final volume of 0.5 mL, adjusting the final volume by weight. At that point, the best extraction parameters had been reached; thus extractions for batch 3 were performed under these optimum conditions, leading to the most reliable results in the study.

3.2. GC-TOF MS analysis

Once the extraction of the volatile compounds from tulip bulbs was performed, their determination was carried out by GC-TOF MS, taking advantage of the accurate-mass full scan spectrum acquisition data. First, analysis was directed towards the target analytes initially selected. Then, a non-target screening was carried out, without the need of re-injecting the samples, in order to detect the presence of other compounds that might be of interest.

3.2.1 Target analysis

The determination of target analytes was carried out in the 162 extracts resulting from the three treatments (coded as ETHYLENE, MCP, BLANCO) and without treatment (coded as CONTROL) of both types of bulbs (non-infested and infested), plus the blank system (coded as EMPTY) at the three different extraction times (24, 48 and 72 h). This was made for the three sets of experiments (batches 1 to 3), for all cases in duplicate.

The results obtained showed that both tulipalin A and α-methyl-γ-butyrolactone were present in almost all extracts coming from tulip bulb samples although with different response intensities. As expected, these compounds were not detected from the blanks of system (EMPTY). ***Figure 2*** shows the identification of tulipalin A in a non-infested tulip bulb sample treated with ambient air stream making use of GC-TOF MS (nw-XIC with mass window 0.02 Da). Experimental EI accurate mass spectrum and chemical structures proposed for the most abundant fragment ions, together with experimental mass errors (in mDa), are also shown.

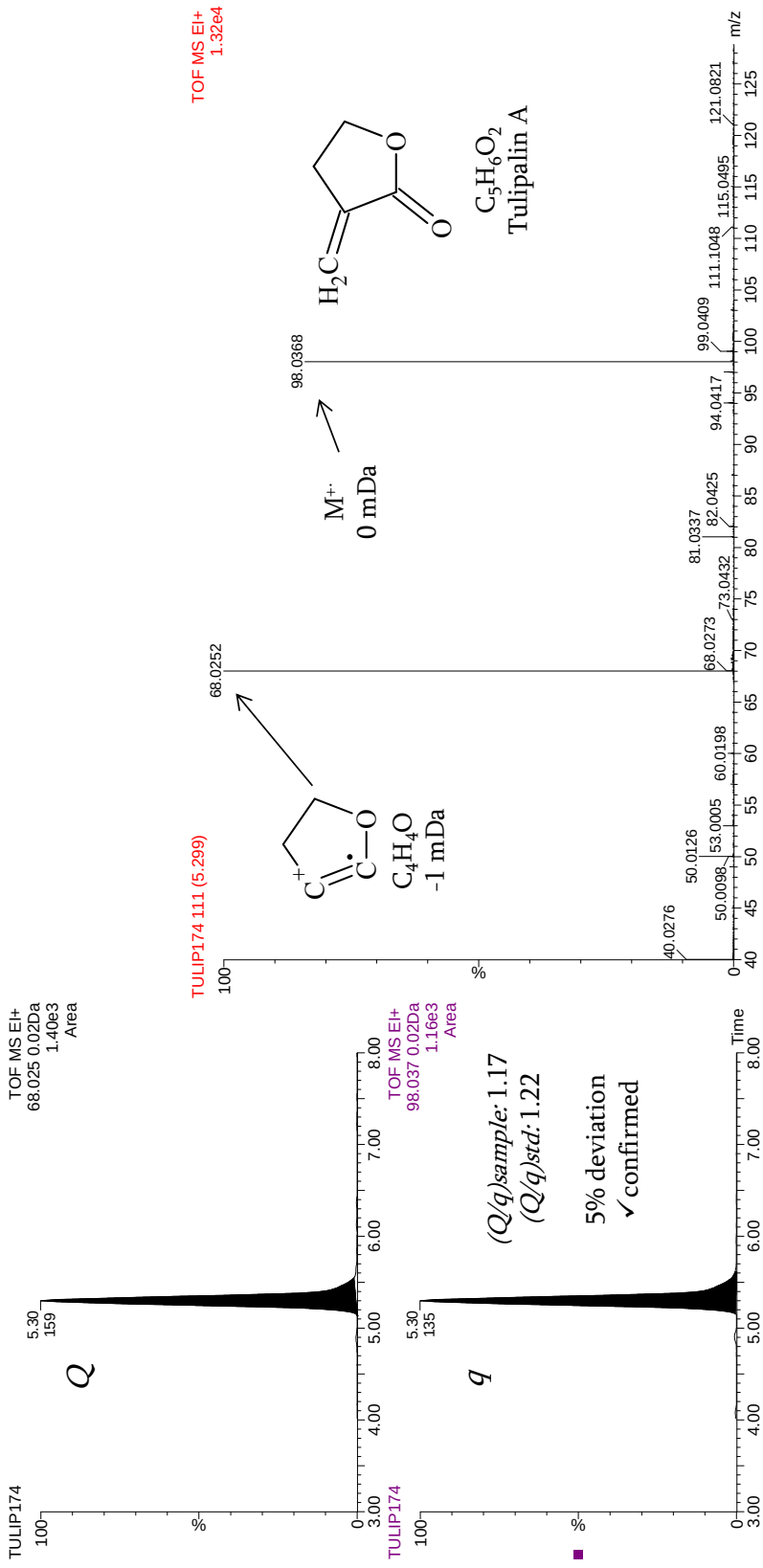

Figure 2. GC-TOF MS nw-extracted ion chromatogram (mass window 0.02 Da) showing the detection of tulipalin A in a non-infested tulip bulb sample treated with ambient air. Experimental EI accurate mass spectrum and chemical structures proposed for the most abundant fragment ions together with experimental mass errors (in mDa) are also shown

3.2.2. Non-target analysis

Non-target analysis were performed only for the extracts obtained from the third set of extractions, batch 3, as a pre-concentration step was applied and the potential analytes were expected to be found at higher concentrations. Data processing was carried out by applying the ChromaLynx Application Manager, which allowed the automated detection of sample components and their subsequent identification making use of the accurate-mass full-scan spectra data acquired.

A large number of potential candidates were found although the reference standards were required in a subsequent step for confirmation of their identity. R-Limonene was one of the compounds tentatively identified in several samples. After acquisition of the reference standard, R-limonene could be identified only in one of these samples as after comparing the mass spectra, higher mass errors were found and/or the interference of the background ions, surely due to their relatively low concentration in those samples, and for this reason, strictly, they could not be confirmed. ***Figure 3*** shows the positive of R-limonene found in a infested tulip bulb sample treated with 1-MCP in the 72 hours of extraction (third Tenax trap). The deconvoluted chromatographic peak is shown together with the experimental and theoretical EI mass spectra, and the chemical structures proposed for four representative fragment ions together with their mass errors (in mDa).

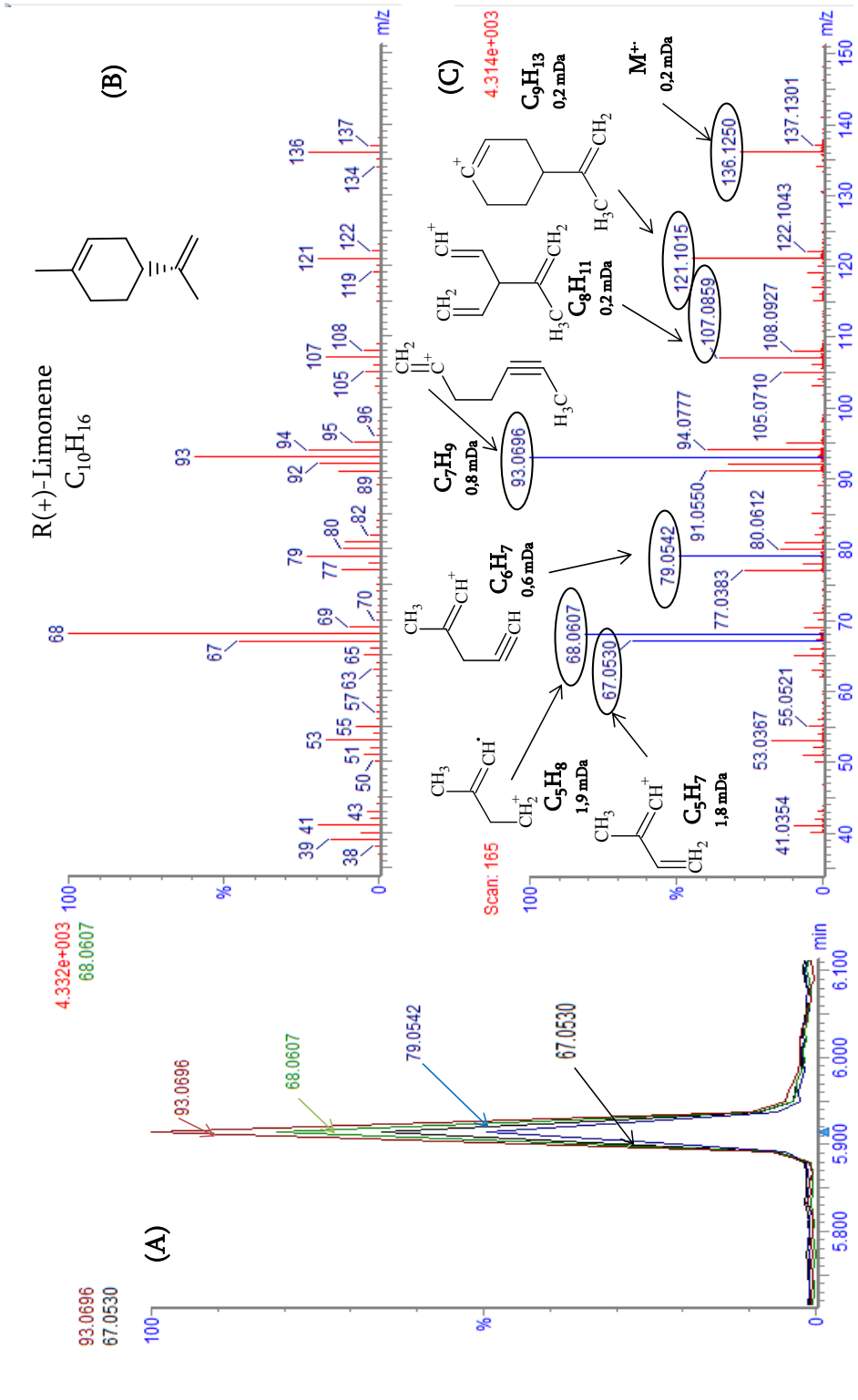

Figure 3. Identification of non-target R(+)-limonene. (A) Extracted ion chromatograms for ions used for deconvolution. (B) Library mass spectrum of R(+)-limonene at nominal masses (match 828). (C) Deconvoluted accurate mass spectrum of R(+)-limonene from the sample and the chemical structures proposed for representative EI fragment ions together with mass errors (in mDa)

Another tentatively identified compound was 3-carene, which could be confirmed by comparison with the reference standard in the non-infested tulip bulbs treated with ethylene, in the first 24 hours of sampling (first Tenax trap), and in the infested tulip bulbs treated with 1-MCP, in the last 72 hours.

Benzaldehyde was also confirmed in several samples: the clean tulip bulbs after 1-MCP treatment, and the infested ones without any treatment (CONTROL) in the first 24 hours, as well as in the clean tulip bulbs with 1-MCP treatment (sampling in the next 48 hours (second Tenax trap)) and in infested tulip bulbs with the ambient air treatment.

Ethyl-4-ethoxybenzoate was tentatively identified but its EI mass spectrum was similar to the isomers ethyl-2-ethoxybenzoate and ethyl-3-ethoxybenzoate. For this reason, the three mentioned reference standards were acquired in order to confirm which of these isomers were truly present in the samples. By comparison with the retention time and the mass spectra, the identity of the ethyl-4-ethoxybenzoate was unequivocally confirmed. ***Figure 4*** shows the identification of ethyl-4-ethoxybenzoate in a non-infested tulip bulb sample treated with 1-MCP by GC-TOF MS showing the nw-XIC for several *m/z* ions (with mass window 0.02 Da) and its experimental EI mass spectra with the chemical structures proposed for the fragment ions together with their mass errors (in mDa).

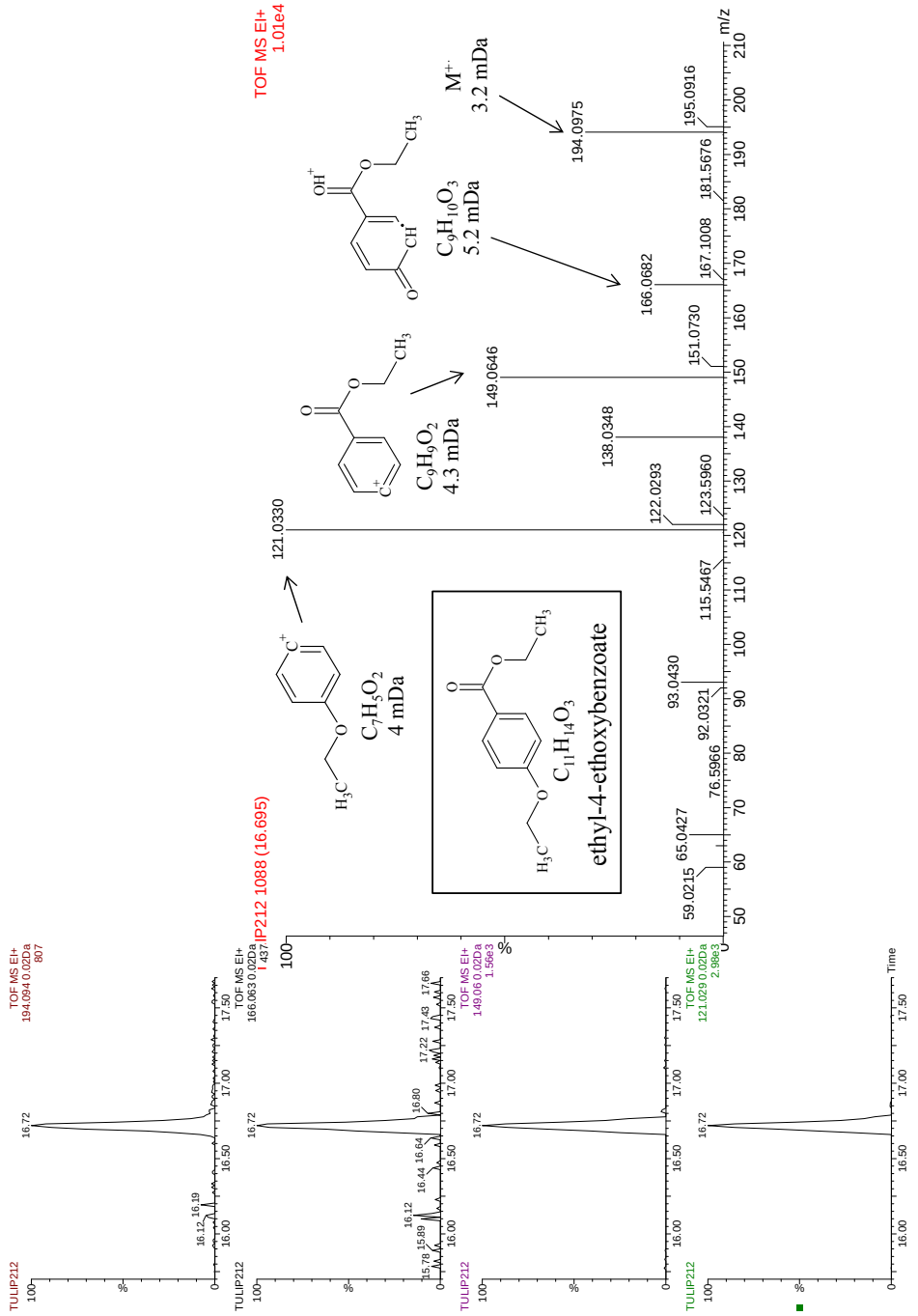

Figure 4. GC-TOF MS nw-extracted ion chromatogram (mass window 0.02 Da) showing the presence of ethyl-4-ethoxybenzoate with experimental EI accurate mass spectrum and chemical structures proposed for the most abundant fragment ions together with experimental mass errors (in mDa)

Table 3. List of automatically non-target compounds detected for the third experiment samples in their last 72 hours of extraction

Treatment	Ethylene, Clean bulbs	1-MCP, Clean bulbs	Ambient air, Clean bulbs	Not-treated, Clean bulbs
Candidates	Methyl ester hexanoic acid (526/ 4 ions/ 2,9 mDa) Benzothiazole (567/ 2 ions/ 1,9 mDa) **Ethyl-4-ethoxybenzoate** (558/ 4 ions/ 4 mDa)	Methyl ester hexanoic acid (680/ 5 ions/ 4 mDa) Acetophenone (624/ 3 ions/ 0,6 mDa) (S)-(+)-6-Methyl-1-octanol (718/ 5 ions/ 2,3 mDa) p-tert-Butyl-phenol (509/ 4 ions/ 3 mDa)	Methyl ester hexanoic acid (676/ 4 ions/ 0,9 mDa) α-Phellandrene (524/ 5 ions/ 0,6 mDa) **α–Methyl-γ–butyrolactone** (764/ 5 ions/ 3,6 mDa) **Tulipalin A** (667/ 3 ions/ 4 mDa) 2-Ethyl-1-hexanol (758/ 5 ions/ 2,9 mDa) Acetophenone (797/ 3 ions/ 0,7 mDa) 6-Methyl-1-octene (778/ 5 ions/ 2,4 mDa) 1- Nonanol (765/ 5 ions/ 2,3 mDa) 3-Methoxy-benzaldehyde (657/ 5 ions/ 1,7 mDa)	**α–Methyl-γ–butyrolactone** (589/ 5 ions/ 4 mDa) **Tulipalin A** (685/ 5 ions/ 3,9 mDa) 2-Ethyl-1-hexanol (691/ 5 ions/ 3,3 mDa) Acetophenone (863/ 3 ions/ 0,7 mDa) (S)-(+)-6-Methyl-1-octanol (817/ 5 ions/ 2,4 mDa) 1- Nonanol (782/ 5 ions/ 3,8 mDa) m-*tert*-Butyl-phenol (585/ 5 ions/ 3,9 mDa)
Treatment	**Ethylene, Infested bulbs**	**1-MCP, Infested bulbs**	**Ambient air, Infested bulbs**	**Not-treated, Infested bulbs**
Candidates	**Ethyl-4-ethoxybenzoate** (614/ 4 ions/ 3,2 mDa) 3-Ethyl-5-(2-ethylbutyl)-octadecane (599/ 5 ions/ 2,8 mDa)	Methyl ester hexanoic acid (821/ 4 ions/ 1,2 mDa) (1-Methylhexadecyl)-benzene (587/ 5 ions/ 1,4 mDa) **3-Carene** (725/5 ions/1 mDa) Propyl-benzene (633/ 5 ions/ 3,1 mDa) **R-limonene** (878/ 5 ions/ 1,9 mDa)	Methyl ester hexanoic acid (687/ 5 ions/ 4,1 mDa) **Ethyl-4-ethoxybenzoate** (637/ 5 ions/ 1 mDa) 2,6,10,15-Tetramethyl-heptadecane (685/ 5 ions/ 3 mDa)	3-Ethyl-3-methylpentane (822/ 5 ions/ 3,8 mDa) m-*tert*-Butyl-phenol (536/ 5 ions/ 1,3 mDa)

In brackets: library forward match/number of compatible ions/maximum experimental mass errors. In Bold the compounds correctly identified by comparison with a reference standard

Table 3 shows as an illustrative example, the possible candidates in the samples of the third experiment (batch 3) for the extracts obtained with the last 72 hours of extraction (third Tenax trap), highlighting those which could be identified using their reference standards. All these compounds were found by application of a non-target analysis, making use of the deconvolution software (ChromaLynx XS). The absence of some of these compounds in the samples does not necessarily mean that they were not present in these samples, as the software gave an automatic response for the most abundant peaks showing higher responses. For this reason, for those considered as the most interesting compounds (since a biological point of view) a deeper study was needed making use of a target analysis directed towards the potential candidates and using a more sensitive technique.

3.3. GC-MS/MS QqQ analysis

A quantitative and sensitive method was developed for some of the afore mentioned interesting compounds and also for the two initial target analytes (*Table 2*). Despite the right identification of the isomer ethyl-4-ethoxybenzoate in some samples, the other two isomers (ethyl-2-ethoxybenzoate and ethyl-3-ethoxybenzoate) were also included in the method. Analysis was performed by GC-MS/MS QqQ in the extracts obtained from the Tenax extractions. The optimized method for the analytes selected as well as the MS/MS transitions and collision energy is shown in *Table 2*.

In order to optimize the SRM transitions, a mixture containing the reference standards in solvent was used. Full scan spectrum was firstly acquired

for all analytes with the aim of selecting the most abundant ions (typically the base peak or other intense peaks) as precursor ions. In a second step, these precursors ions were fragmented with different values of collision energy (between 5 and 30 eV) to perform their fragmentation. Thus, the most abundant product ion was selected to create a transition at the adequate collision energy from the precursor ion. Finally, the SRM method was created acquiring two transitions per compound. For pentachlorobenzene, the injection internal standard, only one transition was acquired.

The quantification was made using a standard calibration in solvent from 5 to 1000 μg/L, with the relative response of analyte versus the injection standard (at 2 mg/L). The confirmatory criteria were the presence of the two transitions at the expected retention time.

Table 4 shows the results for the compounds detected and quantified in the sample extracts from the tulip bulbs for the third experiment. The value shown is the sum of the total mass extracted for the three extracts obtained after 24, 48 and 72 hours, giving a general overview of the amount extracted in the whole experiment.

The results showed that tulipalin A and α-methyl-γ-butyrolactone were present in all samples (unless in one of the two replicates) and no present in the extraction of the empty jar. Unexpectedly, as the other compounds were also present from the jars without bulbs, their presence could not be unequivocally attributed to the tulip bulbs and a reliable explanation about the relation between the compounds and their behaviours could not be achieved.

Table 4. Quantitative results obtained by GC-MS/MS QqQ after application of the overall procedure (concentration expressed in ng extracted)

TREATMENT	Compounds detected											
	α-Methyl-γ-butyrolactone		Tulipalin A		Ethyl-4-ethoxybenzoate		Benzaldehyde		Acetophenone		p-*tert*-Butyl-phenol	
	A[1]	B[2]	A	B	A	B	A	B	A	B	A	B
EMPTY	-	-	-	-	85	101	39	32	26	27	55	55
ETHYLENE-C[3]	43	718	58	478	81	74	44	49	31	97	61	55
MCP-C[3]	363	4557	261	761	69	69	47	55	176	278	48	52
BLANCO-C[3]	1466	915	536	698	86	57	49	106	379	791	61	110
CONTROL-C[3]	826	113	333	125	98	78	56	36	250	48	60	51
ETHYLENE-I[4]	92	565	78	668	85	82	46	58	32	43	56	63
MCP-I[4]	251	232	181	163	130	98	59	49	39	34	63	63
BLANCO-I[4]	10	144	25	98	68	68	42	33	32	27	56	57
CONTROL-I[4]	524	175	281	127	102	73	38	40	31	29	58	56

[1] Replicate A

[2] Replicate B

[3] Samples corresponding to healthy tulip bulbs

[4] Samples corresponding to infested tulip bulbs

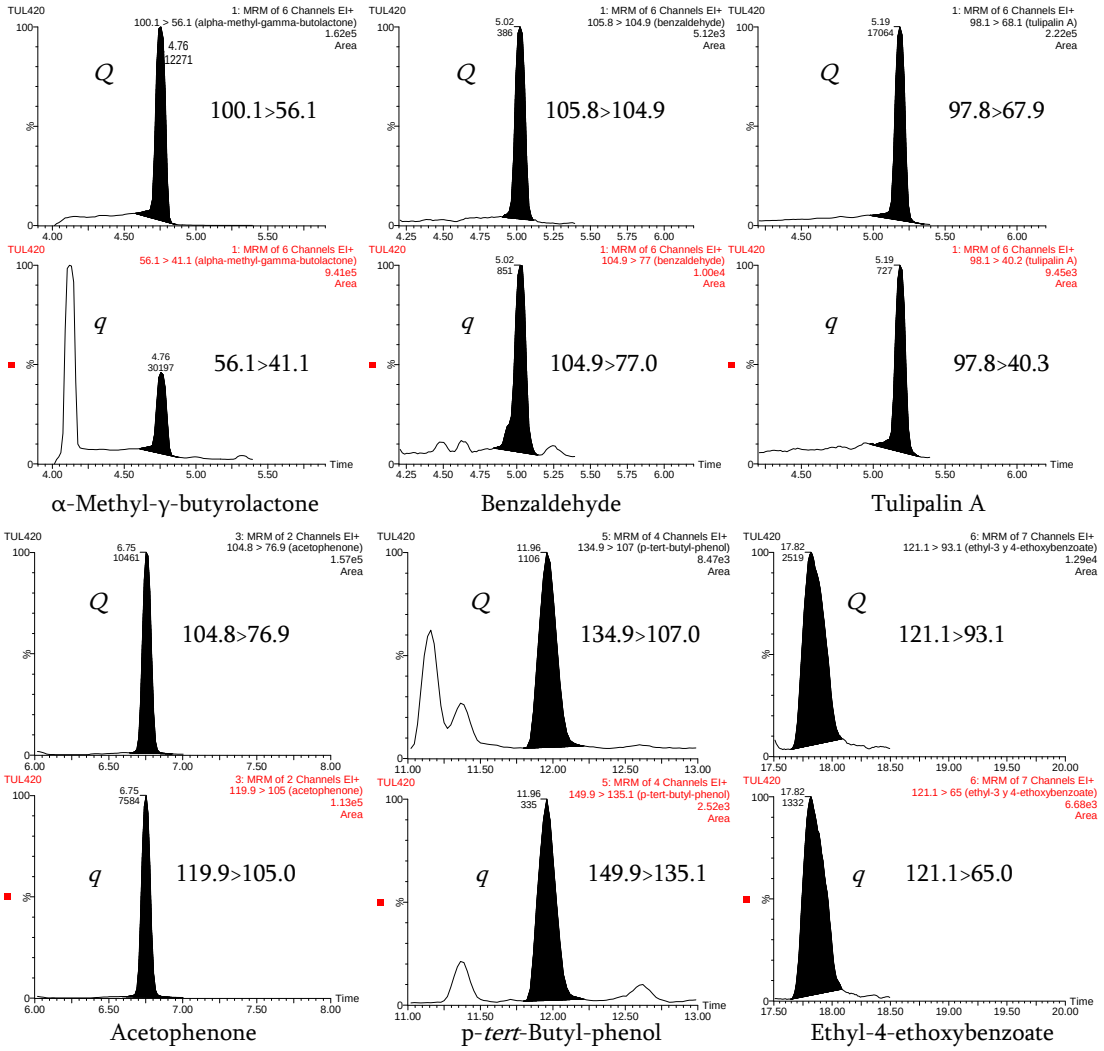

Figure 5. GC-MS/MS QqQ chromatograms for volatile compounds detected in a non-infested tulip bulb sample treated with 1-MCP in the last 72 h of extraction. (Q) Quantification transition, (q) confirmation transition

Figure 5 shows the GC-MS/MS QqQ chromatograms for some volatile organic compounds detected in a non-infested tulip bulb sample treated with 1-MCP in the last 72 h of extraction.

Further statistical studies about tulip bulbs behaviour with current results still need to be performed after obtaining quantitative data from different batches of tulip bulbs samples analysis, including infested and non-infested by *Aceria tulipae*, which had been treated with ethylene, 1-MCP and ambient air previously; or without treatments, in order to try to find some conclusion between the treatments applied and the infestation, over time.

4. CONCLUSIONS

In our study, different approaches were used to investigate the presence of volatile compounds emitted from infested and non-infested tulip bulb samples. The extraction method was designed in order to maintain the original conditions in the storage location of tulip bulbs without any further damage by stress, and to trap the volatile compounds which are commonly emitted. Darkness and appropriate temperature was chosen to avoid the degradation of some volatile analytes, as for example tulipalin A.

The potential of GC-TOF MS has been used in a first exploratory analysis for identification of both target and non-target compounds that may be of interest in the tulips bulb study regarding their composition in order to know their profile of volatiles. In addition, the use of GC-MS/MS QqQ enabled us to quantify the most interesting compounds identified (or tentatively identified) in previous GC-TOF MS analysis with excellent sensitivity and selectivity.

Regarding quantitative results, tulipalin A and α-methyl-γ-butyrolactone showed a marked tendency in their relation, as the higher the proportion of tulipalin A in the mixture, the more attractive the tulip odour was.

Acknowledgments

The authors are very grateful to the SCIC of University Jaume I (Castellón, Spain) for the use of the time-of-flight mass spectrometer. The authors acknowledge the financial support of Generalitat Valenciana (research group of excellence PROMETEO/2009/054; ISIC 2012/016). M.I. Cervera also acknowledges to the Ministry of Economy and Competitiveness of Spain for her pre-doctoral grant and for the financial support for her short stay in the Netherlands.

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4. Discusión de los resultados

El **Capítulo 4** está dedicado al desarrollo de métodos de análisis para la determinación de compuestos volátiles orgánicos, extrayéndolos desde dos matrices muy distintas. Así, en el **Artículo científico 5** se ha desarrollado un método cuantitativo para la determinación de 23 VOCs, que podrían estar presentes en las aguas, mediante extracción por HS-SPME y posterior análisis por GC-MS/MS QqQ. En el **Artículo científico 6** se utiliza un montaje experimental diseñado para atrapar los compuestos volátiles orgánicos que emiten los bulbos de tulipán simulando su etapa de almacenamiento, de modo que tras la identificación de dichos compuestos se pueda estudiar su comportamiento frente a los tratamientos que reciben los tulipanes para mejorar su producción. La extracción se llevó a cabo mediante P&T y para los análisis se combinó el uso de GC-TOF MS con fines cualitativos/elucidativos con el potencial del GC-MS/MS QqQ para fines cuantitativos.

En el **Artículo científico 5** los análisis se llevaron a cabo mediante GC-MS/MS QqQ, que permitió aplicar un método MS/MS con mejoras en sensibilidad y selectividad en comparación con analizadores más convencionales como cuadrupolo. El método fue diseñado para 23 VOCs, algunos de ellos incluidos en listas de contaminantes prioritarios en aguas superficiales (Directive 2013/39/UE). Dado que los límites regulados para estos compuestos son del orden de pocos µg/L, se consideró que el diseño basado en tándem MS podría ser ventajoso para alcanzar estos niveles de concentración y llevar a cabo la correcta cuantificación.

Debido al carácter volátil de los compuestos objeto de estudio, la etapa de extracción se llevó a cabo mediante HS-SPME, atrapándolos en un sorbente

adecuado y desorbiéndolos en el inyector del GC-MS. A causa de que algunos analitos eluían antes que el disolvente, apareció un problema en el análisis de los patrones en solvente y es que, al establecer un *solvent delay* para prevenir la rotura del filamento, la pérdida de información era inevitable. Así, la optimización del trabajo, en especial del método SRM, se llevó a cabo mediante sucesivas extracciones por HS-SPME, de un agua HPLC fortificada a 1 µg/L, en lugar de realizar la inyección directa de los patrones de referencia. Las extracciones mediante HS-SPME permitían obtener un cromatograma limpio con todos los analitos, ya que al no inyectar disolvente no era necesario limitar el tiempo inicial de adquisición del detector. Del mismo modo, la cuantificación se realizó mediante extracción (por duplicado) por HS-SPME del calibrado externo preparado en agua HPLC.

Para la optimización de las transiciones SRM, se realizó en primer lugar el análisis por GC-(EI)QqQ MS en modo *full scan* de la mezcla de los patrones en solvente y tras observar el espectro de masas experimental se seleccionaron aquellos iones considerados buenos candidatos como iones precursores y crear la transición SRM. Normalmente, si el ion molecular estaba presente, éste se seleccionó como ion precursor. En algunos VOCs, como *chloroform*, *1,1,1-trichloroethane*, *carbon tetrachloride, 1,2-dichloroethane, 1,2-dichloropropane, bromodichloromethane, 1,1,2-trichloroethane, dibromochloromethane* y *bromoform*, la ausencia o escasa abundancia del ion molecular llevó a seleccionar el pico base, u otro ion intenso, como ion precursor para generar iones producto con valores de *m/z* no demasiado bajos, que no sufrieran interferencias con el ruido de fondo. Posteriormente, una vez seleccionados los potenciales iones precursores, se realizaron análisis en modo de barrido de iones producto (*product ion scan)* aplicando diferentes energías de colisión desde 5 a 40 eV. Para la

mayoría de compuestos, no hubo problemas en la selección de los iones producto, escogiendo normalmente los más intensos obtenidos a una energía de colisión determinada, para poder crear una transición SRM. Sin embargo, para algunos de los iones precursores seleccionados se observó que, incluso con bajas energías de colisión, sufrían extensiva fragmentación, que provocaba la desaparición de los iones de mayor *m/z*. Así, para 6 de los 23 compuestos estudiados, utilizando EI, no se pudieron crear transiciones SRM al no haber encontrado iones precursores y/o productos adecuados para ello.

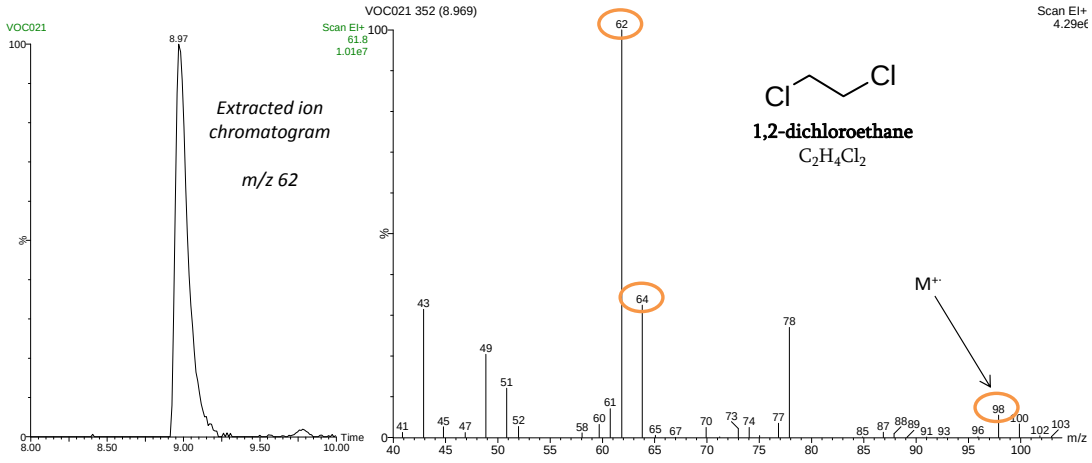
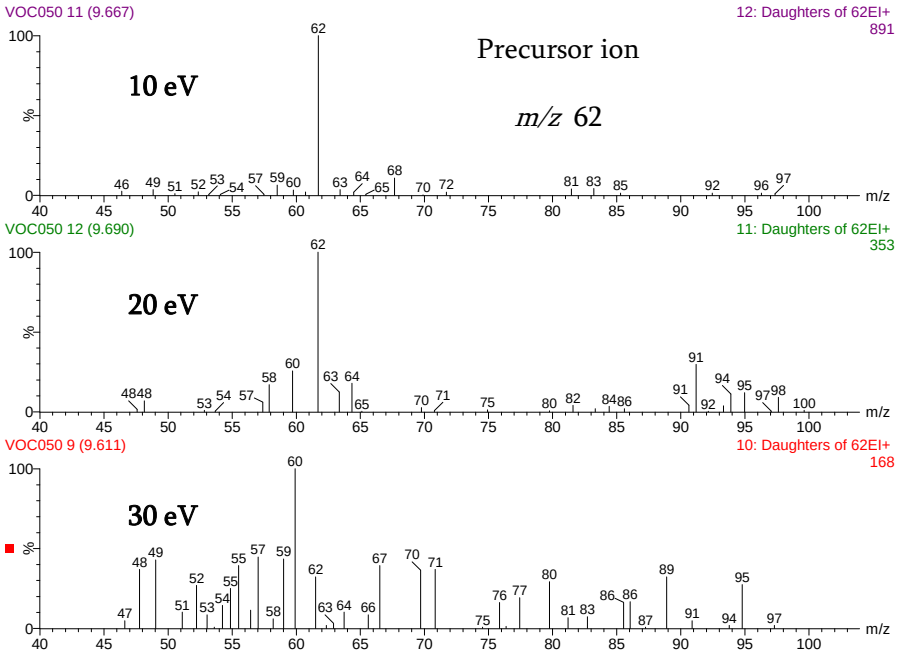


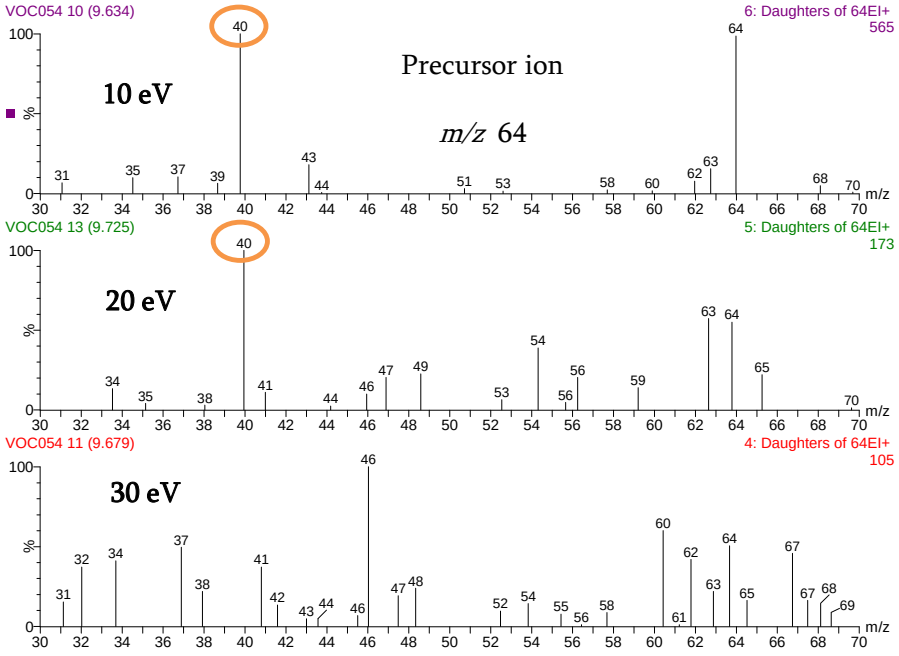
Figura 2. Cromatograma y espectro de masas obtenidos mediante GC-(EI)QqQ MS adquiridos en modo *scan* para el *1,2-dichloroethane* (10 µg/L) seleccionando los iones *m/z* 62, 64 y 98 como iones precursores

A modo de ejemplo, en la **Figura 2** se muestra el espectro de masas experimental adquirido en modo *scan* para *1,2-dichloroethane*. A la vista del espectro, se podrían seleccionar como posibles precursores los iones *m/z* 62 y 64, así como el ion molecular *m/z* 98, resultando este último de menor intensidad. Estos iones se seleccionaron para realizar un segundo análisis en modo de barrido

de iones producto (*product ion scan*) a diferentes energías de colisión (10, 20 y 30 eV). Los diferentes espectros de masas de barrido de iones producto se pueden observar en la **Figura 3**.



(Acquisition window 40-100)



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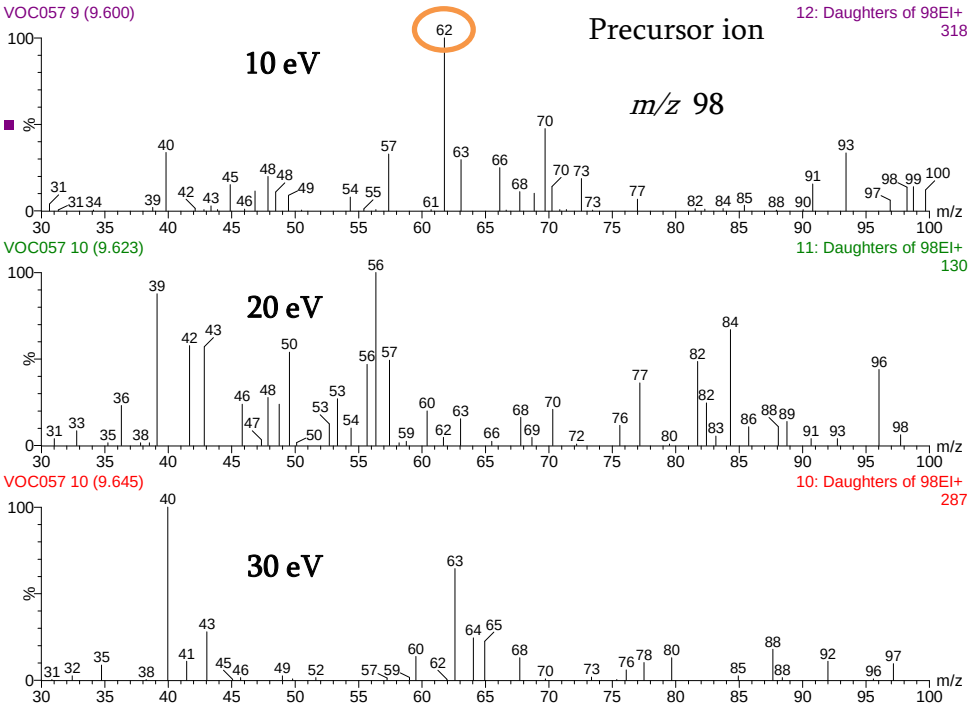

Figura 3. Espectros de masas obtenidos mediante GC-QqQ MS en modo *product ion scan* para los iones precursores *m/z* 62, 64 y 98 a distintas energías de colisión

Se observa que los espectros EI presentan un alto grado de fragmentación, razón por la que los iones precursores seleccionados no producen iones producto abundantes para una adecuada y sensible transición SRM. Se probaron en modo SRM las posibles transiciones 64>40 y 98>62, incluso con menor energía de colisión (5 eV), pero no se obtuvieron buenos picos que pudieran usarse para un método analítico sensible y específico.

Hasta 6 de los 23 compuestos estudiados presentaron esta misma problemática, con lo que se diseñó un método SIM para ellos, seleccionando 3 iones característicos para cada uno. Trabajando en modo SIM no se adquieren transiciones MS/MS, ya que únicamente se monitorizan los iones en el primer

cuadrupolo, mientras que la celda de colisión y el segundo cuadrupolo se encuentran en modo de transmisión. Los resultados fueron adecuados, aunque resultaba necesario trabajar en modo SIM (para algunos compuestos) junto con el modo SRM (para el resto), con el fin de aprovechar el incremento de sensibilidad y selectividad aportado con este último modo, para la mayoría de VOCs. Tras una búsqueda bibliográfica de casos similares se encontró una solución para aquellos compuestos en los que usar una transición SRM resultaba una tarea complicada o imposible. Se usó lo que se conoce como pseudo-SRM, monitorizando en el primer cuadrupolo un ion, y sin aplicar ninguna energía o un valor mínimo en la celda de colisión, monitorizando el mismo ion en el segundo cuadrupolo, creando así una pseudo-transición (Haug, 2009; Ajibola, 2014; Sancho, 2000).

Si se comparan ambas aproximaciones, SIM y pseudo-SRM, para el caso concreto del *1,2-dichloroethane* (**Figura 4**) después de aplicar extracción mediante HS-SPME (agua fortificada a 50 μg/L), la intensidad de los picos es mayor en el método SIM, entendible dado que en SRM pasa un doble filtro. Sin embargo, se observa una clara mejora de la relación S/N en pseudo-SRM, la cual sería previsiblemente más notoria en el análisis de muestras reales, eliminando las coeluciones que puedan surgir. Por otro lado, para el ion *m/z* 49 del *1,2-dichloroethane*, en modo SIM, se observa una mala forma de pico, que podría complicar la confirmación del compuesto detectado en las muestras, ya que se requeriría la presencia de los 3 iones para cumplir con los puntos de identificación necesarios (Commission Decision 2002/657/EC).

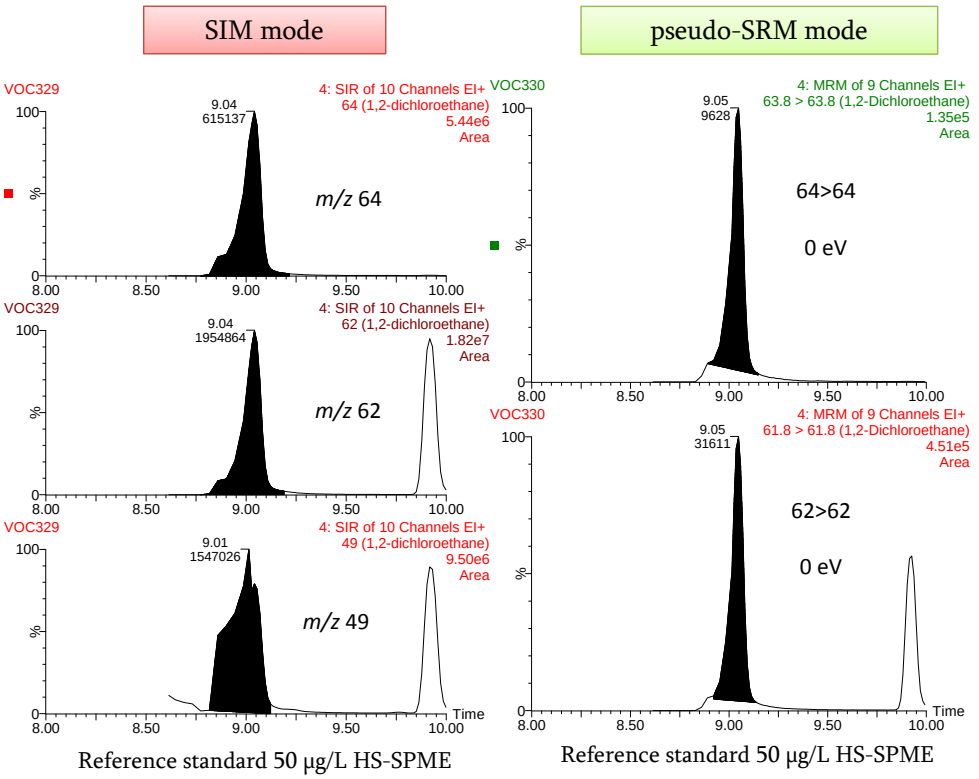

Figura 4. Cromatogramas GC-QqQ MS comparando modo SIM vs modo pseudo-SRM para el *1,2-dichloroethane* (patrón de referencia de 50 µg/L)

Finalmente, usando modo SRM para la mayoría de VOCs y modo pseudo-SRM para 6 de ellos, se consiguió en un único análisis toda la información necesaria para la correcta cuantificación e identificación de los compuestos investigados. En la ***Tabla 1*** del **Artículo científico 5**, se muestran las condiciones experimentales del método optimizado.

Para corregir las posibles desviaciones durante la etapa de extracción así como la variabilidad instrumental se usó como patrón interno *benzene-d*$_6$, que presenta una volatilidad intermedia entre los 23 compuestos seleccionados. Las respuestas se calcularon como áreas relativas con la respuesta el analito frente la respuesta del patrón interno.

Una vez creado el método instrumental, se procedió a la optimización del procedimiento experimental, mediante un diseño estadístico (*full factorial*) utilizando el software estadístico StatGraphics. Mediante esta aproximación se optimizaron de forma combinada la mayoría de parámetros involucrados en la extracción, en lugar de proceder a la optimización secuencial de cada uno de los factores, lo cual generaría un número elevado de experiencias y un gran consumo de tiempo.

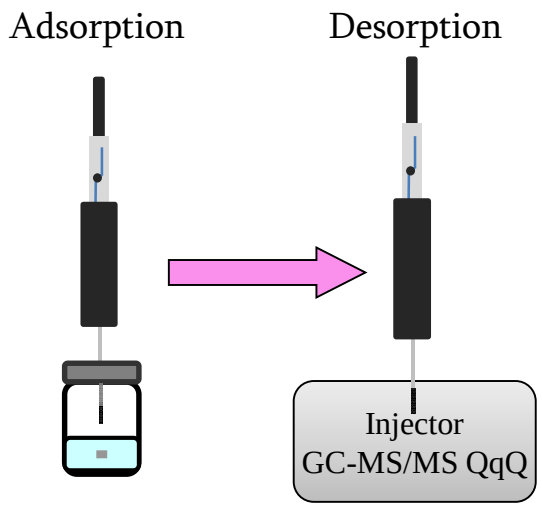

Figura 5. Esquema del proceso de extracción de los analitos mediante HS-SPME

La extracción se realizó mediante SPME en su modalidad de HS, al tratarse de compuestos volátiles (ver **Figura 5**). El primer parámetro optimizado fue la fibra de SPME, y se llevó a cabo de forma individual. Las fases estudiadas fueron *carboxen / polydimethylsiloxane* (CAR/PDMS) y *divinylbenzene / carboxen / polydimethylsiloxane* (DVB/CAR/PDMS). Se comparó la eficiencia de extracción de ambas, de un agua fortificada a 10 µg/L del patrón de referencia. Tal y como se muestra en la ***Figura 1*** del **Artículo científico 5**, se puede apreciar que la fibra de

CAR/PDMS fue la más adecuada para la mayoría de compuestos, de forma más marcada para aquellos más apolares; al contrario que para los menos apolares donde las respuestas fueron ligeramente mejores con la fibra DVB/CAR/PDMS. Llegando a un compromiso, se seleccionó finalmente la fibra CAR/PDMS ya que fue mejor para la mayoría, consiguiendo respuestas aceptables para todos ellos.

Los siguientes parámetros considerados en la optimización fueron la temperatura de extracción, volumen de muestra, volumen de HS, tiempo de incubación y cantidad de NaCl añadida. Para realizar los experimentos se fijó un tiempo de extracción de 30 minutos y agitación a una velocidad intermedia. Se diseñó un experimento de cribado con todos los parámetros para seleccionar cuáles de ellos presentaban un efecto significativo. El diseño estadístico dio lugar a una secuencia de 22 extracciones con diferentes parámetros seleccionados (en un intervalo de valores pre-establecidos) según indica la ***Tabla 2A*** del **Artículo científico 5**. Tras cuantificar los 23 compuestos, las áreas relativas calculadas fueron introducidas en el programa estadístico como funciones de respuesta proporcionando un diagrama (***Figura 2***, **Artículo científico 5**) que mostraron visualmente los efectos de los parámetros estudiados; señalando que volumen de muestra (V_{sample}) y temperatura (T) fueron los más significativos. Con ambos parámetros se realizaron experimentos posteriores, pero para el resto se fijó el valor que indicaba la estadística como óptimo, dependiendo de su tendencia (positiva o negativa). Se repitió la estadística de forma focalizada, con un diseño de superficie de respuesta de los dos factores significativos. Con esta experiencia de 12 extracciones (***Tabla 2B,*** **Artículo científico 5**) se procedió del mismo modo, obteniendo la gráfica de superficie de respuesta. Sobre ella se decidió que valores de cada parámetro maximizaban la respuesta y eran por tanto los mejores para la extracción. En la ***Figura 2C*** del **Artículo científico 5**, se indica para el volumen de

muestra una tendencia positiva, por lo que se tomó como valor óptimo el máximo valor ensayado (4 mL). Aunque para la temperatura la tendencia positiva no fue tan marcada, también se escogió el máximo valor (50 °C).

Posteriormente, se optimizó el tiempo de extracción seleccionando valores desde 5 hasta 120 minutos, mediante extracciones por HS-SPME con los parámetros ya optimizados, por duplicado. En la ***Figura 3*** del **Artículo científico 5** se presenta una gráfica con las señales obtenidas para los analitos seleccionados, observándose para todos ellos que la extracción llegó a un tiempo de equilibrio a partir del cual ya no se extraía más cantidad de analito. Finalmente, se eligió 30 minutos como compromiso entre la eficiencia de extracción y agilidad en el proceso.

Con los parámetros más relevantes debidamente optimizados, se llevó a cabo la validación del método a tres niveles de concentración (0.1, 5 y 50 μg/L). Los resultados fueron satisfactorios y se lograron LODs entre 0.004 y 2 μg/L y LOQs entre 0.01 a 5 μg/L, dependiendo de cada compuesto (ver ***Tabla 3***, **Artículo científico 5**). Los LODs se calcularon como la concentración de analito que produce una señal del pico cromatográfico de 3 veces la relación señal/ruido (S/N) y los LOQ de 10 S/N, en los cromatogramas correspondientes al nivel de concentración más bajo ensayado.

Tras quedar clara la mejora obtenida para patrones de referencias con SRM (o pseudo-SRM) frente a modo SIM, se procedió al análisis de muestras reales de agua. En la ***Figura 6*** del **Artículo científico 5** se muestran los comportamientos encontrados en muestras reales analizadas de ambos modos. Se observan, en general, mejoras en sensibilidad, relación S/N y especialmente una notable mejora

de la selectividad cuando se trabaja en modo SRM frente al trabajo en SIM, sobre todo en muestras más complejas.

La **Figura 6** muestra dos positivos encontrados en un influente urbano (IWW) de una planta de tratamiento de aguas residuales, obtenidos mediante modo SIM y SRM, destacando las evidentes mejoras con este último modo. Además, el positivo de *1,2-dichloroethylene* no se hubiera reportado en modo SIM (falso negativo), al no detectar los 3 iones que requieren los parámetros confirmatorios. En cambio, en SRM las dos transiciones adquiridas se observan sin ninguna dificultad con una buena S/N a pesar de tener poca intensidad. En el positivo de tolueno, la presencia de 3 iones en modo SIM podría ser admisible aunque seguramente no guardaría la relación iónica esperada (*Q/q*) al comparar con el patrón de referencia, por presentar una inferencia que dificulta su correcta integración.

Finalmente, con el método mostrado en el **Artículo científico 5** para determinación de VOCs mediante HS-SPME con GC-MS/MS QqQ se ha evidenciado la capacidad de reportar valores cuantitativos de forma fiable, adquiriendo la información adecuada (tanto cualitativa como cuantitativa) para todos los analitos en una única inyección, lo cual presenta una gran ventaja.

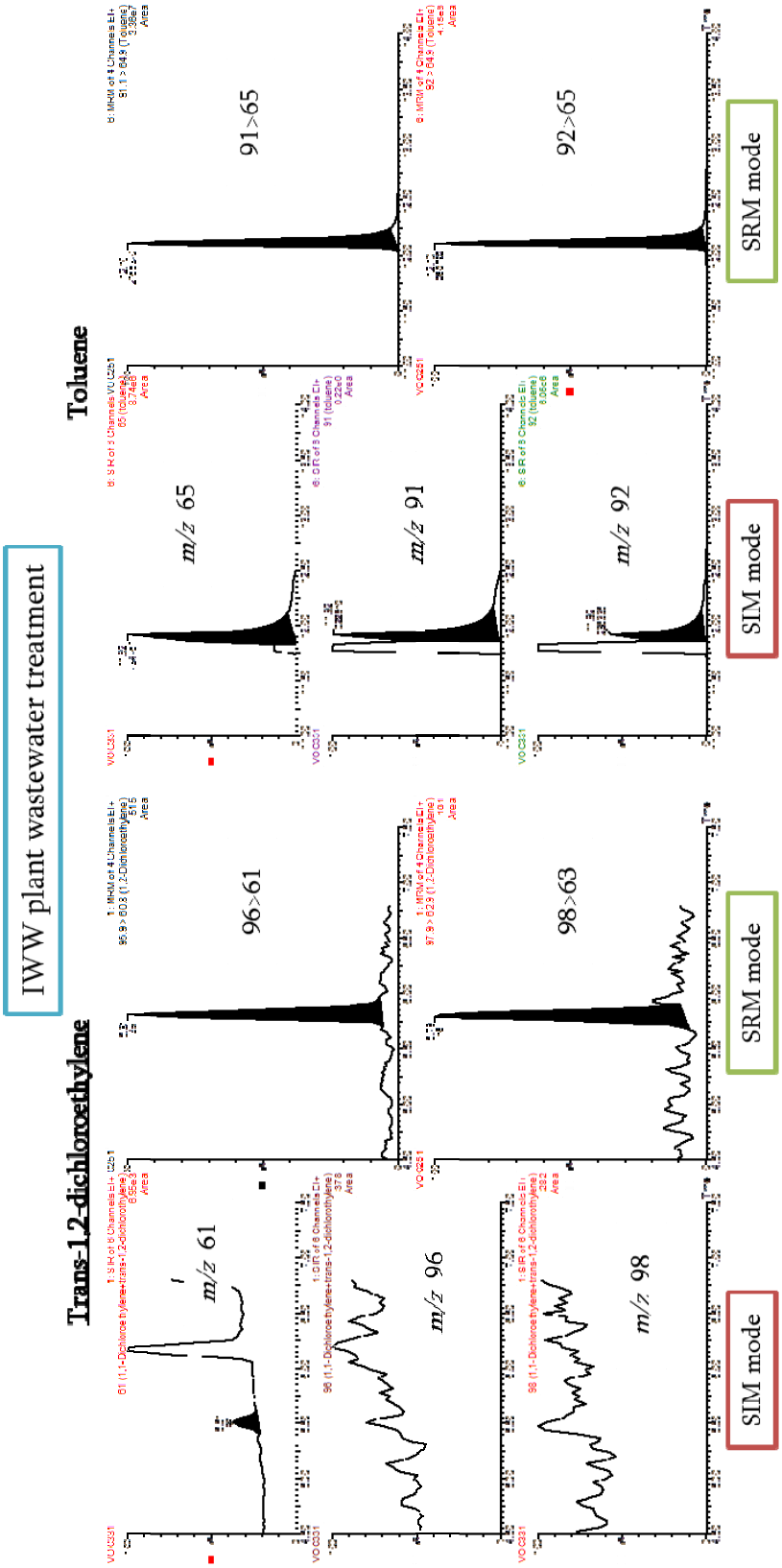

Figura 6. Cromatogramas GC-QqQ MS en modo SIM y SRM para dos positivos en una muestra de agua residual (IWW) de una planta de tratamiento de aguas residuales

Para realizar el trabajo presentado en el **Artículo científico 6** se preparó un cuidadoso montaje que permitía efectuar la extracción de modo simultáneo de los compuestos emitidos por los bulbos de tulipán. Cada lote de muestras estaba constituido por dos grupos de bulbos, bulbos sanos y bulbos infestados de un ácaro herbívoro (*Acearia Tulipae*). Está bien referenciado que durante el almacenaje y transporte los bulbos emiten una elevada cantidad de etileno que provoca daños severos en los mismos. Una forma adecuada para prevenir estos daños es una ventilación masiva, aunque con un coste excesivo de energía. En estudios específicos sobre este hecho (Liou, 2011; Gude, 2005) se muestra que el compuesto 1-metilciclopropeno (1-MCP) puede ayudar a contrarrestar dicho efecto, inhibiendo de forma efectiva la acción del etileno de las plantas. Con el fin de estudiar el comportamiento en condiciones similares a las del almacenaje y/o transporte en contenedores, en el centro proveedor de las muestras a ambos tipos de bulbos, se les aplicaron tres tratamientos durante 24 horas antes de realizar el proceso de extracción con el montaje de laboratorio: etileno, 1-MCP y corriente de aire. Además, se tomó una representación de bulbos blanco (sin tratar) sanos e infestados. Todos ellos se trasladaron al lugar de extracción donde se dispusieron en los distintos frascos, junto con un frasco sin bulbos como blanco de sistema, y todo ello preparado por duplicado, resultando un total de 18 frascos tal como como se puede observar en la **Figura 7**.

Las condiciones de extracción están detalladas en el **Artículo científico 6**, y en el esquema presentado en la ***Figura 1***. Con este tipo de extracción se favorece la captura de los analitos que se desprenderían de los tulipanes si se encontrasen en su lugar habitual de almacenamiento.

Figura 7. Montaje del sistema de extracción por purga y trampa

Tras la etapa de extracción de los bulbos, realizada en tres lotes diferenciados, se procedió a la investigación de los compuestos volátiles mediante GC-(EI)TOF MS. En primer lugar, se realizó el análisis en modo *target* con el fin de detectar los dos compuestos seleccionados. Estos compuestos *target* fueron *Tulipalin A y α-methyl-γ-butyolactone*, cuya presencia ya había sido reportada en tulipanes. Ambos analitos se detectaron en la mayoría de las muestras analizadas, por lo que se planteó un análisis adicional cuantitativo, con el fin de obtener más información de interés. Este análisis cuantitativo se llevó a cabo mediante GC-MS/MS usando analizador QqQ, que proporciona mejor sensibilidad y permite alcanzar menores límites de detección que el TOF MS.

Los extractos también fueron sometidos a un análisis *non-target*, sin necesidad de reinyectar las muestras, procesando de nuevo los datos adquiridos, aplicando un software de deconvolución (*ChromaLynx*). De este modo, se obtuvo de forma automática una lista de candidatos por la similitud de su espectro de masas con el espectro de masas teórico, así como por la compatibilidad de los iones fragmento observados, con errores de masa relativamente bajos (hasta 3 mDa).

Para la confirmación de la identidad fue necesario adquirir los patrones de referencia y comparar el tiempo de retención así como el espectro de masas experimental obtenido en las muestras con aquel obtenido con los patrones. Además, se realizó un estudio más detallado de los positivos detectados, para calcular los errores de masas, intentando elucidar las posibles estructuras de los iones fragmento y calcular su masa exacta teórica.

A modo de ejemplo en la **Figura 8** se muestra la detección de un compuesto volátil, el *3-carene,* con el software utilizado. Se puede observar que el primer candidato para el pico detectado es el compuesto mencionado, aunque seguido por otros candidatos, como el *α-pinene,* con el mismo porcentaje de similitud con la librería teórica (*match*). Los 5 iones fragmento observados en el espectro, compatibles con la estructura química del compuesto, presentaron errores de masa menores de 2.7 mDa, por lo que se asumió que la estructura propuesta por el software ChromaLynx era bastante acertada.

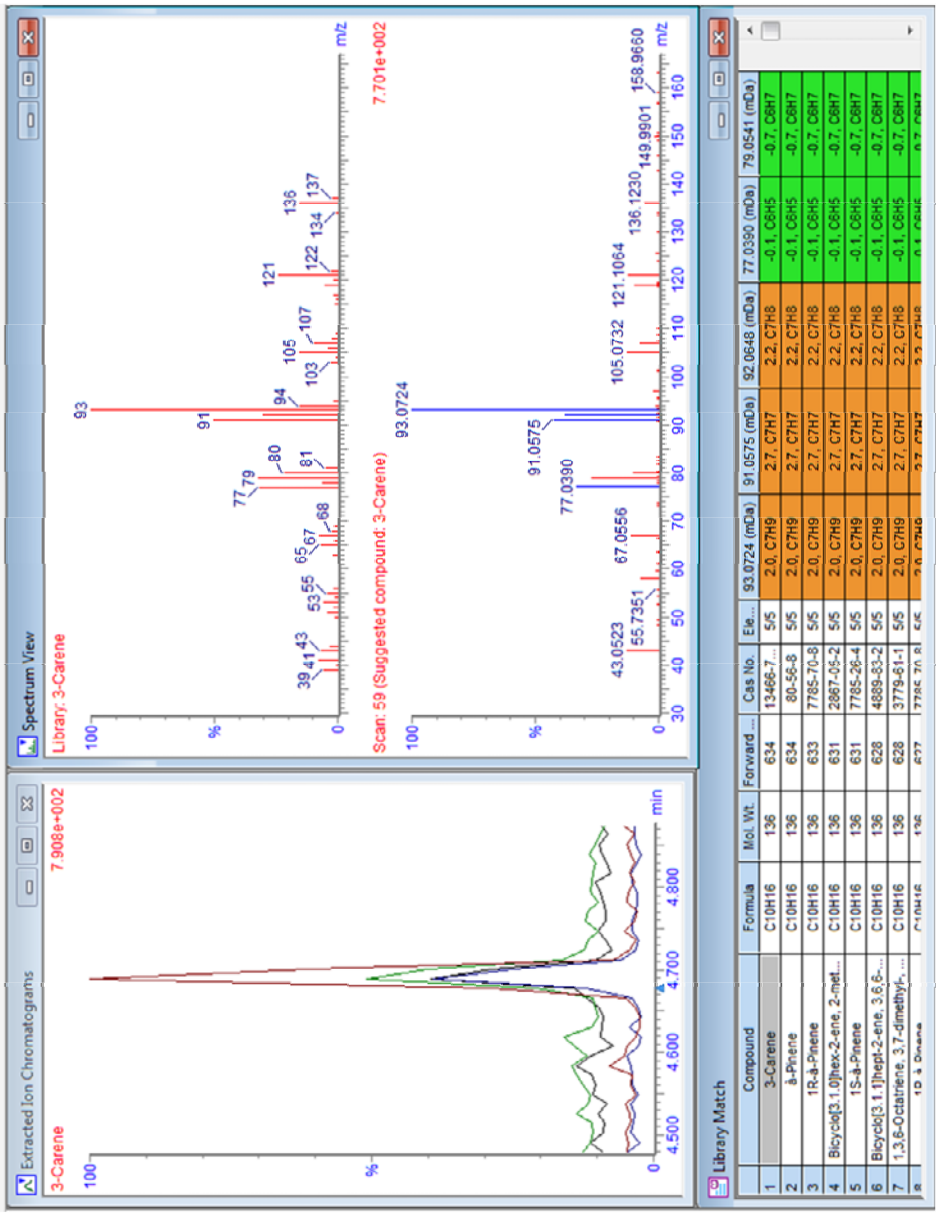

Figura 8. Detección automática del *3-carene* con ChromaLynx mostrando el cromatograma deconvolucionado junto con los espectros de masas experimental y teórico, así como una lista de los posibles candidatos para el compuesto "desconocido" detectado

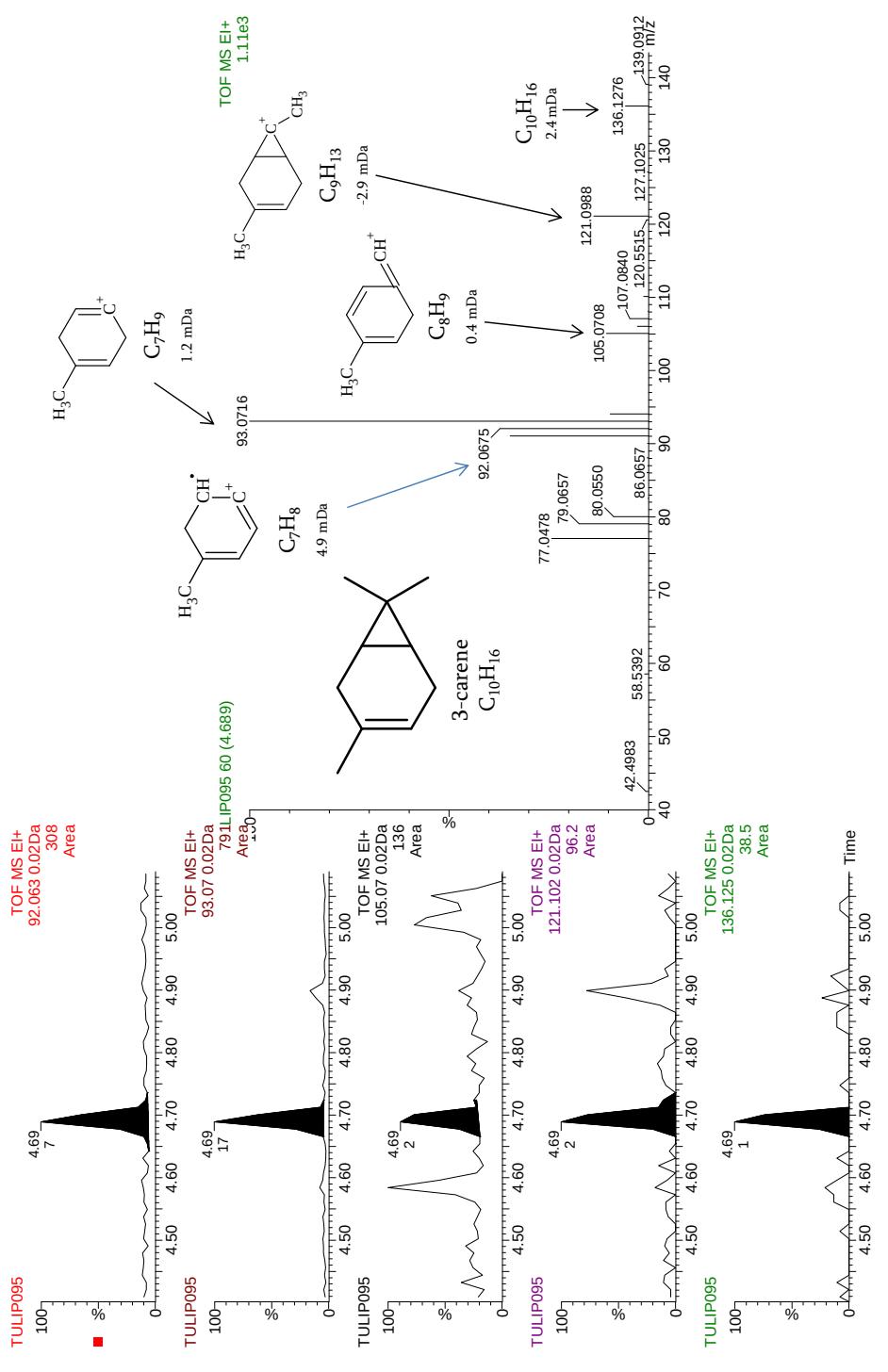

Figura 9. Cromatograma obtenido mediante GC-TOF MS en una ventana de masa de 0.02 Da (nw-XIC) con la presencia del *3-careno* y su espectro de masas experimental con las posibles estructuras propuestas para los iones fragmento más abundantes junto con su errores de masa (en mDa)

Después de la identificación tentativa del compuesto, se adquirió su patrón de referencia y se inyectó en GC-TOF MS. Tras comparar el tiempo de retención, así como su espectro de masas, y calcular los errores de masa de sus iones fragmento empíricamente observados en el patrón, se pudo confirmar la presencia de *3-carene* en una muestra de bulbos sanos, después de aplicar *ethylene* como tratamiento. En la **Figura 9** se puede observar el cromatograma GC-TOF MS (nw-XIC) con una ventana de masas estrecha de 0.02 Da, mostrando la presencia del *3-carene,* con el espectro de masas obtenido en modo EI, las posibles estructuras propuestas para los iones fragmento más abundantes, y los errores de masas en mDa.

Teniendo en cuenta que algunos de los compuestos detectados mediante aproximación *non-target* presentaron cierto interés biológico, se realizó una segunda etapa del estudio con el objetivo de cuantificarlos. Por ello, se seleccionaron los compuestos de interés y se adquirieron sus patrones de referencia. Además, se incluyeron los dos compuestos *target* iniciales, para conocer también la cantidad emitida en las muestras, ya que estos datos podrían ser significativos en cuanto al tratamiento aplicado a los bulbos 24 horas anteriores de su extracción. Esta etapa formaría parte de un futuro estudio más detallado por parte del grupo de biólogos holandeses en el campo de los tulipanes.

Se diseñó un método GC-MS/MS QqQ en modo SRM, que se optimizó siguiendo las pautas habituales, incluyendo la selección de iones precursores e iones producto, y se analizaron de nuevo todos los extractos mediante GC-MS/MS. En la ***Figura 5*** del **Artículo científico 6** se observan numerosos positivos encontrados en una muestra de bulbos sanos, tratados con 1-MCP, en las últimas 72 horas de extracción.

A modo de resumen, en la **Figura 10** se puede observar la cantidad total extraída (en ng) para los dos compuestos *target* inicialmente seleccionados en las 3 extracciones consecutivas realizadas en el tercer lote de muestras, para cada tratamiento recibido, comparando los resultados para bulbos sanos y bulbos infestados. En cuanto al comportamiento de estos dos compuestos, se deduce que ambos están presentes en los dos tipos de bulbos, y que, independientemente del tratamiento recibido, ambos se emiten en mayor cantidad por parte de bulbos sanos que infestados con el ácaro herbívoro.

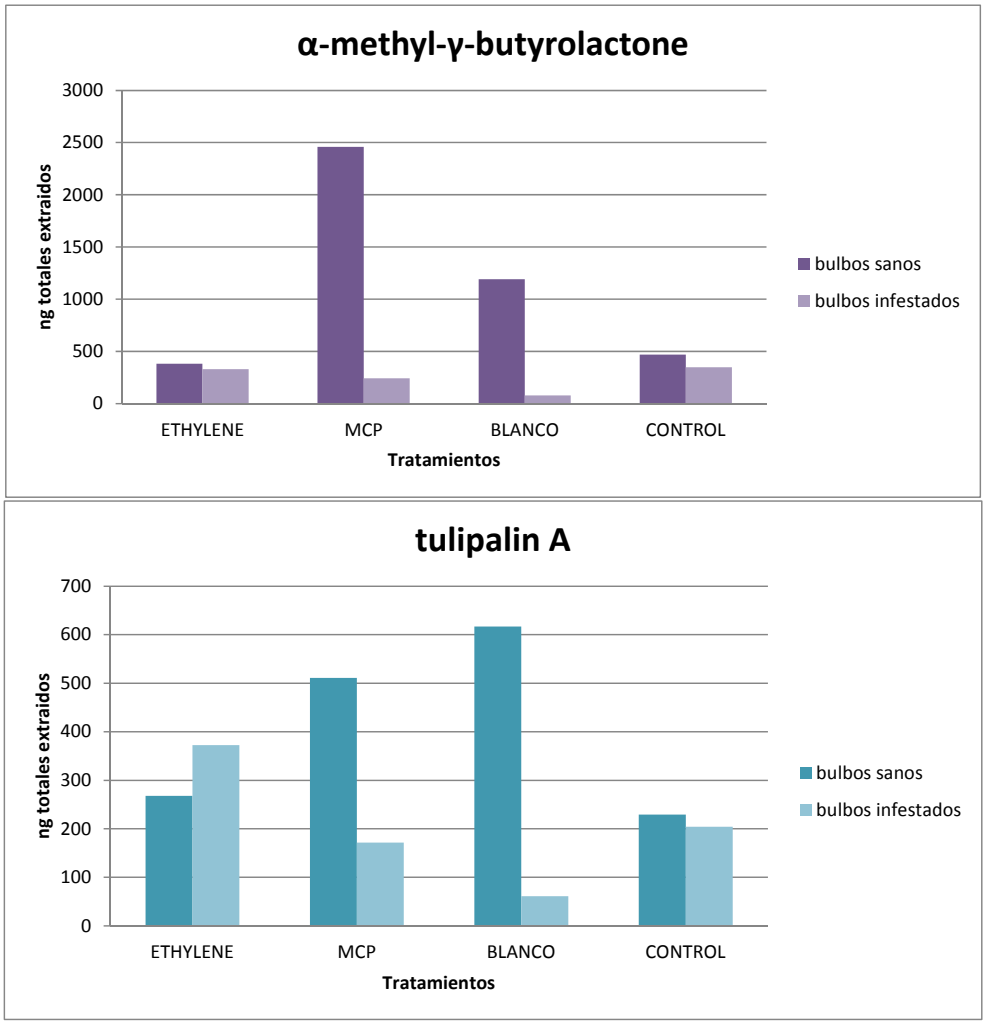

Figura 10. Gráficas de los ng totales de *tulipalin A* y de *α-methyl-γ-butyolactone* frente a los diversos tratamientos recibidos.

En el presente capítulo, debido a las particulares características de los analitos y a la problemática abordada, se ha utilizado un protocolo de extracción específico y ajustado a este tipo de muestras y analitos, ya que la etapa de extracción de muestra resulta muy importante en este caso. En cuanto a las técnicas instrumentales utilizadas, GC-MS/MS QqQ y GC-TOF MS, ambas dieron

buenos resultados para los dos estudios incluidos en este capítulo. Si bien, cabe mencionar que en el desarrollo del método GC-MS/MS con fuente EI, la extensiva fragmentación experimentada en esta fuente hizo complicado en algunos casos la selección de transiciones adecuadas. Seguramente, este inconveniente se podría solventar usando técnicas de ionización más suaves, como APCI en GC-MS/MS. De acuerdo con la experiencia de nuestro grupo de investigación en fuentes APCI usadas en sistemas GC-MS, los analitos apenas sufren fragmentación. Esto permite seleccionar el ion molecular o la molécula protonada como ion precursor, mucho más abundante que en fuentes EI, con lo que se podrían desarrollar métodos analíticos más sensibles y específicos. Este tema será sin duda, objeto de estudios posteriores a la realización de esta Tesis Doctoral.

CAPÍTULO 5

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CAPÍTULO 6

CONCLUSIONES

CONCLUSIONES

A partir del trabajo realizado en esta **Tesis Doctoral** se pueden extraer las siguientes ***conclusiones:***

1. La técnica GC-MS/MS QqQ es una de las más poderosas para la determinación de residuos de plaguicidas en muestras de interés alimentario y medioambiental. Su robustez y excelente sensibilidad y selectividad la convierten en la opción preferida para la cuantificación de residuos de plaguicidas de media-alta volatilidad y baja polaridad en modo convencional *target*.

2. Los métodos de GC-MS/MS QqQ desarrollados en esta **Tesis** permiten no sólo la correcta cuantificación, sino también la identificación/confirmación fiable del compuesto detectado acorde a las regulaciones existentes. Sobre la base de la Decisión Europea 2002/657/EC, en la que se requieren, al menos 4 puntos de identificación (o 3, dependiendo de si el compuesto está prohibido o autorizado), el uso de GC-MS/ MS QqQ permite cumplir con creces este requisito, al adquirir normalmente 2 transiciones MS/MS y confirmar la identidad del analito en una única inyección. Del mismo modo, los métodos desarrollados permiten la correcta confirmación de acuerdo con las guías SANCO, de aplicación en análisis de residuos de

plaguicidas (PRA), por cumplir con los parámetros de adquisición de transiciones, relaciones iónicas y tiempo de retención requeridos.

3. El acoplamiento GC-MS/MS QqQ ha demostrado ser una poderosa herramienta para el análisis multiresidual de un elevado número de plaguicidas en matrices vegetales. El método se ha validado correctamente para los plaguicidas seleccionados en muestras de naranja, nectarina y espinaca a dos niveles de concentración (0.01 y 0.05 mg/kg). El límite de cuantificación (LOQ) objetivo, establecido en 0.01 mg/kg, se ha cumplido para la mayoría de los casos logrando unos límites de detección (LOD) que varían desde 0.0001 hasta 0.01 mg/kg.

4. El estudio del efecto matriz para un buen número de matrices vegetales (naranja, nectarina, espinaca, mango, pasas, pimentón, col, pera, arroz, legumbres y pepinillo) ha demostrado que para la mayoría de combinaciones analito/matriz existe una exaltación de la señal. La cuantificación mediante un calibrado en matriz ha permitido la correcta cuantificación, al contrarrestar el mencionado efecto matriz. Además, el uso de patrones internos (añadidos como *surrogates)* ha ayudado a corregir posibles errores en la extracción y la deriva del instrumento, mejorando la cuantificación de los analitos.

5. Se ha demostrado el potencial de la técnica GC-TOF MS para análisis cuantitativo en matrices de frutas y verduras, como naranja, manzana, zanahoria y tomate, tras haber validado el método para 55 plaguicidas seleccionados a tres niveles de concentración (0.01, 0.05 y 0.5 mg/kg). Además de los compuestos *target*, el análisis de muestras ha permitido identificar otros contaminantes orgánicos no incluidos en la lista

inicial, para los que no se disponía de patrones de referencia. Esto ha sido posible gracias al espectro completo de masas, medido en masa exacta, suministrado por el analizador TOF MS. La adquisición posterior de patrones de referencia ha permitido la confirmación de los positivos tentativamente identificados.

6. El uso de la nueva fuente de ionización APCI en GC, junto con el acoplamiento a un analizador híbrido QTOF MS, ha permitido el rápido *screening* de residuos de plaguicidas (130 compuestos *target*) en muestras de diversas matrices vegetales, como fresas, naranjas, manzanas, zanahorias, lechugas, calabacines, pimientos rojos y tomates. La posibilidad de basar la detección en el ion molecular/molécula protonada, muy abundante en los espectros de APCI, facilita el *screening*, con un nivel notable de sensibilidad y selectividad. Además, la adquisición en el analizador QTOF en modo MS^E facilita no sólo la detección (en la función de baja energía), sino también la identificación fiable sobre la base de la información obtenida sobre iones fragmento en masa exacta (función de alta energía).

7. Gracias a la información espectral adquirida en el analizador QTOF MS, se ha podido ampliar el *screening* para más de 250 nuevos plaguicidas (en modo *post target*), examinando la presencia del ion molecular y la molécula protonada en la función de baja energía. En presencia de pico cromatográfico, el estudio de sus posibles fragmentos y errores de masa permitió la identificación tentativa del compuesto detectado, el cual se pudo confirmar posteriormente con el patrón de referencia.

8. Además del potencial de GC-(APCI)QTOF MS para fines de *screening*, se ha demostrado que esta técnica permite también una adecuada cuantificación para 15 plaguicidas encontrados en las muestras sometidas a *screening*. El método de análisis ha sido validado cuantitativamente a dos niveles de concentración (0.01 y 0.1 mg/kg), alcanzando para la mayoría de las combinaciones analito/matriz un LOQ de 0.01 mg/kg.

9. La extracción de los compuestos volátiles orgánicos (VOCs) en aguas mediante microextracción en fase sólida (SPME) en modo espacio de cabeza (HS) ha sido una opción eficiente para atrapar los analitos en un sorbente e inyectarlos directamente en el sistema GC-MS. De este modo, se minimizan los interferentes de la matriz y se lleva a cabo una extracción limpia, fácil, rápida y sin la utilización de disolventes orgánicos. Con la optimización estadística multivariante se ha logrado la eficaz selección de los parámetros involucrados en la extracción.

10. La técnica HS-SPME junto con la utilización de GC-MS/MS QqQ ha permitido alcanzar valores de LOD y LOQ tan bajos como 0.004 y 0.01 μg/L, para los VOCs estudiados. El método de análisis, novedoso en lo que se refiere al uso de GC-MS/MS QqQ para VOCs, ha quedado satisfactoriamente validado a tres niveles de concentración (0.1, 5 y 50 μg/L) y ha sido aplicado al análisis de muestras de aguas superficiales y residuales.

11. En el estudio de los compuestos volátiles orgánicos emitidos por los bulbos del tulipán, el sistema experimental diseñado para la extracción de VOCs mediante la técnica de purga y trampa ha permitido mantener

condiciones originales, en oscuridad y a temperatura ambiente, con una extracción eficiente de los compuestos emitidos.

12. El uso combinado de GC-TOF MS y GC-MS/MS QqQ es una estrategia muy adecuada para la investigación de compuestos orgánicos de interés en matrices vegetales, como es el caso de los compuestos emitidos por los bulbos de la flor del tulipán, a consecuencia de su mecanismo de defensa contra los ataques de insectos herbívoros, que atraen insectos predadores. La búsqueda de compuestos de interés mediante analizador de TOF se ha realizado tanto en modo *target* como *non-target*. Posteriormente, los análisis mediante GC-MS/MS QqQ han permitido confirmar su identidad y realizar la cuantificación a los niveles en que están presentes en las muestras.

CHAPTER 6

CONCLUSIONS

CONCLUSIONS

As a result of the research performed in this **Doctoral Thesis**, the following ***conclusions*** can be reached:

1. GC-MS/MS QqQ is one of the most powerful techniques for pesticide residue determination in food and environmental samples. Its robustness and excellent sensitivity and selectivity make it one of the most efficient techniques for quantification of pesticide residues with medium or high volatility and low polarity in target analytical methodologies.

2. GC-MS/MS QqQ methods developed in this **Thesis** allow not only the accurate quantification but also the reliable identification/confirmation of the compounds according to the current regulations. Based on European Decision 2002/657/EC, at least 4 or 3 identification points are required for confirmation, depending on if the compound is banned or authorized, respectively. The use of GC-MS/MS QqQ allows easily reach these requirements, by acquiring 2 MS/MS transitions per compound, with the analyte being quantified and identified in one single analysis. Similarly, the methods developed in this **Thesis** allow confirmation of the compounds identity according to the SANCO

guidelines for pesticide residue analysis (PRA) by acquisition of two MS/MS transitions and ion ratios and retention times evaluation.

3. GC-MS/MS QqQ has been tested as a powerful tool for the multiresidue analysis of a large number of pesticides in vegetable matrices. The method has been developed satisfactorily for more than 100 selected pesticides in orange, nectarine and spinach samples at two concentration levels (0.01 and 0.05 mg/kg). The limit of quantification (LOQ) objective, stablished in 0.01 mg/kg, has been accomplished in most cases reaching limits of identification (LOD) from 0.0001 to 0.01 mg/kg.

4. Matrix effects have been evaluated for a notable number of vegetables matrices (orange, nectarine, spinach, mango, raisin, paprika, cabbage, pear, rice, legume and gherkin), showing signal enhancement for majority of analyte/matrix combinations. Quantification with matrix-matched calibration standards allowed the accurate quantification, compensating the mentioned matrix effects. Besides, the use of internal standards (added as surrogates) helped us to correct losses in the extraction step or instrument deviations, improving analyte quantification.

5. The potential of GC-TOF MS has been demonstrated for quantitative analysis in fruits and vegetables matrices as orange, apple, carrot and tomato, for which the method was validated for 55 selected pesticides at three concentrations (0.01, 0.05 and 0.5 mg/kg). In addition to the target compounds, sample analysis by GC-TOF MS allowed to identify other organic contaminants not included in the initial list, for which

the reference standards were unavailable. This was possible thanks to the accurate mass full scan spectra acquisition provided by this analyzer. The subsequent acquisition of reference standards permitted the confirmation of the identity of those positives tentatively identified.

6. The use of the new ionization source APCI in GC, coupled to a hybrid analyser QTOF MS, has allowed the fast screening of pesticide residues (130 target compounds) in different vegetable samples, as strawberries, oranges, apples, carrots, lettuces, courgettes, red peppers and tomatoes. The possibility of detecting the molecular ion or the protonated molecule, highly abundant in APCI spectra, facilitates the screening, with a remarkable improvement of sensitivity and selectivity. In addition, QTOF acquisition in MS^E mode allowed the detection (low energy function, LE) and the reliable identification with the information obtained about *m/z* fragment ions (high energy function), both in accurate mass.

7. The useful and complete spectral information acquired in QTOF MS allowed the screening to be widened for 250 more pesticides (in post target mode), looking for the presence of the molecular ion and/or the protonated molecule at the low energy function. When a chromatographic peak was observed at the LE function, further studies of the fragmentation (HE) and mass errors permitted the tentative identification of the compound detected, which their identity could be confirmed in a subsequent step by acquisition of the reference standard.

8. In addition to the high potential of GC-(APCI)QTOF MS for screening purposes, it has been demonstrated that this technique also allowed the satisfactory quantification for 15 pesticides found in the screening of food samples. The method was quantitatively validated at two concentration levels (0.01 and 0.1 mg/kg), reaching for most analyte/matrix combinations a LOQ objective of 0.01 mg/kg.

9. Volatile organic compounds (VOCs) extraction in waters using solid phase microextraction (SPME) in head space (HS) mode has been an efficient option to trap the analytes in the sorbent and to inject them directly in the GC-MS system. Using this extraction mode, matrix interferences are minimized and the extraction is performed in a clean, easy and fast way, without using organic solvents. An efficient selection of the parameters involved in the extraction has been satisfactorily reached using multivariate statistical optimization.

10. HS-SPME together with GC-MS/MS QqQ has permitted to reach LODs and LOQs as low as 0.004 and 0.1 μg/L, for the studied VOCs. The method of analysis, novel regarding to the use of GC-MS/MS QqQ for VOCs determination, has been satisfactorily validated at three concentrations (0.1, 5 y 50 μg/L) and applied to surface and waste water samples.

11. In the study of the volatile compounds emitted by the tulip bulbs, the experimental design used for extraction of VOCs by purge and trap has allowed to keep the original conditions, in darkness and room temperature, with an efficient extraction of the emitted compounds.

12. The combined use of GC-TOF MS and GC-MS/MS QqQ is a suitable strategy for investigation of volatile organic compounds in vegetal samples, as occurs for compounds emitted by tulip bulbs, which attract predator insects as a result of the defence mechanism against herbivorous insect attacks. The search of compounds of potential interest using TOF MS analyser has been carried out in both target and non-target modes. Afterwards, analysis with GC-MS/MS QqQ has allowed to confirm their identity and to perform quantification at the levels that they are present in the samples.

SUGERENCIAS PARA FUTUROS TRABAJOS

En esta **Tesis Doctoral** se ha investigado el potencial de la cromatografía de gases acoplada a la espectrometría de masas con analizadores de QqQ, TOF y QTOF para la determinación de diversos compuestos orgánicos, principalmente compuestos orgánicos volátiles y residuos de plaguicidas, en matrices de interés alimentario y medioambiental. A la vista de los resultados obtenidos, se pueden proponer diversos trabajos futuros de interés como continuación de los presentados en la presente memoria:

1. Explorar las posibilidades analíticas, cualitativas y cuantitativas, que ofrece GC-(EI)TOF MS, tanto en aproximaciones *target* como *non-target*, estudiando las ventajas de la adquisición del espectro de masas completo en masa exacta en EI. Explorar posibles aplicaciones en los campos de trabajo abordados en esta **Tesis**.

2. Explorar las posibilidades de aplicación de GC-(APCI)QTOF MS en otros campo de trabajo, centrándose en métodos de *screening* para el control de adulterantes, desinfectantes, aditivos, contaminantes derivados del envasado alimentario; o para drogas veterinarias, micotoxinas o biotoxinas marinas; así como control antidoping, o drogas de abuso. Desarrollar y validar métodos cualitativos de

screening. Investigar la presencia de compuestos desconocidos y elucidar sus posibles estructuras.

3. Explorar las posibilidades analíticas, cualitativas y cuantitativas, que ofrece GC-(EI)TOF MS, en los campos de trabajo anteriormente mencionados.

4. Investigar la posibilidad de desarrollar métodos cuantitativos de análisis basados en GC-(APCI)QTOF MS, aprovechando la mayor sensibilidad y selectividad aportada por esta nueva fuente de ionización en comparación con EI TOF MS.

5. Explorar las aplicaciones de la fuente de APCI en sistemas GC-MS/MS QqQ centrándose en métodos cuantitativos, con especial énfasis en compuestos que sufren una extensiva fragmentación en la fuente de EI, en campos de aplicación como seguridad alimentaria y contaminación medioambiental principalmente.

6. Avanzar en el conocimiento de compuestos volátiles emitidos por plantas como mecanismo de defensa frente a depredadores, usando una combinación de técnicas analíticas, como GC-(EI)TOF MS, GC-(APCI)QTOF MS y GC-(APCI)MS/MS QqQ.

SUGGESTIONS FOR FUTURE WORKS

In this **Doctoral Thesis** the strong potential of gas chromatography coupled to mass spectrometry with QqQ, TOF and QTOF analysers has been explored for investigation of organic contaminants, mainly volatile compounds and pesticide residues, in food and environmental matrices. From the results obtained, further studies can be suggested:

1. Explore the qualitative and quantitative capabilities that GC-(EI)TOF MS offers in target and non-target approaches, taking profit of the accurate-mass full-spectrum acquisitions, in the application fields studied in this **Thesis**.

2. Explore the screening potential of GC-(APCI)QTOF MS in other applied fields, e.g. monitoring of adulterants, disinfectants, additives or packaging contaminants in food safety; veterinary drugs, mycotoxins or marine toxins in food safety too; doping control in sport; illicit drugs of abuse in toxicology, etc. Develop and validate screening qualitative methods. Investigate the presence of unknown compounds and elucidate their potential structures.

3. Explore the analytical capability, from a qualitative and quantitative point of view, of GC-(EI)TOF MS in the applied fields mentioned above.

4. Investigate the possibility of developing quantitative methods based on GC-(APCI)QTOF MS, taking profit of the improvements in sensitivity and selectivity offered by this new source in comparison with EI TOF MS.

5. Explore the quantitative applications of APCI source in GC-MS/MS QqQ systems with emphasis in those compounds extensively fragmented in EI, in applied fields as food safety and environmental pollution.

6. Advance in the knowledge of volatile compounds emitted by plants as result of defense mechanisms against predators, using a combination of analytical techniques, as GC-(EI)TOF MS, GC-(APCI)QTOF MS y GC-(APCI)MS/MS QqQ.

RELACIÓN DE ARTÍCULOS CIENTÍFICOS

Los artículos científicos que componen la presente **Tesis Doctoral** son los siguientes:

ARTÍCULO CIENTÍFICO 1

The role of GC-MS/MS with triple quadrupole in pesticide residue analysis in food and the environment

F. Hernández, M.I. Cervera, T. Portolés, J. Beltrán, E. Pitarch

Analytical Methods, ***2013***, 5, 5875-5894

ARTÍCULO CIENTÍFICO 2

Multi-residue determination of 130 multiclass pesticides in fruits and vegetables by gas chromatography coupled to triple quadrupole tandem mass spectrometry

M.I. Cervera, C. Medina, T. Portolés, E. Pitarch, J. Beltrán, E. Serrahima, L. Pineda, G. Muñoz, F. Centrich, F. Hernández

Analytical and Bioanalytical Chemistry, ***2010***, 397, 2873-2891

ARTÍCULO CIENTÍFICO 3

Application of gas chromatography time-of-flight mass spectrometry for target and non-target analysis of pesticide residues in fruits and vegetables

M.I. Cervera, T. Portolés, E. Pitarch, J. Beltrán, F. Hernández

Journal of Chromatography A, ***2012***, 1244, 167-177

ARTÍCULO CIENTÍFICO 4

Screening and quantification of pesticide residues in fruits and vegetables making use of gas chromatography-quadrupole time-of-flight mass spectrometry with atmospheric pressure chemical ionization

M.I. Cervera, T. Portolés, F.J. López, J. Beltrán, F. Hernández

Analytical and Bioanalytical Chemistry, ***2014***, 406, 6843-6855

ARTÍCULO CIENTÍFICO 5

Determination of volatile organic compounds in water by head space-solid-phase microextraction gas chromatography coupled to tandem mass spectrometry with triple quadrupole analyzer

M.I. Cervera, J. Beltrán, F.J. López, F. Hernández

Analytica Chimica Acta, ***2011***, 704, 87-97

ARTÍCULO CIENTÍFICO 6

Capturing chemical signals emitted by tulip bulbs before and after induction by herbivorous mites (Acari: Eriophyidae)

M.I. Cervera, F. van der Wielen, I. Lesna, J. Beltrán, F. Hernández, M.W. Sabelis, P. de Voogt

In process, ***2015***

OTROS ARTÍCULOS CIENTÍFICOS RELACIONADOS

Advancing towards universal screening for organic pollutants in waters

F. Hernández, M. Ibáñez, T. Portolés, M. I. Cervera, J. V. Sancho, F. J. López

Journal of Hazardous Materials, ***2015,*** 282, 86-95

Screening and quantification of organic pollutants in surface and ground water using liquid and gas chromatography coupled to triple quadrupole and quadrupole time of flight mass spectrometry analyzers

E. Pitarch, M.I. Cervera, T. Portolés, M. Ibañez, M. Barreda, I. Morell, F. Hernández, F. Albarrán

In process

Instrumental classification of olive oils by GC-(APCI)QTOF MS and metabolomic based statistical approach

C. Sales, M.I. Cervera, R. Gil, T. Portolés, J. Beltran, F. Hernández

In process

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