



Universitat Jaume I
Departament de Ciències Experimentals
Química Analítica

Institut Universitari de Plaguicides i Aigües

**POTENCIAL DE LA CROMATOGRÀFIA LÍQUIDA ACOPLADA A ESPECTROMETRÍA DE
MASAS CON ANALIZADORES DE TRIPLE CUADRUPOLO Y DE TIEMPO DE VUELO
PARA LA ELUCIDACIÓN, CUANTIFICACIÓN Y CONFIRMACIÓN DE PLAGUICIDAS Y
PRODUCTOS DE TRANSFORMACIÓN EN MUESTRAS AMBIENTALES**

Tesis Doctoral
MARÍA IBÁÑEZ MARTÍNEZ
2007

Dr. Félix Hernández Hernández, Catedrático de Química Analítica, y Dr. Juan Vicente Sancho Llopis, profesor Titular de Química Analítica, de la Universitat Jaume I de Castellón,

Certifican que: la Tesis Doctoral "*Potencial de la cromatografía líquida acoplada a espectrometría de masas con analizadores de triple cuadrupolo y de tiempo de vuelo para la elucidación, cuantificación y confirmación de plaguicidas y productos de transformación en muestras ambientales*" ha sido desarrollada bajo su dirección, en el área de Química Analítica del Departamento de Ciències Experimentals de la Universitat Jaume I de Castelló, por María Ibáñez Martínez. Tras la creación del Instituto Universitario de Plaguicidas y Aguas (IUPA) por Decreto 260/2004, de 19 de Noviembre, la parte experimental de la Tesis Doctoral se finalizó en las instalaciones del mencionado instituto.

Lo que certificamos para los efectos oportunos en Castellón de la Plana, a 15 de Noviembre de 2006

Fdo. Dr. Félix Hernández Hernández

Fdo. Dr. Juan Vicente Sancho Llopis

Este trabajo responde al compromiso adquirido con la Conselleria de Empresa, Universitat i Ciència de la Comunitat Valenciana por la concesión de una beca predoctoral para la Formación de Personal Investigador (FPI), desde el 15 de Julio de 2002.

María Ibáñez Martínez ha sido beneficiaria de una beca Bancaixa concedida por la Universitat Jaume I, para la realización de una estancia en el *Industrial Marketing Group* del *Mass Spectrometry Technologies Centre (Waters-Micromass Corporation)* en Manchester, llevada a cabo desde el 3 de Mayo al 3 de Agosto de 2005. El trabajo de investigación llevó por título: "*Rapid screening of drinking water for multiple pesticide residue by UPLC-TOF MS*" bajo la dirección de Ramesh Rao, Gordon Kearney y Daniel McMillan, y permitió ampliar los conocimientos de la doctoranda en el análisis de contaminantes orgánicos en aguas mediante una técnica tan novedosa como la cromatografía líquida de ultra resolución (UPLC) acoplada a la espectrometría de masas con analizadores de tiempo de vuelo de nueva generación (TOF MS). Fruto de este trabajo se presentaron tres comunicaciones orales en el *American Society of Mass Spectrometry, Waters User Meeting* celebrado en San Antonio (Texas), en el *1st International Workshop on Liquid Chromatography-Tandem Mass Spectrometry for screening and trace level quantitation in environmental and food samples* celebrado en Barcelona, ambos en Junio del 2005. Además se presentó un póster en el *Florida Pesticide Residue Workshop* celebrado en Julio del mismo año. Finalmente los resultados de este trabajo fueron también presentados en *Waters Food Safety Summits*, una serie de congresos en el campo de la salud alimentaria, celebrados en París, India, China y Tailandia durante los meses de Septiembre y Octubre del 2005, y en el *European Pesticide Residue Workshop (Corfu, 2006)*. Por otra parte, se realizó una estancia de dos semanas en el *Pharmaceutical Marketing Group* del mismo centro bajo la supervisión de Jose Castro Pérez, que permitió ampliar los conocimientos de la doctoranda en la realización de experiencias de metabolismo *in vivo* e *in vitro* y posterior tratamiento de los datos obtenidos. Fruto de este trabajo se presentó una comunicación en el *13th North American International Society of Study for Xenobiotics/20th Japanese Society of Study for Xenobiotics Meeting* celebrado en Maui (Hawaii), en Octubre del año 2005.

Los trabajos realizados en la presente Memoria han sido en parte financiados por los proyectos del Ministerio de Ciencia y Tecnología *“Aplicación del acoplamiento cromatografía líquida-espectrometría de masas (LC-MS/MS y LC-QTOF MS) a la identificación de metabolitos de plaguicidas en muestras de interés ambiental y toxicológico”* (Ref: BQU2003-02685) y *“Aprovechamiento de nuevos avances en instrumentación analítica para la realización de estudios sobre el impacto de residuos de plaguicidas en la calidad de las aguas subterráneas”* (REN2002-01818).

Esta Tesis ha sido realizada, y consecuentemente será defendida para la obtención del título de *Doctorado Europeo*.

Previamente a la defensa de la Tesis Doctoral, este trabajo ha sido evaluado por tres censores extranjeros independientes, Prof. Michel W. F. Nielen (*Institute of Food Safety, Wageningen, Netherlands*), Prof. Janna Hajšlová (*Department of Food Chemistry and Analysis, Institute of Chemical Technology, Prague*) y Prof. Susan Richardson (*National Exposure Research Laboratory, U.S. Environmental Protection Agency, USA*).

Aquesta és una bona oportunitat per poder agrair a molta gent el seu suport, ja que si aquesta Tesi ha arribat a la seua fi és resultat, entre altres coses, de l'ajuda i col·laboració de molts.

Començant pel principi vull donar les gràcies als directors d'aquesta Tesi: a Félix per l'oportunitat que em va donar de formar part d'aquest grup d'investigació i apropiarme del QTOF així com per tot el temps que m'ha dedicat; a Juanvi, no sols per tutoritzar-me, sinò també per saber escoltar-me, per la seua paciència i per tants moments compartits. Gràcies als dos per fer possible que aquesta Tesi haja tingut un principi i un acabament.

També a tots els professors de l'àrea, sempre disposats a ajudar: Paco, Ximo, Toni, Roque i Elena. Gràcies per tot.

I als meus companys, ara ja amics:

A Óscar (el nostre 112, sempre disposat a solucionar qualsevol problema en qualsevol moment les 24 hores del dia), Carlos, Mercedes, Tania, Cru, Jose, Ángel, Susana, Cecília, Arantxa, Elena S., Sonia E., Miguel Ángel, Sandra, Carmen i M^a Carmen.

Als nostres últims fitxatges: Edu, Jaime, Jose i Ramón.

A tots els que han treballat en el grup d'investigació en alguna etapa d'aquesta Tesi: Sonia F., Alicia, Ángela, Tati, Ana, Robert, Luna, Oihana, Mireia.

A tots ells, agrair-los les paraules d'ànim i disponibilitat d'ajuda, així com la seua amistad i alegria.

Vull agrair també a Cristian, Lourdes, Cristina, Laura i a tota la gent del SCIC la seua col·laboració, així com l'interés d'altres companys universitaris de diferents departaments.

Thanks to Ramesh Rao for allowing me to be one more of his investigation group. Thanks to my fellow co-workers from Waters in Manchester (Tim, Gordon, Peter, Keith, Jose, Audrey) for their kindness and patience, and especially to Dan, for devoting part of his time to me and teaching me how to use the UPLC.

Thanks to Lorraine MacIntosh for her corrections and comments.

Gràcies també a M^a José Lechón (Hospital La Fe, València), a Marta Miquel i Carlos Aragó (Departament de Psicobiologia de la Universitat Jaume I), a Ignacio Morell (Departament d' Hidrogeoquímica de la Universitat Jaume I) per la seua ajuda tècnica en alguna de les ètapes d'aquest treball.

I per últim, a la meua família i amics: als meus pares, per estar sempre ahí, pel seus ànims i consells a tota hora; al meu germà, per tota la seua ajuda infomàtica (es que me passen unes coses...); als meus cosins i tios pels seus ànims; als xiquets de la casa, Oscar, Andrea, Aleix, Iciar per ajudar-me a desconectar i alegrar-me tots els momentets que passem junts. I como no al meu xiquet, Rubén, pel seu suport incondicional. Gràcies per fer-me somriure!

A tots vosaltres, i a algú més que segur se m'oblida, moltíssimes gràcies

María

Resumen

Dentro de la problemática ambiental, los plaguicidas son, posiblemente, una de las familias de contaminantes que más preocupan en la actualidad, debido a su toxicidad y elevado uso en todo el planeta, así como a la posibilidad de contaminación de distintos tipos de muestras (suelos, aguas, aire, productos alimenticios). El elevado número de plaguicidas actualmente en uso, junto con la gran variabilidad que presentan en cuanto a sus propiedades físico-químicas, dificulta de forma considerable su análisis a nivel de residuos. Aunque se han realizado numerosos estudios ambientales, la mayoría de programas de control de estos contaminantes, tanto en el medio ambiente, como en alimentos o seres vivos expuestos, se centran en tan sólo unos pocos productos (normalmente el plaguicida inalterado), a pesar de que se conocen algunos productos de transformación/metabolitos relativamente estables, persistentes y tóxicos.

Esta carencia de información generalizada sobre la presencia de productos de transformación (TPs) en el medio ambiente es debida, principalmente, a la falta de metodología analítica. Así pues, parece necesario generar información sobre las rutas de degradación/transformación de estos compuestos, desarrollar metodología analítica que permita determinar sus niveles en el medio ambiente, así como evaluar su impacto en el mismo, especialmente en aguas superficiales y subterráneas. Para este fin, es necesario utilizar técnicas modernas y poderosas, en especial la espectrometría de masas, que en los últimos años se ha mostrado como una técnica indispensable en muchas áreas de la Química Analítica.

En esta Tesis se ha explorado el potencial analítico y las aplicaciones del acoplamiento instrumental cromatografía líquida-espectrometría de masas en tándem (LC-MS/MS) con analizadores de triple cuadrupolo (QqQ) e híbrido cuadrupolo-tiempo de vuelo (QTOF) en la determinación de plaguicidas, principalmente en el campo medioambiental, poniendo especial énfasis en los aspectos cualitativos, es decir, en la correcta confirmación de la identidad de los compuestos detectados, siguiendo los criterios de las actuales guías europeas.

El trabajo ha sido estructurado en cuatro partes. En primer lugar, se ha explorado el potencial del acoplamiento LC-QTOF MS en el análisis cualitativo. La versatilidad de la LC, junto con la elevada exactitud de masa del QTOF y la posibilidad de obtener espectros de iones producto con masa exacta, ha permitido la detección, identificación y elucidación de TPs/metabolitos de plaguicidas seleccionados. Inicialmente, se seleccionó como caso de estudio una familia de herbicidas ampliamente utilizada (triazinas). En vista del éxito de los

resultados obtenidos, se decidió ampliar el trabajo a otros plaguicidas aplicados en la Comunidad Valenciana, como por ejemplo, bromacilo, diuron o diazinon. En el caso del diazinon, además, se llevaron a cabo estudios *in vitro* (con microsomas hepáticos humanos) e *in vivo* (con ratas), con el fin de elucidar sus principales metabolitos, demostrando así la gran capacidad analítica de esta técnica tanto en el campo ambiental como biológico. Adicionalmente, la metodología desarrollada fue ligeramente modificada para el estudio de compuestos desconocidos (*non-target*), tras la inyección directa de muestras acuosas de diferentes orígenes. La aplicación de esta metodología permitió la identificación de varios contaminantes en muestras de agua de la Comunidad Valenciana.

En segundo lugar, se ha estudiado el potencial del acoplamiento LC-QqQ MS para la determinación cuantitativa de residuos de plaguicidas y de sus TPs en muestras ambientales. Se han puesto a punto dos métodos basados en SPE-LC-MS/MS: el primero de ellos para la determinación multiresiduo de plaguicidas seleccionados y de sus TPs en muestras de agua, y el segundo para la determinación de compuestos altamente polares como el glifosato, su producto de transformación (AMPA) y el glufosinato, ante la imposibilidad de incluirlos en el método multiresiduo anteriormente descrito. Los métodos fueron optimizados y validados tanto en muestras de agua subterránea como superficial siguiendo el "espíritu" de las Buenas Prácticas de Laboratorio, según los criterios de las guías SANCO de la Unión Europea, estableciendo los parámetros de linealidad, especificidad, exactitud y precisión. En el caso del glifosato y AMPA, el método fue además validado en muestras de suelo. Posteriormente, la metodología se aplicó al análisis de muestras reales, con el fin de obtener información sobre el nivel de contaminación de las aguas de la Comunidad Valenciana.

En tercer lugar, se ha comparado el potencial de los distintos equipos instrumentales utilizados en cuanto a su poder de confirmación de plaguicidas y TPs en muestras reales de agua, de acuerdo con las actuales guías europeas (2002/657/EC). Se han mostrado ejemplos ilustrativos de muestras reales de agua, sobre las que se han detectado diferentes plaguicidas y TPs, y se han discutido las posibilidades de reportar falsos positivos o falsos negativos. En general, se ha demostrado que el triple cuadrupolo puede utilizarse de manera eficiente para la confirmación de muestras positivas siempre y cuando se adquieran varias transiciones MS/MS. El uso de 2 transiciones SRM (*Selected Reaction Monitoring*) es una alternativa válida, siempre que se realice una selección adecuada considerando su especificidad. Por lo que se refiere al QTOF, su modo de operar permite monitorizar todos los iones producto simultáneamente sin necesidad de preseleccionarlos previamente, lo que, unido a las medidas de masa exacta, le proporciona un extraordinario potencial con fines identificativos. El QTOF, sin embargo, presenta una

limitación respecto al QqQ en modo SRM debido a su menor sensibilidad, lo que puede imposibilitar la confirmación de ciertos positivos a bajas concentraciones.

Finalmente, se han efectuado dos revisiones científicas en las que se muestran diferentes estrategias para afrontar la determinación de plaguicidas por LC-MS/MS. La estrategia a seguir dependerá del objetivo perseguido y del instrumento disponible, ya que los requisitos del método a utilizar diferirán dependiendo de si se quiere detectar, cuantificar o confirmar la presencia de un posible residuo. A modo de resumen, el triple cuadrupolo se considera muy adecuado para la cuantificación y el cribado (*screening*) de compuestos predefinidos (*target analytes*). Esta aproximación presenta, sin embargo, una limitación debido a la necesidad de preseleccionar los compuestos objeto de estudio. En cuanto a la confirmación, el uso de transiciones no específicas, como por ejemplo pérdidas de agua, puede resultar esporádicamente en falsos positivos incluso adquiriendo dos transiciones, ya que aumenta la probabilidad de encontrar interferentes que compartan la misma transición. Por otra parte, también podrían resultar falsos negativos en el caso de que una de las transiciones fuera compartida por un interferente, ya que afectaría a la relación de abundancias iónicas experimental. Esta posibilidad aumenta cuando el analito se encuentra a niveles de concentración del orden de sub-ppb. Por este motivo, se considera que la mejor aproximación para una confirmación segura de residuos es la adquisición del mayor número posible de transiciones disponibles.

El QTOF en cambio, debido a su elevada sensibilidad en modo *scan* y a la posibilidad de realizar experiencias en modo MS y MS/MS con medidas de masa exacta, es ideal para la búsqueda de compuestos desconocidos (sin necesidad de ser preseleccionados) y para la elucidación estructural. En cuanto a la confirmación de positivos, el QTOF puede considerarse como la herramienta ideal que permite la identificación inequívoca del analito. Sin embargo, existe la posibilidad de reportar falsos negativos si se encuentra presente en la muestra algún interferente isobárico que coeluya con el analito. En este caso, la mejor opción sería mejorar la separación cromatográfica, lo cual demuestra que ésta juega un papel fundamental en los métodos analíticos, incluso cuando va acoplada a técnicas poderosas de medida como MS/MS con analizadores de QTOF.

En esta Tesis se ha establecido una metodología de trabajo moderna, altamente especializada y con herramientas analíticas poderosas, que podría aplicarse a otros campos de interés relacionados con el impacto de contaminantes orgánicos sobre el medio ambiente, alimentos, y, en definitiva, sobre los seres vivos.

Summary

Potential of liquid chromatography coupled to mass spectrometry with triple quadrupole and time of flight analysers for the elucidation, quantification and confirmation of pesticides and transformation products in environmental samples

Nowadays, within the environmental field, pesticides are probably one of the families of contaminants of most concern, due to their toxicity and worldwide use, as well as to the possibility of contamination of different types of matrices (soil, water, air, food). The high number of pesticides used at present, together with the high variability in their physico-chemical properties, cause considerably difficulty with their analysis at the residue levels. Although numerous environmental studies have been carried out, mostly monitoring programmes, in the environment, in food or in exposed population, they have only focused on a few compounds (typically the unchanged pesticides), despite the fact that several persistent and toxic transformation products (TPs) are well known.

This lack of information about the presence of pesticide TPs in the environment is mainly due to lack of analytical methodology. Thus, it is necessary to generate information about the degradation/transformation pathways of these compounds and to develop analytical methodology to determine their levels in the environment, as well as to evaluate their impact, especially in surface water and groundwater samples. For this purpose, the use of modern and powerful techniques are necessary, especially mass spectrometry, which in recent years has become an essential tool in many areas of analytical chemistry.

In this Thesis, the analytical potential and applications of the coupling between liquid chromatography and tandem mass spectrometry (LC-MS/MS), with triple quadrupole (QqQ) and hybrid quadrupole-time of flight (QTOF) analysers, have been explored in pesticide residue analysis. This work has been mainly carried out in the environmental field, with special emphasis on qualitative aspects, i.e., in the accurate confirmation of the identity of the detected compounds, following the criteria of the current European Union Guidelines.

This work has been structured in four parts. Firstly, the potential of the coupling LC-QTOF MS has been explored for qualitative analysis. The versatility of LC, together with the high mass accuracy provided by QTOF and the possibility of performing MS/MS experiments with accurate mass has allowed the detection, identification and elucidation of pesticide TPs. Initially, a widely used family of herbicides (triazines) was selected as a case of study. Observing the success of the

results obtained, the study was expanded to other pesticides applied in the Valencia region, for example, bromacil, diuron or diazinon. In the case of diazinon, *in vitro* studies (with human liver microsomes) and *in vivo* studies (with rats) were also carried out, to elucidate their main metabolites, demonstrating the high analytical capabilities of this technique in both environmental and biological samples. Additionally, the developed methodology was slightly modified and applied to the study of unknown compounds (non-target), after direct injection of aqueous samples. The application of this methodology allowed identification of several non-target contaminants in water samples from the Valencia region.

Secondly, the potential of the coupling LC-QqQ MS has been investigated for the quantitative determination of pesticide residues and their TPs in environmental samples. Two SPE-LC-MS/MS methods have been developed: the first for the multi-residue determination of selected pesticides and their TPs in water samples, and the second for the determination of highly polar compounds due to the impossibility of including these analytes within the multi-residue method previously described. An example of such highly polar compounds is glyphosate, its transformation product (AMPA) and glufosinate. Both methods were optimised and validated in both ground water and surface water samples, following the principles of "Good Laboratory Practice" , and on the basis of the European Union SANCO Guidelines. Parameters such as linearity, specificity, accuracy and precision were established. In the case of glyphosate and AMPA, the method was also validated in soil samples. The analytical methodology was then applied to the analysis of real samples to obtain information about the level of contamination in water samples from the Valencia region.

Thirdly, the potential of the different instruments has been compared to the confirmation of pesticides and TPs according to the recent European Commission Guidelines (2002/657/EC). Illustrative examples of actual positive water samples have been shown, and the possibility of reporting false positives or false negatives has been discussed. In general, triple quadrupole can be used efficiently for the confirmation of positive samples if several product ions are selected, allowing the acquisition of various MS/MS transitions. The use of two SRM (Selected Reaction Monitoring) transitions is a valid alternative when an adequate selection is carried out considering their specificity. With QTOF, its operation mode allows monitoring of all product ions simultaneously without needing to preselect them. This fact, together with the accurate mass measurement, provides extraordinary potential for identification purposes. The main limitation of QTOF compared to QqQ in SRM mode comes from its lower sensitivity, which makes the confirmation of positives at low concentrations troublesome.

Finally, two scientific reviews have been carried out, showing different strategies for the monitoring of pesticides by LC-MS/MS. The strategy to be applied will depend on the objective pursued and the available instrument, as method requirements will differ, depending on whether it is intended simply to detect, to quantify or to confirm the presence of a possible residue. In summary, triple quadrupole is considered the best option for the screening and quantification of predefined compounds (target analytes). However, this approach presents a limitation due to the need for preselecting the analytes. For confirmation, the use of non-specific transitions, for example loss of water, may sporadically result in false positives, even acquiring two transitions, as the probability of finding an interferent that shares the same transition increases. On the other hand, false negatives may also result if one of the transitions is shared by an interferent, as experimental ion ratio would be affected. This possibility increases when the analyte is present at low concentrations (sub-ppb). For this reason, the best approach to obtain a safe confirmation of residues is the acquisition of the highest number of available transitions.

Conversely QTOF, due to its high sensitivity in full scan mode and the possibility of performing both MS and MS/MS experiments with accurate mass measurement, is ideal for screening unknown compounds (without need of pre-selection) and for structural elucidation. For confirmation of positives, the use of QTOF can be considered as the ideal tool for the unequivocal identification of the analyte. However, there is a possibility of reporting false negatives if some co-eluting interferent is present in the sample. In this case, the best option is to improve the chromatographic separation, which shows the importance of the chromatographic process even when using powerful hyphenated techniques, like LC-MS/MS with QTOF analysers.

In this Thesis, a modern and highly specialised analytical methodology has been established for pesticide residue analysis in water. While this work specially focus on pesticide residues it could be applied to other fiels of interest. Given the huge importance of knowing the impact of organic contaminants in the environment, in food, and their effect on human health, this methodology may provide an important tool for gaining further knowledge in this field.

Índice general

Objetivos, metodología y plan de trabajo	1
Objectives, methodology and work plan	9

CAPÍTULO 1.

Introducción general

1.1 Introducción.....	15
1.2 Determinación de residuos de plaguicidas y productos de transformación/ metabolitos en muestras de interés ambiental y toxicológico.....	19
1.2.1 Determinación de residuos de plaguicidas y productos de transformación en muestras de interés ambiental.....	20
1.2.2 Determinación de residuos de plaguicidas y metabolitos en muestras de interés toxicológico.....	23
1.3 Tratamiento de muestra.....	26
1.3.1 Étaps del análisis.	28
1.3.2 Parámetros de preconcentración.....	30
1.4 Cromatografía líquida acoplada a espectrometría de masas (LC-MS).....	32
1.4.1 Cromatografía líquida.....	33
1.4.2 Interfases.....	34
1.4.3 Espectrometría de masas.....	39
Analizador de cuadrupolo.....	40
Analizador de triple cuadrupolo.....	41
Analizador de tiempo de vuelo.....	44
Analizador híbrido cuadrupolo-tiempo de vuelo.....	46
1.5 Problemáticas analíticas asociadas a la determinación por LC-MS/MS.....	47
1.5.1 Cuantificación	48
1.5.2 Especificidad.....	52
1.6 Bibliografía.....	54

CAPÍTULO 2.

Identificación y elucidación de productos de transformación/metabolitos de plaguicidas mediante espectrómetro de masas híbrido cuadrupolo-tiempo de vuelo (QTOF). Aplicación al análisis de compuestos desconocidos

2.1	Introducción.....	65
2.1.1	Obtención de masa exacta: Calibración y <i>lock mass</i>	68
2.1.2	Aplicaciones del QTOF.....	72
2.1.3	Uso del software MetaboLynx.....	73
2.2	Experimental.....	75
2.3	Elucidación de productos de transformación/metabolitos de plaguicidas.....	76
2.3.1	Introducción.....	76
2.3.2	Resultados y discusión.....	82
	Artículo científico 1.....	93
	<i>The efficient use of quadrupole-time of flight mass spectrometry in environmental analysis: elucidation of transformation products of triazine herbicides in water after UV exposure</i> Anal. Chem. (2004) 76: 1328-1335	
	Artículo científico 2.....	111
	<i>Use of liquid chromatography quadrupole time-of-flight mass spectrometry in the elucidation of transformation products and metabolites of pesticides. Diazinon as a case of study</i> Anal. Bioanal. Chem. (2006) 384: 448-457	
2.3.3	Bibliografía.....	135
2.4	Identificación de compuestos desconocidos (<i>non-target</i>) en muestras de agua.....	139
2.4.1	Introducción.....	139
2.4.2	Resultados y discusión.....	142
	Artículo científico 3.....	143
	<i>Use of quadrupole-time of flight mass spectrometry in the elucidation of unknown compounds present in environmental water</i> Rapid Commun. Mass Spectrom. (2004) 19: 169-178	

Identificación de interferencias reveladas en etapas de confirmación.....	165
Método de cribado (<i>screening</i>) para plaguicidas. Búsqueda de plaguicidas desconocidos (<i>non target</i>).....	169
Nota de aplicación 1	187
<i>UPLC-oo TOF MS for rapid screening of multiple pesticide residues</i>	
Waters application note (2006)	
2.4.3 Bibliografía.....	199

CAPÍTULO 3.

Cuantificación de plaguicidas y productos de transformación mediante LC-MS/MS con analizador de triple cuadrupolo (QqQ)

3.1	Introducción.....	201
3.2	Experimental.....	205
3.3	Determinación multiresiduo de plaguicidas y productos de transformación en aguas mediante preconcentración en línea (SPE-LC).....	207
3.3.1	Introducción.....	207
3.3.2	Desarrollo y validación de un método multiresiduo en aguas.....	210
3.3.3	Resultados y discusión.....	220
	Artículo científico 4.....	225
	<i>Liquid chromatography-mass spectrometry role in the elucidation and determination of transformation products of pesticides in water</i> (En preparación)	
3.3.4	Bibliografía.....	249
3.4	Determinación individual de plaguicidas y productos de transformación en aguas mediante preconcentración en línea (SPE-LC).....	250
3.4.1	Introducción.....	250
3.4.2	Resultados y discusión.....	252
	Artículo científico 5.....	271
	<i>Residue determination of glyphosate, glufosinate and AMPA in water and soil samples by liquid chromatography coupled to electrospray tandem mass spectrometry</i> J. Chromatogr. A (2005) 1081: 145-155	
	Artículo científico 6.....	297
	<i>Re-evaluation of glyphosate determination in water by liquid chromatography coupled to electrospray tandem mass spectrometry</i> J. Chromatogr. A (2006) 1134: 51-55	
3.4.3	Bibliografía.....	311

CAPÍTULO 4.

QqQ versus QTOF en el proceso de confirmación de contaminantes orgánicos

4.1	Introducción.....	313
4.2	Experimental.....	317
4.2.1	Triple cuadrupolo.....	317
4.2.2	(Q) TOF.....	317
4.2.3	Comparación "233 XL" versus "ASPEC XL".....	319
4.3	Resultados y discusión.....	320
4.3.1	Triple cuadrupolo.....	320
4.3.2	Tiempo de vuelo.....	323
4.3.3	Cuadrupolo-tiempo de vuelo.....	325
	Artículo científico 7.....	333
	<i>Comparison of different mass spectrometric techniques combined with liquid chromatography for confirmation of pesticides in environmental waters</i> Anal. Chem. (2004) 76: 4349-4357	
	Artículo científico 8.....	355
	<i>Confirmation of organic micropollutants detected in environmental samples by liquid chromatography tandem mass spectrometry: achievements and pitfalls</i> Trends Anal. Chem. (2006) 15: 1030-1042	
4.4	Bibliografía.....	379

CAPÍTULO 5.

Estrategias para la determinación de plaguicidas por LC-MS(/MS)

5.1	Introducción.....	381
5.2	Resultados y discusión.....	387
	Artículo científico 9.....	389
	<i>Strategies for quantification and confirmation of multi-class polar pesticides and transformation products in water by LC-MS/MS using triple quadrupole and hybrid quadrupole-time of flight analysers</i>	
	Trends Anal. Chem. (2005) 24: 596-612	
	Artículo científico 10.....	421
	<i>Potential of liquid chromatography/time of flight mass spectrometry for the determination of pesticides and transformation products in water.</i>	
	Anal. Bioanal. Chem. (2006) 386: 987-997	
5.3	Bibliografía.....	447

CAPÍTULO 6

Conclusiones	449
Conclusions	455
Sugerencias para posteriores trabajos.....	461
Artículos científicos presentados en la Tesis Doctoral.....	463
Otros artículos científicos relacionados.....	467
Apéndice 1.....	469

General index

Objectives, methodology and work plan	9
--	---

CHAPTER 1.

General introduction

1.1 Introduction.....	15
1.2 Residue determination of pesticides and transformation products/metabolites in environmental and toxicological samples.....	19
1.2.1 Residue determination of pesticides and transformation products in environmental samples.....	20
1.2.2 Residue determination of pesticides and metabolites in toxicological samples.....	23
1.3 Sample treatment.....	26
1.3.1 Phases of analysis.....	28
1.3.2 Preconcentration parameters.....	30
1.4 Liquid chromatography coupled to mass spectrometry (LC-MS).....	32
1.4.1 Liquid chromatography.....	33
1.4.2 Interfaces.....	34
1.4.3 Mass spectrometry	39
Quadrupole analyser	40
Triple quadrupole analyser.....	41
Time of flight analyzer.....	44
Hybrid quadrupole-time of flight analyser	46
1.5 Analytical problems associated to the determination by LC-MS/MS.....	47
1.5.1 Quantification.....	48
1.5.2 Specificity	52
1.6 Bibliography	54

CHAPTER 2.

Identification and elucidation of pesticide transformation products/metabolites by hybrid quadrupole-time of flight mass (QTOF) spectrometer. Application to the analysis of unknown compounds

2.1	Introduction.....	65
2.1.1	Mass exact achievement: Calibration and <i>lock mass</i>	68
2.1.2	QTOF applications.....	72
2.1.3	Use of MetaboLynx software.....	73
2.2	Experimental.....	75
2.3	Elucidation of pesticide transformation products/metabolites.....	76
2.3.1	Introduction.....	76
2.3.2	Results and discussion	82
	Article 1.....	93
	<i>The efficient use of quadrupole-time of flight mass spectrometry in environmental analysis: elucidation of transformation products of triazine herbicides in water after UV exposure</i> Anal. Chem. (2004) 76: 1328-1335	
	Article 2.....	111
	<i>Use of liquid chromatography quadrupole time-of-flight mass spectrometry in the elucidation of transformation products and metabolites of pesticides. Diazinon as a case of study</i> Anal. Bioanal. Chem. (2006) 384: 448-457	
2.3.3	Bibliography.....	135
2.4	Identification of unknown compounds in water samples.....	139
2.4.1	Introduction.....	139
2.4.2	Results and discussion	142
	Article 3.....	143
	<i>Use of quadrupole-time of flight mass spectrometry in the elucidation of unknown compounds present in environmental water</i> Rapid Commun. Mass Spectrom. (2004) 19: 169-178	

Identification of interferences revealed in confirmation process.....	165
Screening method for pesticides. Search of unknown pesticides (non target).....	169
Application Note 1.....	187
<i>UPLC-MS/MS for rapid screening of multiple pesticide residues</i>	
Waters application note (2006)	
2.4.3 Bibliography.....	199

CHAPTER 3.

Quantification of pesticides and transformation products by LC-MS/MS with a triple quadrupole analyser (QqQ)

3.1	Introduction.....	201
3.2	Experimental.....	205
3.3	Multi-residual determination of pesticides and transformation products in water samples by on line preconcentración (SPE-LC).....	207
3.3.1	Introduction.....	207
3.3.2	Development and validation of a multi-residue method in water samples.....	210
3.3.3	Results and discusión.....	220
	Article 4.....	225
	<i>Liquid chromatography-mass spectrometry role in the elucidation and determination of transformation products of pesticides in water</i> (In preparation)	
3.3.4	Bibliography.....	249
3.4	Individual determination of pesticides and transformation products in water samples by on line preconcentración (SPE-LC).....	250
3.4.1	Introduction.....	250
3.4.2	Results and discussion	252
	Article 5.....	271
	<i>Residue determination of glyphosate, glufosinate and AMPA in water and soil samples by liquid chromatography coupled to electrospray tandem mass spectrometry</i> J. Chromatogr. A (2005) 1081: 145-155	
	Article 6.....	297
	<i>Re-evaluation of glyphosate determination in water by liquid chromatography coupled to electrospray tandem mass spectrometry</i> J. Chromatogr. A (2006) 1134: 51-55	
3.4.3	Bibliography.....	311

CHAPTER 4.

QqQ versus QTOF in pesticide confirmation

4.1 Introduction.....	313
4.2 Experimental.....	317
4.2.1 Triple quadrupole.....	317
4.2.2 (Q) TOF.....	317
4.2.3 Comparison "233 XL" versus "ASPEC XL".....	319
4.3 Results and discussion.....	320
4.3.1 Triple quadrupole.....	320
4.3.2 TOF.....	323
4.3.3 (Q) TOF.....	325
Article 7.....	333
<i>Comparison of different mass spectrometric techniques combined with liquid chromatography for confirmation of pesticides in environmental waters</i> <i>Anal. Chem. (2004) 76: 4349-4357</i>	
Article 8.....	355
<i>Confirmation of organic micropollutants detected in environmental samples by liquid chromatography tandem mass spectrometry: achievements and pitfalls</i> <i>Trends Anal. Chem. (2006) 15: 1030-1042</i>	
4.4 Bibliography.....	379

CHAPTER 5.

Strategies for the determination of pesticides by LC-MS(/MS)

5.1 Introduction.....	381
5.2 Results and discussion.....	387
Article 9.....	389
<i>Strategies for quantification and confirmation of multi-class polar pesticides and transformation products in water by LC-MS/MS using triple quadrupole and hybrid quadrupole-time of flight analysers</i>	
Trends Anal. Chem. (2005) 24: 596-612	
Article 10.....	421
<i>Potential of liquid chromatography/time of flight mass spectrometry for the determination of pesticides and transformation products in water.</i>	
Anal. Bioanal. Chem. (2006) 386: 987-997	
5.3 Bibliography	447

CHAPTER 6

Conclusions	455
Suggestions for future work	461
Articles presented in this Doctoral Thesis	463
Other related articles	467
Apendix 1	469

Índice de acrónimos

AMPA	<i>AminoMethylPhosphonic Acid</i>
API	<i>Atmospheric Pressure Interface</i>
APCI	<i>Atmospheric Pressure Chemical Ionization interface</i>
APPI	<i>Atmospheric Pressure Photoionization Interface</i>
BCH	<i>BromoCycloHexane</i>
C-1	<i>first Column in bidimensional liquid chromatography</i>
C-2	<i>second Column in bidimensional liquid chromatography</i>
CID	<i>Collision Induced Dissociation</i>
DAD	<i>Diode Array Detector</i>
DC	<i>Direct Current</i>
DDT	<i>Dichloro-Diphenyl-Trichloroethane</i>
DEP	<i>DiEthyl Phosphate</i>
DIA	<i>DesIsopropylAtrazine</i>
ECD	<i>Electron Capture Detector</i>
EPA	<i>Environmental Protection Agency</i>
EI	<i>Electron Impact</i>
ESI	<i>ElectroSpray Interface</i>
EU	<i>European Union</i>
FAO	<i>Food and Agriculture Organization</i>
FD	<i>Fluorescence Detection</i>
FMOC	<i>9-FluorenylMethOxyCarbonyl chloride/ 9-Fluorenylmethyl chloroformate</i>
FPD	<i>Flame Photometric Detector</i>
FWHM	<i>Full-Width Half Maximum</i>

GC	<i>Gas Chromatography</i>
GLP	<i>Good Laboratory Practice</i>
GUS	<i>General Unknown Screening</i>
HFBA	<i>HeptaFluoroButyric Acid</i>
HPLC	<i>High Performance Liquid Chromatography</i>
HV-1	<i>first High pressure Valve in bidimensional liquid chromatography</i>
HV-2	<i>second High pressure Valve in bidimensional liquid chromatography</i>
IMP	<i>2-Isopropyl-6-Methyl-4-Pyrimidinol</i>
IP	<i>Identification Product</i>
IT	<i>Ion Trap</i>
LC	<i>Liquid Chromatography</i>
LC-LC	<i>Coupled column Liquid Chromatography</i>
LLE	<i>Liquid-Liquid Extraction</i>
LOD	<i>Limit Of Detection</i>
LOQ	<i>Limit Of Quantification</i>
M-1	<i>First mobile phase in bidimensional liquid chromatography</i>
M-2	<i>Second mobile phase in bidimensional liquid chromatography</i>
MRL	<i>Maximum Residue Level</i>
MS	<i>Mass Spectrometry</i>
MS/MS	<i>Tandem Mass Spectrometry</i>
m/z	<i>mass/charge ratio</i>
NCBA	<i>N-Cyclohexyl-2-BenzothiAzolamine</i>
NPD	<i>Nitrogen Phosphorus Detector</i>
NMR	<i>Nuclear Magnetic Resonance</i>
OCs	<i>OrganoChlorine pesticides</i>

OPs	<i>OrganoPhosphorus pesticides</i>
PAH	<i>PolyAromatic Hidrocarbons</i>
PB	<i>Particle Beam interface</i>
PEGs	<i>PoliEthylenGlicol</i>
ppm	<i>Part Per Million</i>
PTV	<i>Programmable Temperature Vaporizer</i>
Q	<i>Quadrupole analyser</i>
QC	<i>Quality Control</i>
QqQ	<i>Triple Quadrupole analyser</i>
QTOF	<i>hybrid Quadrupole-Time Of Flight analyser</i>
RF	<i>RadioFrequency voltage</i>
RSD	<i>Relative Standard Deviation</i>
SIM	<i>Single Ion Monitoring</i>
S/N	<i>Signal/ Noise ratio</i>
SPE	<i>Solid Phase Extraction</i>
SRM	<i>Single Reaction Monitoring</i>
TIC	<i>Total Ion Current</i>
TOF	<i>Time Of Flight analyser</i>
TP	<i>Transformation Product</i>
USDA	<i>United States Department of Agriculture</i>
UV	<i>Ultraviolet Detection</i>
WADA	<i>World Anti-Doping Agency</i>
WHO	<i>World Health Organisation</i>
XIC	<i>eXtracted Ion Chromatogram</i>

Índice de figuras

Figura 1.1	Esquema general de las vías de contaminación difusa por la aplicación de plaguicidas.....	19
Figura 1.2	Representación esquemática de un acoplamiento SPE-LC. C-1: precolumna ; C-2: columna analítica; P-1: bomba enriquecedora de LC; P-2: bomba de LC; HV-1, HV-2: válvulas de alta presión de 6 vías; D: detector; W: deshecho.....	27
Figura 1.3	Etapas de un procedimiento con acoplamiento SPE-LC (A) Acondicionamiento y carga de la muestra, (B) Transferencia, (C) Separación cromatográfica y detección. C-1: precolumna enriquecedora; C-2: columna analítica; D ₁ ,D ₂ : disolventes acondicionadores de C-1; A ₁ , analitos de la muestra 1 (A ₁₁ ,A ₁₂); A ₂ : analitos de la muestra 2; P-1: bomba enriquecedora de LC; P-2: bomba de LC; HV-1, HV-2: válvulas de alta presión de 6 vías; D: detector; W: deshecho.	29
Figura 1.4	Esquema del mecanismo de formación de los iones en ESI.....	35
Figura 1.5	Esquema del mecanismo de formación de los iones en APCI.....	35
Figura 1.6	Rango de aplicación de las distintas interfases en función de la polaridad del compuesto y de su masa molecular.....	37
Figura 1.7	Esquema de la interfase electrospray empleada.....	38
Figura 1.8	Evolución de la espectrometría de masas.....	39
Figura 1.9	Esquema de un cuadrupolo.....	40
Figura 1.10	Diagrama de estabilidad.....	41
Figura 1.11	Esquema de un triple cuadrupolo.....	42
Figura 1.12	Esquema de los modos de barrido utilizados en esta Tesis.....	43
Figura 1.13	Esquema de un TOF con reflectrón.....	45
Figura 1.14	Esquema de un TOF con doble reflectrón.....	45
Figura 1.15	Esquema de un QTOF con doble refrectrón.....	46
Figura 2.1	Resolución obtenida con el TOF.....	65
Figura 2.2	Resolución obtenida con un triple cuadrupolo y con un tiempo de vuelo para dos compuestos cuyas masas difieren 27.6 mDa..	66
Figura 2.3	Calibración en modo de ionización negativo.....	68
Figura 2.4	Introducción del <i>lock mass</i> via <i>lock spray</i>	70
Figura 2.5.	Introducción post columna del <i>lock mass</i>	71

Figura 2.6	Estructura del compuesto 3,5-diido-L-tyrosina, usado como <i>lock mass</i>	71
Figura 2.7	Procesamiento de datos con ayuda del MetaboLynx: (a) cromatograma en modo <i>full scan</i> de la muestra control y de la muestra fortificada, tras 4 días de irradiación; (b)XIC a m/z 240 de la muestra control y de la muestra analito.....	74
Figura 2.8	Estructuras de las triazinas seleccionadas.....	80
Figura 2.9	Montaje experimental utilizado en las experiencias de fotodegradación.....	81
Figura 2.10	Estructura del diazinon.....	82
Figura 2.11	(a) XIC a la masa correspondiente al diuron (m/z 233.02) con una ventana de 0.2 Da, (b) espectros correspondientes a cada uno de los picos: (arriba) interferencia y (abajo) diurón.....	165
Figura 2.12	(a) espectro combinado y posteriormente suavizado, (b) espectro centrado, correspondientes al compuesto interferente de la Figura 2.11.....	166
Figura 2.13	(a) Cromatograma LC-MS/MS, (b) Espectro MS/MS	168
Figura 2.14	Espectros GC-MS de (a) 2-amino-3-ciano-4,4 α ,5,6,7,8,8 α ,9-octahidronaftol [2,3b]tiofene, (b) N-ciclohexil-2-benzotiazolamina.....	168
Figura 2.15	Curva de Vam Deemter, ilustrando la evolución del tamaño de partícula a lo largo de las últimas décadas.....	170
Figura 2.16	Estructura de la leucina encefalina.....	182
Figura 2.17	Información incluida en la librería para cada compuesto.....	183
Figura 3.1	Efecto del <i>dwell time</i> y del aumento del número de transiciones sobre la señal y la forma de pico cromatográfico.....	203
Figura 3.2.	Esquema del equipo instrumental utilizado IP: inyector automático con un loop de 4300 μ L; HV: válvula de 6 vías; P-1: bomba binaria HPLC; P-2: bomba cuaternaria HPLC; C-1: cartucho SPE; C-2: columna analítica; MS/MS: detector de masas en tándem; PC: adquisición de datos; W: desecho.....	205
Figura 3.3	Efecto del tiempo de lavado sobre la recuperación en aguas subterráneas para 2-OH-terbutilazina y terbacilo.....	222
Figura 3.4.	Estructuras de (a) glifosato, (b) glufosinato y (c) AMPA.....	250
Figura 3.5.	Espectros MS (arriba) y MS/MS (abajo) del glifosato en (a) modo de ionización negativo y (b) modo de ionización positivo.....	253
Figura 3.6	Espectros MS (arriba) y MS/MS (abajo) obtenidos tras cromatografiar un patrón de (a) glifosato, (b) glufosinato y (c) AMPA a una concentración de 10 μ g/mL en modo de ionización negativo.....	254
Figura 3.7	Ruta de fragmentación MS/MS en ESI negativo propuesta para el glifosato bajo las condiciones optimizadas.....	255

Figura 3.8	Ruta de fragmentación MS/MS en ESI negativo propuesta para el glufosinato bajo las condiciones optimizadas.....	255
Figura 3.9	Ruta de fragmentación MS/MS en ESI negativo propuesta para el AMPA bajo las condiciones optimizadas.....	256
Figura 3.10	Espectros MS (arriba) y MS/MS (abajo) obtenidos tras cromatografiar un patrón de (a) glifosato, (b) glufosinato y (c) AMPA a una concentración de 10 µg/mL en modo de ionización positivo.....	257
Figura 3.11	Ruta de fragmentación MS/MS en ESI positivo propuesta para el glifosato bajo las condiciones optimizadas.....	258
Figura 3.12	Ruta de fragmentación MS/MS en ESI positivo propuesta para el AMPA bajo las condiciones optimizadas.....	259
Figura 3.13	Ruta de fragmentación MS/MS en ESI positivo propuesta para el glufosinato bajo las condiciones optimizadas.....	260
Figura 3.14	Efecto del pH en la sensibilidad y forma de pico del glifosato, glufosinato y AMPA: (a) pH de la muestra = 6, (b) pH de la muestra = 3, (c) pH de la muestra = 1.5 y (d) pH de la muestra=1.5 y pH de la fase móvil = 2.5.....	262
Figura 3.15	Diagrama de flujo de la etapa de derivatización para la determinación de glifosato, glufosinato y AMPA en (a) muestras de agua y (b) muestras de suelo.....	264
Figura 3.16	Puntos de muestreo. Determinación de glifosato en aguas de la Comunidad Valenciana (2003).....	266
Figura 3.17	Cromatogramas LC-MS/MS obtenidos para la determinación de glifosato en muestras reales de suelo: (a) patron de 5ppb, (b) muestra de suelo conteniendo 2.29 mg/kg.....	269
Figura 3.18	Resultados obtenidos por nuestro laboratorio en el análisis de aguas superficiales.....	294
Figura 3.19	Resultados obtenidos por nuestro laboratorio en el análisis de aguas subterráneas.....	294
Figura 4.1	Representación esquemática del equipo instrumental utilizado en el acoplamiento SPE-LC-QTOF MS (ASPEC XL, Gilson, modificado). D: disolventes acondicionadores; M: muestras de agua; P-1: bomba preconcentradora; C-1: columna/cartucho preconcentrador; P-2: bomba para la elución y separación cromatográfica; C-2: columna analítica; LP: válvula de baja presión; HV-1 y HV-2: válvulas de alta presión; IP: puerto de inyección para inyecciones mediante <i>loop</i> . W: deshecho.....	319
Figura 4.2	Análisis realizado por SPE-LC-MS/MS (QqQ). Confirmación de los plaguicidas detectados en una muestra de agua subterránea procedente de Carcaixent (11-07-03). (a) Muestra, (b) Patrón. (Q) transición de cuantificación y (q) transición de confirmación.....	322

Figura 4.3	Confirmación mediante SPE-LC TOF MS de la presencia de terbutilazina, simazina, terbumetona y terbutrina en un muestra de agua subterránea de Carcaixent. (a) XIC's a las correspondientes m/z (b) Espectro combinado del pico obtenido.....	324
Figura 4.4	Confirmación mediante SPE-LC-QTOF MS de la presencia de terbutilazina y terbumetona en una muestra de agua subterránea de Carcaixent. (a) Cromatogramas en modo MS/MS; (b) Espectros combinados de los picos obtenidos.....	326

Índice de tablas

Tabla 1.1	Acontecimientos más importantes en el desarrollo y uso de los plaguicidas.....	17
Tabla 2.1.	Productos de transformación del bromacilo identificados por LC-QTOF MS.	84
Tabla 2.2	Productos de transformación del glifosato identificados por LC-QTOF MS....	85
Tabla 2.3	Productos de transformación del benfuracarb identificados por LC-QTOF MS.....	86
Tabla 2.4	Productos de transformación del diuron identificados por LC-QTOF MS.....	87
Tabla 2.5	Productos de transformación del imazalil identificados por LC-QTOF MS.....	89
Tabla 2.6	Productos de transformación de la carbendazima identificados por LC-QTOF MS.....	90
Tabla 2.7	Productos de transformación de la bentazona identificados por LC-QTOF MS.....	91
Tabla 2.8	Productos de transformación del terbacilo identificados por LC-QTOF MS...	91
Tabla 2.9	Composiciones elementales resultantes tras aplicar la estrategia basada en el uso de abundancias isotópicas.....	167
Tabla 2.10	Compuestos estudiados por UPLC-TOF MS.....	172
Tabla 3.1	Resumen de los resultados obtenidos en la determinación de triazinas y sus productos de transformación en aguas subterráneas y superficiales de la Comunidad Valenciana (2003). Número total de muestras analizadas 147.....	208
Tabla 3.2	Triazinas y productos de transformación detectados en los muestreos procedentes de Carcaixent.....	209
Tabla 3.3	Compuestos seleccionados en el método multiresiduo.....	213
Tabla 3.4	Resultados obtenidos en el análisis de aguas de la Comunidad Valenciana.....	267
Tabla 3.5	Resultados obtenidos en el análisis de suelos.....	268
Tabla 3.6	Resultados obtenidos en el análisis de plantas decorativas.....	268
Tabla 4.1	Relación entre las distintas técnicas MS y sus IPs asociados.....	315
Tabla 4.2	Tolerancias máximas permitidas en la medida de relaciones iónicas para la confirmación de contaminantes orgánicos en muestras de origen animal.....	316
Tabla 4.3	Desviaciones obtenidas en el <i>ion ratio</i> en la confirmación por triple cuadrupolo de plaguicidas detectados en el agua subterránea de Carcaixent (11-07-03)	322
Tabla 4.4	Resultados obtenidos en la confirmación por TOF de plaguicidas detectados en el agua subterránea de Carcaixent (11-07-03).....	325

Tabla 4.5	Resultados obtenidos en la confirmación por QTOF de los herbicidas terbutilazina, terbumetona, simazina y terbutrina detectados en el agua subterránea de Carcaixent (11-07-03)	327
Tabla 4.6	Desviaciones obtenidas en el <i>ion ratio</i> en la confirmación de los herbicidas terbutilazina, terbumetona, simazina y terbutrina detectados en el agua procedente de Carcaixent (11-07-03).....	329

Objetivos, metodología y plan de trabajo

Objetivos

El **objetivo principal** de la presente Tesis Doctoral es explorar las capacidades analíticas del potente acoplamiento instrumental cromatografía líquida-espectrometría de masas en tándem (LC-MS/MS) con analizadores de triple cuadrupolo (QqQ) e híbrido cuadrupolo-tiempo de vuelo (QTOF) en el análisis de residuos de plaguicidas, principalmente en el campo medioambiental.

En primer lugar, se investigará el potencial de LC-MS/MS con un analizador QTOF para la elucidación estructural de productos de transformación (TPs)/metabolitos de plaguicidas seleccionados, así como la identificación de compuestos desconocidos (*non-target*) en muestras de aguas de diferentes tipos. En segundo lugar, se estudiará el potencial de LC-MS/MS con analizadores QqQ para la determinación cuantitativa de residuos de plaguicidas y de sus TPs en muestras ambientales. Finalmente, se compararán las dos técnicas estudiadas, con especial énfasis en cuanto al poder de confirmación de la identidad de un analito.

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

1. Desarrollar metodología analítica basada en LC-QTOF MS para la identificación y elucidación estructural de TPs de plaguicidas, usando como ejemplo representativo herbicidas de la familia de las triazinas, a partir de experiencias de fotodegradación en laboratorio.

2. Ampliar la metodología propuesta a otros grupos de plaguicidas (por ejemplo fungicidas o insecticidas) pertenecientes a otras familias químicas, como organofosforados, carbamatos, benzimidazoles o uracilos.

3. Explorar y adaptar la metodología desarrollada al campo biológico, mediante la realización de ensayos *in vitro* con microsomas hepáticos humanos e *in vivo* con ratas, usando el diazinon como caso de estudio.

4. Adaptar la metodología desarrollada a la elucidación de compuestos totalmente desconocidos (*non target*) en muestras de agua de diferentes tipos.

5. Utilizar la información obtenida en los estudios de laboratorio mediante LC-QTOF MS con el fin de establecer los compuestos que deben investigarse para proceder a un control más realista del nivel de contaminación de las aguas, a través de la determinación de TPs relevantes.

6. Desarrollar metodología analítica avanzada para la determinación multiresiduo de plaguicidas y TPs en aguas mediante extracción en fase sólida (SPE) acoplada en línea al sistema LC-MS/MS con analizador de QqQ con el fin de minimizar el volumen de agua utilizado y el tratamiento de muestra. Para algunos de los TPs, la evaluación del nivel de contaminación

será únicamente semicuantitativa debido a la falta de patrones de referencia disponibles comercialmente.

7. Puesta a punto de un método basado en SPE-LC-MS/MS con analizador de QqQ para la determinación de compuestos objetivo, altamente polares e iónicos, y de extraordinaria dificultad analítica, en muestras de agua y suelos. En este apartado se incluyen los herbicidas glifosato y glufosinato, así como el producto de transformación AMPA, ninguno de los cuales puede ser determinado mediante el método multiresiduo.

8. Realizar ensayos de campo con parcelas experimentales que permitan comprobar la validez de los resultados obtenidos en experiencias de laboratorio frente a una situación real de campo. Aplicación de plaguicidas seleccionados, a las dosis recomendadas y con las técnicas de aplicación habituales, en parcelas experimentales provistas de lisímetros, para investigar los procesos que sufren estos compuestos en el suelo y zona no saturada.

9. Aplicar la metodología analítica desarrollada en esta Tesis a muestras reales (aguas subterráneas y superficiales del litoral mediterráneo, caracterizado por una elevada actividad agrícola) con el fin de comprobar la existencia de los compuestos estudiados y así obtener una visión más real del estado de contaminación de la zona, con especial atención a las aguas subterráneas.

10. Estudio de distintas aproximaciones analíticas para confirmar la identidad de los compuestos detectados en las aguas mediante el uso de instrumentos de QqQ y QTOF.

11. Comparación de los distintos instrumentos disponibles en cuanto a su potencial para la elucidación, cuantificación y confirmación de plaguicidas y TPs.

12. Establecer una metodología general de trabajo moderna, altamente especializada y con herramientas analíticas poderosas, que pueda aplicarse a otros campos de interés, relacionados con el impacto de contaminantes orgánicos en el medio ambiente y la salud de las personas, la cual permita obtener información relevante para una evaluación más correcta del impacto ambiental a través de la determinación de aquellos productos que realmente se encuentren presentes en las muestras.

Metodología y plan de trabajo

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

1. Inyección de la muestra en el LC-TOF: adquisición de los datos en continuo en modo barrido (*scan*).
2. Procesamiento de los datos con el software adecuado (MetaboLynx). Este software compara el cromatograma de una muestra problema con el de una muestra control exhaltando las diferencias entre ellos. Los picos encontrados se atribuyen a los compuestos de interés, de los cuales se obtiene el espectro con masa exacta.
3. Cálculo de las posibles composiciones elementales. Los parámetros se seleccionan en función del problema analítico, siendo mucho más restringidos en el caso de las experiencias de fotodegradación, donde se pretenden identificar TPs de plaguicidas seleccionados, que en la búsqueda de compuestos totalmente desconocidos.
4. Estudio de la fragmentación MS/MS con QTOF, obteniendo los espectros de iones producto con masa exacta.
5. En el caso de disponer de patrones comerciales, comparación de sus masas exactas, tiempos de retención y espectros MS/MS.

Para el desarrollo de los métodos analíticos cuantitativos propuestos en la presente Tesis Doctoral, se ha seguido la siguiente **metodología**:

1. Estudio de las condiciones óptimas de MS mediante el uso de patrones. Se establecerá, en primer lugar, el modo de ionización y voltaje de cono con el fin de definir el ion precursor del análisis. Una vez definido, aislamiento del ion precursor y optimización de la energía de colisión para la obtención de los iones productos característicos.
2. Optimización de la separación cromatográfica mediante la inyección de patrones de concentración adecuada. Selección de la composición de las fases móviles y del gradiente, en su caso, que permitan obtener picos cromatográficos y tiempos de retención adecuados. Evaluación de la sensibilidad del método, a partir de la cual se puede predecir el factor de preconcentración necesario para alcanzar los límites de detección objetivo.
3. Aplicación de SPE-LC *on-line* para la preconcentración/purificación automatizada de la muestra.

4. Análisis de muestras blanco fortificadas y de patrones de la misma concentración para evaluar el posible efecto matriz producido sobre el analito. Si el efecto matriz es elevado, estudio de las distintas aproximaciones para su eliminación o minimización, dando prioridad a aquellas en las que se simplifique el tratamiento de muestra.

5. Validación de los métodos optimizados basándose en los criterios de las guías SANCO de la Unión Europea, estableciendo los parámetros de linealidad, especificidad, exactitud y precisión mediante ensayos de recuperación a varios niveles.

6. Aplicación de la metodología analítica desarrollada a muestras reales y discusión de los resultados.

En términos de análisis confirmatorio se ha seguido la siguiente **metodología**:

- En el caso de utilizar instrumentos de triple cuadrupolo:

1. Estudio de la fragmentación de cada compuesto mediante infusión de los patrones individuales.

2. Selección de los iones producto más selectivos, evitando, en los casos en que sea posible, las transiciones debidas a pérdidas poco selectivas, como por ejemplo de agua, ácido fórmico, cloro, etc.

3. Adquisición de al menos dos transiciones por compuesto.

4. Cálculo del *ion ratio* o de la *concentration ratio*, tanto de la muestra como del patrón. En el primer caso, se calcula la relación entre las intensidades de señal de las dos transiciones seleccionadas. En el segundo caso, se calcula la relación entre las concentraciones obtenidas a partir de la curva de calibrado de cada transición.

5. Cálculo de la desviación entre el valor teórico (patrón) y el experimental (muestra).

6. Comparación de las desviaciones obtenidas frente a las permitidas por las guías vigentes.

- En el proceso de confirmación de muestras positivas por QTOF:

1. Inyección de la muestra en LC-TOF MS: adquisición de los datos en continuo en modo barrido (*scan*) en masa exacta.

2. Comparación de la masa exacta del ion $[M+H]^+/[M-H]^-$ teórica (patrón) y experimental (muestra).

3. Inyección de la muestra en LC-QTOF MS: adquisición de todos los iones producto disponibles en masa exacta.

4. Estudio de la masa exacta de todos los iones producto disponibles en una muestra, y comparación con los correspondientes a patrones de referencia.

5. Estudio de la intensidad relativa de todos los iones producto disponibles en una muestra, y comparación con los correspondientes a patrones de referencia.

6. Comparación de las desviaciones obtenidas frente a las permitidas por las guías vigentes.

El **plan de trabajo** seguido se indica a continuación:

1. Revisión bibliográfica sobre el estado actual de la determinación de plaguicidas polares, del uso del acoplamiento LC-MS para el análisis de muestras ambientales y biológicas, así como de cuestiones prácticas relevantes relacionadas con la técnica.

2. Selección de los plaguicidas a estudiar principalmente en función de su mayor utilización como productos fitosanitarios en la Comunidad Valenciana, así como de la posibilidad de ionización en LC-MS.

3. Desarrollo de una estrategia analítica moderna y fiable, basada en el uso de la masa exacta y de la abundancia isotópica observada, para la identificación y elucidación de TPs/metabolitos de los plaguicidas seleccionados mediante LC-QTOF MS.

4. Aplicación de la estrategia desarrollada en el apartado anterior a estudios de laboratorio de fotodegradación y transformación ambiental de una familia de herbicidas de amplio uso en la Comunidad Valenciana como son las triazinas.

5. Ampliación de la estrategia anteriormente descrita al estudio de otras familias de plaguicidas, como por ejemplo organofosforados y carbamatos.

6. Realización de ensayos de laboratorio en otros tipos de matrices, aplicando experiencias *in vitro* con microsomas hepáticos humanos o *in vivo* con ratas, con el fin de obtener información sobre el metabolismo de los plaguicidas

7. Estudio de la posibilidad de utilizar la estrategia desarrollada en el punto 3 en situaciones no controladas: elucidación de compuestos desconocidos (*non-target*) en muestras reales de agua (superficial, subterránea, residual y depurada).

8. Desarrollo y validación de un método analítico multiresiduo basado en SPE-LC-MS/MS para la determinación simultánea de plaguicidas y de los TPs anteriormente elucidados en muestras medioambientales

9. Desarrollo y validación de un método SPE-LC-MS/MS específico para la determinación de otros compuestos ampliamente utilizados, y que no puedan ser incluidos en el método multiresiduo descrito anteriormente. Se incluyen en este apartado compuestos como el glufosinato, glifosato y su TP (AMPA).

10. Comprobación de la validez de los resultados de laboratorio en experiencias de degradación/metabolismo frente a una situación real de campo mucho más compleja, donde intervienen diversos parámetros, mediante la aplicación de plaguicidas en parcelas experimentales.

11. Aplicación de la metodología analítica desarrollada al análisis de muestras de aguas subterráneas y superficiales con el fin de comprobar la existencia en las aguas de los productos identificados en las experiencias de fotodegradación.

12. Confirmación de la presencia de plaguicidas y sus TPs en muestras de agua superficial así como de aguas subterráneas mediante distintas técnicas LC-MS (MS/MS y (Q)TOF MS).

13. Estudio comparativo de los dos instrumentos utilizados en la presente Tesis en cuanto a su potencial con fines de cuantificación, identificación y elucidación estructural.

14. Elaboración de las principales conclusiones derivadas de las investigaciones llevadas a cabo.

Objectives, methodology and work plan



Objectives

The **main objective** of this thesis is to investigate the analytical capabilities of coupling liquid chromatography and tandem mass spectrometry (LC-MS/MS) using triple quadrupole (QqQ) and hybrid quadrupole-time of flight (QTOF) analysers of in the analysis of pesticide residues, mainly in the environmental field.

Firstly, potential of the powerful instrumental coupling LC-QTOF MS will be investigated for the structural elucidation of transformation products (TPs) and metabolites of selected pesticides, as well as the identification of unknown compounds (non-target) in different kinds of water samples. Secondly, the potential of the instrumental coupling LC-QqQ MS will be studied for quantitative determination. Finally, both techniques will be compared, with emphasis on confirmation aspects.

To reach the main goal, more **specific objectives** are shown:

1. Development of analytical methodology based on LC-QTOF MS for the identification and structural elucidation of TPs of selected pesticides, obtained in the photodegradation experiments in the laboratory, using triazinic herbicides as a representative example.

2. Extension of the proposed methodology to other pesticide groups, for example fungicides or insecticides, belonging to other chemical families, such as organophosphates, carbamates, benzimidazols or uracils.

3. Application of the developed methodology to the biological field, by performing *in vitro* studies with human liver microsomes and *in vivo* studies with rats, using diazinon as a case of study.

4. Application of the developed methodology to the elucidation of unknown compounds in different water samples.

5. Use of the information obtained in the laboratory studies by LC-QTOF MS to establish the compounds (and TPs) that should be investigated to obtain a more realistic view of the contamination level in water (by determining important TPs).

6. Development of advanced analytical methodology for the multi-residue determination of pesticides and TPs in water by using on line solid phase extraction coupled to a LC-MS/MS system with triple quadrupole analysers, to minimise the water volume and the sample pre-treatment. For some of the TPs, evaluation of the contamination level will be semi quantitative due to the lack of commercially available reference standards.

7. Development of a SPE-LC-MS/MS method with triple quadrupole analyser for the determination of highly polar and ionic target analytes in water samples. In this section, herbicides glyphosate and glufosinate are included, as well as the transformation product AMPA, as none of them can be determined using the multi-residue method.

8. Use of agriculture field experiments to check the validity of laboratory results versus a real scenario. Pesticides will be applied in experimental fields provided with lisimeters, following standard agricultural practices in the particular area at the recommended dosage, to investigate the transformation processes that can affect to these compounds in the soil and in the non-saturated zone.

9. Application of the analytical methodology developed in this thesis to real samples (ground water and surface water samples from the Mediterranean littoral, characterised by extensive citric activity) to check the existence of the studied compounds and therefore obtain a more realistic view of the contamination of water in the area, with special focus on the groundwater samples.

10. Study of different analytical approaches to confirm the identity of detected compounds in water samples using triple quadrupole instruments and hybrid quadrupole-time of flight analysers.

11. Comparison of the different instruments available relative to their potential in elucidation, quantitation and confirmation of pesticides and TPs.

12. Development of general, modern, highly specialised methodology, which can be applied to other fields of interest, related to the impact of organic contaminants in the environment and human health. It is intended to obtain relevant information and evaluate their environmental impact by analysing those compounds which are actually present in the samples.

Methodology and work plan

The **general methodology** used in this thesis for qualitative analysis is shown:

1. Injection of the sample in LC-TOF system. Acquisition of the data in continuum in scan mode.
2. Processing of the data with suitable software (MetaboLynx). This software compares the chromatograms of the analyte sample and the control sample, and highlights differences in the presence of compounds, which could be attributed to transformation processes.
3. Calculation of all possible elemental compositions. Parameters are selected depending on the analytical problem, this being much more restrictive in the case of photodegradation experiments, where TPs of selected pesticides are determined, rather than in the search of unknown compounds.
4. Study of the MS/MS fragmentation with QTOF, obtaining product ion spectra with accurate mass.
5. If commercially reference standards are available, exact mass, retention time and MS/MS spectra are compared.

The **general methodology** used in this thesis for quantitative method development is shown:

1. Study of optimum MS conditions by infusion of standards, establishing, first, the ionisation mode and the cone voltage to define the precursor ion. Then, the precursor ion is isolated and the collision energy is optimised in order to obtain characteristic product ions.
2. Optimisation of the chromatographic separation by injecting standard solutions of adequate concentration level. Selection of the mobile phase composition and gradient, if necessary, which obtains satisfactory chromatographic peaks and retention times. Evaluation of method sensitivity. Estimation of the preconcentration factor required to achieve the detection limit objective.
3. Application of on-line SPE-LC, if necessary, for the automated sample preconcentration/purification.

4. Analysis of spiked blank samples and standards of the same concentration to evaluate possible matrix effect on analyte ionisation. If high matrix effect is observed, then different approaches for its elimination are investigated, with priority of those that minimise sample pre-treatment.

5. Validation of the optimised methods based on the SANCO Guidelines from the European Union, by establishing the main analytical characteristics (linearity, specificity, accuracy and precision) from recovery experiments at different concentration levels.

6. Application of the developed analytical methodology to real samples and discussion of the results obtained.

Regarding confirmation, the **general methodology** is shown:

- If triple quadrupole is used:

1. Study of the fragmentation of each compound, by infusion of individual reference standards.

2. Determination of the most selective transitions, avoiding, if possible, transitions due to low selective losses, as for example, water or formic acid.

3. Acquisition of at least two transitions for each compound.

4. Calculation of the ion ratio or concentration ratio, of both sample and standard. Ion ratio is calculated as the relation between the signal intensities of both transitions. Concentration ratio is calculated as the relation between the concentrations obtained from the calibration curve of each transition.

5. Calculation of the deviation between the theoretical value (standard) and the experimental (sample).

6. Comparison of the obtained deviations with those allowed by the actual Guidelines.

- When confirming positive samples by QTOF:

1. Injection of the sample in LC-TOF system. Acquisition of the data in continuum in scan mode in exact mass.

2. Comparison of the theoretical exact mass $[M+H]^+/[M-H]^-$ (standard) and experimental mass (sample).

3. Injection of the sample in LC-QTOF system. Acquisition of all available ion products in exact mass.

4. Study of the exact mass of all available product ions in the sample, and comparison with those corresponding to a standard.

5. Study of the relative intensity of all available product ions in the sample, and comparison with those corresponding to a standard.

6. Comparison of the obtained deviations with those allowed by the actual legislation.

The **work plan** is shown:

1. Bibliographic revision of the state-of-the-art on the determination of polar pesticides, the use of LC-MS for environmental and biological samples, and practical aspects related to the technique.

2. Selection of pesticides to be studied, based mainly on two main parameters: their use in the study area and their feasibility to be ionised in LC-MS.

3. Development of a modern and reliable analytical strategy, based on the use of exact mass and observed isotopic abundance, for the identification and elucidation of TPs/metabolites of selected pesticides by LC-QTOF MS.

4. Application of the previously developed strategy to experiments of photodegradation and environmental transformation of a family of herbicides widely used in the Valencia region, triazines.

5. Application of the previously developed methodology to other families of pesticides, for example, organophosphates compounds and carbamates.

6. Development of laboratory studies with other matrices, applying *in vitro* experiments with human liver microsomes or *in vivo* experiments with rats, in order to obtain information about the metabolism of pesticides.

7. Study of the possibility of using the strategy developed in point three, in non-controlled situations: elucidation of unknown compounds (non-target) in real water samples (surface water, ground water, sewage effluent and waste water treatment plant influent and effluent).

8. Development and validation of an analytical multi-residue method based on SPE-LC-MS/MS for the simultaneous determination of pesticides and TPs previously elucidated in environmental waters.

9. Development and validation of a specific SPE-LC-MS/MS method for the determination of other widely used compounds, not included in the multi-residue method. This method includes compounds as for example glufosinate, glyphosate and its transformation product (AMPA).

10. Verification of the validity of the results obtained in the experiments of photodegradation/metabolism in the laboratory versus a real situation, where there is more complexity and where several parameters can have an effect. This was done by applying pesticides in experimental fields.

11. Application of the developed methodology to the analysis of both ground water and surface water samples to check for the existence of the products identified in the photodegradation experiments.

12. Confirmation of selected positive results by different LC-MS techniques (MS/MS and (Q)TOF MS) in both surface water and groundwater samples.

13. Comparative study of the two instruments used in this thesis, in relation to quantitation, identification and structural elucidation.

14. Elaboration of the main conclusions from the research carried out.

Capítulo 1.

Introducción general

- 1.1 Introducción
- 1.2 Determinación de residuos de plaguicidas y productos de transformación/ metabolitos en muestras de interés ambiental y toxicológico
 - 1.2.1 Determinación de residuos de plaguicidas y productos de transformación en muestras de interés ambiental
 - 1.2.2 Determinación de residuos de plaguicidas y metabolitos en muestras de interés toxicológico
- 1.3 Tratamiento de muestra
 - 1.3.1 Étaps del análisis
 - 1.3.2 Parámetros de preconcentración
- 1.4 Cromatografía líquida acoplada a espectrometría de masas (LC-MS)
 - 1.4.1 Cromatografía líquida
 - 1.4.2 Interfases
 - 1.4.3 Espectrometría de masas
 - Analizador de cuadrupolo
 - Analizador de triple cuadrupolo
 - Analizador de tiempo de vuelo
 - Analizador híbrido cuadrupolo-tiempo de vuelo
- 1.5 Problemáticas analíticas asociadas a la determinación por LC-MS/MS
 - 1.5.1 Interfases
 - 1.5.2 Espectrometría de masas
- 1.6 Bibliografía

1.1 Introducción

Los **plaguicidas** son sustancias de origen natural o sintético que sirven para combatir todo tipo de enfermedades y parásitos que dañan directamente o indirectamente los cultivos, o que pueden transmitir enfermedades a seres humanos. La FAO/WHO da la siguiente definición de plaguicida: *“Sustancia o mezcla de ellas, estudiada para prevenir, controlar o destruir cualquier especie de planta o animal indeseable, incluyendo también aquellas destinadas a regular el crecimiento de las plantas, defoliantes y desecantes”* (Barberá, 1989).

Los plaguicidas se pueden clasificar según diferentes criterios, como, por ejemplo, la función que desarrollan o su estructura química. Según la función desarrollada, se integran en esta definición, además de insecticidas, otros grupos de compuestos como herbicidas, fungicidas, acaricidas, nematocidas, desinfectantes de suelo o reguladores de crecimiento. En cuanto a la estructura química se pueden clasificar en inorgánicos, que englobaría a los polisulfuros, compuestos de azufre y sales de cobre, y orgánicos, en el que se incluirían compuestos organofosforados (OPs), organoclorados (OCs), carbamatos, triazinas, fenilureas o fenoles, entre otros, y compuestos de origen biológico.

La historia ofrece innumerables ejemplos de destrucción masiva de cultivos provocados por enfermedades o insectos (Ware, 1982). Por ejemplo, en el periodo comprendido entre 1845 y 1851, tuvo lugar la hambruna de la patata de Irlanda como resultado de la infección masiva de las patatas por un hongo, *Phytophthora Infestans*. Esto provocó la pérdida de casi un millón de vidas y una emigración masiva. Otro ejemplo se remonta a 1930, cuando el 30% del cultivo de trigo de Estados Unidos se perdió por putrefacción del tallo, enfermedad que, veinte años más tarde, destruiría 3 millones de toneladas en Canadá. Del mismo modo, muchas enfermedades de personas y animales son causadas por organismos transportados por insectos. Así, desde la primera epidemia de la peste bubónica, más de 65 millones de personas han muerto por la enfermedad transmitida por la pulga de las ratas, *Nosopsyllus fasciatus*.

La primera referencia del uso de un material como plaguicida se remonta posiblemente a la Antigua Grecia (año 1000 a.C.), donde la quema de azufre era empleada para fumigar las casas. Sin embargo, no fue hasta mediados del siglo XIX, cuando las plagas fueron controladas con cierto éxito gracias al uso de productos químicos.

Los plaguicidas fueron introducidos de modo masivo a escala mundial a partir de los años 20-30 del siglo XX, y especialmente a partir de la segunda Guerra Mundial, coincidiendo con la obtención de los primeros compuestos orgánicos sintéticos. El primer plaguicida utilizado, el DDT, fue presentado al mundo como la solución para todas las plagas sin efectos negativos para el hombre, e hizo a su descubridor merecedor del Premio Nobel. Su facilidad de obtención y aplicación, la rapidez de sus resultados y su reducido coste extendieron rápidamente su uso indiscriminado, sin sospechar los efectos negativos sobre los seres vivos y el ambiente, efectos que aún hoy –más de 20 años después de su prohibición– persisten. Muy cercanos químicamente al DDT, aparecieron otros compuestos orgánicos sintéticos con cloro en su fórmula, los organoclorados. Estos compuestos, contrariamente a lo que ocurría con los inorgánicos, penetraban a través de los tejidos de las plantas, con lo que su actuación era mucho más efectiva.

Hacia finales de la década de los 50 aparecieron los plaguicidas organofosforados, con una mayor potencia de acción que los anteriores pero que provocaban efectos más graves en los seres humanos, y más tarde los carbamatos. Ambos grupos se han usado en grandes cantidades. En la Tabla 1, se muestran algunos de los acontecimientos más importantes en el desarrollo y uso de los plaguicidas. Como puede observarse, los plaguicidas inicialmente desarrollados han ido dando paso a otros compuestos menos persistentes, que tienden a degradarse y metabolizarse más rápidamente que los primeros.

El empleo sistemático de plaguicidas, principalmente para prevenir la acción de parásitos agrícolas, ha supuesto, sin lugar a dudas, una gran mejora en la calidad de vida de los pueblos. No obstante, a los espectaculares resultados obtenidos al comienzo de su empleo de forma intensiva siguieron otros problemas al observarse que la aplicación masiva e indiscriminada de estos productos tenía consecuencias sobre la salud humana, el medio ambiente, e incluso, sobre la efectividad del producto. Aún hoy, en muchos casos se siguen empleando los plaguicidas de forma inadecuada, aplicando dosis mayores a las necesarias, empleando sustancias que no siempre son las idóneas, e incluso utilizando formas de aplicación incorrectas. Se estima que en el año 2000 la cantidad de plaguicidas aplicados a nivel mundial se situó alrededor de unos 2.3 millones de toneladas, de los cuales 36% correspondían a herbicidas, 25% a insecticidas y 10% a fungicidas (EPA, 2006).

Tabla 1.1 Acontecimientos más importantes en el desarrollo y uso de los plaguicidas.

1939	Descubrimiento del DDT como insecticida (Paul Müller, Suiza).
1941	Descubrimiento del BCH como insecticida (Francia).
1943	Lanzamiento comercial del primer fungicida ditiocarbamato, zineb.
1945	Lanzamiento comercial del primer insecticida clorado ciclodieno, clordano. Aparece el primer herbicida carbamato, profam.
1947	Aparece el insecticida toxafeno (se convertirá en el insecticida más usado en la historia de la agricultura de Estados Unidos).
1948	Aparición de los insecticidas aldrin y dieldrin.
1949	Aparición del primer fungicida dicarboximida, captan.
1956	Introducción del primer insecticida carbamato, carbaril.
1958	Aparición de la atrazina (primer herbicida triazínico) y paraquat (primer herbicida bipyridínico).
1969	La USDA adopta una política sobre plaguicidas para evitar el uso de materiales persistentes cuando se encuentran disponibles métodos de control efectivos y no-residuales.
1970	Formación de la Agencia de Protección del Medio Ambiente (EPA).
1973	Aparición del primer piretroide sintético fotoestable, permetrin. Cancelación de los usos del DDT por la EPA.
1975	Cancelación de los usos del aldrin y dieldrin, excepto como termicidas.
1976	Cancelación por la EPA de la mayoría de los usos de los compuestos de mercurio como plaguicidas. La EU crea lista sobre sobre los contaminantes más peligrosos del medio acuático, la llamada "lista negra".
1984	Prohibición de todos los usos del endrin.
1985	Cancelación de todos los usos registrados como plaguicidas del hexaclorobenceno y del mirex.
1987	Prohibición de todos los usos del dieldrin.
1989	Prohibición de todos los usos del DDT.
1990	Cancelación de todos los usos del toxafeno.
1996	La Comunidad Europea elimina los bifenilospoliclorados y policloroterfenilos.
1997	Finaliza la producción del heptacloro.
1998	La Comunidad Europea firma protocolo de Aarhus sobre los contaminantes orgánicos persistentes, que incluye 11 plaguicidas.
2001	Se crea la comisión nacional de Buenas Prácticas Agrarias.

Así pues deben vigilarse los daños, tanto ambientales como en la salud humana, ocasionados por el uso de estos compuestos a gran escala, para lo cual es necesario controlar la presencia de este tipo de sustancias, tanto en muestras ambientales como biológicas.

El análisis de estos plaguicidas a nivel de residuos resulta muy problemático debido a la gran variedad de propiedades físico-químicas que pueden presentar, a la complejidad de algunas de las matrices objeto de estudio y a los bajos niveles permitidos por la legislación, normalmente del orden de pocos $\mu\text{g/L}$. Este control requiere, por tanto, métodos muy sensibles y específicos. Con este fin, se deben utilizar técnicas analíticas avanzadas, normalmente basadas en acoplamientos instrumentales, como una alternativa a las más convencionales utilizadas hasta la fecha que requerían complejos y tediosos tratamientos de muestra. La metodología analítica moderna permite un alto grado de automatización del procedimiento analítico global y un mayor grado de fiabilidad tanto desde el punto de vista cualitativo como cuantitativo.

En esta Tesis se estudiará el potencial de la cromatografía líquida acoplada a la espectrometría de masas en tándem (LC-MS/MS) con analizadores de triple cuadrupolo (QqQ) e híbridos cuadrupolo-tiempo de vuelo (QTOF) para la identificación, confirmación y cuantificación de plaguicidas y productos de transformación en muestras de interés ambiental. Por otro lado, aunque en menor extensión, se explorará el potencial de estas técnicas en la determinación de plaguicidas y metabolitos en muestra de origen biológico.

En el presente capítulo de Introducción se efectúa una discusión general sobre los temas más relevantes tratados en esta Tesis, sin el ánimo de realizar una revisión crítica de la bibliografía (artículos científicos) relativa a los distintos trabajos experimentales llevados a cabo. Posteriormente, en los capítulos 2-4 y, especialmente, en el capítulo 5 se encuentran los antecedentes bibliográficos, recogidos principalmente en los artículos científicos que se han publicado a partir de los trabajos realizados en la Tesis.

1.2 Determinación de residuos de plaguicidas y productos de transformación/metabolitos en muestras de interés ambiental y toxicológico

Aunque resulta innegable que los plaguicidas han beneficiado la producción agrícola y la lucha contra enfermedades humanas y animales, como la malaria, la fiebre amarilla, el dengue y numerosas parasitosis, el continuo y a menudo desproporcionado uso de plaguicidas en todo el mundo está haciendo que se multiplique su presencia en numerosos ámbitos. Uno de los más afectados es, sin duda, el medio ambiente, ya que el uso inadecuado de plaguicidas puede provocar daños a animales y plantas, perturbando gravemente los ecosistemas al crear desequilibrios entre las poblaciones de animales. De esta manera, se ha asociado la mortandad masiva de peces en una paraje natural de la Comunidad Valenciana con la acumulación excesiva de plaguicidas ampliamente usados en esa zona (Morell y Hernández, 2000). En cuanto a la salud humana, ésta puede verse afectada por los plaguicidas en diferentes aspectos. Por un lado, la toxicidad aguda, causada en su manipulación, aplicación o por intentos suicidas. Por otro lado, la toxicidad crónica, derivada de una prolongada exposición a dosis muy bajas a uno o varios de estos productos, provocada generalmente por la presencia de estos compuestos en diversos tipos de muestras como aguas, suelos, aire, alimentos...

En la Figura 1.1 se muestra un esquema en el que se señalan las posibles vías de contaminación, una vez aplicado el plaguicida.

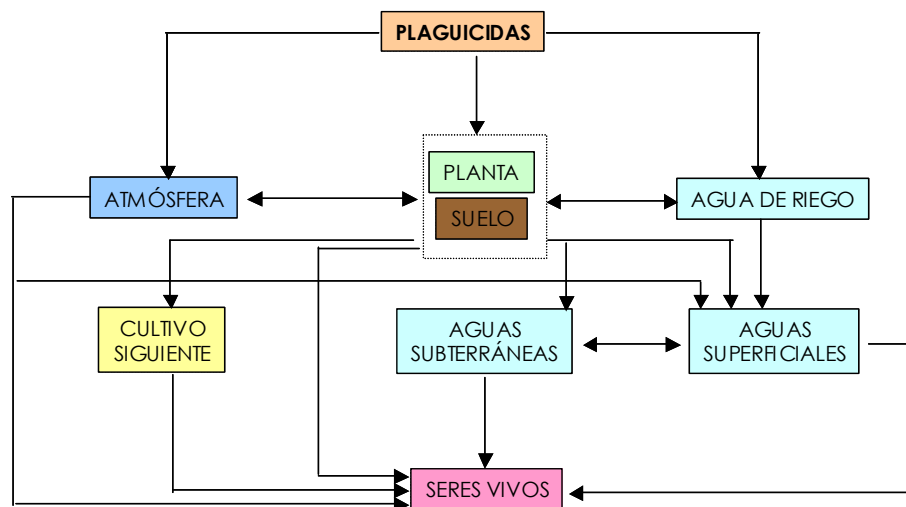


Figura 1.1 Esquema general de las vías de contaminación difusa por la aplicación de plaguicidas.

Como se observa en la Figura 1.1 los plaguicidas pueden alcanzar tanto las aguas superficiales como las subterráneas a través de diferentes vías de contaminación. De los factores decisivos, en cuanto a contaminación medioambiental, los más relevantes en el caso de las aguas son la solubilidad del plaguicida en este medio, su persistencia, el tipo de suelo, los factores climatológicos y las prácticas agrícolas. Por otra parte, la presencia de estos contaminantes en el medio ambiente hace que puedan ser bioacumulados por organismos vivos pasando de esta manera a la cadena alimenticia y llegando finalmente al ser humano. De este modo, los plaguicidas pueden estar presentes en las aguas, así como en muchos alimentos, tanto de origen vegetal como animal.

1.2.1 Determinación de residuos de plaguicidas y productos de transformación en muestras de interés ambiental

La preocupación por los efectos nocivos derivados de la amplia utilización de plaguicidas en las actividades principalmente agrícolas es una constante de nuestra sociedad actual. De todos los posibles efectos adversos sobre el medio ambiente, la contaminación de las aguas es uno de los problemas que, en la actualidad, genera más preocupación debido a los riesgos toxicológicos que conlleva.

Además, el número de plaguicidas usados en la actualidad es muy alto y su composición química muy variada, por lo que los efectos tóxicos y los problemas ambientales derivados de su amplia utilización son complejos y muy variados. La toxicidad de los plaguicidas presentes en el medio ambiente obliga a disponer de métodos analíticos fiables, rápidos, económicos y suficientemente sensibles que permitan el control de estos compuestos tóxicos. No es de extrañar, por lo tanto, que desde las últimas décadas del siglo XX se hayan empezado a desarrollar cada vez más métodos con el fin de determinar los niveles de plaguicidas en muestras ambientales.

Sin embargo, el desarrollo de esta metodología analítica presenta diversos problemas. Como ya hemos comentado, el elevado número de plaguicidas usados en la actualidad junto con la gran variabilidad en sus características físico-químicas dificulta la determinación simultánea de la mayoría de ellos en un solo método multiresiduo. Además, se debe tener presente la restrictiva legislación comunitaria que, en el caso de aguas destinadas al consumo humano, establece un límite máximo de 0.1 µg/L por plaguicida individual y de 0.5 µg/L por plaguicida total, lo que obliga a usar métodos analíticos que presenten límites de cuantificación de este orden o inferior.

Tradicionalmente, los métodos utilizados para este fin se han basado tanto en cromatografía líquida (LC) como en cromatografía de gases (GC), usando detectores de ultravioleta (UV) o fluorescencia (FD) en el primer caso, mientras que en GC se han empleado típicamente detectores de nitrógeno-fósforo (NPD), fotométrico de llama (FPD), captura de electrones (ECD) o espectrometría de masas (MS).

La técnica más aplicada para la determinación de plaguicidas en muestras ambientales ha sido, sin lugar a dudas, la GC. En los años 60, esta técnica revolucionó el análisis de residuos de plaguicidas debido a su elevada sensibilidad, selectividad y a la posibilidad de separación de un gran número de compuestos simultáneamente, surgiendo así los primeros métodos multiresiduo de análisis. Más tarde, en los años 70 se introdujo la GC capilar con la incorporación de detectores muy sensibles y selectivos, como ECD para el caso de los compuestos halogenados, NPD para los plaguicidas organonitrogenados y FPD para los compuestos organofosforados u organosulfurados. Poco a poco estos detectores fueron cediendo paso al acoplamiento con la MS, tanto GC-MS como GC-MS/MS que, posiblemente, es la técnica más usada en este campo en la actualidad.

Sin embargo, los métodos basados en GC presentan algunas limitaciones en el análisis de aguas, como por ejemplo el elevado tratamiento de muestra necesario derivado de la imposibilidad de inyección directa de muestras acuosas. Este tratamiento conlleva normalmente un cambio de disolvente además de un proceso de preconcentración con el fin de alcanzar la sensibilidad requerida. Por otro lado, la incompatibilidad de algunos compuestos con la técnica de GC, debido a su inestabilidad térmica, baja volatilidad y/o alta polaridad, hace que muchos de estos compuestos no estén incluidos en los métodos multiresiduo al no poder determinarse fácilmente por GC. Así los herbicidas clorofenoxiácidos, carbamatos y fenilureas son ejemplos típicos de compuestos cuya determinación por GC requiere, generalmente, procedimientos complicados de derivatización previos a la inyección (Wang-Hsien et al, 2000).

Con el fin de simplificar el tratamiento de muestra se han desarrollado métodos utilizando microextracción en fase sólida (Hernández et al, 2000) o la inyección directa de grandes volúmenes en un inyector de temperatura programable (Forcada et al, 2000; Nakamura et al, 2001).

Fue precisamente la imposibilidad de determinar compuestos muy polares por GC, lo que impulsó en los años 80 el uso de la LC. La LC ha sido tradicionalmente acoplada a detectores clásicos de UV (Chen et al, 2002; Hogendoorn et al, 1993) o FD (Morrica et al, 2005; Zamora et al, 2004). La principal desventaja de estos detectores es su limitada aplicación, ya que para que un compuesto absorba en la zona del UV o presente fluorescencia tiene que

presentar una conjugación importante en su estructura. Otras limitaciones son su baja sensibilidad (sobre todo en el caso de detectores UV), necesitándose generalmente una etapa de preconcentración, y su baja especificidad, por lo que estos métodos podrían ser útiles con fines de *screening* pero requerirían de una técnica adicional de confirmación.

En los últimos años, al igual que la GC, la LC ha hecho uso de MS como sistema de detección (LC-MS), implantándose también MS en tándem. Con esta técnica se han solventado algunos de los problemas existentes en GC-MS y LC-UV. Así es posible, por ejemplo, determinar ciertos plaguicidas mediante inyección directa de muestras acuosas. En el caso de ser necesaria una etapa de preconcentración, el tratamiento más habitual es la extracción en fase sólida (SPE) o la extracción líquido-líquido (LLE). Aunque inicialmente se utilizó la LLE, actualmente se prefiere la SPE como técnica de preconcentración debido, principalmente, a la posibilidad de completa automatización y al menor volumen de disolventes empleado. Esta técnica, que se comentará con más detalle en el apartado 1.3 del presente capítulo, puede ser utilizada en su modalidad *off-line* o *on-line*. En la modalidad *off-line* se pueden usar cómodamente mayores volúmenes de muestra, alcanzándose límites de detección del orden de ng/L, como en la determinación de triazinas, acetanilidas y fenilureas (D'Ascenzo et al, 1998) o de plaguicidas polares (Volmer et al, 1994) en muestras de agua mediante LC-MS preconcentrando volúmenes de entre 100-1000 mL. En lo referente a la determinación por MS/MS se obtuvieron resultados similares para la determinación de herbicidas ácidos (Koppen y Spliid, 1998) o plaguicidas polares y productos de transformación (Steen et al, 1999) tras la preconcentración de 1 L de muestra. En la modalidad *on-line* se utilizan normalmente volúmenes menores de muestra. Así se han determinado 15 fenilureas mediante LC-MS tras preconcentrar en línea 50 mL de agua obteniendo LODs de entre 5-20 ng/L (Bagheri et al, 1993) y 40 plaguicidas y 10 productos de transformación mediante LC-MS/MS tras preconcentrar 1.3 mL de muestra (Sancho et al, 2004) obteniendo límites de detección de 25 ng/L. En este sentido, se han publicado varias revisiones que resumen esta temática (Geerdink et al, 2002).

Otras aproximaciones que permiten la total automatización del proceso son el uso de columnas acopladas (LC-LC) o la inyección de grandes volúmenes en una única columna. Así, se han determinado plaguicidas en muestras de agua tras inyectar 2 mL de muestra (Hernández et al, 1997), alcanzando límites de detección del orden de 0.1-0.5 µg/L.

En la presente Tesis se explorará la capacidad analítica de una técnica tan potente y novedosa como LC-MS/MS con analizadores QTOF en la elucidación de TPs de plaguicidas seleccionados en muestras ambientales. Por otro lado, se abordará la determinación y confirmación de estos plaguicidas y TPs en aguas y suelos, mediante LC-MS/MS con analizadores de triple cuadrupolo e híbridos cuadrupolo-tiempo de vuelo.

1.2.2 Determinación de residuos de plaguicidas y metabolitos en muestras de interés toxicológico

Por lo que se refiere al campo biológico, el elevado uso de plaguicidas en diferentes actividades humanas hace prácticamente imposible impedir la exposición a estos compuestos de la población en general y de los trabajadores en contacto con ellos en particular.

Una vez el plaguicida entra en el organismo a través de la ingestión, inhalación o absorción dérmica, está sujeto a una serie de mecanismos (metabolismo) que transforman el plaguicida en otros compuestos, normalmente más polares que la molécula original, y por consiguiente, más fácilmente excretables por el organismo. Por tanto, la mayoría de las transformaciones metabólicas de los plaguicidas son procesos de detoxificación. Sin embargo, está bien establecido que el metabolismo conduce en algunas ocasiones a compuestos más activos, como es el caso de la conversión del paration o malation a las formas oxon (Chapalamadugu et al, 1992). Este tema será comentado con más detalle en el capítulo 2 de la Tesis.

El mejor modo de evaluar el grado de exposición humana a plaguicidas es mediante la determinación de la concentración de indicadores biológicos. Recibe el nombre de indicador biológico, el contaminante, metabolito o componente bioquímico indicativo, directa o indirectamente, de la exposición a un determinado plaguicida. Los indicadores se clasifican en tres categorías. Con los indicadores de exposición se mide el contaminante químico o sus metabolitos presentes en algún tipo de tejido o fluido biológico. Con los indicadores de dosis efectiva se determina la unión del analito, o sus metabolitos, a un receptor biológico. Finalmente, con los indicadores de efecto se mide el efecto que tiene el plaguicida sobre alguna actividad biológica en concreto. Aunque los indicadores elegidos para evaluar la exposición a un determinado plaguicida dependerán de la naturaleza de éste, los indicadores de exposición suelen ser los más adecuados, dada su mayor especificidad. Consecuentemente, el objetivo del control biológico suele ser la determinación del agente contaminante o de sus metabolitos en sangre, orina, tejido u otras muestras biológicas como la saliva, las heces, el aire

exhalado, la leche materna, etc., para lo cual es necesario conocer el metabolismo de los analitos.

Uno de los primeros factores a tener en cuenta para evaluar la exposición a un determinado plaguicida es la elección de la matriz, lo cual dependerá principalmente de la naturaleza del plaguicida. En el caso de los compuestos organoclorados (OCs), los residuos del compuesto o de sus metabolitos tienden a acumularse en el tejido adiposo debido al marcado carácter apolar de estos plaguicidas, a su persistencia, así como a su baja solubilidad en agua. Es por lo tanto normal encontrar residuos en la fracción lípida de fluidos humanos como la leche materna, la sangre o el suero. Las características de estos plaguicidas condicionan su determinación, que suele llevarse a cabo por GC acoplada a distintos detectores. Así, se han desarrollado métodos para la determinación de OCs como endosulfan, α -HCB, β -HCB, γ -HCB, DDE o DDT en muestras de suero (Minelli y Ribeiro, 1996; Burse et al, 1990) o tejido graso (Gallelli et al, 1995, Fidalgo-Used et al, 2003, Zrostlikova et al, 2002) utilizando detectores de ECD. Estos compuestos también han sido analizados con detectores de MS (Pitarch et al, 2003; Pauwels et al, 1999) o de alta resolución (Ward et al, 2000). En este sentido, se han publicado varias revisiones que resumen esta temática (Barr y Needham, 2002; Aprea et al, 2002).

Los OCs fueron prohibidos en los años 70 debido a su gran persistencia en el medio y sustituidos por otras familias de plaguicidas menos persistentes, como por ejemplo carbamatos y organofosforados. Paralelamente han ido apareciendo numerosos compuestos como herbicidas triazínicos, fenoxiácidos... Estos plaguicidas tienden a degradarse y raramente se bioacumulan en la cadena trófica. La reactividad de estos grupos hace que sean más fácilmente metabolizados, siendo posible utilizar como indicadores biológicos tanto el plaguicida intacto como sus metabolitos.

Así se han descrito diversos métodos para la determinación de plaguicidas no persistentes y sus metabolitos en suero y orina. Por ejemplo, los metabolitos de la atrazina han sido determinados en orina mediante GC-NPD (Catenacci et al, 1993). En cuanto a la determinación de metabolitos de compuestos OPs en orina, se han descrito varios métodos para alquilfosfatos, utilizando GC-FPD (Aprea et al, 1996), GC-MS (Hardt et Angerer, 2000) o GC-MS/MS (Bravo et al, 2002), tras la necesaria derivatización.

En el caso de HPLC, el insecticida carbaril ha sido determinado en sangre mediante HPLC-UV (Ward et al, 1987), mientras que uno de sus metabolitos ha sido detectado en orina mediante HPLC-FD (Sancho et al, 2003).

La introducción del acoplamiento LC-MS y, más concretamente de LC-MS/MS, en este tipo de análisis está produciendo grandes avances ya que permite simplificar el tratamiento de muestra, además de minimizar los errores asociados a esta etapa y reducir el tiempo de análisis. Así se han descrito diversos métodos para la determinación de plaguicidas no persistentes y sus metabolitos en suero y orina por LC-MS(MS). En el caso de los herbicidas triazínicos, se ha determinado la presencia de dos herbicidas como la simazina y la atrazina, así como uno de sus metabolitos, en orina por LC-MS (Pozzebon et al, 2003). Los metabolitos de las triazinas se han determinado en orina mediante LC-MS/MS (Baker et al, 2000; Beeson et al, 1999; Hernández et al, 2005) obteniendo LODs de unos pocos $\mu\text{g/L}$. Algunos metabolitos de compuestos organofosforados también han sido determinados satisfactoriamente en orina (Beeson et al, 1999; Baker et al, 2000; Hernández et al, 2002, 2004a) por LC-MS/MS.

Así pues, aunque la GC ha sido la técnica más ampliamente utilizada para la determinación de plaguicidas y metabolitos en muestras de tejidos grasos y fluidos biológicos, la LC, especialmente acoplada a MS, ha permitido ampliar notablemente este campo de trabajo extendiendo las aplicaciones a otras familias de compuestos más polares y sobretodo a sus metabolitos, lo cual se ha visto favorecido ampliamente desde la introducción del acoplamiento LC-MS/MS.

En la presente Tesis se explorará el potencial analítico de LC-MS/MS con analizadores QTOF en la elucidación de metabolitos de plaguicidas seleccionados en muestras de interés biológico, ya que aunque se conoce la relación entre la exposición a plaguicidas y varios efectos nocivos para la salud humana, el conocimiento de los metabolitos presentes en el organismo en contacto con un plaguicida es mucho menor.

1.3 Tratamiento de muestra

El tratamiento de muestra habitual en la determinación de plaguicidas consiste en una etapa de extracción, seguida normalmente de una purificación, con el fin de eliminar los interferentes que pueden afectar a la determinación, y/o una etapa de preconcentración, con el fin de alcanzar la sensibilidad adecuada.

Una de las grandes ventajas de la LC-MS es la posibilidad de determinar plaguicidas en aguas por inyección directa, mientras que en el caso de GC-MS la inyección de muestras acuosas es problemática, siendo necesario un cambio de solvente. En el caso de inyectar muestras con cierto contenido orgánico, hay que tener en cuenta que, idealmente, el extracto ha de presentar un contenido acuoso superior al inicial del gradiente, con el fin de obtener una buena separación cromatográfica. Por otra parte, la inyección directa de los extractos suele llevar asociada la introducción en la interfase de compuestos no volátiles que pueden depositarse en el cono de muestreo, minimizando la respuesta, por lo que sólo es recomendable en matrices relativamente limpias. En el caso de muestras acuosas, la mayoría de los interferentes son sales, que pueden ser eliminados del sistema mediante el uso de la función conocida como *solvent delay*.

En aquellos casos en los que es necesario alcanzar mayor sensibilidad o eliminar interferentes, es preferible utilizar alguna etapa de preconcentración/purificación automatizada, como por ejemplo la extracción en fase sólida (SPE). Esta técnica permite reducir los interferentes de la matriz, mejorando asimismo la sensibilidad del método, minimizando tanto la limpieza del equipo instrumental como los errores asociados al tratamiento de muestra, sin necesidad de aumentar el tiempo de análisis. El principal inconveniente de esta aproximación con respecto a la inyección directa es el tiempo que se consume y que supone una cierta manipulación de muestra, especialmente en el modo *off-line*, por lo que aumenta la posibilidad de que se produzcan errores por pérdida del analito o por contaminación de la muestra. Finalmente, en algunos casos, dependiendo de la combinación matriz-analito, también se pueden preconcentrar algunos interferentes aumentando, por tanto, el efecto matriz.

El tratamiento de muestra seguido en la presente Tesis ha consistido en la inyección directa de las muestras, tras una simple centrifugación, o en una extracción en fase sólida en modo *on-line*, excepto en la determinación del glifosato, donde, por sus especiales características fue necesaria una etapa de derivatización previa.

La SPE es una técnica muy adecuada para el tratamiento de muestras acuosas. Consiste en la utilización de una pequeña columna enriquecedora para retener a los analitos, previamente a la separación analítica en la segunda columna de LC. Así, los analitos son atrapados por una fase adsorbente adecuada que se encuentra empaquetada en columnas de extracción, o en cartuchos desechables, a través de las cuales se hace pasar la muestra acuosa.

La SPE puede utilizarse en su modalidad *off-line*, de manera que la preparación de la muestra se lleva a cabo completamente separada del análisis cromatográfico, o bien de modo *on-line*, mediante conexión directa de la precolumna enriquecedora al sistema cromatográfico (acoplamiento SPE-LC, también conocida como *on-line trace enrichment*). En el primer caso, se utilizan cartuchos desechables, a través de los cuales se pasa la muestra. Los analitos retenidos son posteriormente eluidos con el disolvente adecuado, y habitualmente, se introduce alguna etapa de evaporación previamente a la determinación cromatográfica. En la SPE *on-line* la muestra se introduce en la primera columna del sistema mediante un autoinyector/bomba preconcentradora. Posteriormente, los analitos retenidos se transfieren directamente y en línea a la columna analítica. Esta técnica ha sido ampliamente comentada y revisada por diversos autores (Hennion, 1999). En la Figura 2 se muestra un esquema básico de acoplamiento SPE-LC.

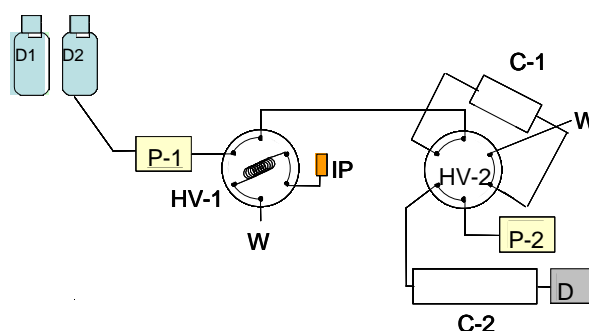


Figura 1.2 Representación esquemática de un acoplamiento SPE-LC.

C-1: precolumna ; C-2: columna analítica; P-1: bomba enriquecedora de LC; P-2: bomba de LC;

HV-1, HV-2: válvulas de alta presión de 6 vías; D: detector; W: deshecho

1.3.1 Etapas del análisis

Todo método analítico basado en el acoplamiento SPE-LC se divide en una serie de etapas (esquemáticas en la Figura 1.3), que se comentan a continuación:

A.1) Acondicionamiento de la precolumna C-1: El acondicionamiento de la precolumna enriquecedora C-1 se lleva a cabo con disolventes adecuados (D_1 , D_2) que se introducen mediante la bomba P-1. Los disolventes más utilizados en esta etapa son metanol y/o acetonitrilo, seguidos de agua de calidad HPLC.

A.2) Carga de la muestra en C-1: Una vez acondicionada la precolumna C-1, un determinado volumen de muestra (normalmente entre 10 y 200 mL) se introduce en el sistema mediante un inyector automático o una bomba de cromatografía líquida (P-1), de modo que los analitos quedan retenidos en la primera columna.

A.3) Lavado: Con el fin de purificar la muestra se suele realizar una etapa de lavado con un disolvente adecuado, de manera que se eliminen el mayor número posible de interferencias sin que ningún analito sea desorbido. Por ejemplo, en muestras acuosas, el lavado suele llevarse a cabo con agua destilada con el fin de eliminar los compuestos más polares, como sales. Mientras, la fase móvil circula a través de la columna analítica C-2.

B) Etapa de transferencia: Cuando finaliza la etapa de preconcentración se activa la válvula HV-1, de manera que los compuestos preconcentrados son transportados a la segunda columna analítica (C-2), siendo la fase móvil usada en C-2 la responsable de dicha desorción en línea. Para ello, es necesario el uso de una interfase, que suele ser una válvula de 6 vías. La transferencia de estos compuestos puede llevarse a cabo en la dirección de la carga de la muestra (*forward-flush*), aunque es más frecuente realizarla en contracorriente (*backflush*), ya que los analitos preconcentrados y retenidos, idealmente en cabeza de columna, se transfieren de este modo con menor ensanchamiento de bandas (Hernández y Sancho, 2004b).

C) Separación cromatográfica y detección: Una vez transferidos los analitos a C-2, en esta columna se produce la separación final de los distintos analitos entre sí, o entre ellos y los interferentes que presenta la matriz. En este sentido, no existen diferencias entre la cromatografía en C-2 y la cromatografía convencional.

Transcurrido el tiempo necesario para que eluyan todos los analitos desde C-1 hasta C-2 se vuelve a activar la válvula HV-1, de modo que se pueda empezar a procesar la siguiente muestra tras acondicionar nuevamente la precolumna C-1 mientras en la columna C-2 se lleva a cabo la separación cromatográfica y los analitos separados pasan al detector.

La posibilidad de que se produzca superposición entre la etapa de separación cromatográfica y la de preconcentración de la siguiente muestra es interesante ya que permite disminuir de manera considerable el tiempo de procesamiento de cada muestra y, por lo tanto, el tiempo global de análisis.

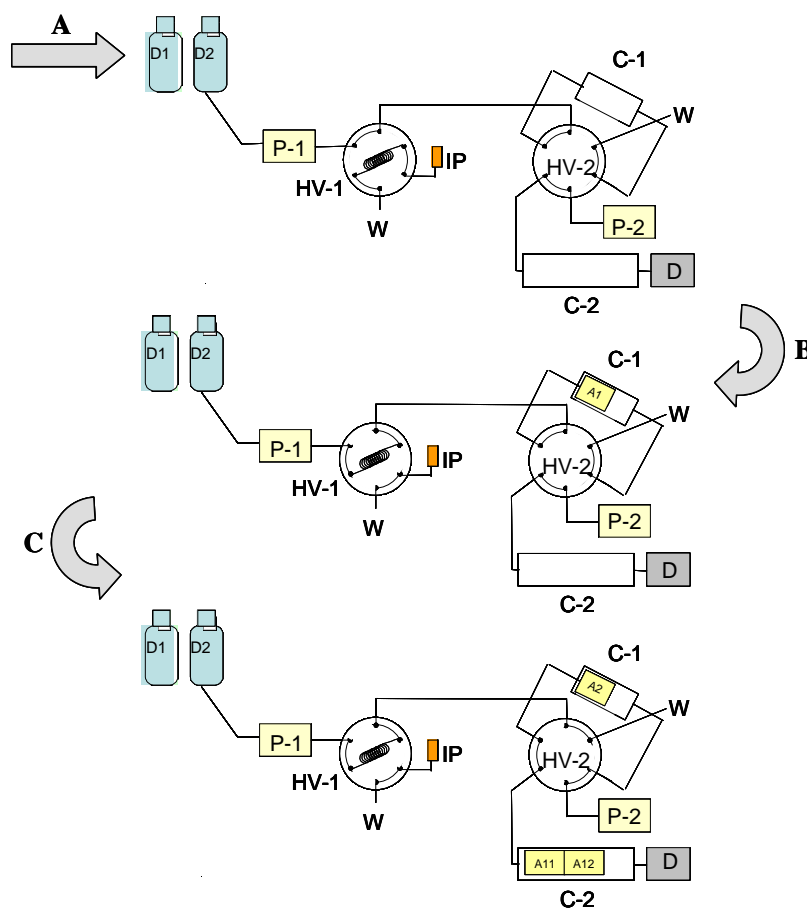


Figura 1.3. Etapas de un procedimiento con acoplamiento SPE-LC: (A) Acondicionamiento y carga de la muestra, (B) Transferencia, (C) Separación cromatográfica y detección.
 C-1: precolumna enriquecedora; C-2: columna analítica; D₁, D₂: disolventes acondicionadores de C-1; A₁, A₂: analitos de la muestra 1 (A₁₁, A₁₂); A₂: analitos de la muestra 2; P-1: bomba enriquecedora de LC; P-2: bomba de LC; HV-1, HV-2: válvulas de alta presión de 6 vías; D: detector; W: deshecho

1.3.2 Parámetros de preconcentración

En el acoplamiento SPE-LC, las pequeñas precolumnas o cartuchos usados como C-1 permiten utilizar flujos relativamente grandes manteniendo una capacidad de retención razonable.

Entre los parámetros más importantes a tener en cuenta en todo el procedimiento de acoplamiento SPE-LC se encuentran el volumen de ruptura, el tamaño de la precolumna utilizada y la elección de la fase adsorbente.

- **Estimación del volumen de ruptura:** A medida que la muestra acuosa se preconcentra, los analitos más retenidos quedan en cabeza de columna mientras que los menos retenidos pueden desplazarse a través de C-1, pudiendo llegar a ser eluidos por la propia muestra, a pesar de la poca fuerza eluotrópica del agua. En el momento en que los analitos empiezan a eluir de la primera columna se produce la ruptura de los mismos (*breakthrough*) y el volumen de muestra preconcentrado hasta ese instante se conoce como volumen de ruptura. Este volumen, que indica el volumen de muestra que puede ser manejado eficazmente en el proceso de preconcentración sin pérdidas de analito, es una variable importante en los métodos desarrollados, que conviene determinar experimentalmente.

- **Selección de la fase adsorbente:** Tradicionalmente, la SPE se ha utilizado como método de preconcentración y purificación alternativo a la extracción líquido-líquido aplicada a compuestos relativamente no polares. Por ello, los rellenos más utilizados han sido de C₁₈ (Hennion, 1999; Hernández et al, 2001). Sin embargo, en los últimos años se ha incrementado el uso de nuevos sorbentes que permiten la retención de gran variedad de analitos, desde no polares a altamente polares. Entre ellos, cabe destacar los cartuchos poliméricos para la retención de compuestos polares (Geerdink et al, 1999), los de intercambio iónico o formadores de pares iónicos, para la retención de analitos que puedan estar en forma catiónica o aniónica (Patsias et al, 2001), los de exclusión por tamaños, con el fin de purificar la muestra de moléculas de gran tamaño como proteínas o ácidos húmicos (Onnerfjord et al, 1996), los cartuchos diseñados especialmente para retener un compuesto concreto, como pueden ser los basados en inmunoadfinidad (Piochon et al, 2004) o en polímeros impresos molecularmente (Chapuis et al, 2004), así como una nueva generación de polímeros híbridos, OASIS (Ibáñez et al, 2005).

- **Tamaño de la precolumna:** Idealmente, las dimensiones de C-1 en cuanto a diámetro interno deben ser iguales a las utilizadas en C-2 para minimizar los efectos de la etapa de transferencia. Por otra parte, y dado que el objetivo de esta columna no es la separación de los analitos sino su retención, la longitud debe ser la mínima posible con el fin de disminuir el

ensanchamiento de pico. Las dimensiones típicas de C-1 en procedimientos SPE-LC son 5-15 mm de longitud y 1-4.6 mm de diámetro interno.

En cuanto al empacado, las precolumnas de tamaño de partícula de 5 μm no permiten flujos muy elevados, trabajando generalmente a 1-2 mL/min. Esto hace que el tiempo de análisis aumente sustancialmente si se desean inyectar volúmenes elevados de muestra. Para evitar que este proceso sea la etapa limitante del análisis en cuanto a tiempo, la tendencia actual es utilizar columnas con tamaño de partícula 15-40 μm .

En esta Tesis se hará uso de la técnica SPE-LC como método de preconcentración de muestras de agua, con fines de determinación y/o confirmación de plaguicidas a niveles de concentración de sub ppb.

1.4 Cromatografía líquida acoplada a espectrometría de masas (LC-MS)

La técnica híbrida LC-MS ha sido una de las que mayor crecimiento ha experimentado en las últimas décadas, ya que permite combinar las características de separación de LC con las excelentes características de detección de MS, obteniendo métodos analíticos de gran sensibilidad y poder de identificación. Sin embargo, el desarrollo de este acoplamiento se produjo con un considerable retraso respecto a su homólogo GC-MS debido, principalmente, a la incompatibilidad de introducir los elevados flujos usados en cromatografía líquida convencional (1 mL/min) con el alto vacío necesario en un detector MS. Otras limitaciones que retrasaron la aplicación de esta técnica fueron la incompatibilidad del detector MS con las fases móviles no volátiles frecuentemente utilizadas en LC y la dificultad de ionizar compuestos no volátiles o lábiles térmicamente. La historia de este acoplamiento y su desarrollo están bien referenciados en varios libros, entre los que destaca "*Liquid Chromatography-Mass Spectrometry: third edition*" (Niessen, 2006) y "*A global view of LC-MS*" (Willoughby, 1998), en los cuales se evidencia la dificultad que entrañó este reto.

Una de las principales ventajas de LC-MS es su versatilidad, que permite determinar moléculas muy polares o incluso iónicas. En este sentido, se han determinado compuestos tan polares como la ciromazina (Sancho et al, 2005), compuestos aniónicos como el fosetil (Hernández et al, 2003) o catiónicos, como plaguicidas con amonios cuaternarios (Castro et al, 2000, 2001). Esta capacidad de determinación de compuestos polares hace de la LC una herramienta ideal para la determinación de TPs. La mayoría de los plaguicidas que se encuentran en el medio ambiente sufren alteraciones por vía química, fotoquímica o bioquímica, formándose TPs relativamente estables y en general más polares que el plaguicida original, y que por tanto, pueden alcanzar las aguas con mayor facilidad. Además, en algunas ocasiones, estos TPs pueden llegar a ser tan tóxicos, o incluso más, que el compuesto de partida. Este tema será comentado con más detalle en el capítulo 2 de la Tesis.

A pesar de las notables ventajas de los métodos basados en LC-MS frente a los GC-MS (menor tratamiento de muestra, posibilidad de analizar compuestos muy polares o análisis directo de muestras acuosas), el acoplamiento LC-MS presentaba inicialmente algunas limitaciones, debido al uso de interfases a presión atmosférica (API) que restringían su aplicación a moléculas con centros susceptibles de (de)protonación. Esta carencia ha impulsado el desarrollo de nuevas interfases que han permitido el análisis de compuestos inabordables hasta la fecha por LC-MS. Todas estas interfases se comentarán con más detalle en el apartado 1.4.2.

1.4.1 Cromatografía líquida

La cromatografía líquida es una técnica de separación física en la cual los componentes de una muestra se separan en función de su distinta afinidad entre dos fases, una fase estacionaria contenida en una columna y una fase móvil líquida, normalmente constituida por una mezcla de disolventes de distinta fuerza eluotrópica, que fluye a través de la columna. El proceso cromatográfico ocurre como resultado de una repetición de etapas de absorción/desorción durante el movimiento de los analitos a través de la fase estacionaria. Finalmente, la separación es consecuencia de los diferentes coeficientes de distribución entre los componentes de una muestra.

Los distintos mecanismos de separación han sido bien estudiados y se encuentran descritos en diversos libros y monografías (Cela et al, 2002; Davrio y colaboradores, 2000; Valcárcel y Gómez, 1994; Poole and Poole, 1991). Aunque la elección de la columna y de la fase móvil es un factor crítico a la hora de desarrollar un método analítico, su discusión no es el objetivo de esta Tesis.

Como ya se ha comentado anteriormente, el desarrollo del acoplamiento LC-MS presentó algunas dificultades en sus inicios debido a la incompatibilidad de introducir los elevados flujos utilizados en LC convencional (1 mL/min) con el alto vacío necesario en un detector MS, a la incompatibilidad de este detector con las fases móviles no volátiles habitualmente usadas en LC y a la dificultad de ionizar compuestos no volátiles o lábiles térmicamente.

En el caso de matrices complejas, la cromatografía con columnas acopladas permite, además de la reducción del tratamiento de muestra de una manera automatizada, la preconcentración de los analitos. Esta técnica consiste en la separación de los componentes de una muestra utilizando dos o más columnas en serie, las cuales difieren en su capacidad o selectividad. Nuestro grupo de trabajo tiene dilatada experiencia en el uso y aplicaciones de LC-LC y SPE-LC, habiendo realizado también algunas revisiones y discusiones críticas sobre la cromatografía líquida multidimensional y su aplicación a la determinación de compuestos orgánicos (Hernández y Sancho, 2004b; Hernández et al, 2006).

En esta Tesis se ha utilizado la técnica acoplada SPE-LC, ya comentada en las páginas precedentes.

1.4.2 Interfases

Como ya se ha indicado anteriormente, el factor limitante para el desarrollo del acoplamiento LC-MS fue la obtención de interfases que permitieran la introducción directa del flujo de LC en el detector MS. Las primeras interfases desarrolladas pusieron especial énfasis en la eliminación del disolvente, con el fin de simular el proceso de evaporación del analito y posterior ionización, al igual que sucedía en el acoplamiento GC-MS. A este tipo pertenece la interfase de haz de partículas (*particle-beam*, PB). Sin embargo, durante el desarrollo de estas interfases se observó que la presencia de disolvente no tenía necesariamente que perturbar el análisis por MS, sino que incluso podía ayudar a la ionización de los analitos. A lo largo del tiempo se propusieron distintas aproximaciones, que culminaron con las dos más utilizadas en la actualidad: la interfase de ionización química a presión atmosférica (APCI) y la interfase electrospray (ESI). Estas dos interfases se empezaron a desarrollar en los años 70, aunque no fue hasta los 90 cuando se implementaron ampliamente. Así, John B. Fenn fue recompensado en el año 2002 con el Premio Nobel de Química por su contribución al desarrollo de la interfase ESI.

Cuando se usa la interfase ESI, el flujo procedente de la LC, en el cual se encuentran disueltos los analitos, pasa a través de un capilar a presión atmosférica, mantenido a alto voltaje (típicamente 3-4 kV para la producción de iones positivos; ligeramente inferior y de polaridad opuesta para la producción de iones negativos). Este alto voltaje dispersa la corriente de líquido, formándose gotas altamente cargadas (nebulización), que son desolvatadas a medida que pasan a través de la región a presión atmosférica de la fuente del espectrómetro de masas. La desolvatación es asistida por una corriente de gas caliente, generalmente nitrógeno. A medida que disminuye el tamaño de las gotas se alcanza un punto en el cual las fuerzas repulsivas entre cargas en la superficie de las gotas son suficientes para superar las fuerzas cohesivas de tensión superficial. Entonces ocurre una "explosión de Coulomb", produciendo gotas más pequeñas. Se producen una serie de explosiones hasta que se alcanza un punto en el cual los iones pasan a fase gas, siendo transferidos a través de una serie de lentes focalizadoras hacia el espectrómetro de masas. Puesto que la ionización tiene lugar directamente en la disolución, las moléculas térmicamente lábiles pueden ser ionizadas sin degradación. Esta teoría se conoce como el *modelo de la carga residual*. En la Figura 1.4 se muestra un esquema del mecanismo de formación de los iones en ESI.

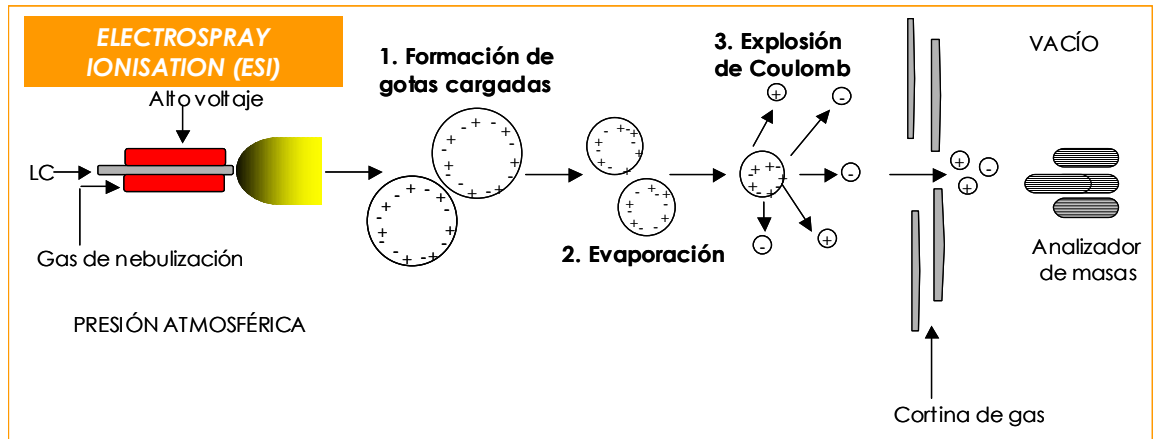


Figura 1.4. Esquema del mecanismo de formación de los iones en ESI

Cuando se utiliza la interfase APCI el flujo de LC procedente del sistema cromatográfico se nebuliza en un tubo vaporizador de sílice fundida caliente (Figura 1.5), donde el solvente es evaporado prácticamente en su totalidad. La mezcla gas-vapor entra en la fuente a presión atmosférica, donde la ionización química es iniciada por los electrones generados en una aguja (corona) por descarga. El vapor de solvente actúa como gas reactivo. Los iones generados son transferidos por el alto vacío del detector MS.

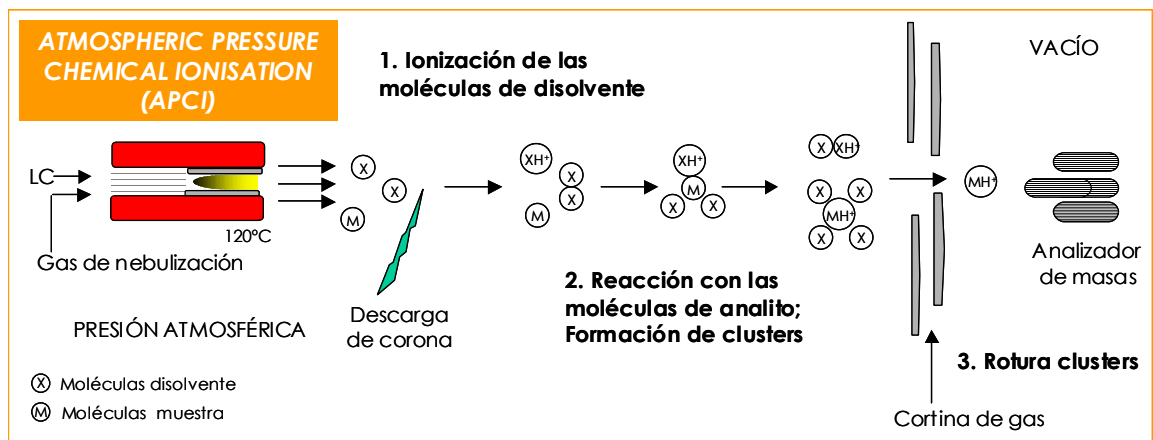


Figura 1.5. Esquema del mecanismo de formación de los iones en APCI

En ambas interfases, las especies más frecuentemente detectadas son $[M+H]^+$, en el caso de que la molécula capte un protón del medio (moléculas básicas), o $[M-H]^-$, en el caso de que la molécula ceda un protón al medio (moléculas ácidas). Así pues, en función de las características físico-químicas del analito, se elegirá el modo de ionización adecuado para que se pueda observar el ion molecular. Además de estos iones, en el caso de ionización positiva se pueden formar aductos con otras especies presentes en el medio, siendo los más comunes los aductos con sodio, potasio o amonio.

Por otro lado, y en contraste con otros modos de ionización, los iones producidos en electrospray pueden estar multicargados. Esto es importante ya que el espectrómetro de masas mide relaciones m/z (masa/carga) de los iones, por lo que el rango de masas de un instrumento puede extenderse por un factor equivalente al número de cargas de la molécula analito y así permitir el estudio de moléculas con pesos moleculares fuera de su rango, es decir, un ion de m/z 1000 con 20 cargas, procedería de un compuesto con un peso molecular de 20000 Da.

Aunque la interfase ESI está más indicada para compuestos iónicos o muy polares y la APCI para los menos polares, hay un amplio rango de polaridades en las que ambas interfases pueden trabajar aceptablemente, dependiendo en estos casos la elección de una u otra en función de la sensibilidad alcanzada y de la matriz de la muestra. Por norma general, la ionización mediante ESI es más efectiva, por lo que suele dar lugar a determinaciones más sensibles.

Por otro lado, hay que destacar la suave ionización producida en LC-MS en contraste con la interfase de impacto electrónico (EI) utilizada en GC, donde la enorme energía con la que son bombardeadas las sustancias que alcanzan la fuente (aproximadamente 70 eV) es suficiente para producir, por un lado, la ionización del compuesto (formación del ion molecular) y además generar un cierto grado de fragmentación del mismo, que suministra una valiosa información acerca de su estructura. En LC-MS, en cambio, es habitual obtener exclusivamente el ion molecular.

Cabe también destacar el hecho de que aún no se dispone de una interfase normalizada para LC-MS, lo que hace que no se puedan utilizar librerías de espectros, como ocurre en GC-MS. La diferencia en el modo y grado de ionización de cada interfase, así como la variabilidad de resultados en función de factores como la composición de la fase móvil o el voltaje de cono aplicado hacen muy difícil la utilización de librerías de compuestos normalizadas con el fin de comparar los resultados obtenidos con otros previamente reportados.

Estas dos interfases no proporcionan una ionización universal, es decir, todavía existen numerosos compuestos que no pueden ser ionizados por estas técnicas, por lo que el desarrollo de nuevas interfases que solventen este problema sigue en constante evolución. En este sentido, la carencia más importante que presenta en la actualidad el análisis por LC-MS mediante las interfases citadas anteriormente es la imposibilidad de determinar moléculas apolares como, por ejemplo, hidrocarburos policíclicos aromáticos (PAHs) u OCs. Una de las últimas interfases desarrolladas ha sido la de fotoionización a presión atmosférica (APPI), que permite determinar moléculas apolares, como por ejemplo los PCBs, compuestos inabordables con las otras dos interfases. En esta interfase se hace incidir un haz de fotones generado por una lámpara ultravioleta sobre el flujo procedente del LC vaporizado. Estos fotones pueden ionizar a la molécula si su potencial de ionización es inferior a la energía del fotón dando lugar al ion molecular M^+ . En presencia de un disolvente prótico, también puede protonarse dando lugar a $[M+H]^+$.

En la Figura 1.6 se muestran los rangos generales de aplicabilidad de las tres interfases en función de la polaridad y la masa molecular del analito a determinar.

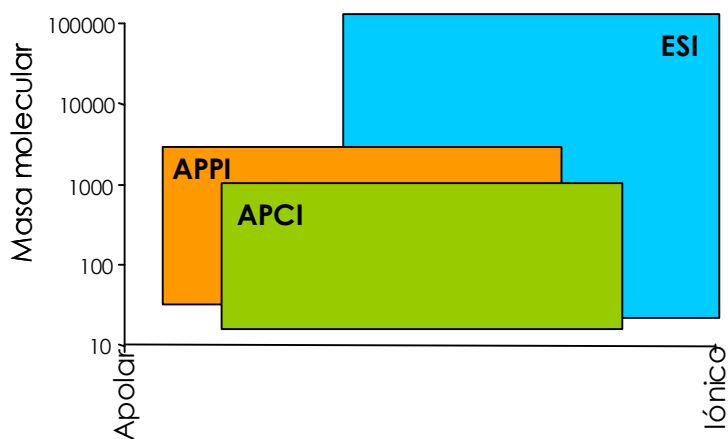


Figura 1.6 Rango de aplicación de las distintas interfases en función de la polaridad del compuesto y de su masa molecular.

En esta Tesis se ha empleado únicamente la interfase electrospray, cuyo esquema se muestra en la Figura 1.7. Una sonda que proporciona un flujo de gas concéntrico a la corriente de fase móvil ayuda a la formación de gotas desde el líquido, permitiendo emplear una velocidad de flujo alrededor de 0.2 mL/min.

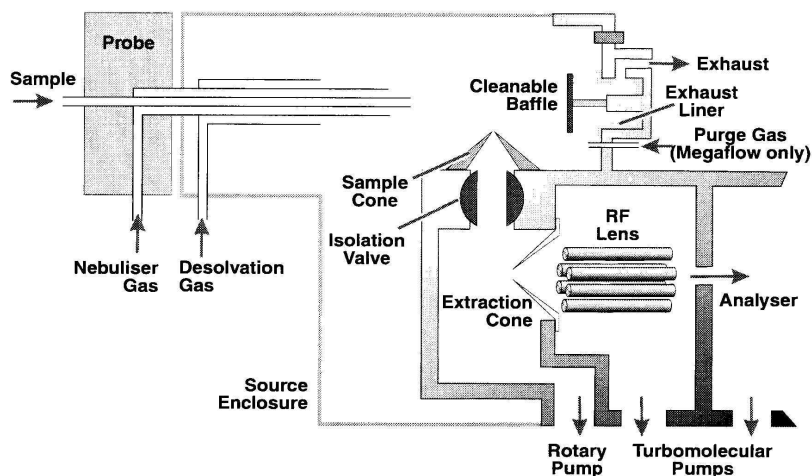


Figura 1.7 Esquema de la interfase electrospray empleada

En general, los analitos que pueden ser estudiados con esta interfase son compuestos iónicos intrínsecamente cargados en disolución, compuestos neutros/polares que puedan ser protonados o deprotonados en las condiciones empleadas (por ejemplo, pH apropiado) y compuestos no polares que experimenten oxidación o reducción en la punta del capilar.

En definitiva, la interfase electrospray es posiblemente la más utilizada ya que es aplicable a una gran variedad de analitos polares y térmicamente lábiles, tanto de bajo como de elevado peso molecular, y además es compatible con un amplio rango de condiciones HPLC.

1.4.3 Espectrometría de masas

Desde sus inicios, hace aproximadamente 100 años, la espectrometría de masas se ha convertido en una de las herramientas más poderosas al alcance de los químicos para el análisis de mezclas orgánicas y bioquímicas complejas. En la Figura 1.8, se muestra de modo esquemático la evolución de la MS.

Un espectrómetro de masas es un instrumento que convierte los compuestos de una muestra en iones gaseosos y los separa en función de su relación masa/carga (m/z). Los espectrómetros de masas se pueden clasificar en función del analizador de masas usado, es decir, en función de cómo se separan los iones. Los cinco analizadores más importantes son: cuadrupolo, tiempo de vuelo, trampa de iones cuadrupolares, sector magnéticos y resonancia de ion-ciclotrón con transformada de Fourier.

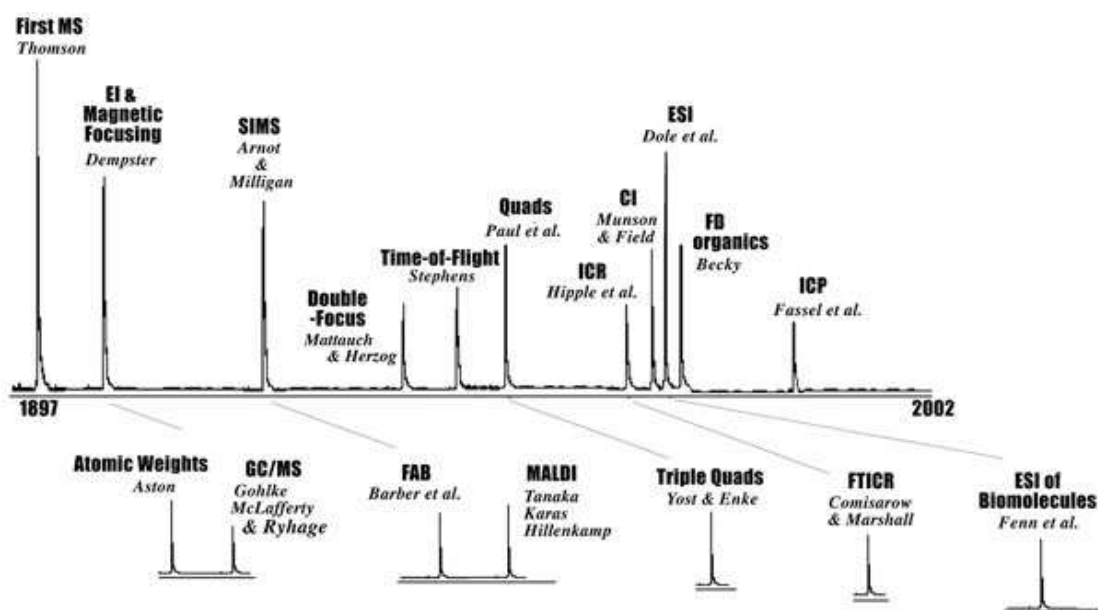


Figura 1.8 Evolución de la espectrometría de masas

En este apartado se comentarán exclusivamente los analizadores de cuadrupolo y de tiempo de vuelo, por ser los utilizados en esta Tesis. Se pretende ofrecer una idea sobre las ventajas e inconvenientes de cada detector, al tiempo que se comentará también la espectrometría de masas en tándem con analizadores de triple cuadrupolo e híbrido cuadrupolo-tiempo de vuelo. Para un estudio más detallado se recomienda la lectura de Niessen (2006).

Analizador de cuadrupolo

El llamado analizador cuadrupolar, filtro de masas cuadrupolar o, simplemente, cuadrupolo, está formado por cuatro barras alineadas paralelamente entre sí y equidistantes una distancia r_0 de un eje central imaginario, tal y como se muestra en la Figura 1.9. Sobre las barras del cuadrupolo se aplican (dos a dos) voltajes de corriente continua (DC) y de radiofrecuencia (RF). Los iones son introducidos en el campo cuadrupolar mediante la aplicación de un potencial, con lo que empiezan a oscilar en un plano perpendicular a las cuatro barras. De esta manera los iones describen una trayectoria que depende directamente de su relación m/z . A un valor específico de DC y RF, sólo los iones con una particular relación m/z siguen una trayectoria estable a través de las barras y alcanzan el detector (Figura 1.10). Así pues, el cuadrupolo actúa como un filtro, ya que sólo deja pasar un número limitado de iones. El espectro de masas se produce, por lo tanto, variando los voltajes RF y DC de manera sistemática, para llevar iones con m/z crecientes o decreciente hacia el detector.

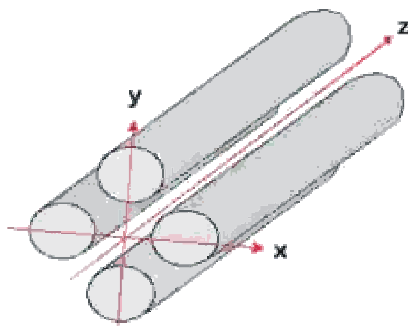


Figura 1.9 Esquema de un cuadrupolo

Dependiendo de la recta DC/RF podrán estabilizarse más trayectorias y por tanto podrán atravesar el cuadrupolo más iones. La resolución de los cuadrupolos está íntimamente ligada con la pendiente de esta recta, ya que a medida que aumenta la pendiente disminuye el número de trayectorias estabilizadas pudiendo separarse compuestos con m/z muy parecidas. Sin embargo, al disminuir el número de trayectorias también disminuye el número de iones que atraviesa el cuadrupolo dando lugar a una pérdida importante de sensibilidad. Como compromiso entre resolución y sensibilidad, se suele trabajar en resolución unidad, es decir, con una pendiente tal que permita separar compuestos cuya relación m/z se diferencie en 1 Da, obteniendo de este modo una buena sensibilidad.

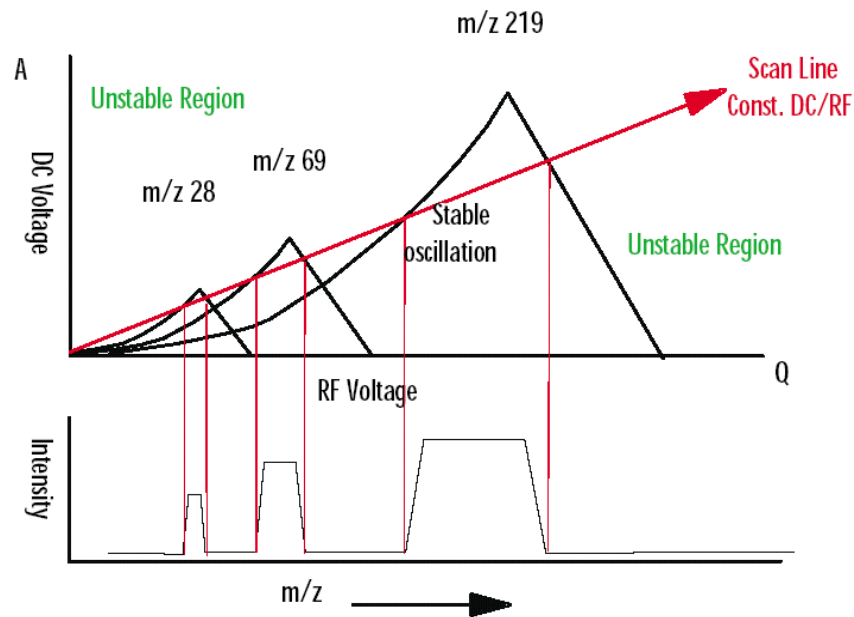


Figura 1.10. Diagrama de estabilidad

Analizador de triple cuadrupolo (QqQ)

Cuando se desea utilizar MS para la elucidación estructural es necesaria una mayor información que la obtenida por los iones generados en el proceso de ionización. Esta información adicional se puede obtener a partir de la fragmentación de estos iones, es decir, a partir de la espectrometría de masas en tándem. En el caso de instrumentos de filtro cuadrupolar, el uso de MS/MS implica necesariamente la adición de dos cuadrupolos extra a un instrumento de cuadrupolo lineal, tal y como se muestra en la Figura 1.11. La fragmentación se produce por colisión del ion seleccionado (ion precursor) con moléculas de gas inerte (generalmente argón). Este proceso recibe el nombre de disociación inducida por colisión (CID) y se produce en dos etapas: en la primera, la energía translacional del ion se convierte en energía interna tras colisionar con las moléculas del gas inerte; en la segunda, esta energía interna se utiliza para romper el ion en varios fragmentos (iones producto). Así, en el primer cuadrupolo se puede aislar un ion de m/z determinada que pasa al segundo cuadrupolo, usado como celda de colisión, donde tiene lugar la fragmentación de los iones al chocar contra las moléculas de gas inerte. El ion se fragmenta en función de la estructura del analito. Es importante que la transmisión de los iones desde la celda de colisión al tercer cuadrupolo sea lo más efectiva posible, es decir, que la mayoría de los iones producto formados lleguen hasta el tercer cuadrupolo, donde se puede realizar un barrido de los iones o seleccionar uno de éstos

con el fin de obtener una transición selectiva del analito. Normalmente, se suele modificar la geometría de la celda de colisión, que suele ser un hexapolo u octapolo con el fin de favorecer la transmisión.

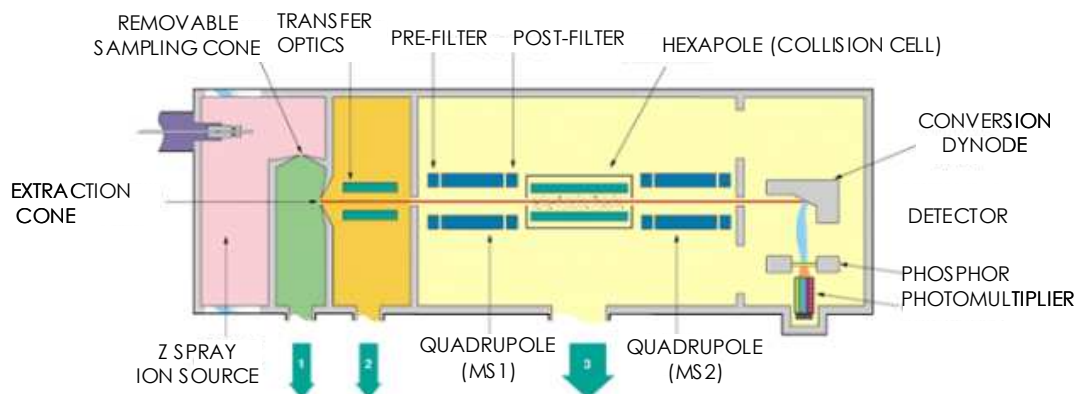


Figura 1.11. Esquema de un triple cuadrupolo

Cuando se trabaja con un triple cuadrupolo se pueden utilizar distintos modos de barrido en función del objetivo final del análisis. Así, en modo MS se puede realizar un barrido de todos los iones (*full scan*) o seleccionar un ion concreto para ser monitorizado (*Selected Ion Monitoring, SIM*). Cuando se trabaja en modo MS/MS se pueden realizar barridos de iones producto, barridos de iones precursores, de pérdidas neutras, o la monitorización de una transición concreta (*Selected Reaction Monitoring, SRM*), aumentando la sensibilidad y la selectividad.

En este apartado nos centraremos exclusivamente en los tres modos utilizados en esta Tesis (esquematisados en la Figura 1.12) explicándolos con más detalle. En el barrido *full scan* todos los iones que alcanzan el detector son monitorizados, generándose cromatogramas en tres dimensiones (tiempo de retención, intensidad y m/z) que son de gran utilidad cuando se desea elucidar la composición de una muestra desconocida o en la optimización de los parámetros instrumentales cuando se inyectan patrones. Si trabajando en este modo se desea observar únicamente el cromatograma generado por una m/z determinada se puede realizar una visualización del cromatograma en dos dimensiones dejando fija la m/z ; a este cromatograma se le denomina XIC (*eXtracted Ion Chromatogram*). Por lo que se refiere al modo barrido de iones producto (*Product Ion Mode*), los iones aislados para una m/z determinada

alcanzan la celda de colisión (Q2), donde se fragmentan. Todos estos fragmentos pueden ser medidos mediante un barrido, dando lugar a un espectro que contiene todos los iones producto que se generan a partir de la m/z aislada (ion precursor). Del mismo modo que en *full scan*, si se desea obtener la información correspondiente a una única m/z se puede realizar un XIC.

Finalmente en el modo SRM se monitoriza una transición concreta, es decir, la fragmentación de un determinado ion precursor para dar un determinado ion producto. Así pues, el primer cuadrupolo (Q1) estabiliza una única m/z correspondiente al ion precursor; en la celda de colisión (Q2) se aplica una energía predeterminada para fragmentar este ion y el último cuadrupolo (Q3) sólo estabiliza una m/z que corresponde a un ion producto concreto del precursor seleccionado. De esta manera, dado que durante todo el tiempo de análisis se está midiendo una o varias transiciones específicas, se consigue una mayor sensibilidad y selectividad. La principal desventaja de este modo, es la imposibilidad de detectar otras especies presentes en la muestra que podrían resultar de interés. Este modo de trabajo ha sido el utilizado para la determinación cuantitativa de los analitos considerados en esta Tesis.

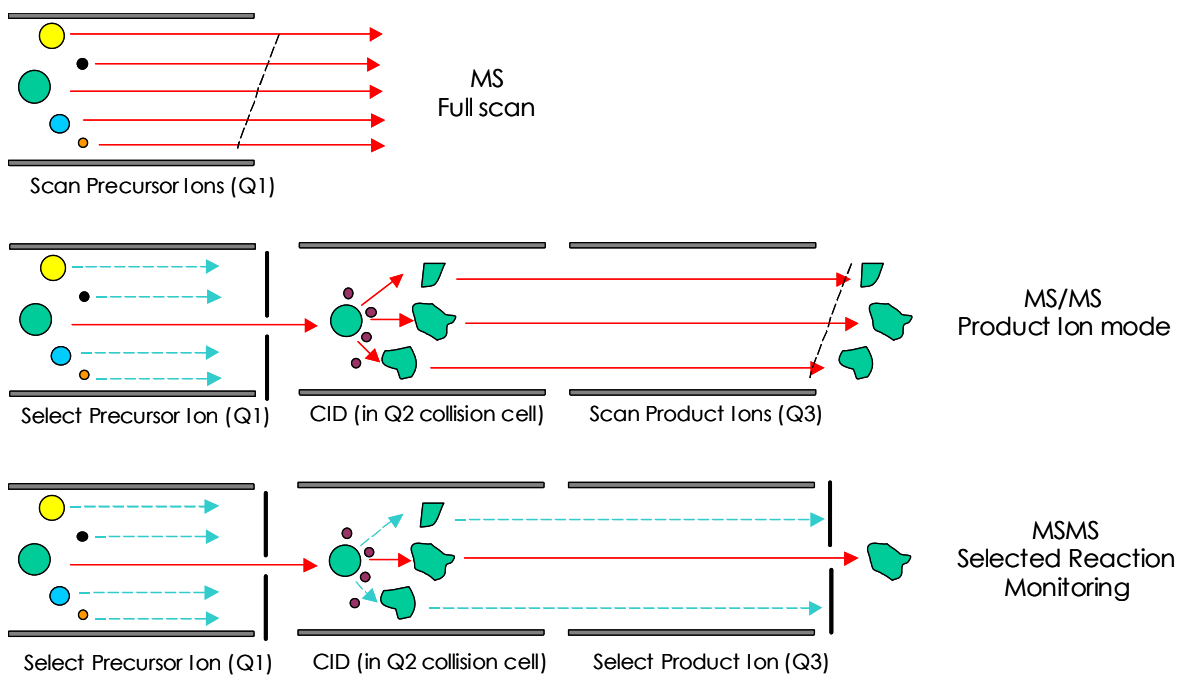


Figura 1.12 Esquema de los modos de barrido utilizados en esta Tesis

A modo de resumen, las principales características de los triples cuadrupolos son su elevada sensibilidad en modo SRM y su amplio rango lineal, que los hacen ideales para el análisis cuantitativo y especialmente en el análisis de residuos. Sin embargo, su aplicación en el campo cualitativo es limitada debido a su bajo poder de resolución (del orden de 1 Da) y baja sensibilidad en modo *full scan*. En nuestro caso, el modo *scan* se utilizará exclusivamente con el fin de optimizar los parámetros instrumentales de algunos compuestos objeto de estudio.

Analizador de tiempo de vuelo (TOF)

En este analizador los iones son separados en función del tiempo que tardan en atravesar un tubo de vuelo de longitud conocida, el cual depende de la relación m/z . Dado que todos los iones empiezan su viaje al mismo tiempo, o al menos en un intervalo de tiempo lo suficientemente corto, los iones más ligeros llegaran al detector antes que los más pesados. Esto requiere que los iones que llegan continuamente procedentes de la interfase sean enviados al TOF mediante un pulso, lo cual se puede conseguir mediante la aceleración ortogonal de los mismos y muestreando una fracción de este haz de iones. Así pues, estos analizadores operan en modo discontinuo, ya que es necesario esperar a que todos los iones lleguen al detector, antes de volver a lanzar otro pulso de iones. Normalmente, la fracción de iones muestreados es aproximadamente el 25% de los generados. Aún así, este porcentaje es elevado en comparación con los analizadores cuadrupolares trabajando en *full scan*.

La resolución de un TOF ha estado tradicionalmente limitada por la energía de dispersión que los iones adquieren en la fuente, haciendo que iones con la misma m/z puedan llegar al detector a distintos tiempos. Este problema ha sido solucionado con la introducción de reflectrones o espejos iónicos, cuyo papel es el de reenfocar los iones de la misma masa sobre el detector. El reflectrón disminuye la dispersión de velocidades iniciales favoreciendo que iones con igual m/z alcancen el detector al mismo tiempo, es decir, actúa como un espejo de manera que los iones con más energía cinética tardan más en dar la vuelta que aquellos con menor energía cinética, pudiendo alcanzar el detector simultáneamente. La resolución alcanzada con este diseño se ha incrementado notablemente, llegando a alcanzar valores típicos entre 5000-20000 fwhm. Esta resolución ha sido mejorada con la inclusión de más reflectrones aumentando así el camino recorrido por los iones. En la Figura 1.13 y 1.14 se muestra el esquema de un TOF con reflectrón y con doble reflectrón, respectivamente.

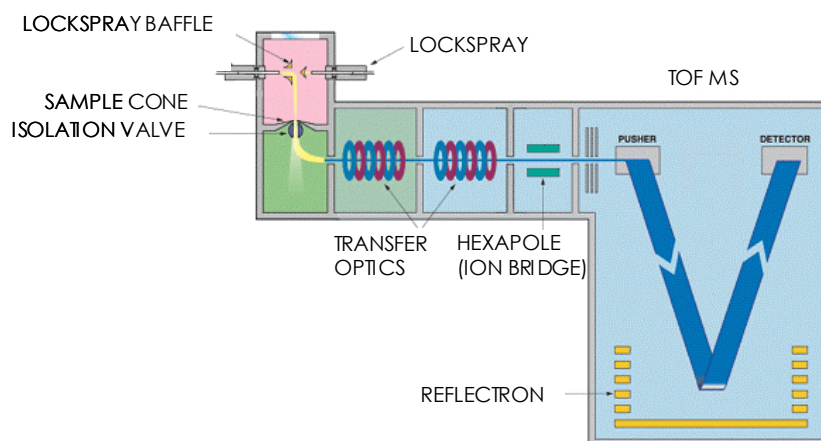


Figura 1.13 Esquema de un TOF con reflectrón

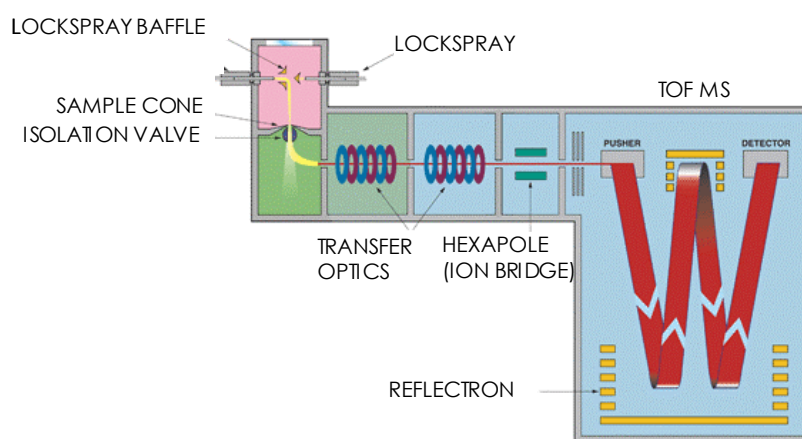


Figura 1.14 Esquema de un TOF con doble reflectrón

La elevada resolución obtenida con el TOF repercute en la posibilidad de obtener medidas con masa exacta de los iones detectados. Sin embargo, debido a la variabilidad que presenta este tipo de instrumentos con respecto a parámetros como, por ejemplo, la temperatura, es necesario, aparte de la calibración diaria, que el equipo esté en constante calibración. Como veremos en el capítulo 2, esto se consigue mediante la adición de un compuesto de masa conocida (*lock mass*) simultáneamente a la entrada de la muestra en la interfase del espectrómetro. La masa del analito se corrige posteriormente en función de la masa del *lock mass*, obteniéndose generalmente errores de masa inferiores a 2 mDa, lo cual suele permitir la asignación de una fórmula molecular concreta a cada ion.

Analizador híbrido cuadrupolo tiempo de vuelo (QTOF)

En el caso del QTOF (Figura 1.15), el trabajo en modo MS/MS se realiza con dos analizadores distintos, un cuadrupolo y un TOF, por lo que constituye un instrumento híbrido. Este diseño es similar al de un triple cuadrupolo, en el que se ha sustituido el último cuadrupolo por un TOF. De esta manera, una vez fragmentado el ion precursor en la celda de colisión, todos los iones producto son determinados mediante el TOF (*Product Ion Mode*). La posibilidad de obtener el espectro de iones producto con masa exacta aumenta la calidad de los datos.

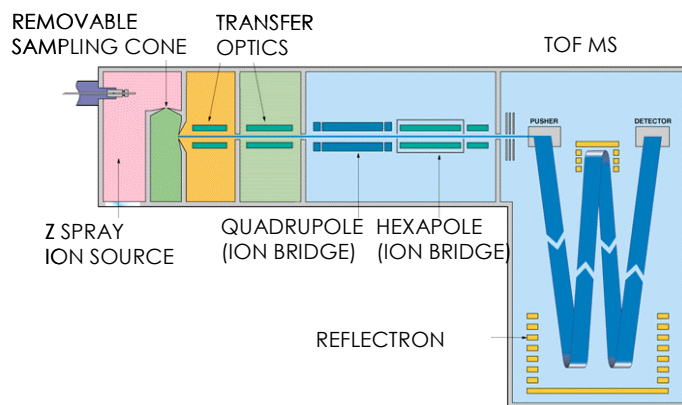


Figura 1.15 Esquema de un QTOF con doble reflectrón

Las principales ventajas de los instrumentos QTOF son su elevada sensibilidad en modo de barrido de iones (ya que un gran porcentaje de los iones generados llegan al detector), su elevada velocidad de barrido, su elevado poder de resolución y la posibilidad de obtener medidas con masa exacta de los iones detectados, tanto trabajando en modo MS como MS/MS. Todas estas características lo hacen ideal para el análisis cualitativo, tanto para la búsqueda de moléculas desconocidas como para la elucidación estructural. Sin embargo, su aplicación en el campo cuantitativo, y especialmente en el análisis de residuos es limitada debido a su bajo rango lineal (apenas dos órdenes de magnitud) y su menor sensibilidad con respecto a instrumentos cuadrupolares monitorizando un ion. Estos instrumentos han sido tradicionalmente usados en el campo farmacéutico, ya que su elevado coste ha limitado su uso en el campo ambiental.

1.5 Problemáticas analíticas asociadas a la determinación por LC-MS/MS

Cada vez es más evidente la necesidad de garantizar la calidad de las determinaciones analíticas con el fin de asegurar, por un lado, que los datos de concentración reportados son fiables y, por otro, evitar la presencia tanto de falsos positivos como de falsos negativos.

En el caso de residuos de plaguicidas, la Unión Europea ha publicado dos guías donde se definen los criterios que deben cumplir los métodos analíticos desarrollados con fines de pre-registro (SANCO/3029/99 rev 4) y de post-registro (SANCO/825/00 rev 7). En el caso de estudios pre-registro, la normativa obliga a efectuarlos en cumplimiento con los principios de las Buenas Prácticas de Laboratorios (BPLs), mientras que en el caso de análisis post-registro es necesario una acreditación de cumplimiento de la norma ISO 17025. En ambas guías se abordan los criterios de calidad relativos a la especificidad, linealidad, exactitud (estimada a partir de ensayos de recuperación), precisión y límite de cuantificación.

Las BPLs (equivalen al término inglés GLP, *Good Laboratory Practice*) se definen como un sistema de calidad relacionado con los procesos organizativos y las condiciones bajo las cuales los estudios no clínicos de seguridad sanitaria y medioambiental son planificados, realizados, controlados, registrados, archivados o informados. Los principios de buenas prácticas de laboratorio son aplicables a todos los estudios no clínicos de seguridad sanitaria y ambiental requeridos reglamentariamente con el fin de registrar o autorizar productos farmacéuticos, plaguicidas, aditivos destinados a la alimentación humana y animal, cosméticos, medicamentos veterinarios y productos similares, así como para la regulación de las sustancias químicas industriales (BOE, 2000).

Nuestro laboratorio (LARP) tiene la certificación GLP desde 2001, lo cual nos ha permitido aplicar el "espíritu" de este sistema de calidad en muchos de nuestros trabajos de investigación. Así, los métodos multiresiduo desarrollados en esta Tesis se han optimizado y validado teniendo en cuenta los principios de las GLPs.

Dos de los aspectos fundamentales a la hora de garantizar la calidad de un resultado analítico son la cuantificación y la especificidad de la determinación. Sobre estos dos parámetros analíticos se va a profundizar en las páginas siguientes, en los aspectos relativos al uso de LC-MS/MS.

1.5.1 Cuantificación

Las guías SANCO definen claramente los requisitos necesarios para la validación de un método. A continuación se resumen las características analíticas más importantes:

Linealidad: Según las guías SANCO, el calibrado debe cubrir un rango de concentraciones adecuado, desde al menos un 20% menor que el límite de cuantificación (LOQ) hasta un valor del 20% mayor que el máximo esperado. Se acepta una calibración en la que se inyecten al menos tres niveles de concentración por duplicado, o una inyección de cinco o más niveles de concentración. Aunque ninguna de las dos guías habla de los criterios de aceptación, se suele utilizar el coeficiente de correlación como indicativo.

Exactitud: La exactitud del método se estima normalmente mediante ensayos de recuperación de cada analito en la matriz de interés. Estos ensayos se suelen realizar a partir de muestras fortificadas, siendo necesarios al menos dos niveles de concentración. Uno de estos niveles será obligatoriamente el LOQ mientras que el otro se suele establecer en 10 veces el LOQ. La recuperación se calcula como la media de las recuperaciones obtenidas en el análisis de 5 réplicas fortificadas al mismo nivel. Se considera aceptable si la media se encuentra en el rango 70-110%. En el caso de analitos o matrices complejas, se permiten recuperaciones inferiores siempre que la precisión sea aceptable.

Precisión: La precisión del método se suele medir mediante la repetibilidad obtenida en los ensayos de recuperación y viene expresada como RSD. Se aceptan valores inferiores al 20%. Ambas guías permiten descartar un punto como máximo para cada nivel, siempre que esté justificado por algún método estadístico.

LOQ: Se define como la concentración más baja a la que se puede determinar el analito en la matriz de interés con las condiciones de exactitud y precisión comentadas (recuperación entre 70-110% y RSD<20%).

LOD: Se define como la concentración más baja a la que se puede detectar el analito en la matriz de interés con las condiciones de exactitud y precisión comentadas.

Los métodos optimizados en el capítulo 3, tanto para la determinación multiresiduo de plaguicidas y TPs en aguas como para la determinación de glifosato, glufosinato y AMPA en muestras ambientales, han sido validados teniendo en cuenta los requisitos indicados en este apartado.

La cuantificación es uno de los aspectos más críticos a la hora de asegurar la calidad de los resultados obtenidos mediante cualquier técnica. Con algunos detectores como los basados en UV, es relativamente frecuente que los componentes de la matriz también absorban en la misma zona de longitud de onda que el analito, especialmente en muestras de matriz compleja. Algo análogo ocurre con detectores de simple cuadrupolo. En el caso de LC-MS/MS los posibles interferentes de la matriz, aunque pudieran tener la misma masa nominal que el analito (interferentes isobáricos), no suelen compartir la misma transición que el analito y por lo tanto, normalmente sólo aparece un pico en el cromatograma perteneciente a la molécula de interés. Sin embargo, en algunos casos, cuando se comparan las áreas correspondientes a los picos obtenidos para un patrón y para una muestra fortificada al mismo nivel, se puede observar la falta de concordancia entre las mismas. Ello es debido a la presencia de interferentes en la matriz, invisibles en la detección ya que no comparten la misma transición que el analito, pero que afectan a la ionización de éste haciendo que la respuesta sea distinta en ausencia o presencia de la matriz. La explicación de este fenómeno sigue siendo motivo de controversia. Aunque normalmente el resultado de estos efectos es una menor respuesta para el analito cuando la matriz está presente, también puede producirse el efecto contrario, es decir, una exaltación de la señal debida a la presencia de la matriz. En todo caso, las consecuencias de este efecto matriz se traducen en importantes errores en la cuantificación si se usan patrones externos en solvente. El efecto matriz depende principalmente de las características físico-químicas del analito (acidez o basicidad), de la matriz objeto del análisis, de la interfase utilizada y especialmente de las características de los interferentes que eluyan de la columna al mismo tiempo de retención que el analito (Sancho et al, 2002; Dijkman et al, 2001). Debido a la importancia del efecto matriz en la cuantificación resulta necesario eliminarlo o, al menos, minimizarlo con el fin de que los errores cometidos sean aceptables. Para ello, se han propuesto varias alternativas que se basan en la compensación de este efecto (teniéndolo en cuenta a la hora de la cuantificación) o en la eliminación de los interferentes.

a) Uso de patrones internos: La principal ventaja de este tipo de corrección es la minimización de la etapa de procesamiento de muestra, pudiendo inyectarse directamente el extracto en muchos casos. Para que un patrón interno pueda corregir el efecto matriz, su ionización debe verse afectada por los mismos interferentes y del mismo modo que el analito. Para ello, tiene que presentar una estructura química y un tiempo de retención similares a los del analito. Por esta razón, el patrón interno ideal para corregir el efecto matriz es el mismo analito marcado isotópicamente. Sin embargo, hay que tener en cuenta que el porcentaje del compuesto marcado presente en la naturaleza sea bajo con el fin de que no interfiera en la

correcta cuantificación. Por este motivo, no es habitual usar patrones marcados con ^{37}Cl o ^{34}S debido a su elevado porcentaje natural (aproximadamente 30% y 5%, respectivamente). Aunque se suelen preferir compuestos marcados con ^{13}C o D, la utilización de estos isótopos también presenta una serie de limitaciones. En el caso del D, éste ha de estar en una posición que no permita su intercambio rápido con los protones del medio, evitando así posibles irreproducibilidades en la señal del patrón interno. En el caso del ^{13}C , su presencia en la naturaleza es alrededor del 1%, con lo que una muestra positiva por la presencia del analito aportaría un 1% de su concentración a la añadida del patrón interno, en el caso de que éste tuviera un único átomo marcado. Por esta razón es aconsejable que el compuesto marcado utilizado como patrón interno presente más de 1 átomo de ^{13}C . Sin embargo, a medida que aumenta el número de átomos marcados, aumentan las diferencias entre la molécula del patrón interno y la de analito, pudiendo llegar a no corregir adecuadamente el efecto matriz. De esta manera, los compuestos marcados utilizados como patrones internos suelen presentar entre 2 y 5 átomos de ^{13}C o D.

A pesar de todo esto, la limitación más importante a la hora de utilizar estos compuestos como patrones internos es, sin duda, su disponibilidad comercial. Así, cuando no se puede disponer del compuesto marcado, se suelen utilizar análogos estructurales con el fin de corregir el efecto matriz. Otra limitación del uso de patrones internos es su aplicación en métodos multiresiduo, pues, como ya se ha comentado, el efecto matriz experimentado por un analito depende en buena medida del tiempo de retención al que eluye. Por lo tanto, cada uno de los analitos, que van eluyendo secuencialmente de la columna, puede verse afectados por un efecto matriz distinto.

A pesar de todas estas limitaciones, el uso de patrones internos (isotópicamente marcados si están disponibles) es el método de corrección del efecto matriz más aconsejable, siempre que sea posible, ya que minimiza la etapa de pretratamiento de muestra (Olsson et al, 2003, 2004)

b) Calibrado en matriz: Cuando no es posible disponer de un patrón interno adecuado para la corrección del efecto matriz, se puede conseguir este mismo objetivo realizando la cuantificación mediante calibrados realizados en matriz de muestra blanco. En este caso, todos los analitos sufren el efecto matriz, incluidos los patrones usados para la calibración y, por lo tanto, la cuantificación se realiza teniendo en cuenta este efecto. Esta práctica es bastante habitual en el análisis de matrices biológicas como suero u orina (Lee et Kerns, 1999), donde siempre se dispone de un organismo control al que no se le ha suministrado el fármaco, o en los

ensayos de residuos con fines de registro de plaguicidas en vegetales (Sancho et al, 2005), donde se dispone de un campo control, con muestras blanco, en el que no se ha aplicado el plaguicida. Sin embargo, esta aproximación presenta algunas limitaciones que la hacen inviable en programas de control, especialmente en muestras de interés ambiental como suelos o aguas, ante la imposibilidad de obtener blancos representativos debido a las diferencias que pueden existir en la composición de las distintas muestras analizadas.

c) Dilución: Cuando no se puede corregir el efecto matriz debido a la no disponibilidad de patrones internos adecuados o a la imposibilidad de realizar calibrado en matriz blanco, se debe intentar eliminar o minimizar el efecto matriz con el fin de hacer comparables las respuestas en las muestras y en los patrones. El método más sencillo para ello es la dilución de la muestra. Al diluir la muestra con el solvente en el que están preparados los patrones, se minimiza el contenido de interferentes presentes en la matriz y por tanto las muestras se pueden llegar a hacer comparables con los patrones, en cuanto a su respuesta en LC-MS. Este es el caso, por ejemplo, de la determinación de fosefil-Al en lechugas (Hernández et al, 2003), donde se aplicó un factor de dilución de cinco a los extractos con el objetivo de igualar todas las matrices y poder aplicar después calibrado en matriz para la correcta cuantificación. Esto fue necesario a causa de las diferencias observadas en las recuperaciones, dependiendo del origen y variedad de la muestra analizada, debido al distinto efecto matriz observado para distintas variedades de lechuga.

d) Pretratamiento de muestra: Otro método utilizado con el fin de minimizar el efecto matriz es la aplicación de una etapa de purificación de la muestra. Para ello se suelen utilizar técnicas como la SPE o la LLE, tanto en muestras ambientales como biológicas (Henion et al, 1998). Uno de los principales inconvenientes que presenta esta aproximación con respecto a las anteriores es el tiempo que se consume en esta operación. Por otro lado, el mayor tratamiento de muestra puede aumentar la posibilidad de errores por pérdidas de analito así como por contaminación de la muestra. Finalmente, en algunos casos, la técnica de purificación puede preconcentrar algunos interferentes, generando el efecto contrario al deseado. Una aproximación adecuada para eliminar el efecto matriz sin algunos de los inconvenientes mencionados en el pretratamiento de muestra es la realización de un proceso automatizado. En este contexto destaca la LC con columnas acopladas, como por ejemplo LC-LC o SPE-LC (Hernández y Sancho, 2004b; Hogendoorn et al, 1993). Como ya se ha comentado, estos

procesos permiten la purificación automatizada del extracto así como mejorar la sensibilidad del método sin invertir tanto tiempo como en las técnicas de purificación convencionales.

En esta Tesis se ha hecho uso de algunas de las estrategias comentadas anteriormente. Así en el análisis de glifosato y AMPA en aguas fue necesario el uso de patrón interno, con el fin de corregir el efecto matriz observado. En el caso de suelos, además, fue necesaria una dilución de las muestras, con el fin de minimizar el contenido de interferentes presentes en la matriz. Por otro lado, se ha hecho uso de la SPE-LC para la optimización de métodos en la determinación multiresiduo de plaguicidas y TPs en aguas, lo que ha permitido obtener una elevada sensibilidad.

e) Método de adiciones estándar: Esta sería otra alternativa a la hora de cuantificar correctamente muestras con un importante efecto matriz. Sin embargo, su principal limitación es el mayor número de inyecciones a realizar por muestra, así como la necesidad de preveer el nivel esperado de residuo para realizar las adiciones correctas. Además, esta previsión debería realizarse para cada uno de los analitos considerados.

1.5.2 Especificidad

Cuando se utilizan métodos basados en LC-MS/MS, podría parecer que el uso de un ion precursor y de un ion producto, junto con el tiempo de retención, proporciona la especificidad adecuada en la detección. Sin embargo, en algunos casos, se observa la presencia de algún interferente de la matriz que presenta las mismas propiedades espectrométricas que el analito, pudiendo dar lugar a falsos positivos. Este hecho puede producirse con más facilidad cuanto más compleja sea la matriz y menos específica sea la transición. Sin embargo, las normativas vigentes en la determinación de residuos de plaguicidas no contemplan estos problemas en los programas de control, ya que aunque señalan la necesidad de utilizar técnicas de confirmación como GC-MS ó HPLC-DAD para demostrar la especificidad, considera que dada la elevada especificidad de LC-MS/MS, no se necesita un método adicional de confirmación.

Debido, posiblemente, al mayor uso de LC-MS/MS en el campo farmacéutico y veterinario, la legislación sobre determinación de residuos en este tipo de muestras va por delante de la de muestras de alimentos o medioambientales. Recientemente se ha publicado una Decisión Europea (2002/657/EC) donde se establecen los criterios para la cuantificación y confirmación de contaminantes y residuos orgánicos en muestras de origen animal, con el fin de garantizar un control seguro y eficaz. Esta decisión hace uso de una nueva aproximación para

unificar criterios en la confirmación espectrométrica de contaminantes y residuos orgánicos, basada en el uso de puntos de identificación (IPs), de tal forma que a cada técnica se le asigna un número de IPs en función de su especificidad inherente.

En el capítulo 5 se discuten con detalle los aspectos relativos a la confirmación de la identidad de los analitos detectados en una muestra, utilizando los distintos analizadores MS, a la vez que se propone una revisión de ciertos conceptos, como por ejemplo la asignación de IPs para instrumentos de alta resolución en función de la exactitud de la masa obtenida y no de la resolución del instrumento, o la asignación de distinto número de IPs en función de la especificidad de la transición utilizada.

1.6. Bibliografía

2002/657/CE Decisión de la Comisión de 12 de Agosto de 2002, por la que se aplica la Directiva 96/23/CE del Consejo en cuanto al funcionamiento de los métodos analíticos y la interpretación de los resultados. Official Journal of the European Communities L221 (2002) pp 8-36, Bruselas, Bélgica

Aprèa C, Sciarra G, Bozzi N

Analytical method for the determination of urinary alkylphosphates in subjects occupationally exposed to organophosphorus pesticides and in the general population

J. Anal. Toxicol. 20 (1996) 559-563

Aprèa C, Colosio C, Mammome T, Minoia C, Maroni M

Biological monitoring of pesticide exposure: a review of analytical methods (Review)

J. Chromatogr. B 769 (2002) 191-219

Bagheri H, Brouwer ER, Ghijsen RT, Brinkman UATH

On-line low-level screening of polar pesticides in drinking and surface waters by liquid chromatography-thermospray mass spectrometry

J. Chromatogr. 647 (1993) 121-129

Baker SE, Barr DB, Driskell WJ, Beeson MD, Needham LL

Quantification of selected pesticide metabolites in human urine using isotope dilution high-performance liquid chromatography/tandem mass spectrometry

J. Exposure Anal. Environ. Epidemiol. 10 (2000) 789-798

Barberá C

Plaguicidas Agrícolas. Cuarta revisión, revisada y ampliada

Ediciones Omega (1989)

Barr DB, Needham LL

Analytical methods for biological monitoring of exposure to pesticides: a review (Review)

J. Chromatogr. B 778 (2002) 5-29

Beeson MD, Driskell WJ, Barr DB

Isotope Dilution High-Performance Liquid Chromatography/Tandem Mass Spectrometry Method for Quantifying Urinary Metabolites of Atrazine, Malathion, and 2,4-Dichlorophenoxyacetic Acid

Anal. Chem. 71 (1999) 3526-3530

BOE Num. 173, 20 julio 2000, Real Decreto 1369/2000, 25832-25838

- Bravo R, Driskell WJ, Whitehead RD, Needham LL, Barr DB
Quantitation of dialkyl phosphate metabolites of organophosphate pesticides in human urine using GC-MS/MS with isotopic internal standards
J. Anal. Toxicol. 26 (2002) 245-252
- Burse VW, Head SL, Korver MP, McClure PC, Donahue JF, Needham LL
Determination of selected organochlorine pesticides and polychlorinated biphenyls in human serum.
J. Anal. Toxicol. 14 (1990) 137-142
- Castro R, Moyano E, Galcerán MT
On-line ion-pair solid-phase extraction-liquid chromatography-mass spectrometry for the analysis of quaternary ammonium herbicides
J. Chromatogr. A 869 (2000) 441-449
- Castro R, Moyano E, Galcerán MT
Determination of quaternary ammonium plaguicidas by liquid chromatography-electrospray tandem mass spectrometry
J. Chromatogr. A 914 (2001) 111-121
- G. Catenacci, F. Barbieri, M. Bersani, A. Ferioli, D. Cottica, M. Maroni
Biological monitoring of human exposure to atrazine
Toxicol. Lett. 69 (1993) 217-222
- Cela R, Lorenzo RA, Casais MC
Técnicas de Separación en Química Analítica
Editorial Síntesis (2002)
- Chapalamadugu S, Chaudhry GR
Microbiological and biotechnological aspects of metabolism of carbamates and organophosphates.
Crit. Rev. Biotechnol. 12 (1992) 357-389.
- Chapuis F, Pichon V, Lanza F, Sellergren B, Hennion MC
Retention mechanism of analytes in the solid-phase extraction process using molecularly imprinted polymers - Application to the extraction of triazines from complex matrices
J. Chromatogr. B 804 (2004) 93-101

Chen ZL, Megharaj M, Naidu R

On-line solid phase extraction of pesticide residues in natural water, coupled with liquid chromatography and UV detection, using various sorbents

J. Liq. Chromatogr. Related Technol. 25 (2002) 1779-1790

D'Ascenzo G, Gentili A, Marchese S, Marino A, Pret D

Simultaneous determination of base/neutral and acid herbicides in natural water at the part per trillion level

Chromatographia 48 (1998) 497-505

Davrio MV y colaboradores

Cromatografía y electroforesis en columna

Springer-Verlag Ibérica (2000)

Dijkman E, Mooibroek D, Hoogerbrugge R, Hogendoorn E, Sancho JV, Pozo O, Hernández F

Study of matrix effects on the direct trace analysis of acidic pesticides in water using various liquid chromatographic modes coupled to tandem mass spectrometric detection

J. Chromatogr. A 926 (2001) 113-125

EPA, 2006- U.S. Environmental Protection Agency

www.epa.gov/oppead1/pestsales/01pestsales/usage2001.html (fecha de consulta abril, 2006)

Forcada M, Beltran J, Lopez FJ, Hernández F

Multiresidue procedures for determination of triazine and organophosphorus pesticides in water by use of large-volume PTV injection in gas chromatography

Chromatographia 51 (2000) 362-368

Fidalgo-Used N, Centineo G, Blanco-Gonzalez E, Sanz-Medel A

Solid-phase microextraction as a clean-up and preconcentration procedure for organochlorine pesticides determination in fish tissue by gas chromatography with electron capture detection.

J. Chromatogr. A 1017 (2003) 35-44

Gallelli G, Mangini S, Gerbino C

Organochlorine residues in human adipose and hepatic tissues from autopsy sources in northern Italy

J. Toxicol. Environ. Health 43 (1995) 293-300

Geerdink RB, Kooistra-Sijpersma A, Tiesnitsch J, Kienhuis PGM, Brinkman UATH

Determination of polar pesticides with atmospheric pressure chemical ionization mass spectrometry-mass spectrometry using methanol and/or acetonitrile for solid-phase desorption and gradient liquid chromatography.

J. Chromatogr. A 863 (1999) 147-155

Geerdink RB, Niessen WMA, Brinkman UATH

Trace-level determination of pesticides in water by means of liquid and gas chromatography (Review)

J. Chromatogr. A 970 (2002) 65-93

Hardt L, Angerer J

Determination of dialkyl phosphates in human urine using gas chromatography-mass spectrometry

J. Anal. Toxicol. 24 (2000) 678-684

Henion J, Brewer E, Rule G

Sample preparation for LC/MS/MS: analysing biological and environmental samples.

Anal. Chem. 70 (1998) 650A-656A

Hennion MC

Solid Phase Extraction: method development, sorbents and coupling with LC (Review)

J. Chromatogr. A 856 (1999) 3-54

Hernández F, Hidalgo C, Sancho JV, López FJ

New method for the rapid determination of triazine herbicides and some of their main metabolites in water by using coupled-column liquid chromatography and large volume injection

J. Chromatogr. A 778 (1997) 171-181

Hernández F, Beltran J, Lopez FJ, Gaspar JV

Use of solid phase microextraction for the quantitative determination of herbicides in soil and water samples

Anal. Chem. 72 (2000) 2313-2322

Hernández F, Sancho JV, Pozo O, Lara A, Pitarch E

Rapid direct determination of pesticides and metabolites in environmental water samples at sub- $\mu\text{g/L}$ level by on-line solid-phase extraction-liquid chromatography-electrospray tandem mass spectrometry

J. Chromatogr. A 939 (2001) 1-11

Hernández F, Sancho JV, Pozo O

Direct determination of alkyl phosphates in human urine by liquid chromatography/electrospray-tandem mass spectrometry

Rapid Commun. Mass Spectrom. 16 (2002) 1766-1773

Hernández F, Sancho JV, Pozo O, Villaplana C, Ibáñez M, Grimalt S

New method for the rapid determination of fosetyl aluminium residues based on liquid chromatography-electrospray tandem mass spectrometry

J. AOAC Int. 86 (2003) 832-838

Hernández F, Sancho JV, Pozo O

An estimation to the exposure to organophosphorus pesticides throughout the simultaneous determination of their main metabolites in urine by liquid chromatography tandem mass spectrometry

J. Chromatogr. B (2004a) 229-239

Hernández F, Sancho JV

Multidimensional Liquid Chromatography

Encyclopedia Analytical Science (2004b) 197-205

Hernández F, Sancho JV, Pozo O

Critical review of the application of liquid chromatography/mass spectrometry to the determination of pesticide residues in biological samples (Review)

Anal. Bioanal. Chem. 382 (2005) 934-946

Hernández F, Sancho JV, Pozo O

Application of multidimensional LC-MS for the determination of pesticides and their degradation products in environmental and biological samples

Encyclopedia of Mass Spectrometry (2006) In press

Hogendoorn EA, Brinkman UATh, Van Zoonen P

Coupled-column reversed-phase liquid chromatography - UV analyser for the determination of polar pesticides in water.

J. Chromatogr. 644 (1993) 307-314

Koppen B, Spliid NH

Determination of acidic herbicides using liquid chromatography with pneumatically assisted electrospray ionization mass spectrometric and tandem mass spectrometric detection

J. Chromatogr. A 803 (1998) 157-168

Ibáñez M, Pozo OJ, Sancho JV, López FJ, Hernández F

Residue determination of glyphosate, glufosinate and aminomethylphosphonic acid in water and soil samples by liquid chromatography coupled to electrospray tandem mass spectrometry.

J. Chromatogr. A 1081 (2005) 145-155

Lee MS, Kerns EH

LC-MS applications in drug development (Review)

Mass Spectrom Rev 18 (1999) 187-279

Minelli EV, Ribeiro ML

Quantitative Method for the Determination of Organochlorine Pesticides in Serum

J. Anal. Toxicol. 20 (1996) 23-26

Morell I, Hernández F

El agua en Castellón: un reto para el siglo XXI

Publicacions de la Universitat Jaume I (2000)

Morrice P, Fidente P, Seccia S

Liquid chromatographic determination of nine N-methylcarbamates in drinking water

Biomed. Chromatogr. 19 (2005) 107-110

Nakamura S, Yamagami T, Daishima S

Determination of organochlorine pesticides in river water by gas chromatography-negative-ion chemical-ionization mass spectrometry using large volume injection

Analyst 126 (2001) 1658-1662

Niessen WMA

Liquid Chromatography-Mass spectrometry: Third edition

Chromatographic Science Series 97 (2006) Ed. Marcel Dekker

Olsson AO, Nguyen JV, Sadowski MS, Barr DB

A liquid chromatography/electrospray ionization-tandem mass spectrometry method for quantification of specific organophosphorus pesticide biomarkers in human urine.

Anal. Bioanal. Chem. 376 (2003) 808-815

- Olsson AO, Baker SE, Nguyen JV, Romanoff LC, Udunka SO, Walker RD, Flemmen KL, Barr DB
A liquid chromatography - tandem mass spectrometry multiresidue method for quantification of specific metabolites of organosphosphorus pesticides, synthetic pyrethroids, selected herbicides, and DEET in human urine.
Anal. Chem. 76 (2004) 2453-2461
- Onnerfjord P, Barcelo D, Emneus J, Gorton L, Marko-Varga G
On-line solid-phase extraction in liquid chromatography using restricted access pre-columns for the analysis of s-triazines in humic-containing waters
J. Chromatogr. A 737 (1996) 35-45
- Patsias J, Papadopoulou A, Papadopoulou-Mourkidou E
Automated trace level determination of glyphosate and aminomethyl phosphonic acid in water by on-line anion-exchange solid-phase extraction followed by cation-exchange liquid chromatography and post-column derivatization
J. Chromatogr. A 932 (2001) 83-90
- Pauwels A, Wells DA, Covaci A, Schepens PJC
Improved sample preparation method for selected persistent organochlorine pollutants in human serum using solid-phase disk extraction with gas chromatographic analysis
J. Chromatogr. B 723 (1999) 117-125
- Piochon V, Krasnova AI, Hennion MC
Development and characterization of an immunoaffinity solid-phase-extraction sorbent for trace analysis of propanil and related phenylurea herbicides in environmental waters and in beverages
Chromatographia 60 (2004) Suppl. S S221-S226
- Pitarch E, Serrano R, Lopez FJ, Hernández F
Rapid multiresidue determination of organochlorine and organophosphorus compounds in human serum by solid-phase extraction and gas chromatography coupled to tandem mass spectrometry
Anal. Bioanal. Chem. 376 (2003) 189-197
- Poole CF and Poole SK
Chromatography today
Elsevier (1991)

Pozzebon JM, Vilegas W, Jardim ICSF

Determination of herbicides and a metabolite in human urine by liquid chromatography-electrospray ionization mass spectrometry

J. Chromatogr. A 987 (2003) 375-380

SANCO/3029/99 rev 4 11/07/00

Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, section 4) and Annex III (part A, section 5) of directive 91/141

European Commission, Directorate General Health and Consumer Protection

SANCO/825/00 rev 6 20/06/00

Guidance document on residue analytical methods

European Commission, Directorate General Health and Consumer Protection

Sancho JV, Pozo OJ, López FJ, Hernández F

Different quantitation approaches for xenobiotics in human urine samples by liquid chromatography/electrospray tandem mass spectrometry

Rapid Commun. Mass Spectrom. 16 (2002) 639-645

Sancho JV, Cabanes RA, Lopez FJ, Hernández F

Direct determination of 1-naphthol in human urine by coupled-column liquid chromatography with fluorescence detection.

Chromatographia 58 (2003) 565-569

Sancho JV, Pozo OJ, Hernández F

Liquid chromatography and tandem mass spectrometry: a powerful approach for the sensitive and rapid multiclass determination of pesticides and transformation products in water

Analyst 129 (2004) 38-44

Sancho JV, Ibáñez M, Grimalt S, Pozo OJ, Hernández F

Residue determination of cyromazine and its metabolite melamine in chard samples by ion-pair liquid chromatography coupled to electrospray tandem mass spectrometry

Anal. Chim. Acta 530 (2005) 237-243

- Steen RJCA, Hogenboom AC, Leonards PEG, Peerboom RAL, Cofino WP, Brinkman UATH
Ultra-trace level determination of polar pesticides and their transformation products in surface and estuarine water samples using column liquid chromatography-electrospray tandem mass spectrometry
J. Chromatogr. A 857 (1999) 157-166
- Valcárcel Cases M, Gómez Hens A
Técnicas analíticas de separación
Editorial Reverté (1994)
- Volmer D, Levsen K, Wünsch G
Thermospray liquid chromatographic-mass spectrometric multi-residue determination of 128 polar pesticides in aqueous environmental samples
J. Chromatogr. A 660 (1994) 231-248
- Wang-Hsien D, Chi-Hung L, Shiow-Ping Y
Analysis of chlorophenoxy acid herbicides in water by large-volume on-line derivatization and gas chromatography-mass spectrometry
J. Chromatogr. A 896 (2000) 111-116
- Ware GW
Pesticides. Theory and Application
W.H. Freeman and Company (1982)
- Ward EM, Schulte P, Grajewski B, Andersen A, Patterson Jr. DG, Turner W, Jellum E, Deddens JA, Friedland J, Roeleveld N, Waters M, Butler MA, DiPietro E, Needham LL
Serum Organochlorine Levels and Breast Cancer: A Nested Case-Control Study of Norwegian Women
Cancer Epidem Biomark Prev 9 (2000) 1357-1367
- Ward SA, May G, Branch RA
Quantitative determination of carbaryl and carbaryl metabolites by reversed-phase high-performance liquid chromatography.
J. Chromatogr. 388 (1987) 462-466
- Willoughby R, Sheehan E, Mitrovich S
A global view of LC-MS: How to solve your most challenging analytical problems
Global View Publishing (1998)

Zamora T, Hidalgo C, López FJ, Hernández F

Determination of fungicide residues in fruits by coupled-column liquid chromatography

J. Sep. Sci. 27 (2004) 645-652

Zrostlikova J, Lehotay SJ, Hajslova J

Simultaneous analysis of organophosphorus and organochlorine pesticides in animal fat by gas chromatography with pulsed flame photometric and micro-electron capture detectors

J. Sep. Sci. 25 (2002) 527-537

Capítulo 2.

Identificación y elucidación de productos de transformación/metabolitos de plaguicidas mediante espectrómetro de masas híbrido cuadrupolo-tiempo de vuelo (QTOF). Aplicación al análisis de compuestos desconocidos

2.1 Introducción

2.1.1 Obtención de masa exacta: calibración y *lock mass*

2.1.2 Aplicaciones del QTOF

2.1.3 Uso del software MetaboLynx

2.2 Experimental

2.3 Elucidación de productos de transformación/metabolitos de plaguicidas

2.3.1 Introducción

2.3.2 Resultados y discusión.

Artículo científico 1

Use of quadrupole time-of-flight mass spectrometry in environmental analysis: elucidation of transformation products of triazine herbicides in water after UV exposure
Anal. Chem. (2004) 76: 1328-1335

Artículo científico 2

Use of liquid chromatography quadrupole time-of-flight mass spectrometry in the elucidation of transformation products and metabolites of pesticides. Diazinon as a case study
Anal. Bioanal. Chem. (2006) 384: 448-457

2.3.3 Bibliografía

2.4 Identificación de compuestos desconocidos (*non target*) en muestras de agua

2.4.1 Introducción

2.4.2 Resultados y discusión

Artículo científico 3

Use of quadrupole time-of-flight mass spectrometry in the elucidation of unknown compounds present in environmental water

Rapid Commun. Mass Spectrom. (2005) 19: 1-10

-Identificación de interferencias reveladas en etapas de confirmación

-Método de cribado (*screening*) para plaguicidas. Búsqueda de plaguicidas desconocidos (*non target*)

Nota de aplicación 1

UPLC-oo TOF MS for rapid screening of multiple pesticide residues

Waters application note (2006)

2.4.3 Bibliografía

2.1 Introducción

Como ya se ha comentado en la introducción, los espectrómetros de masas de tiempo de vuelo separan los distintos iones en función del tiempo que tardan en atravesar un tubo de vuelo, el cual depende de la relación m/z (ver Figura 1.13 del capítulo 1). Estos instrumentos presentan una serie de ventajas en el análisis cualitativo frente a otro tipo de analizadores de masas como, por ejemplo, los triples cuadrupolos o las trampas de iones, debido a su elevada resolución y exactitud en la medida de masa.

La resolución se define como la capacidad para distinguir entre dos picos con m/z muy próximas. Se suele utilizar FWHM (*full width at half maximum*), es decir, la anchura del pico a la mitad de su altura, tal y como se muestra en la Figura 2.1.

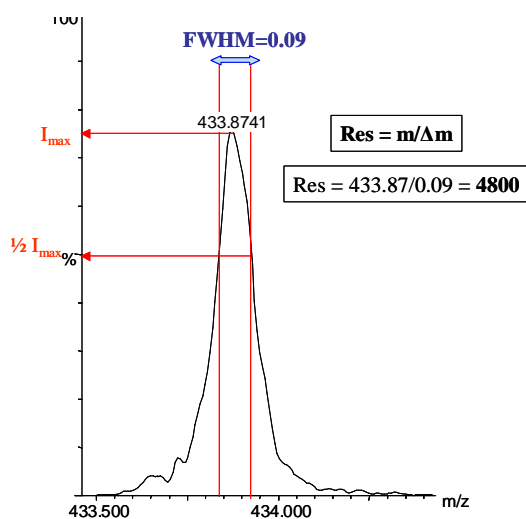


Figura 2.1 Resolución obtenida con el TOF

En los instrumentos cuadrupolares se suele trabajar con resolución unidad, es decir, sólo se pueden diferenciar masas que estén separadas entre sí 1 uma, mientras que en los instrumentos de tiempo de vuelo la resolución suele ser alrededor de 5000, es decir, a una masa m/z de 500 se podría diferenciar entre dos masas separadas 0.1 umas. A modo de ejemplo, en la figura 2.2 se muestran los espectros obtenidos con un triple cuadrupolo y con un TOF para dos compuestos cuya masa exacta difiere 27.6 mDa. Como podemos ver, la resolución del cuadrupolo no es suficiente para diferenciar la presencia de los dos compuestos. El TOF, en cambio, debido a su mayor poder de resolución, muestra claramente la presencia de dos picos.

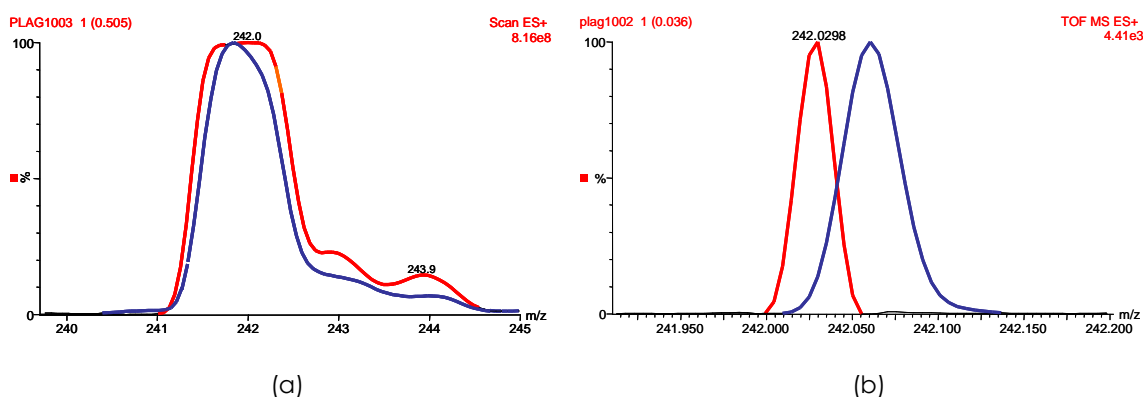


Figura 2.2 Resolución obtenida con un triple cuadrupolo y con un tiempo de vuelo para dos compuestos cuyas masas difieren 27.6 mDa

Es precisamente esta elevada resolución la que ofrece la posibilidad de obtener medidas con masa exacta de los iones detectados. Tal y como veremos a continuación, este parámetro es de gran importancia. Se define como masa nominal, la masa de un ion calculada usando la masa entera del isótopo más abundante de cada elemento (obviando el defecto de masa, ej. H = 1, C = 12, O = 16, etc). La masa monoisotópica es la masa de un ion calculada usando la masa exacta del isótopo más abundante de cada elemento (teniendo en cuenta el defecto de masa, ej. H = 1.0078, C = 12.0000, O = 15.9949, etc). La masa promedio es la masa de un ion calculada a partir de la masa isotópica media relativa de cada elemento (ej. C = 12.0111, H = 1.00797, O = 15.9994, etc). Por último, se define como abundancia isotópica, la distribución natural de los diferentes isótopos de un elemento, por ejemplo, $^{12}\text{C} = 12.0000 = 98.9\%$, $^{13}\text{C} = 13.0034 = 1.1\%$.

Así pues, es posible encontrar combinaciones de átomos que tengan la misma masa nominal pero diferente masa exacta, por lo que si esos compuestos pueden ser medidos con suficiente exactitud de masa, será posible distinguirlos. Por ejemplo, $\text{CO} = 27.9949$, $\text{N}_2 = 28.0061$ ($\Delta m = 11.2$ mDa) y $\text{C}_2\text{H}_4 = 28.0313$ ($\Delta m = 36.4$ mDa). Estas moléculas tienen la misma masa nominal pero diferente masa exacta, por lo que utilizando un cuadrupolo (medida de masa nominal) no podrían ser distinguidas. En el caso de analizadores de tiempo de vuelo, la exactitud de la masa suele ser menor de 2 mDa ó 5 ppm, por lo que estos compuestos sí serían diferenciados. Se define ppp (partes por millón) como $\Delta m/m \times 10^{-6}$.

Por lo tanto, la masa exacta es una herramienta analítica muy útil, ya que permite distinguir entre compuestos de masa similar. Sin embargo, en el caso de que aparezcan varios picos espectrales juntos, a parte de esta exactitud de masa, es necesaria una buena resolución para poder diferenciarlos.

Por otra parte, la masa exacta puede ayudar a identificar compuestos desconocidos, al poder predecir su composición elemental. A modo de ejemplo, para una m/z 226.1668, asumiendo C_{0-50} H_{0-100} O_{0-10} N_{0-10} S_{0-5} F_{0-10} P_{0-10} y un error de 50 ppm, habría 18 composiciones elementales posibles, mientras que con un error de 5 ppm, disminuirían a 3. En el caso de encontrarse presentes en la molécula átomos con un *pattern* isotópico característico (como por ejemplo átomos de Cl o de Br), el número de posibles composiciones elementales disminuye. Así, para una m/z 230.1172, asumiendo la presencia de un átomo de cloro, es decir, C_{0-50} H_{0-100} O_{0-10} N_{0-10} S_{0-5} F_{0-10} P_{0-10} Cl_{1-1} , y errores de 50 y 5 ppm, el número de composiciones elementales sería 9 y 1, respectivamente.

En el caso del QTOF, la presencia de un filtro cuadrupolar previo permite realizar experiencias en tándem a voluntad, e incluso, en la actualidad, se puede llevar a cabo de modo automático el cambio de MS a MS/MS. Por lo tanto, se pueden realizar experiencias en barrido total de iones (*full scan*) con mucha mayor sensibilidad que con un filtro cuadrupolar (10-100 veces), y sobretodo con una elevada resolución y masa exacta (5 ppm). Esto facilita la elucidación de la estructura del analito así como diferenciar entre compuestos isoméricos, que, obviamente, presentan la misma masa exacta.

2.1.1 Obtención de masa exacta: Calibración y lock mass

Aunque para la obtención de masa exacta es necesaria la elevada resolución de los analizadores TOF, ésta puede no ser suficiente, ya que para tener seguridad en la exactitud de la medida, el equipo ha de estar calibrado. Para ello se utilizan sustancias de masa conocida y se ajusta la respuesta del detector a estas masas. Aunque se puede utilizar cualquier mezcla de compuestos que posea masas en todo el rango en el que se desea calibrar, lo más habitual es formar aductos empleando una mezcla de polietilenglicoles (PEGs), Csl o HCOONa. En nuestro caso, utilizamos esta última mezcla (aductos de sodio/formiato) debido, entre otras cosas, a la posibilidad de utilizarla tanto en modo de ionización positivo como negativo. Por otra parte, esta mezcla no presenta *pattern* isotópico, minimizando la posibilidad de asignar erróneamente algún pico durante la calibración. Finalmente, presenta un número elevado de señales distribuidas de forma equidistante a lo largo de todo el rango de masas, por lo que el error en la calibración será menor. El único inconveniente de esta mezcla es la introducción de sales. A modo de ejemplo, en la figura 2.3 se muestra una calibración en modo de ionización negativo. Como puede verse, el error de masa medio en la calibración es menor de 0.2 mDa.

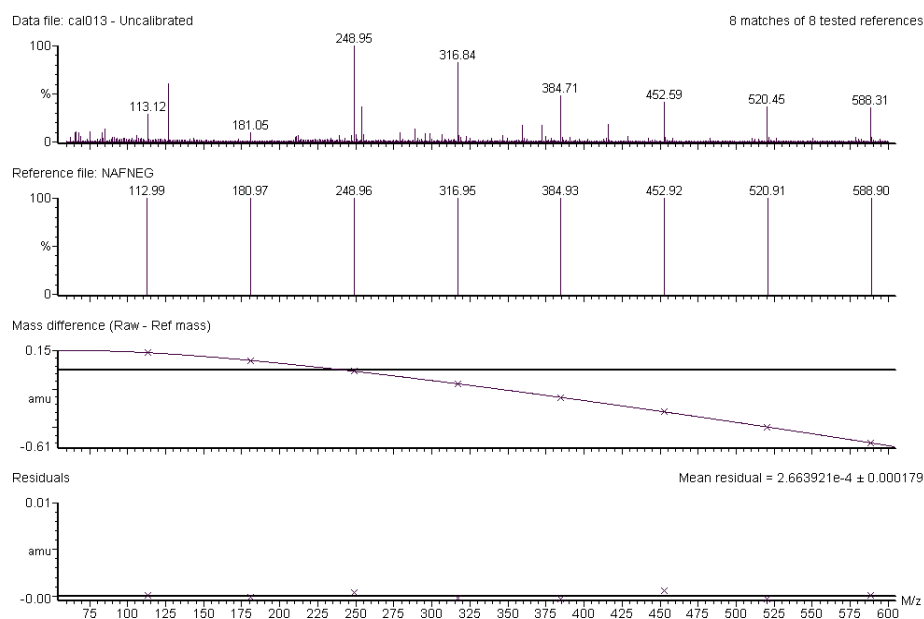


Figura 2.3 Calibración en modo de ionización negativo

Con la calibración se consigue una medida de masa fiable en el tiempo cuando se trabaja con cuadrupolos (en el rango de 1 uma). Sin embargo, con los analizadores de tiempo de vuelo, si se quiere obtener exactitud en la medida de masa en el rango de 2 mDa, aparte de la calibración diaria, es necesario que el instrumento esté en constante calibración. Esto es debido a la variabilidad que presentan las medidas del tiempo de vuelo de los iones respecto a parámetros como, por ejemplo, la temperatura, por posible dilatación del tubo de vuelo. En el modo MS, ésto se consigue mediante la adición post-columna de un compuesto de masa conocida (denominado *lock mass*) previa a la entrada de la muestra en la interfase del espectrómetro, de manera que todas las masas obtenidas se recalculan posteriormente teniendo en cuenta la masa conocida de éste. En el modo MS/MS, las masas de los iones producto se recalculan en función de la masa exacta del ion precursor.

Algunos instrumentos TOF han sido recientemente diseñados con una fuente de electrospray dual (ver Figura 2.4), lo que permite la continua introducción del *lock mass* via un segundo capilar, proporcionando masa exacta tanto en MS como en MS/MS. La principal ventaja de este diseño es que la adquisición del *lock mass* se realiza en una función diferente a la del analito, obteniendo por tanto un espectro más limpio. De este modo, el *lock mass* no interfiere en la medida (ni por masa ni por efecto matriz) y por lo tanto se puede utilizar, en principio, cualquier compuesto. La principal desventaja de este dispositivo es su mayor coste.

En nuestro caso dada la imposibilidad de disponer de un sistema de lock spray, utilizamos una aproximación basada en la introducción del *lock mass* en modo post-columna mediante una T (ver Figura 2.5).

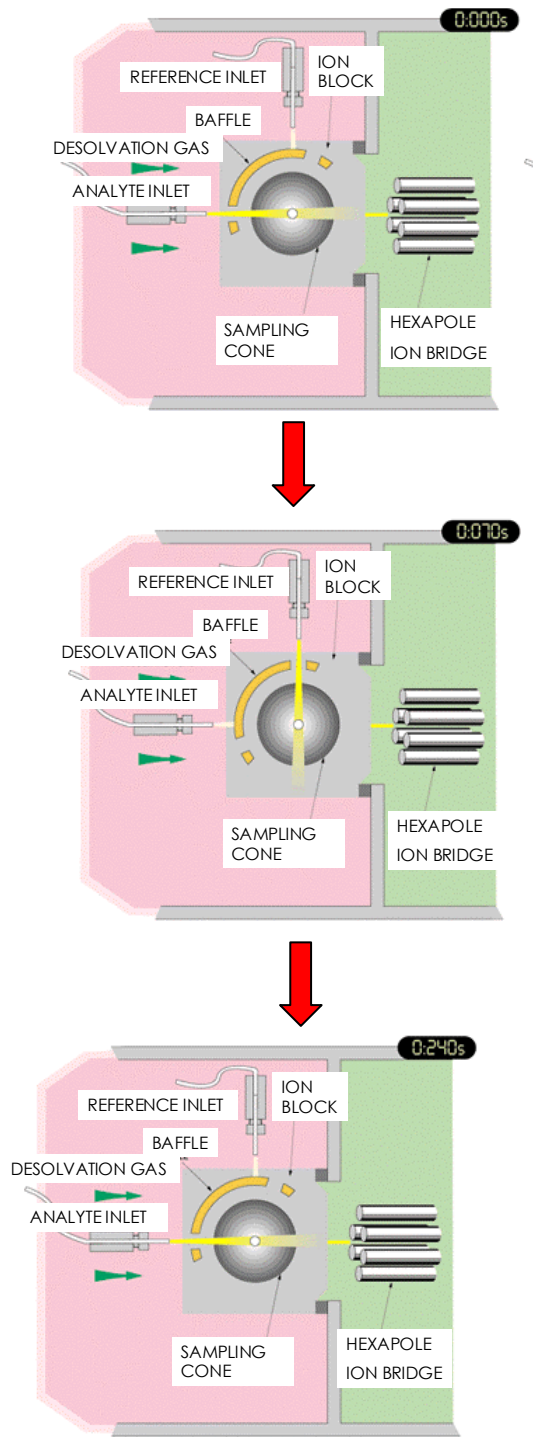


Figura 2.4 Introducción del lock mass via lock spray.

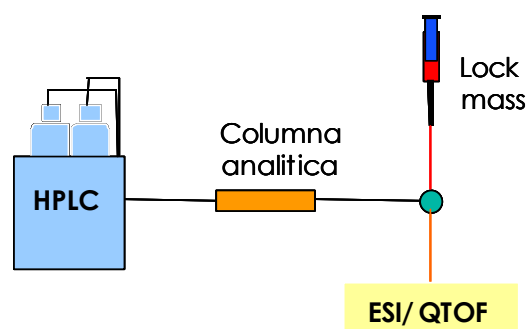


Figura 2.5 Introducción post columna del *lock mass*

Esta aproximación presenta algunas limitaciones prácticas tales como competencias en la ionización o interferencias producidas por la masa del *lock mass*, por lo que se debe seleccionar concienzudamente la molécula que actúa como tal. Tras estudiar varios compuestos, finalmente se eligió el 3,5-diiodo-L-tyrosina (Figura 2.6). Este compuesto es ideal para este propósito debido a su ionización tanto en modo positivo como negativo ($[M+H]^+$ m/z 433.8750, $[M-H]^-$ m/z 431.8594). Además, al incorporar dos átomos de yodo presenta defecto de masa, siendo por tanto más difícil su interferencia con algún posible compuesto de interés en el espectro de masas. Por otro lado, presenta poca fragmentación en la fuente y no posee un *pattern* isotópico abundante, lo que simplifica el espectro de MS. Finalmente, se ha observado que no produce un efecto matriz importante.

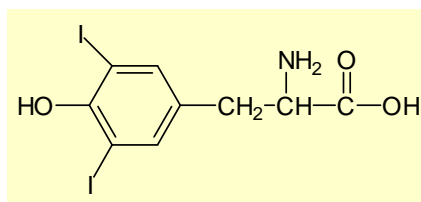


Figura 2.6 Estructura del compuesto 3,5-diido-L-tyrosina, usado como *lock mass*

En esta Tesis, el *lock mass* se preparó en metanol, ya que por una parte se favorecía su ionización y por otra, permitía mantener el porcentaje de modificador orgánico más o menos constante durante los gradientes utilizados, de modo que se obtenía una señal estable a lo largo de toda la inyección.

Para obtener espectros con masa exacta, los cromatogramas se adquirirían en modo continuo, y más tarde se centraban, teniendo en cuenta la masa exacta del *lock mass*. Es importante comentar que para una buena corrección de la masa, las cuentas del analito y las del *lock mass* habían de ser del orden de unas 400 por segundo. Así, en aquellos picos cromatográficos grandes, el algoritmo de corrección no podía corregir las desviaciones obtenidas, y por lo tanto se utilizaron los datos de la cola del pico, es decir, donde la intensidad de la señal era menor. En el caso de picos pequeños, no fue necesaria ninguna consideración especial.

2.1.2 Aplicaciones del QTOF

Lamentablemente, si los sistemas LC-MS/MS de triple cuadrupolo presentan como principal desventaja su elevado coste, los equipos LC-QTOF son todavía más costosos, por lo que las posibilidades de acceso a este tipo de instrumentos son más bien limitadas. Esta es seguramente la principal causa de que sean los campos farmacéutico, biomédico y biotecnológico los que más han utilizado esta potente técnica analítica para la elucidación estructural, siendo muy escasas sus aplicaciones en el campo ambiental.

Está ampliamente aceptado en la comunidad científica internacional que el acoplamiento LC-QTOF MS es una de las técnicas más poderosas en la actualidad y más adecuadas en el campo farmacológico y bioquímico, ya que basándose en la información obtenida sobre masas exactas, se pueden resolver numerosos problemas de identificación de compuestos desconocidos en diversos tipos de muestras. Sus aplicaciones en otros campos, como el ambiental o alimentario, están prácticamente inexploradas, pero parece evidente el enorme potencial de esta técnica, especialmente con fines cualitativos.

En los artículos científicos incluidos en el capítulo 5 se comentan con más detalle las aplicaciones de LC-(Q)TOF en el análisis de residuos de plaguicidas.

2.1.3 Uso del software MetaboLynx

Como ya se ha indicado, una de las principales aplicaciones del QTOF ha sido en el estudio de metabolismo de fármacos. En este campo se suele utilizar softwares específicos como MetaboLynx, aplicación que compara el archivo LC-MS de una muestra conteniendo el análisis con el de una muestra control, resaltando las diferencias entre ellos, que son entonces atribuidas a los metabolitos originados. En nuestro caso, y con el fin de procesar la gran cantidad de información generada en los análisis, se utilizó este mismo software. Sin embargo, dado que esta aplicación está originariamente diseñada para el estudio del metabolismo de fármacos (es decir, búsqueda de metabolitos más o menos esperados) fue necesario un estudio previo con el fin de optimizar todos los parámetros, de manera que el software fuera útil para nuestros objetivos en el campo ambiental.

A continuación, mostraremos el potencial del MetaboLynx con un ejemplo ilustrativo. En la Figura 2.7(a) se muestra el cromatograma obtenido en la degradación del herbicida terbumetona tras 4 días de irradiación de un agua superficial fortificada con dicho compuesto. Asimismo, en la parte superior se muestra el cromatograma para la muestra control a este mismo tiempo. A simple vista, sólo se distinguen dos picos, uno a 20 minutos (correspondiente al pesticida original) y otro a 17 minutos que corresponderá a algún TP originado, ya que no aparece en la muestra control. Sin embargo tras procesar estos datos con el MetaboLynx se detectaron otros TPs, que no habían sido observados anteriormente. A modo de ejemplo, en la figura 2.7(b) se muestra el cromatograma para m/z 240. Se observa la presencia de 2 picos a 9.7 y 20.6 minutos, respectivamente, correspondientes a TPs de la terbumetona. Estos picos quedaban ocultos en el TIC de la muestra fortificada (indicadas como “?” en Figura 2.7(a)), por lo que no hubieran sido detectados sin ayuda de esta aplicación.

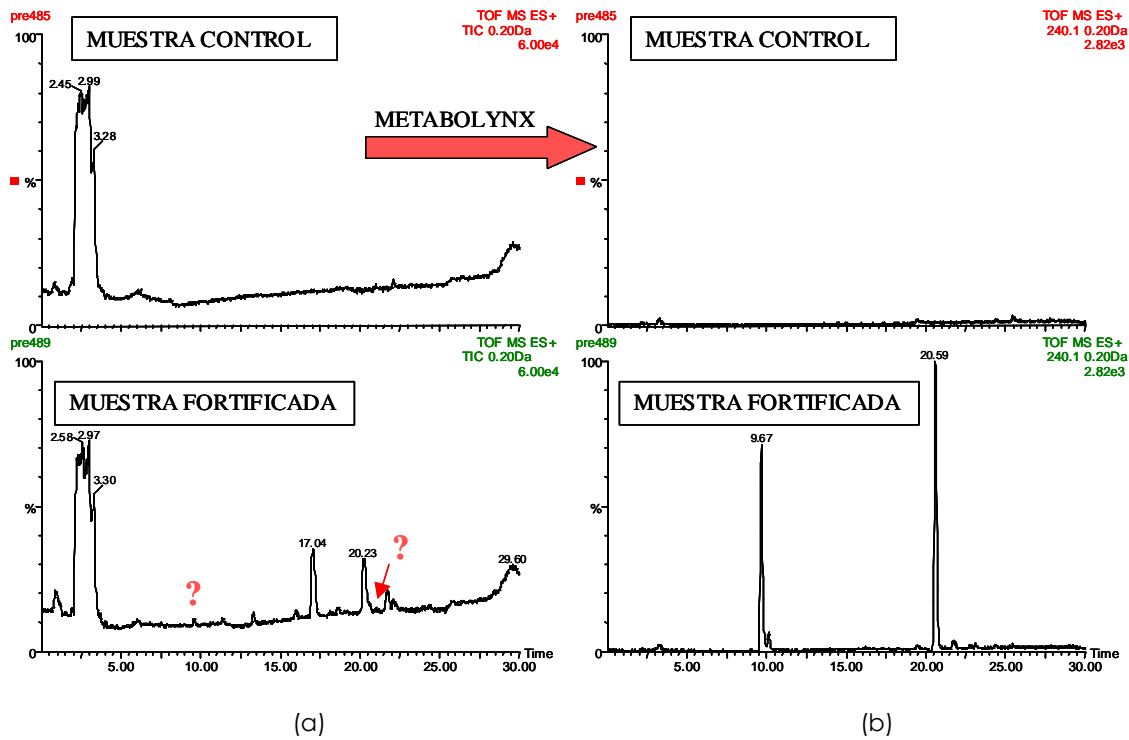


Figura 2.7 Procesamiento de datos con ayuda del MetaboLynx: (a) cromatograma en modo *full scan* de la muestra control y de la muestra fortificada, tras 4 días de irradiación; (b) XIC a m/z 240 de la muestra control y de la muestra analito

En los apartados siguientes se explorará el potencial analítico de la cromatografía líquida acoplada a la espectrometría de masas con analizadores híbridos cuadrupolo-tiempo de vuelo principalmente en el análisis medioambiental. Además, se mostrará de modo conciso el potencial de este acoplamiento en el análisis biológico. En todos los casos, los estudios realizados se han centrado en plaguicidas y sus metabolitos/TPs.

2.2. Experimental

Se utilizó una bomba HPLC Waters Alliance 2695 (Waters, Milford, USA) acoplada a un espectrómetro de masas híbrido cuadrupolo-tiempo de vuelo (QTOF I, Micromass, Manchester, UK) usando una interfase electrospray octogonal de Z-spray.

Como gas de desolvatación y de nebulización se utilizó nitrógeno, generado a partir de aire presurizado en un generador de nitrógeno (Peak Scientific, Inchinnan, Scotland). Los flujos de gas de nebulización y de desolvatación se ajustaron a aproximadamente 15 L/h y 500-600 L/h, respectivamente. La resolución del TOF-MS era aproximadamente 5000 (FWHM). El voltaje del detector MCP se ajustó a 2700 V.

Para trabajar en modo MS/MS, se utilizó argón como gas de colisión (99.995%, Carburos Metalicos, Valencia, Spain) con una presión de aproximadamente 2.10^{-5} mbar en la celda de colisión. Se seleccionó un cono de 25 V y un capilar de 3.5 kV tanto en modo positivo como negativo. La temperatura de la interfase fue de 120°C. Se seleccionó un *solvent delay* de 3 minutos y un tiempo de monitorización de 1 s/scan. El software utilizado fue Masslynx NT v.4.0 software. Se eligió un *MS profile* adecuado.

El equipo se calibró diariamente usando una bomba de jeringa (Model 11, Harvard, Holliston, MA) directamente conectada a la interfase, en el mismo rango con el que después se iba a trabajar, normalmente de m/z 50 a m/z 500. La calibración se llevó a cabo usando una mezcla NaOH 0.1M-HCOOH 10% (50:50) diluída (1:50) con acetonitrilo-agua (80:20) a un flujo de 10 µL/min, en ambos modos de ionización.

El *lock mass* (3,5-diiodo-L-tirosina) se introdujo en modo post-columna, a una concentración de 1 mg/L, usando una bomba HPLC isocrática (2150, LKB, Bromma, Sweden) a un flujo de 100 µL/min mediante una unión en forma de T.

Para la separación cromatográfica se utilizó una columna analítica X-Terra C₁₈ 5µm, 2.1 x 250 mm (Waters) a un flujo de 200 µL/min. La fase móvil utilizada fue un gradiente agua 0.01%HCOOH-metanol 0.01%HCOOH. El porcentaje de modificador orgánico varió linealmente del siguiente modo: 0 min.,5%; 0.1 min.,5%; 20 min.,95%; 23 min.,95%; 24 min.,5%; 29 min., 5%. El volumen de inyección fue 50 µL.

2.3. Elucidación de productos de transformación/metabolitos de plaguicidas

2.3.1 Introducción

El uso generalizado de los plaguicidas en la agricultura, así como en la industria, caminos, carreteras, vías férreas y en aplicaciones domésticas, está haciendo que se multiplique su presencia en numerosos ámbitos, pudiendo llegar a ocasionar efectos nocivos tanto para el medio ambiente como para la salud humana. Sin embargo, a pesar de los numerosos estudios realizados para conocer los niveles de los plaguicidas y sus efectos sobre el medio ambiente, las consecuencias de su elevado uso no siguen siendo del todo conocidas ya que hasta ahora la mayor parte de los trabajos se han centrado en el plaguicida original (Geerdink, 2002), a pesar de que la mayoría de los plaguicidas que se encuentran en el medio ambiente suelen sufrir alteraciones debido a reacciones con la luz solar (fotodegradación) o con organismos presentes en el medio (biodegradación). En muchos casos, estas alteraciones no llegan a degradar por completo a los compuestos, formándose TPs relativamente estables y persistentes (Barceló 1997, Kolpin 1998-2000-2001-2004, Scribner, 2000; Rebich 2004).

En los últimos años está aumentando la preocupación por la posible presencia en las aguas de los TPs de plaguicidas, ya que estas reacciones de degradación suelen ser oxidaciones, hidroxilaciones, hidrólisis o dealquilaciones, en las que se suelen formar TPs más polares que los productos de partida, por lo que su movilidad y posibilidades de contaminar las aguas son generalmente mayores que las de los plaguicidas inalterados. Así, algunos estudios muestran la presencia cada vez más frecuente de productos de TPs en aguas subterráneas. Por ejemplo, tras analizar muestras de agua procedentes de 88 pozos, Kolpin et al (1998) encontraron que 9 de los 10 compuestos más frecuentemente detectados eran TPs. Además, mientras los herbicidas inalterados fueron detectados en un 50% de las muestras, sus TPs lo fueron en un 75%. El mismo grupo de investigación observó que mientras el compuesto de partida (cianacina) era sólo detectado en un 3% de las muestras analizadas, sus TPs eran detectados entre un 17-33% (Kolpin et al, 2001). Aunque no es frecuente, en algunos casos estos TPs pueden llegar a ser tan tóxicos o incluso más que los plaguicidas inalterados (Belfroid, 1998; La Clair, 1998). Esto ocurre por ejemplo con el fenitrooxon (TP del fenitrotion), 1-naftol (TP del carbaril) o la etilentiourea (TP de fungicidas ditiocarbamatos).

En los últimos años es habitual realizar programas de control de residuos de plaguicidas en aguas, pero la mayoría de los métodos multiresiduo desarrollados hasta la fecha se centran en un número determinado de plaguicidas con características físico-químicas y polaridades relativamente similares, siendo poco frecuente que se incluyan TPs o compuestos altamente polares. Las principales razones por la que los TPs no suelen ser incluidos en los métodos

multiresiduo son la poca información disponible sobre ellos unido a su elevada polaridad, que dificulta su análisis junto con otros compuestos, así como la poca disponibilidad comercial de sustancias de referencia. Por otra parte, no hay que olvidar la carencia de listas prioritarias de TPs a controlar en las distintas áreas de interés. Así, por ejemplo, la "lista negra" de contaminantes elaborada por la Unión Europea incluye 39 plaguicidas, cuyo control en las aguas se considera prioritario. Sin embargo, en esta lista no se incluyen TPs. Al respecto, la Directiva 76/464/CEE establece un límite máximo por plaguicida individual y productos relacionados en aguas potables, aunque no deja claro qué son "productos relacionados", que bien podrían definirse como aquellos TPs que también son tóxicos.

Así pues parece necesario generar información sobre las rutas de degradación/transformación de estos compuestos y desarrollar metodología analítica que permita determinar sus niveles en el medio ambiente. La aplicación de estos métodos a programas de control permitiría tener una visión más realista de la contaminación de las aguas, especialmente en las subterráneas a causa de la mayor movilidad de los TPs en el suelo/zona no saturada.

La mayor parte de los estudios de degradación realizados hasta la fecha se han centrado en comprobar la utilidad de determinados procedimientos que permitan degradar al plaguicida original con fines "depurativos", más que en la elucidación de los TPs formados (Drożdżewska, 2004). En otros casos, la finalidad de estos estudios consiste en evaluar aspectos cinéticos del proceso de degradación del plaguicida (Lányi, 2005; Parra, 2004).

En cuanto al campo biológico, el elevado uso de plaguicidas en diferentes actividades puede suponer un riesgo para la salud humana, tanto de los trabajadores del sector agrícola, por exposición directa, como de la población en general a través de sus residuos en alimentos o en las aguas potables.

Uno de los indicadores más utilizados para evaluar la exposición a plaguicidas es la determinación de la actividad de las dos enzimas colinesterásicas: la acetilcolinesterasa en los eritrocitos o la pseudocolinesterasa en suero o plasma. Sin embargo, este indicador indirecto presenta limitaciones ya que sólo da información de la sobreexposición a dos grupos de plaguicidas (organofosforados y carbamatos), no es específico (pueden existir otros factores que también provoquen la disminución de las colinesterasas en sangre) y quizás, el más importante, la actividad colinesterásica en sangre está relacionada únicamente con la toxicidad aguda y subaguda de los plaguicidas, pero no da información sobre los efectos crónicos. Es por eso que el control biológico de la exposición a plaguicidas debe contemplar

otras medidas alternativas de control, para lo cual hay que hacer referencia al metabolismo o biotransformación de los tóxicos que han ingresado en el organismo. Hay una serie de etapas que constituyen las vías metabólicas que siguen la mayoría de las sustancias exógenas.

Los mecanismos de biotransformación de xenobióticos se dividen generalmente en dos grandes grupos, que reciben el nombre de fase I (primaria) y fase II (secundaria o metabolismo de conjugación). La fase I comprende la formación de un metabolito libre por medio de transformaciones de tipo no sintético, que implican normalmente reacciones de oxidación, reducción, hidrólisis, dehalogenación, desulfuración o nitrosación. La fase II implica transformaciones de tipo sintético con la formación de un producto a partir de la sustancia original, o uno de sus metabolitos, y un compuesto endógeno. El caso más general es que la molécula original sufra una transformación del tipo I (según las disponibilidades enzimáticas de la especie y del individuo) y la molécula resultante, en general más hidrosoluble, sea conjugada con un componente endógeno, como por ejemplo, el glutatión, el ácido glucurónico o el ion sulfato. El objetivo del control biológico va a ser, por lo tanto, determinar el agente contaminante o, en muchos casos, sus metabolitos en sangre, orina, tejidos u otras muestras biológicas como la saliva, las heces, el aire exhalado o la leche materna.

Aunque se conoce la relación entre la exposición a plaguicidas y varios efectos nocivos para la salud, el conocimiento de los metabolitos presentes en un organismo expuesto a un plaguicida es mucho menor, a pesar de la importancia de este tema. De hecho, en la actualidad no existe demasiada información publicada sobre el metabolismo de plaguicidas, a pesar de ser un requisito indispensable a la hora de registrar un nuevo producto agroquímico. Ello significa que, aunque muchas veces existen estudios muy completos realizados por las compañías fabricantes de productos fitosanitarios, la información no está normalmente disponible en el circuito científico. Es necesario, por tanto, estudiar el metabolismo de los plaguicidas con el objeto de conocer los compuestos (metabolitos) que deben determinarse para realizar correctamente la evaluación de la exposición humana y realizar estudios de control, tanto en población expuesta como no expuesta, e incluso como análisis rutinario para el control de la salud de los trabajadores del sector agrícola. Para ello se debe aplicar una metodología analítica que permita la elucidación de estos metabolitos de una manera fiable y segura.

Una herramienta analítica muy útil en estos dos campos (ambiental y biológico) es el acoplamiento LC-QTOF. Por una parte, se pueden realizar experiencias de barrido total de iones (*full scan*) con mucha mayor sensibilidad que con un filtro cuadrupolar (10-100 veces), y sobretodo con una elevada resolución y masa exacta, lo que permitirá, con el error habitual de estos analizadores (5 ppm), establecer la fórmula empírica del compuesto desconocido. Además, para discernir entre los posibles candidatos que puedan satisfacer esa fórmula empírica, se pueden realizar experiencias en tándem, seleccionando el ión precursor de interés y obteniendo su espectro de iones producto con masa exacta, lo que posibilitará la obtención de las fórmulas empíricas de los fragmentos y, por lo tanto, la elucidación final de la estructura del compuesto desconocido (Nielen, 2001; Lopes, 2002).

Aunque existen antecedentes sobre el uso de LC-QTOF como una de las técnicas más poderosas para la elucidación estructural, las posibilidades de acceso a este tipo de instrumentos son más bien limitadas para la mayoría de laboratorios. Esta es la causa principal de que sean los campos farmacéutico, biomédico y biotecnológico los que más han utilizado esta potente técnica analítica (Borchers, 1999; Bateman, 2002), y que sean muy escasas las aplicaciones desarrolladas en otros campos, como el ambiental.

En el caso del análisis de plaguicidas, LC-TOF se ha utilizado con fines confirmatorios en combinación con otras técnicas analíticas. Así, Maizels and Budde (2001) mostraron la utilidad del LC-TOF MS para la determinación de carbamatos y fenilureas haciendo uso del tiempo de retención, masa exacta y abundancia relativa de algunos isótopos. De manera similar, Hogenboom et al (1999) aplicaron LC-TOF MS al análisis de aguas de río fortificadas con plaguicidas a concentraciones de 0.1-10 µg/L. En lo referente a estudios de degradación, se han encontrado muy pocas referencias bibliográficas que hagan uso del (Q)TOF. Así, Thurman et al (2002) analizaron productos de degradación del acetoclor y alaclor (ácidos etanosulfónicos) en patrones y en un agua subterránea, usando HPLC-TOF MS en ambos modos de ionización en combinación con fragmentación inducida en la fuente. En el agua subterránea sólo se detectaron dos de los cuatro productos de degradación a pesar de encontrarse presentes en la muestra (concentraciones de alrededor de 15 µg/L). Este mismo grupo de investigación (Thurman et al, 2003) combinó el uso del TOF con una trampa de iones para la identificación de dos nuevos productos de degradación del acetoclor, alaclor y metolaclor. Hogenboom et al (2000), por su parte, combinaron LC-TOF con experiencias MS/MS usando un triple cuadrupolo con el fin de identificar las estructuras de los TPs del alaclor. Malato et al (2003) identificaron los TPs del diuron combinando TOF MS y trampa de iones. Más recientemente se ha estudiado la fotodegradación del diazinon (Kouloumbos et al, 2003) y del carbofuran (Detomaso et al, 2005) haciendo uso de QTOF, identificando 6 y 7 productos de transformación, respectivamente.

En los dos artículos científicos que se presentan a continuación, se muestra el potencial de LC-MS con analizadores híbridos QTOF para la elucidación de productos de transformación/metabolitos de plaguicidas seleccionados. Se ha aplicado una metodología de trabajo que incluye ensayos de laboratorio de distintos tipos (fotodegradación, ensayos *in vitro* y ensayos *in vivo*) junto con la posterior investigación de las muestras con equipos del LC-QTOF MS con el fin de identificar los productos resultantes.

En primer lugar, se estudió la fotodegradación de una familia de herbicidas ampliamente utilizada en el sector cítrico como son las triazinas. Concretamente, se estudió la fotodegradación de la terbutilazina, simazina, terbutrina y terbumetona, cuyas estructuras se muestran en la Figura 2.8.

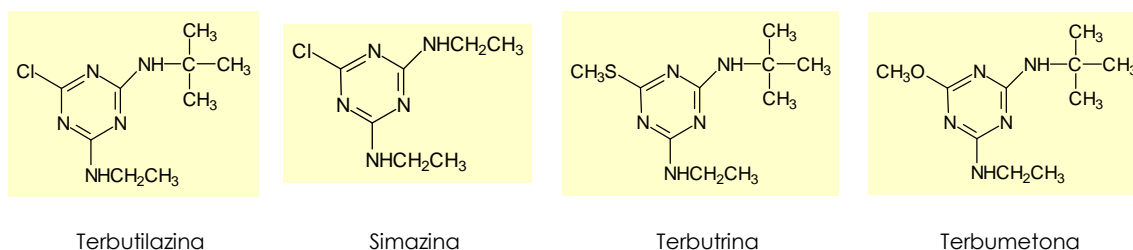


Figura 2.8 Estructuras de las triazinas seleccionadas

Así, se fortificaron diferentes aguas superficiales con los compuestos seleccionados y se irradiaron con una lámpara de mercurio durante, aproximadamente, 15 días. Adicionalmente, se irradió una muestra de agua superficial sin fortificar, que fue usada como muestra control. El montaje experimental se muestra en la Figura 2.9. Cada cierto tiempo se tomaron alícuotas, que fueron directamente inyectados en el LC-ESI-QTOF MS. Tras adquirir los cromatogramas de la muestra control y de la muestra fortificada en modo *scan*, los datos se procesaron con ayuda del MetaboLynx con el fin de visualizar los picos correspondientes a posibles TPs. En este caso se seleccionó como precursor el compuesto objeto de degradación.

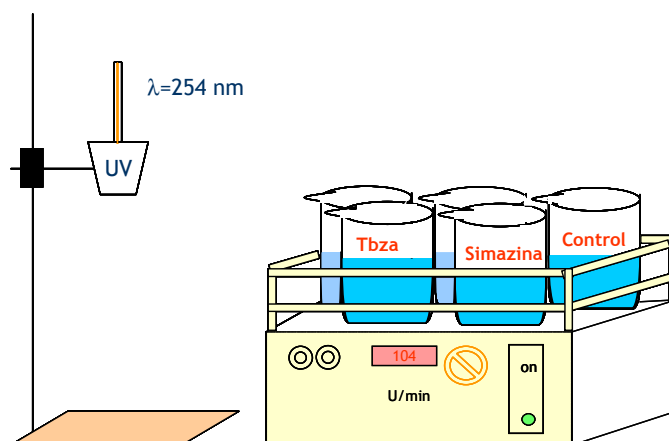


Figura 2.9 Montaje experimental utilizado en las experiencias de fotodegradación

Una vez obtenida la masa exacta de los picos encontrados, se calcularon las posibles composiciones elementales, con el fin de obtener la fórmula empírica del compuesto. Los parámetros seleccionados (valores máximo y mínimo) se establecieron en función de la estructura del plaguicida original. Así, por ejemplo, en el caso de encontrarse presentes en la molécula átomos de elementos halogenados, éstos se ajustaron en función de su *pattern* isotópico. En los casos en que no se disponía de patrones comerciales, la posibilidad de llevar a cabo experiencias de MS/MS con masa exacta, nos ayudó a elucidar sus estructuras, al proporcionar información tanto de los iones producto como de las pérdidas neutras.

Posteriormente, se decidió ampliar el campo de trabajo de esta técnica y explorar su potencial en el campo biológico. Se estudió tanto la fotodegradación como el metabolismo de un insecticida organofosforado ampliamente utilizado, diazinon, que se tomó como modelo para desarrollar una metodología general de trabajo (Figura 2.10). Las experiencias de fotodegradación se llevaron a cabo de manera análoga a las triazinas. Por otro lado, con el fin de determinar los principales metabolitos de este insecticida se realizaron dos tipos de experiencias. En primer lugar, se llevaron a cabo experiencias *in vitro* con microsomas hepáticos humanos, en colaboración con el Hospital La Fe de Valencia. Con estos estudios se pretendía obtener una visión aproximada del metabolismo humano. Aunque en principio la idea era llevar a cabo experiencias *in vitro* con hepatocitos humanos, la elevada infraestructura requerida y la complejidad de los cultivos imposibilitó llevar a cabo estas experiencias. Posteriormente, el estudio de metabolismo fue completado con experiencias *in vivo* con el fin comprobar la validez de los resultados obtenidos en un escenario más real. Para ello se realizaron experiencias con ratas, en colaboración con el Departamento de Psicobiología de la Universidad Jaume I.

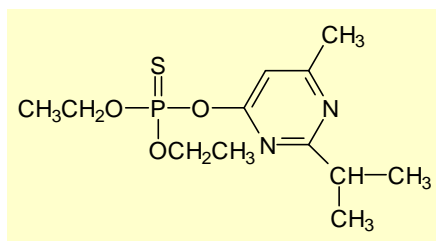


Figura 2.10 Estructura del diazinon

En vista de los resultados satisfactorios obtenidos, y siguiendo la misma metodología empleada para diazinon y triazinas, estudiamos la fotodegradación de otros plaguicidas utilizados en la zona, los cuales ya habían sido detectados en numerosas ocasiones en aguas de la Comunidad Valenciana analizadas en nuestro laboratorio. Los estudios se llevaron a cabo sobre los herbicidas glifosato, diuron, bromacilo y terbacilo, sobre los fungicidas imazalil y carbendazima, y sobre el insecticida benfuracarb (ver capítulo 3).

2.3.2 Resultados y discusión

Estos trabajos han demostrado el extraordinario potencial de LC-QTOF MS para la identificación/elucidación de productos de transformación/metabolitos de plaguicidas, tanto en el campo medioambiental como en el biológico. La versatilidad de la cromatografía líquida, junto con la elevada exactitud de masa del QTOF y la posibilidad de obtener espectros de iones producto con masa exacta, ha permitido la detección e identificación de TPs/metabolitos tras la inyección directa de muestras acuosas, sin necesidad de efectuar ningún tratamiento de muestra y, por lo tanto, minimizando posibles errores durante el proceso analítico. Sin embargo, esta aproximación presenta algunas limitaciones. Por un lado, la interfase utilizada restringe su aplicación a aquellos compuestos ionizables con esta interfase, que suelen ser de naturaleza polar a iónica. Por otra parte, en algunas ocasiones, la obtención de un espectro MS/MS rico en información se convierte en un factor determinante a la hora de poder elucidar una estructura.

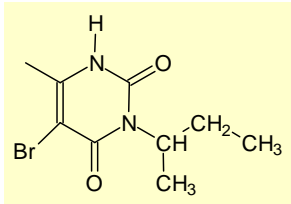
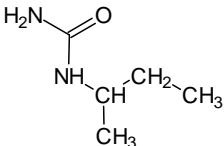
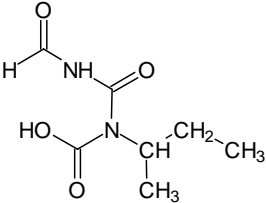
En el caso de las triazinas se detectaron 16 productos de transformación, siendo los más importantes los correspondientes a dehalogenaciones, hidroxilaciones, dealquilaciones o combinaciones de estos procesos. A todos los compuestos se les asignó una única estructura, excepto a dos de ellos, para los cuales se obtuvieron dos posibles estructuras. La alta sensibilidad del QTOF permitió, por otro lado, detectar metabolitos poco abundantes, incluso inferiores al 2% del área de pico total (Para más detalle ver artículo científico 1).

Los productos de transformación identificados fueron incluidos en un método multiresiduo basado en LC-MS/MS (QqQ) que se estaba desarrollando en nuestro laboratorio. Como se verá más adelante (capítulo 3), los resultados obtenidos al analizar muestras de agua reales mostraron la utilidad de estos experimentos, ya que se detectó un gran número de estos productos de transformación en las aguas de la Comunidad Valenciana, algunos de ellos en concentración superior a la del compuesto de partida.

Por lo que se refiere al diazinon, los principales procesos observados en los estudios de fotodegradación/metabolismo fueron hidrólisis, oxidación, hidroxilación y combinaciones de los mismos. Los productos de transformación mayoritarios obtenidos en las experiencias de fotodegradación fueron diazoxon, 1-hidroxiisopropil diazinon e hidroxidiazinon. En el caso de los estudios *in vitro*, sólo se identificaron dos metabolitos, 2-isopropil-6-metil-4-pirimidinol (IMP) y 1-hidroxiisopropil diazinon. En las experiencias *in vivo* los principales metabolitos identificados fueron el IMP y los alquilfosfatos. En este caso, únicamente fue imposible asignar una estructura inequívoca para uno de los compuestos detectados (*Para más detalle ver artículo científico 2*).

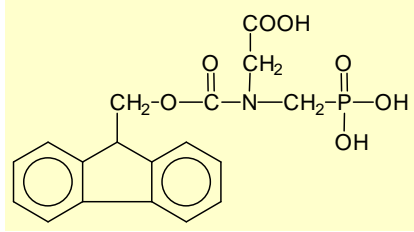
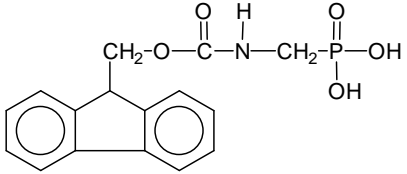
El éxito de los experimentos con triazinas y la detección de numerosos de sus TPs en las aguas analizadas nos llevó a ampliar este estudio a otros compuestos que se habían detectado frecuentemente en aguas, y que se sometieron también a experiencias de fotodegradación en aguas fortificadas. Los TPs identificados se muestran en las tablas 2.1 a 2.8 (*Para más detalle ver capítulo 3*).

Tabla 2.1 Productos de transformación del bromacilo identificados por LC-QTOF MS

Compuesto	Modo ionización	Masa exacta experimental	Masa exacta teórica	error (mDa)
BROMACILO 	ES-	$C_9H_{13}N_2O_2Br$ Ion precursor 259.0085 Iones producto 202.9491 159.9438 78.9203	259.0082 202.9456 159.9398 78.9183	0.3 3.5 4 2
METABOLITO 1 	ES+	$C_5H_{12}N_2O$ Ion precursor 117.1014 Iones producto 61.0247	117.1028 61.0402	-1.4 2.5
METABOLITO 2 	ES+	$C_7H_{12}N_2O_4$ Ion precursor 187.0681 Iones producto 115.0899	187.0719 115.0871	-3.8 2.8

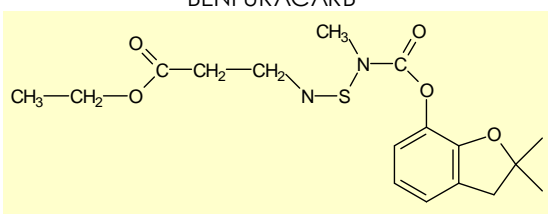
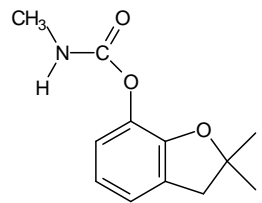
A pesar de que el bromacilo se determinó en modo de ionización negativo, sus TPs, en los cuales se produce ruptura del anillo aromático, se determinaron en modo de ionización positivo.

Tabla 2.2 Productos de transformación del glifosato identificados por LC-QTOF MS

Compuesto	Modo ionización	Masa exacta experimental	Masa exacta teórica	error (mDa)
GLIFOSATO	ES+	C ₁₇ H ₁₈ NPO ₇		
		Ion precursor 392.0881	392.0899	2.6
		Iones producto 214.0096	214.0117	-2.1
		179.0849	179.0861	-1.2
		170.0224	170.0218	0.6
		88.0413	88.0399	1.4
METABOLITO 1 (AMPA)	ES+	C ₁₅ H ₁₆ NPO ₅		
		Ion precursor 334.0845	334.0844	1.9
		Iones producto 179.0811	179.0861	-5
		156.0036	156.0062	-2.6
		112.0145	112.0164	-1.9

La fotodegradación del glifosato condujo a la formación de AMPA como único TP. La determinación de estos dos compuestos en las muestras de agua requirió una derivatización previa, tal y como se verá en el capítulo 3.

Tabla 2.3 Productos de transformación del benfuracarb identificados por LC-QTOF MS

Compuesto	Modo ionización	Masa exacta experimental	Masa exacta teórica	error (mDa)	
BENFURACARB 	ES+	C ₂₀ H ₃₀ N ₂ O ₅ S			
		Ion precursor	411.1941	411.1953	-1.2
		Iones producto	252.0649	252.0694	-4.5
			195.0424	195.0440	-1.6
			190.0843	190.0902	-5.9
			158.1129	158.1181	-5.2
METABOLITO 1 (CARBOFURAN) 	ES+	C ₁₂ H ₁₅ NO ₃			
		Ion precursor	222.1123	222.1130	-0.7
		Iones producto	165.0895	165.0916	-2.1
			123.0427	123.0446	-1.9

Como es bien conocido, el benfuracarb se degrada rápidamente a carbofuran en contacto con el agua. En nuestras condiciones éste fue el único TP detectado, a pesar de que en la bibliografía se suele reportar el carbofuran-3-OH como TP del benfuracarb. Una posible explicación radicaría en que la lámpara de mercurio no puede simular todos los procesos que ocurren en un escenario real, como por ejemplo aquellos debidos a microorganismos.

Tabla 2.4 Productos de transformación del diuron identificados por LC-QTOF MS

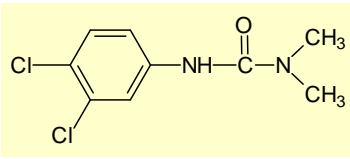
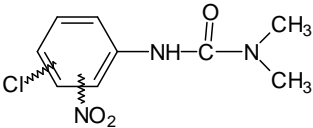
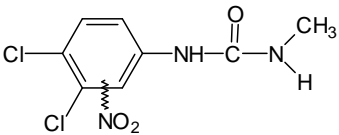
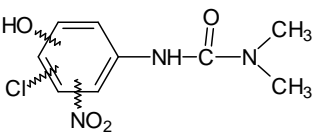
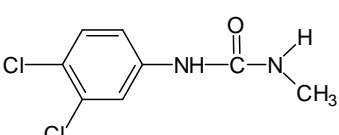
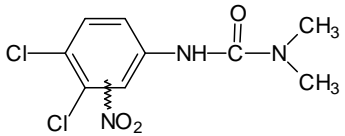
Compuesto	Modo de ionización	Masa exacta experimental	Masa exacta teórica	error (mDa)
<p>DIURON</p> 	ES+	C ₉ H ₁₀ N ₂ O ₂ Cl ₂ Ion precursor 233.0246 Iones producto 187.9612 159.9762 72.0437	233.0248 187.9670 159.9721 72.0449	-0.2 -5.8 4.1 -1.2
<p>METABOLITO 1</p> 	ES+ ES-	C ₉ H ₁₀ N ₃ O ₃ Cl Ion precursor 244.0504 Iones producto 198.0468 153.9906 152.9882 72.0453 Ion precursor 242.0374 Iones producto 196.9977 168.0009	244.0489 198.0560 153.9934 152.9981 72.0449 242.0332 196.9992 168.0090	1.5 -9.2 -2.8 -9.9 0.4 4.2 -1.5 -8.1
<p>METABOLITO 2</p> 	ES+	C ₈ H ₇ N ₃ O ₃ Cl Ion precursor 263.9995 Iones producto 206.9723 188.9617 170.9525 158.9641 109.9790	263.9943 206.9728 188.9622 170.9517 158.9643 109.9690	5.2 -0.5 -0.5 0.8 -0.2 10
<p>METABOLITO 3</p> 	ES-	C ₉ H ₁₀ N ₃ O ₄ Cl Ion precursor 258.0305 Iones producto 212.9690 176.0625 167.9824 151.9919	258.0282 212.9703 176.0586 167.9852 151.9903	2.3 -1.3 3.9 -2.8 1.6
<p>METABOLITO 4</p> 	ES+	C ₈ H ₈ N ₂ OCl ₂ Ion precursor 219.0135 Iones producto 161.9879 127.0186	219.0092 161.9877 127.0189	4.3 0.2 -0.3

Tabla 2.4(cont) Productos de transformación del diuron identificados por LC-QTOF MS

Compuesto	Modo de ionización	Masa exacta experimental	Masa exacta teórica	error (mDa)
METABOLITO 5	ES+	$C_9H_{10}N_3O_3Cl_2$		
		Ion precursor 278.0109	278.0099	1.0
		Iones producto 72.0478	72.0449	2.9

En el caso del diuron, hay que destacar la elevada cantidad de nitroderivados detectados. Estas experiencias de fotodegradación fueron repetidas en agua de consumo humano, no obteniéndose dichos compuestos. Esto indica que los TPs formados pueden depender, en buena medida, de la composición de la matriz, y en este caso en concreto de la concentración de nitratos del agua.

Tabla 2.5 Productos de transformación del imazalil identificados por LC-QTOF MS

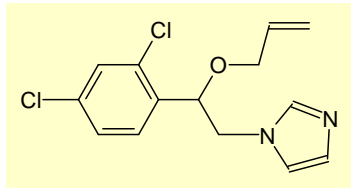
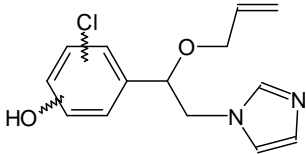
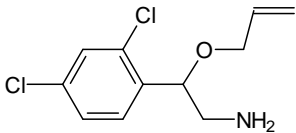
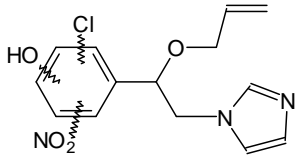
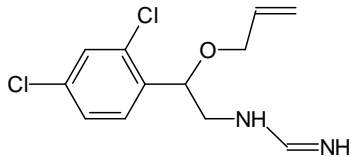
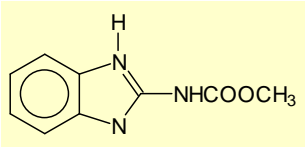
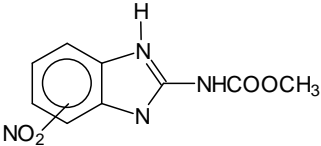
Compuesto	Modo ionización	Masa exacta experimental	Masa exacta teórica	Error (mDa)	
IMAZALIL 	ES+	C ₁₄ H ₁₄ N ₂ OCl ₂			
		Ion precursor	297.0568	297.0561	0.7
		Iones producto	255.0085	255.0092	-0.7
			200.9858	200.9874	-1.6
			158.9738	158.9768	-3.0
			176.0380	176.0393	-1.3
	69.0404	69.0453	-4.9		
METABOLITO 1 	ES+	C ₁₄ H ₁₅ N ₂ O ₂ Cl			
		Ion precursor	279.0916	279.0916	1.6
		Iones producto	261.0780	261.0795	-1.5
			165.0489	165.0471	1.8
			130.0784	130.0783	0.1
			69.0442	69.0453	-1.1
METABOLITO 2 	ES+	C ₁₁ H ₁₃ NOCl ₂			
		Ion precursor	246.0468	246.0452	1.6
		Iones producto	188.0059	188.0034	2.5
			153.0365	153.0345	2.0
			152.0264	152.0267	-0.3
			117.0576	117.0578	-0.2
METABOLITO 3 	ES+	C ₁₄ H ₁₄ N ₃ O ₄ Cl			
		Ion precursor	324.0779	324.0751	2.8
		Iones producto	282.0258	282.0282	-2.4
			263.0382	263.0353	2.9
			185.9958	185.9958	0
			181.0417	181.0420	-0.3
			109.0750	109.0653	9.7
	69.0436	69.0453	-1.7		
METABOLITO 4 	ES+	C ₁₁ H ₉ N ₂ OCl ₂			
		Ion precursor	273.0582	273.0561	2.1
		Iones producto	215.0139	215.0143	-0.4
			188.0025	188.0034	-0.9
			170.9788	170.9768	2.0
			153.0332	153.0345	-1.3
			152.0284	152.0267	1.7
			117.0578	117.0578	0

Tabla 2.6 Productos de transformación de la carbendazima identificados por LC-QTOF MS

Compuesto	Modo ionización	Masa exacta experimental	Masa exacta teórica	error (mDa)	
CARBENDAZIMA 	ES+	C ₉ H ₉ N ₃ O ₃			
		Ion precursor	192.0778	192.0773	0.5
		Iones producto	160.0477	160.0511	-3.4
			132.0534	132.0562	-2.8
METABOLITO 1 	ES+	C ₉ H ₈ N ₄ O ₅			
		Ion precursor	237.0631	237.0624	0.5
		Iones producto	205.0325	205.0362	-3.7
			191.0697	191.0695	0.2
			159.0321	159.0307	1.4

En el caso de la carbendazima, sólo se detectó un TP, que también se trató de un derivado nitrado.

Tabla 2.7 Productos de transformación de la bentazona identificados por LC-QTOF MS

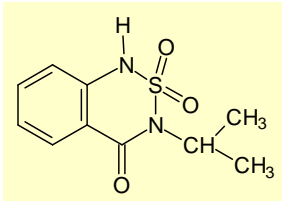
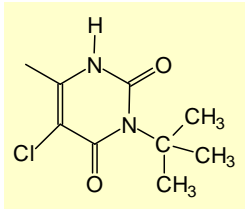
Compuesto	Modo de ionización	Masa exacta experimental	Masa exacta teórica	error (mDa)
BENTAZONA	ES-	$C_9H_9N_3O_3$		
		Ion precursor 239.0488	239.0491	-0.3
		Iones producto 197.0001 175.0855 132.0363	197.0021 175.0871 132.0324	-2.0 -1.6 3.9

Tabla 2.8 Productos de transformación del terbacilo identificados por LC-QTOF MS

Compuesto	Modo ionización	Masa exacta experimental	Masa exacta teórica	Error (mDa)
TERBACILO	ES-	$C_9H_{13}N_2O_2Cl$		
		Ion precursor 215.0587	215.0588	-0.1
		Iones producto 158.9915 115.9953	158.9961 115.9903	-4.6 5

Tanto en el caso de la bentazona como en el terbacilo, no se detectó ningún TP. Esta situación es curiosa, especialmente en el caso del terbacilo, cuya estructura es semejante a la del bromacilo, para el cual sí se detectaron varios TPs. Dado que sí se observó la desaparición del compuesto de partida, posiblemente se formaran TPs, pero no fueron detectables por LC-QTOF, por alguna limitación bien en lo referente a la cromatografía o a la ionización.

Artículo científico 1

Anal. Chem. (2004) 76: 1328-1335

USE OF QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY IN ENVIRONMENTAL ANALYSIS: ELUCIDATION OF TRANSFORMATION PRODUCTS OF TRIAZINE HERBICIDES IN WATER AFTER UV EXPOSURE

María Ibáñez, Juan V. Sancho, Óscar J. Pozo, Félix Hernández

Analytical Chemistry, Experimental Sciences Department, University Jaume I, E-12071, Castellón, Spain

ABSTRACT

The high resolution and exact mass capabilities of hybrid quadrupole time-of-flight (QTOF) mass spectrometry can provide an ultimate confirmation in target analysis mode and aid in discovery and elucidation of unknown analytes. In this paper, the latter approach has been applied to study the transformation products of selected pesticides (terbuthylazine, simazine, terbutryn, terbumeton) in environmental waters. Additionally, the usefulness of software modules, originally designed for drug metabolism discovery, has been tested. Different environmental waters spiked at 0.5 µg/mL level have been irradiated with a mercury lamp for 7 days, sampling 3-mL aliquots every ~ 12 hours. After centrifugation, 50 µL was directly injected in an LC-ESI-QTOF system. The high sensitivity in full-scan mode allowed us to elucidate minor metabolites even below 2% of the total peak area. The mass errors observed in almost every case fell below 2 mDa, allowing assignment of a highly probable empirical formula. Besides, the MS/MS capability of this tandem instrument was very useful for differentiation between isomeric transformation products. This work shows that hyphenated LC-QTOF is a powerful approach for the rapid screening and confirmation of unknown pesticides transformation products in environmental water.

INTRODUCTION

There is a growing concern about the degradation and transformation products (TPs) of organic contaminants in the environment, since they also may be potentially toxic. Both, surface and ground water are the main sources for drinking water production. The quality of this source is influenced by many factors, such as agricultural and industrial effluents, and has to be monitored regularly. Within regular monitoring programs, modern techniques such as gas and liquid chromatography combined with different types of detection are used in order to detect and quantify known (target) pollutants. Pesticides are among the most investigated and priority pollutants in water due to their wide use and toxicity.

The development of liquid chromatography-mass spectrometry (LC-MS) using quadrupole instruments has made analysis of semi-polar and polar pesticides in surface and groundwater a common procedure for this purpose. During the last five years, a number of papers has been published on the analysis of known (target) pesticides and some selected degradation and/or transformation products by quadrupole LC-MS/MS ¹⁻⁵.

LC-MS/MS in the selected reaction monitoring mode has shown its superiority in target determinations ⁶⁻⁷ due to its excellent sensitivity, selectivity and efficiency. However, qualitative information that supports the recognition and structural elucidation of compounds other than the target is still needed. This information can be obtained in full-scan mode, but in addition to the decrease of sensitivity with conventional equipment, the lack of large libraries of LC-MS/MS spectra prevents the identification of real unknowns. However, there are still many unknown TPs in water, which usually present higher mobility (leachability) in soil and which can be even more toxic than the parent pesticides.

Although in some cases, gas chromatography with mass detection (GC-MS) has been a good solution in order to determine pesticides and their transformation products ^{8,9}, LC has been the preferred analytical separation technique, in combination with diode array ¹⁰, single-mass spectrometry ¹⁰ and tandem mass spectrometry detection ¹¹ as the majority of the TPs are much more polar than their parent pesticides, much less volatile, and often also thermolabile. Within the recent advances in mass spectrometry, a new powerful identification tool has become available, the quadrupole time-of-flight (QTOF) mass spectrometer. This type of instrument is often used for the identification of either small or big molecules (pharmaceutical and proteomic applications, respectively) due to the advantages of the ion separation and detection principle of a TOF compared to other mass analysers (quadrupoles, ion traps, etc.). Its main advantages, as elevated resolution (>5000) and high mass accuracy (< 5ppm), allow us to obtain a predicted empirical formula. Additionally, the inherent high sensitivity of the TOF analyser in full-scan mode

seems adequate to study the degradation/transformation of organic pollutants at relevant environmental levels (at ppb levels).

Hybrid QTOF instruments give the possibility of performing tandem MS acquisitions obtaining product ion spectra in exact mass, which became sometimes necessary in order to fully differentiate between isobaric analytes. One of the few drawbacks is the huge amount of data to evaluate. To cope with this problem, application managers have been developed, normally designed for drug metabolism. Thus, the use of LC-QTOF has been successfully applied in metabolism studies in the pharmaceutical field¹²⁻¹³. However, QTOF is rarely used in environmental applications (soil or water samples) due mainly to the high acquisition cost, usually unaffordable to environmental chemists involved in governmental agencies, universities and research institutes. In this field, some applications have been published using LC-TOF as a confirmatory tool due to the high mass accuracy^{14,15}. Unknown groundwater samples were analysed and the retention time and accurate mass spectra of selected peaks were compared with standards for identification. To distinguish between isomers, in-source CID was used¹⁴. In other paper¹⁵, confirmation of 10 pesticides and herbicides was ensured by the exact mass measurement in combination with a reasonable retention time and the abundance ratios of some isotopes. In other cases, LC-TOF was combined with MS/MS experiences with a triple-stage quadrupole in order to really identify proposed structures for transformation products, as for example, transformation products of alachlor¹⁶. When two or more compounds were found to be isomers, structures of the degradation products were proposed by considering the most likely fragmentation patterns in MS/MS experiments. An approach based on SPE-LC-TOFMS was used to obtain accurate mass information for the peaks as well as their fragment ions by applying in-source CID.

In this paper, we explore the possibilities of the combination of LC-QTOF and MetaboLynx software in an LC-MS/MS screening and identification approach based on the direct injection of water samples without any sample pretreatment. Transformation products of a widely used herbicides group, triazines, in surface water samples are chosen as a model example.

The first application in this field using LC-QTOF deals with the screening and identification of six selected model compounds added to surface water extracts. Additionally, applying the developed procedure, the structures of three real unknown compounds present in the water extracts were also elucidated. The structure of a fourth unknown detected remained unresolved¹⁷. To our knowledge, this is the first QTOF application for the identification of TPs of pesticides of concern regarding their potential toxicity and environmental fate.

EXPERIMENTAL

Reagents and chemicals

Terbutylazine (99.8%), simazine (98.5%), terbutryn (98%) and terbumeton (99.7%) reference standards were purchased from Dr Ehrenstorfer (Augsburg, Germany). 3,5-Diiodo-L-tyrosine, used as lock mass, was purchased from Sigma (St Louis, MO). HPLC-grade methanol, HPLC-grade acetone, reagent-grade formic acid, and sodium hydroxide were purchased from Scharlab (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralised water in a Nanopure II system (Barnstead, Newton, MA).

Standard stock solutions were prepared dissolving ~50 mg of powder, accurately weighted, in 100 mL of acetone, obtaining a final concentration of ~500 µg/mL. Standard working solutions were prepared by dilution of the stock solution with either methanol or water depending on their concentration.

Some metabolites of these triazines are commercially available from Dr Ehrenstorfer as desethylterbutylazine, desethyl-2-hidroxyterbutylazine, 2-hidroxyterbutylazine, 2-hidroxy-simazine, desisopropylatrazine, desisopropyl-2-hidroxyatrazine, desethyl-desisopropyl-2-hidroxyatrazine and desethylterbumeton. Stock solutions were dissolved in acetone, except 2-hidroxyterbutylazine and desisopropyl-2-hidroxyatrazine, which needed some droplets of concentrated HCl to complete dissolution. A standard mixture of pesticides and metabolites was prepared by delivering 200 µL of each 50 µg/mL standard solutions to 20 mL HPLC water in order to yield 500 ng/mL standard mixture. This mixture was diluted with water to obtain the working solutions.

Standard stock solution of 3,5-diiodo-L-tyrosine was prepared by dissolving ~500 mg powder in 100 mL of HPLC-grade water obtaining a final concentration of ~500 µg/mL. 1 µg/mL solution was prepared by dilution of the stock solution with methanol.

Instrumentation

A LC system Waters Alliance 2695 (Waters, Milford, M) was interfaced to a hybrid QTOF mass spectrometer (Micromass, Manchester, UK) using an orthogonal Z-spray-electrospray interface. The LC separation was performed using a X-Terra C₁₈ 5µm, 2.1 x 250 mm (Waters) at a flow rate of 200 µL/min. The mobile phase used was water-0.01%HCOOH-methanol-0.01%HCOOH gradient where the percentage of methanol was changed linearly as follows: 0 min.,5%; 0.1

min.,5%; 20 min.,95%; 23 min.,95%; 24 min.,5%; 29 min., 5%. The separations were completed within 26 minutes. The injection volume was 50 μL .

Drying gas as well as nebulising gas was nitrogen, generated from pressurized air in a nitrogen generator from Peak Scientific (Inchinnan, Scotland). The nebuliser gas flow was set to ~ 15 L/h. and the desolvation gas flow to 500-600 L/h. TOF-MS resolution was ~ 5000 (fwhm). MS data were acquired over an m/z range of 30-500. The MCP detector potential was set to 2700 V.

For operation in MS/MS mode, collision gas was Argon 99.995% (Carbueros Metalicos, Valencia, Spain) with a pressure of $\sim 2 \cdot 10^{-5}$ mbar in the collision cell. A cone voltage of 25 V and a capillary voltage of 3.5 kV were used in positive ionization mode. The interface temperature was set to 350°C and the source temperature to 120°C. Dwell times of 1 s/scan were chosen. A solvent delay of 3 minutes was selected to perform a cleanup using the built-in divert valve controlled by time-controlled contact closures using the Masslynx NT v.4.0 software. A suitable MS profile was used.

Calibration experiments were performed daily using a Model 11 single-syringe pump (Harvard, Holliston, MA), directly connected to the interface. Calibration in positive electrospray mode from 30 to 500 m/z was conducted with a mixture of NaOH 0.1M-HCOOH 10% (50:50) diluted (1:50) with acetonitrile-water (80:20) at a flow rate of 10 $\mu\text{L}/\text{min}$.

The lock mass was introduced postcolumn using a model 2150 isocratic HPLC pump from LKB (Bromma, Sweden) at a flow rate of 100 $\mu\text{L}/\text{min}$ by means of a PEEK T-union.

Samples and irradiation procedures

The irradiation procedure applied in this work has been used previously¹⁶. Irradiation was performed using a mercury vapor-lamp with its main output at 254 nm. The 250-mL surface water samples were kept in glass beakers. The distance from the lamp to the surface of the samples was ~ 15 cm. Irradiation was carried out in fume hood at room temperature over a period of ~ 164 h under constant stirring of the samples. Prior to the photolysis experiment, four 250-mL water samples were spiked at a 500 ng/mL level with terbuthylazine, simazine, terbutryn, and terbumeton, respectively, by adding 2.5 mL of 50 $\mu\text{g}/\text{mL}$ standard solutions. Additionally, a 250-mL unspiked water sample was irradiated in the same conditions and used as a control for all experiences. The first 3-mL water aliquots were analysed prior to irradiation ($t=0$). During irradiation, 3-mL water samples were taken from the beakers at different intervals, centrifuged for 10 minutes to remove possible solid particulate, and analysed by LC-QTOF MS. Protective glasses

were used in order to avoid UV exposure. Name, structural formulas, elemental composition and exact mass of the protonated molecule for each model compound are shown in Figure 1.

Analysis

A calibration lock mass was added to the LC effluent post-column to obtain accurate mass measurements for the study samples. 3,5-Diido-L-tyrosine $[(M+H)^+ m/z 433.8750]$ at 1 mg/L concentration was infused post-column at 100 $\mu\text{L}/\text{min}$. Solvent mixing between the chromatographic effluent and infusion solvent was sufficient to provide a stable lock mass response. The infusion flow rate and lock mass concentration were adjusted to avoid possible detector saturation effects.

To confirm identification of some analytes, two matrix-matched standards were prepared. They were performed by delivering 100 μL of 500 ng/mL or 50 ng/mL standard mixtures to 0.9 mL of the water control sample.

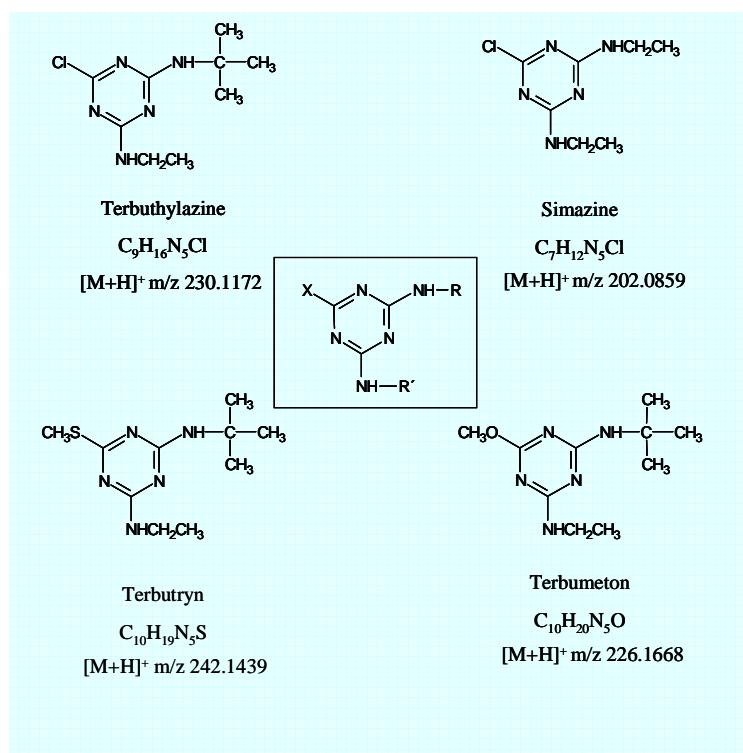


Figure 1. General structures, names, structural formulas, and elemental compositions of the model (parent) compounds.

Data processing

Data were processed using the postacquisition data processing package MetaboLynx application (Micromass v 4.0). To obtain accurate mass measurements, 15 spectra were combined, using an Np value of 0.6 and a mass window ± 0.05 Da. The mass was measured at 80% of centroid top. MetaboLynx compares two LC-MS data files and highlight differences in the presence of compounds, which could be attributed to transformation processes.

Based on the accurate mass, possible elemental compositions of the peaks of interest were calculated using the elemental composition calculator, within the MassLynx software, with a maximum deviation of 5 mDa from the measured exact mass. Parameter settings for all compounds were C 2-14, H 0-50, N 3-7, O 0-3, and additionally in the case of terbumeton S 0-3. For terbuthylazine and simazine, that contain a chlorine atom in their structure, the appropriate number of Cl was determined from the isotopic pattern and added if required. The applied double-bond equivalent (DBE) filter was set between 3.5 and 10. These DBE values as well as number of C and N were chosen as retention of aromatic ring during degradation was assumed. With these restrictions, the first proposed elemental composition was the right option in all cases.

When triazines TPs were commercially available, we could get an additional confirmation comparing retention times of the observed peaks against the measurements of the matrix-matched standards analysed in the same conditions. In the cases where no standards were available, the possibility of performing MS/MS experiences helped us to elucidate the structure of transformation products as we obtained a product ion spectrum with the exact mass of fragments.

To have representative curves between percentage of each compound and irradiation time, the response of each individual compound was related with the total response of all detected compounds by assuming that all compounds gave the same response in the instrument. This approach was used due to the nonavailability of standards for all TPs detected as well as the low linear range of QTOF instruments, which did not allow the correct quantitation of several detected TPs.

RESULTS AND DISCUSSION

Lock mass selection

Some TOF instruments have been recently designed with dual electrospray ion sources, which allow the continuous introduction of a lock mass via a second spray and provide exact mass information in both MS and MS/MS modes. Although this design has obvious advantages, it is also a more costly approach as sometimes there is no possible upgrade. In our case, an optimized lock mass has been introduced postcolumn in order to obtain exact mass measurements due to the simultaneous occurrence of lock mass and analytes in the instrument. This approach would have some practical limitations such as ion suppression due to the high amount of lock mass introduced, lower sensitivity due to the dilution produced by the postcolumn addition, and possible interferences of the lock mass with some potential degradation products. Moreover, software modules in the instrument used are originally designed for drug metabolism, and therefore, we had to adjust very carefully all the parameters in order to obtain satisfactory results. However, despite these potential limitations, the use of an adequate lock mass introduced post-column by means of a PEEK T-union, was satisfactory for achieving successful data.

To find the most adequate lock mass, some compounds were tested. We checked some pesticides, but due to the presence of chlorine atoms and abundant in-source fragmentation, the lock mass interfered with analyte ions. The best solution was the use of a diiodated amino acid, 3,5-diiodo-L-Tyrosine. This compound is mass deficient and therefore does not interfere with analyte ions. Moreover, it ionizes well in both positive ($[M+H]^+$ m/z 433.8750) and negative ($[M+H]^-$ m/z 431.8594) ionization modes, does not present any isotopic pattern and exhibits very little in-source fragmentation. In this study, due to the structure of the selected family (triazines), only positive ionization mode was used.

Firstly, a Model 11 single syringe pump was used to introduce the lock mass at 10 $\mu\text{L}/\text{min}$, but a slight pressure increase within the source did not allow us a constant introduction of the lock mass. Finally, a high-pressure LC pump was used (LKB-2150). Although in this case the dilution was higher as the minimum reliable flow rate was 100 $\mu\text{L}/\text{min}$, the analyte signal was not too much affected.

Lock mass was prepared in MeOH because its ionization as well as ionization of other compounds was favoured, achieving for lock mass a stable signal along the chromatographic time.

Chromatography optimisation

Several methanol-water mixtures were tested to find the best overall sensitivity. With no buffer addition, $[M+Na]^+$ adducts were enhanced over $[M+H]^+$, making their identification difficult. When ammonium acetate was used, the protonation of analytes was improved but a general loss of sensitivity was observed. Finally, the addition of 0.01% formic acid led to enhance protonation without any important signal variation. Formic acid was added to both eluents in order to minimize false peaks due to the gradient, which could interfere in the determination of the analytes. In order to obtain a good chromatographic separation between possible isobaric compounds, a long shallow gradient was chosen (see Instrumentation section).

Survey scan and exact mass measurements

For the determination of accurate mass, the experiments were acquired in continuum mode and afterwards processed by MetaboLynx to centroid data in order to implement lock mass correction and obtain accurately mass measured spectra. For all compounds, exact mass of protonated molecules was determined based on averaged spectra obtained in the survey scan. A time of 0.2 minutes was chosen for background subtraction. Where abundant peaks were observed (>400 counts/s), the digital dead time correction algorithm could not correct deviations obtained, and therefore, data were taken from peak tail, i.e., at lower signal intensity.

Identification of triazines TPs in surface water

The results obtained in our experiments are summarized in Table 1. As Table 1 shows, the four parents compounds studied and 16 TPs were detected over the threshold of 2% of the total peak area. When exact mass was calculated by averaging the mass of all the detections, deviations (see Table 1) lower than 2 mDa were obtained for every compound except for compounds 19 and 20, which had a deviation of ~ 3 mDa. These compounds were detected only in the last seven collection times and with an abundance below 10%. Similar results were obtained by averaging all the individual deviations (" $|\text{mean deviation}|$ " in Table 1). As regards RSD of all the measurements, all values were lower than 1.7 mDa for all compounds except for compound 20 which had an RSD of 2.4 mDa. With these results, we can be confident about the elemental composition of each analyte; however, this information is not sufficient to know the exact structure of the detected TPs as some of them are isomeric compounds (e.g. 2 and 6; 4, 8 and 10). The availability of standards for some of the TPs helped us in the elucidation of several detected compounds by comparing their retention times. However, 8 over 16 TPs detected were

not commercially available; in these cases, MS/MS experiments were necessary to elucidate unknown TPs. For this purpose, the MS/MS fragmentation pathways of all available standards were studied. Studying the MS/MS spectra of the unknown TPs and comparing them with the MS/MS spectra of the available compounds, we could elucidate the different substituents X, R and R' with exact mass (see Figure 1) and therefore predict plausible structures.

Figure 2 shows the elucidation process for three unknown TPs, numbered 14, 18 and 19, that are taken as an example to show the methodology followed in this paper. In the case of 14 (a TP of terbutryn), the exact mass of this unknown compound with retention time 11.73 min was determined to be m/z 168.1228. This mass differed -2.1 mDa from m/z 168.1249, which corresponds to an elemental composition of $C_7H_{14}N_5$, and therefore it is obtained after a loss of 74.0211 mDa (C_3H_6S) from terbutryn $[(M+H)^+ 242.1439, C_{10}H_{20}N_5S]$. Fragmentation upon CID was performed in order to facilitate the identification and structural elucidation of this compound. The MS/MS spectra, acquired at three different collision energies, showed two important fragments for this TP at m/z 112.0605 and 70.0389. The difference between m/z 168.1228 and 112.0605 corresponded to the loss of the group tert-butyl (therefore R = tert-butyl). Thus, fragment A (Figure 2a) corresponded to m/z 112.0605 with an elemental composition of $C_3H_6N_5$ (Δ 1.8 mDa). Thus, R' and X had to be both H. The other fragment of m/z 70.0389 (fragment B) corresponded to $C_2H_4N_3$ (Δ 1.7 mDa), i.e., to the loss CH_2N_2R . Although exact mass can help us in order to have more confident data, this compound could be elucidated by using MS/MS without the use of the exact mass capability of the QTOF instrument (e.g. using a triple quadrupole instrument).

As regards compounds 18 and 19 (TPs of terbumeton), their nominal mass was m/z 240 and the exact masses of these compounds were determined to be m/z 240.1481 (retention time 9.6 min) and 240.1489 (retention time 20.6 min), respectively. The nominal mass increase observed for both compounds respect terbumeton was m/z 14, which could correspond to a N-methylation (+ CH_3-H) or an oxidation (+ $O-2H$), i.e., to an elemental composition of $C_{11}H_{22}N_5O$ (m/z 240.1824) or $C_{10}H_{18}N_5O_2$ (m/z 240.1461), respectively.

Table 1. Exact mass measurements. Mass errors and precision for proposed compounds^a

Proposed compound	ret time (min)	Elemental composition [M+H] ⁺	Mean measured mass	theoretical mass [M+H] ⁺	deviation (mDa)	n	mean deviation	RSD (mDa)
Terbutylazine								
1 (parent)	23.4	C ₉ H ₁₇ N ₅ Cl	230.1176	230.1172	0.4	13	1.5	0.9
2	20.1	C ₇ H ₁₃ N ₅ Cl	202.0856	202.0859	-0.3	12	1.2	1.0
3	13.3	C ₉ H ₁₈ N ₅ O	212.1511	212.1511	0.0	11	1.5	0.7
4	8.6	C ₇ H ₁₄ N ₅ O	184.1196	184.1198	-0.2	9	0.8	0.5
5	6.0	C ₉ H ₁₆ N ₅ O	226.1310	226.1304	0.6	4	1.2	0.6
Simazine								
6 (parent)	20.0	C ₇ H ₁₃ N ₅ Cl	202.0854	202.0859	-0.5	13	2.3	1.4
7	14.9	C ₅ H ₉ N ₅ Cl	174.0530	174.0546	-1.6	12	1.7	1.0
8	9.7	C ₇ H ₁₄ N ₅ O	184.1185	184.1198	-1.3	12	1.3	1.3
9	3.7	C ₅ H ₁₀ N ₅ O	156.0868	156.0885	-1.7	12	1.8	1.4
10	4.9	C ₇ H ₁₄ N ₅ O	184.1195	184.1198	-0.3	8	0.5	0.3
Terbutryn								
11 (parent)	23.3	C ₁₀ H ₂₀ N ₅ S	242.1447	242.1439	0.8	13	2.0	1.3
12	13.3	C ₉ H ₁₈ N ₅ O	212.1516	212.1511	0.5	12	1.4	1.1
13	16.0	C ₉ H ₁₈ N ₅	196.1558	196.1562	-0.4	11	2.0	1.7
14	11.7	C ₇ H ₁₄ N ₅	168.1228	168.1249	-2.1	10	2.1	0.7
Terbumeton								
15 (parent)	20.3	C ₁₀ H ₂₀ N ₅ O	226.1672	226.1668	0.4	13	1.7	0.8
16	17.1	C ₈ H ₁₆ N ₅ O	198.1369	198.1355	1.4	10	2.5	1.6
17	13.4	C ₉ H ₁₈ N ₅ O	212.1519	212.1511	0.8	10	1.5	0.8
18	9.6	C ₁₀ H ₁₈ N ₅ O	240.1481	240.1461	2.0	7	2.0	1.4
19	20.6	C ₁₀ H ₁₈ N ₅ O	240.1489	240.1461	2.8	7	2.8	0.9
20	21.7	C ₁₁ H ₂₂ N ₅ O	256.1803	256.1774	2.9	7	3.5	2.4

^a Deviation (mDa) = 1000 × (mean measured mass-theoretical mass) | mean deviation | = 1000 × [Σ | (measured mass)_i - (theoretical mass) | / n]
 where n indicates the number of findings over time for each compound

The MS/MS spectra of compound 18 showed two important fragments for this TP at m/z 210.1403 and 170.1062. The difference between m/z 240.1481 and 210.1403 corresponded to the loss of the OCH_3 group (therefore $X = \text{OCH}_3$). The fragment at 170.1061, corresponding to an elemental composition of $\text{C}_6\text{H}_{12}\text{N}_5\text{O}$ ($\Delta m = 1.9$ mDa), appeared in the MS/MS spectrum of the parent terbumeton and corresponded to the loss of R' . Therefore, and coinciding with terbumeton structure, R was an ethyl group in the fragment C (see Figure 2b). In the same way, R' was the difference between 240.1481 and 170.1061 which corresponded to an elemental composition of $\text{C}_4\text{H}_6\text{O}$ ($\Delta m = 0.1$ mDa). This difference evidenced an oxidation in the tertbutyl group with an aldehyde as the only possibility.

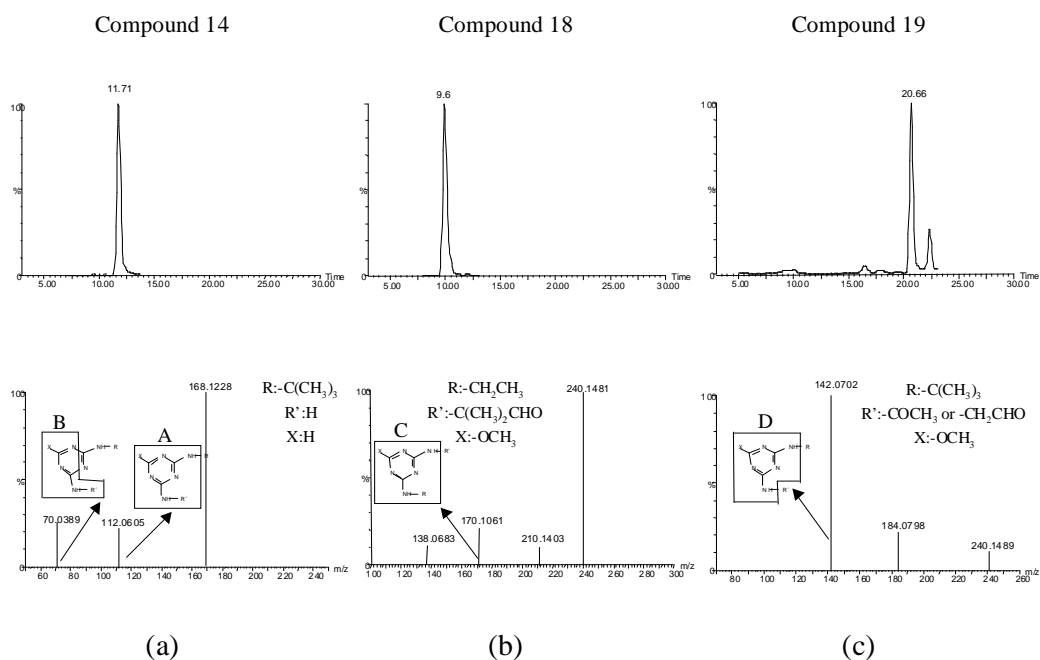


Figure 2. LC-QTOF chromatograms (top) and product ion spectra (bottom) of (a) compound 14 (a TP of terbutyryn), (b) compound 18 (a TP of terbumeton), and (c) compound 19 (a TP of terbumeton).

The MS/MS spectrum of compound 19 showed important fragments at m/z 184.0798 and 142.0702 (Figure 2c). The difference between m/z 240.1489 and 184.0798 corresponded to the loss of tert-butyl (thus, R was tert-butyl). The fragment D at m/z 142.0702, corresponding to an elemental composition of $\text{C}_4\text{H}_8\text{N}_5\text{O}$, also appeared in the MS/MS spectrum of terbumeton and corresponded to the loss of R and R' . Therefore, X had to be the same group as in terbumeton,

OCH₃. Thus, once X and R were elucidated, R' was easily calculated by difference, obtaining an elemental composition of C₂H₃O. However, we could not differentiate if the correct structure was an aldehyde or a ketone.

The possibility of structural elucidation of these compounds against other isobars, evidences the usefulness of exact mass measurements in the unequivocal identification of TPs in the environmental field.

In a similar way, all TPs were identified. Only two compounds showed two possible structures (compounds 10 and 19). All the identified TPs for triazines in water under experimental conditions were proposed for each parent compound (Figure 3).

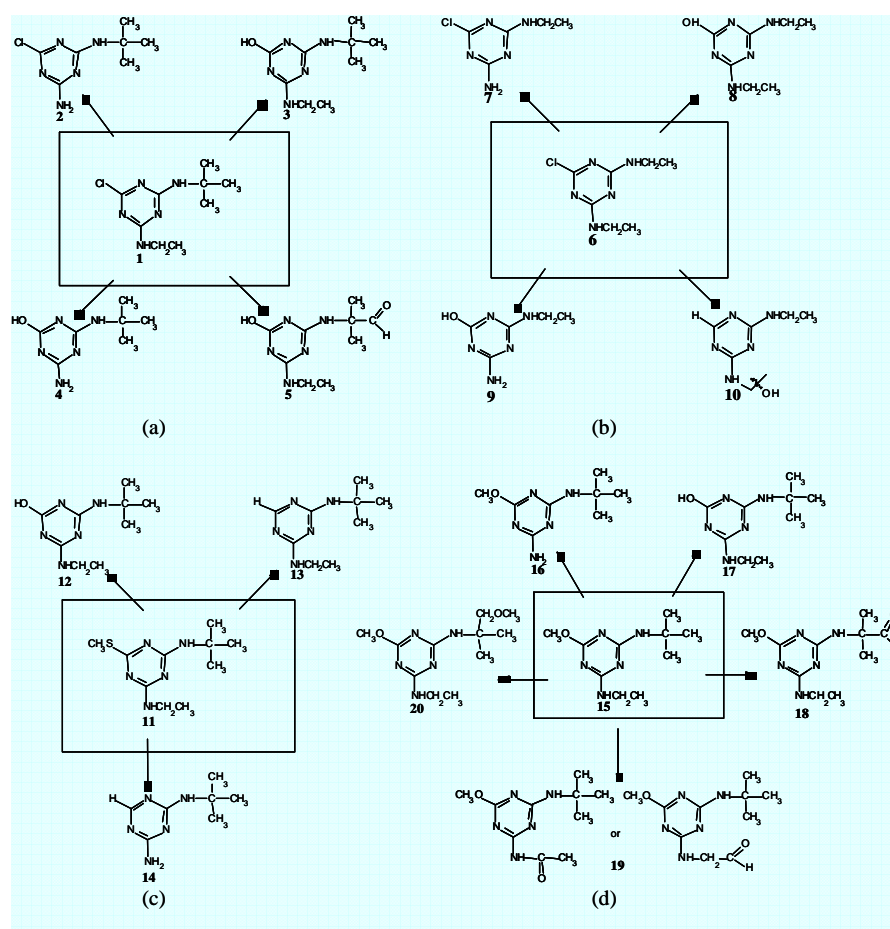


Figure 3. Identified TPs for (a) terbuthylazine, (b) simazine, (c) terbutryn, and (d) terbumeton in water under the experimental conditions applied in this paper

On the other hand, degradation curves were constructed for the four parent compounds and including the information on appearance of the most relevant TPs (Figure 4). The disappearance of terbuthylazine is reported in Figure 4a. Simultaneously to the decrease in concentration of this analyte, the formation of several peaks is observed, obtaining mainly dechlorination plus hydroxylation, deethylation or a combination of both processes. Compounds 2 and 3 were the main TPs, and the parent was degraded along one week ~50%. Simazine shows a photodegradation pattern similar to terbuthylazine: dechlorination plus hydroxylation, deethylation or a combination of both processes, leading to compounds 7 and 8 as the main TPs. Terbumeton exhibits a slower degradation rate, mainly throughout a deethylation process (compound 16). Conversely, the presence of a methylthio moiety in the terbutryn molecule highly increases the degradation rate obtaining two major TPs, the hydroxylation and the loss of the methylthio group (compounds 12 and 13, respectively).

Although Figure 3 only shows the main TPs with peak area higher than 2% of the total area, the high sensitivity of the QTOF allows us to detect and identify other less abundant TPs, such as demethylation of terbuthylazine, simazine and terbumeton or desethylation of terbutryn. An example is showed in Figure 5. The exact mass of a TP of terbutryn, with retention time 21.9 minutes, was determined to be 258.1422 ($C_{10}H_{20}N_5OS$, Δ 3.4 mDa), i.e., an increase of 15.9983 mDa (an atom of O) from terbutryn. The MS/MS spectra for this TP showed fragments at m/z 240.1283, 202.0682, 184.0635, 156.0870 and 138.0732. The last two fragments at m/z 138.0732 and m/z 156.0870 were also obtained when studying the fragmentation pathway of terbutryn and corresponded to the loss of R and, X plus R, respectively. Therefore, if R' = ethyl and X = OH, then R had to be tert-butyl group with a CH_3S substituent. However, this structure did not explain the other fragments at m/z 240.1283, 202.0682 and 184.0635. Studying these fragments, another structure was proposed (see Figure 5): a hydroxylated compound with $X = CH_3S$, $R' =$ ethyl and $R =$ tert-butyl, where the OH group was located in the N of the ethylamino group. This structure explained the fragments at m/z 240.1283 (due to the loss of water) and 202.0682 (loss of tert-butyl), as well as, the fragments at m/z 184.0635, 156.0870 and 138.0732.

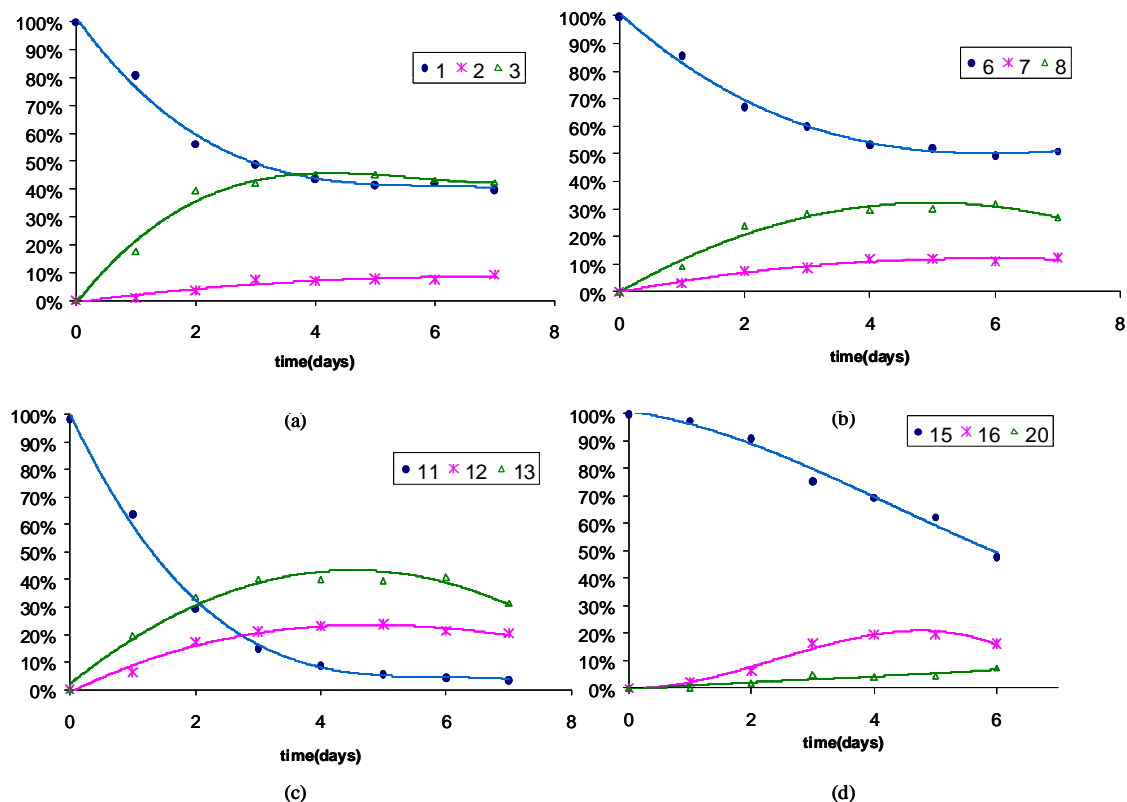


Figure 4. Degradation curves for (a) terbutylazine, (b) simazine, (c) terbutryn and, (d) terbumeton and some of their more relevant TPs

Triazine herbicides have been extensively used for weed control in recent years; thus, it would be worthful to study both parent compounds and degradation products in environmental waters. Although some of the TPs described in this paper have already been included in monitoring programmes, several TPs have not been searched for before. Therefore, it could be of interest to include some of the more relevant TPs found in our work (for example, TPs higher than 10%) in multiresidue methods, although the toxicological evaluation of these compounds would also be necessary in order to establish priorities in monitoring programmes. The methodology applied in this paper, based on irradiation of spiked water samples and direct injection on LC-QTOF MS, and consequently with very little sample manipulation, could be used for degradation studies of other relevant compounds than triazines. Photodegradation experiments using a different source (e.g. based on xenon lamps) and soil experiments would also be helpful in order

to better simulate the sunlight conditions and obtain more realistic results, predicting the behaviour of organic pollutants in the soil-water environment.

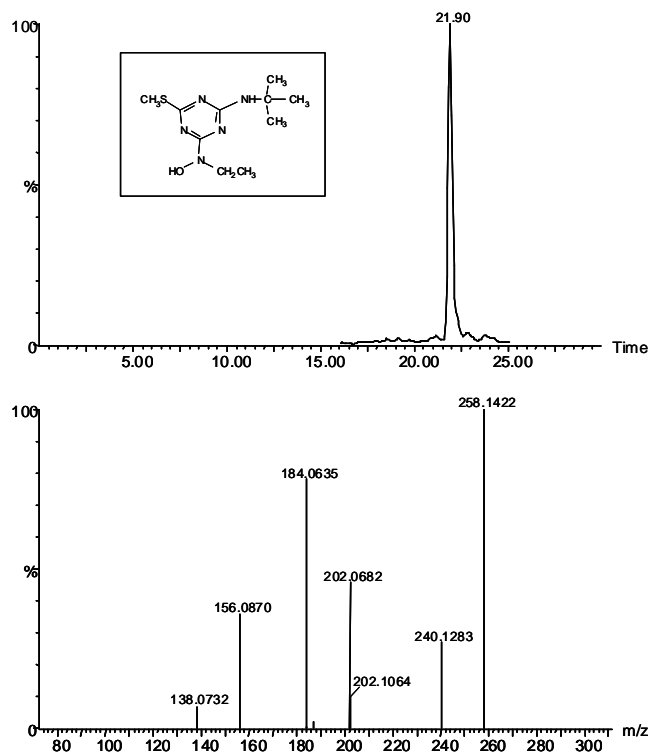


Figure 5. Product ion spectrum of a minor metabolite of terbutryn

CONCLUSION

This work has demonstrated that the use of an hybrid quadrupole time-of-flight mass spectrometer, combined with the MetaboLynx application manager originally designed for drug metabolism, is a valuable tool for the identification of transformation products (TPs) of pesticides. In this study, the widely used group of triazine herbicides was taken as model example. Some triazines TPs were commercially available and, therefore, we could get an additional confirmation using the chromatographic retention times. In those cases where no standards were available, the possibility of performing MS/MS experiences helped us to elucidate the structure of the TPs, as we obtained the product ion spectra with the exact mass of fragments. Additionally, the use of MS/MS allowed us to elucidate between isomeric metabolites that obviously exhibited equal exact mass. Regarding mass errors, and using post-column addition of lock mass, the mean deviations obtained generally remained below 2 mDa, except for minor metabolites. Finally, the high sensitivity attainable with the QTOF allowed us to detect even metabolites with only 2% of the total peak area.

The methodology followed in this paper, based on irradiation of spiked water samples and subsequent investigation of the compounds formed by using LC-QTOF MS, can be applied to other pesticides families or any other organic pollutants present in water. This is one of the most powerful approaches currently existing to investigate the formation of TPs and the presence of unknown compounds in environmental water.

ACKNOWLEDGEMENTS

The authors are very grateful to Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for use of the Q-TOF hybrid quadrupole time-of-flight mass spectrometer. This work forms a part of the research project "Aplicación del acoplamiento cromatografía líquida-espectrometría de masas (LC-MS/MS y LC-QTOF MS) a la identificación de metabolitos de plaguicidas en muestras de interés ambiental y toxicológico" (Ref: BQU2003-02685) developed under financial support of the Ministerio de Ciencia y Tecnología, Spain.

REFERENCES

- (1) Geerdink, R.B.; Kooistra-Sijpersma, A.; Tiesnitsch, J.; Kienhuis, P.G.M.; Brinkman, U.A.Th. *J.Chromatogr. A* **1999**, 863, 147-155
- (2) Thurman, E.M.; Ferrer, I.; Barcelo, D. *Anal. Chem.* **2001**, 73, 5441-5449
- (3) Di Corcia A.; Nazzari, M.; Rao, R.; Samperi, R.; Sebastiani, E. *J.Chromatogr. A* **2000**, 878, 87-98
- (4) Hernandez, F.; Sancho, J.V.; Pozo, O.; Lara, A.; Pitarch, E. *J. Chromatogr. A* **2001**, 939, 1-11
- (5) Di Corcia, A.; Crescenzi, C.; Samperi, R.; Scappaticcio, L. *Anal. Chem.* **1997**, 69, 2819
- (6) Pozo, O.J.; Pitarch, E.; Sancho, J.V.; Hernandez, F. *J. Chromatogr. A* **2001**, 923, 75-85
- (7) Yokley, R.A.; Mayer, L.C.; Huang, S.B.; Vargo, J.D. *Anal. Chem.* **2002**, 74, 3754-3759
- (8) Sanz-Asensio, J.; Plaza-Medina, M.; Martinez-Soria, M.T.; Perez-Clavijo, M. *J. Chromatogr. A* **1999**, 840, 235-247
- (9) Lanyi, K.; Dinya, Z. *Microchemical Journal* **2003**, 75, 1-14
- (10) Santos, T.C.; Rocha, J.C.; Barcelo, D. *J Chromatogr. A* **2000**, 879, 3-12
- (11) Steen, R.J.C.A.; Hogenboom, A.C.; Leonards, P.E.G.; Peerboom, R.A.L.; Cofino, W.P.; Brinkman, U.A.Th *J Chromatogr. A* **1999**, 857, 157-166
- (12) Hopfgartner, G.; Chernushevich, I.V.; Covey, T.; Plomley, J.B.; Bonner, R. *J. Am. Soc. Mass Spectrom.* **1999**, 10, 1305-1314.
- (13) Corcoran, O.; Nicholson, J.K.; Lenz, E.M.; Abou-Shakra, F. *Rapid Commun. Mass Spectrom.* **2000**, 14, 2377-2384.
- (14) Thurman, E.M.; Ferrer, I.; Parry, R. *J Chromatogr. A* **2002**, 957, 3-9
- (15) Maizels, M.; Budde, W.L. *Anal. Chem.* **2001**, 73, 5436-5440.
- (16) Hogenboom, A.C.; Niessen, W.M.A.; Brinkman, U.A.Th. *Rapid Commun. Mass Spectrom.* **2000**, 14, 1914-1924.
- (17) Bobeldijk, I.; Vissers, J.P.C.; Kearney, G.; Major, H.; van Leerdam, J.A. *J Chromatogr. A* **2001**, 929, 63-74.

Artículo científico 2

Anal. Bioanal. Chem. (2006) 384: 448-457

USE OF LIQUID CHROMATOGRAPHY QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY IN THE ELUCIDATION OF TRANSFORMATION PRODUCTS AND METABOLITES OF PESTICIDES. DIAZINON AS A CASE STUDY

María Ibáñez, Juan V. Sancho, Óscar J. Pozo, Félix Hernández

Research Institute for Pesticides and Water, University Jaume I, E-12071, Castellón, Spain

ABSTRACT

Liquid chromatography (LC) coupled to hybrid quadrupole time-of-flight (QTOF) mass spectrometry (MS) is a useful analytical tool in the elucidation and confirmation of transformation products (TPs)/metabolites of pesticides with a wide range of polarity, in both environmental and biological samples. Firstly, the versatility of LC allows the determination of very distinct TPs/metabolites as chromatographic conditions can be easily changed and optimized depending on the analytical problem. Secondly, the mass accuracy provided by the TOF analyser allows the assignment of a highly probable empirical formula for each compound and the differentiation between nominal isobaric compounds. Finally, the possibility of performing MS/MS spectra with accurate mass measurements can be used for the final characterization of the TPs/metabolites detected and for the differentiation of isomeric compounds. In this study, the insecticide diazinon has been used as model compound, and its photodegradation and metabolism have been investigated by LC-QTOF-MS. On one hand, environmental spiked water was irradiated with a mercury lamp for 9 days, sampling 3-mL aliquots approximately every 12 hours. On the other hand, both *in vitro* and *in vivo* metabolism experiments were carried out with different substrate concentrations and incubation times. After centrifugation, and protein precipitation in the *in vitro* and *in vivo* studies, 50- μ L aliquots of both environmental and biological samples were directly injected into the LC electrospray ionization QTOF system. The most important transformation processes were found to be hydrolysis of the ester moiety, hydroxylation in the aromatic ring or in one of the alkylic groups, oxidation of the sulfur atom on the P=S cleavage or a combination of these processes, with the highest number of compounds being found in the photodegradation study. Very polar compounds, such as diethyl phosphate and diethyl thiophosphate, were detected after direct injection of the aqueous sample, which was feasible owing to the characteristics of the LC. In MS mode, mass errors were below 3 mDa,

leading to an empirical formula for each compound. MS/MS spectra with accurate mass were used for the final elucidation of the compounds detected.

INTRODUCTION

Currently, there is growing interest in the degradation pathways of organic contaminants such as pesticides, drugs and pharmaceuticals. In the case of pesticides, the determination of transformation products (TPs) in the environment is necessary as some of them could present similar toxicity to or even higher than the parent compound. Besides, present regulations on drinking water also include TPs within the group of compounds to be monitored. Moreover, the widespread use of pesticides and the potential adverse health effects of pesticide exposure make metabolism studies necessary in order to identify biomarkers of pesticides (normally pesticides metabolites) which might be used for exposure control. The development of analytical methodology for the identification and quantification of pesticide TPs and metabolites is required to properly evaluate the presence and effects of pesticides in both environmental and biological samples.

Data on metabolite formation and on environmental behaviour have to be presented by the phytosanitary companies for the official approval and registration of pesticides. For example, study of the environmental fate implies knowledge of the chemical degradation processes in water and soil. Besides, it is also necessary to know the metabolic pathway of the pesticide in animals and plants [1]. However, most of these data have not been published and are difficult to find in the scientific literature.

One of the commonest approaches to estimate the TP formed in the environment is to study the hydrolytic and/or photolytic degradation of selected pesticides. In relation to metabolism studies, two approaches are the most commonly used: *in vivo* experiments, which allow us to know the metabolic fate in animals, or *in vitro* experiments, e.g. using human liver microsomes. A considerable number of papers can be found in the literature concerning pesticides degradation under environmental, *in vivo* and *in vitro* conditions. However, in most cases these studies focussed on the monitoring of the parent pesticide or a few (normally one or two) preselected metabolites. Conventional chromatographic techniques can be used in these cases to quantify the analytes, such as gas chromatography (GC)-electron capture detection, GC-nitrogen phosphorus detection or liquid chromatography (LC)-UV detection.

However, the elucidation of the metabolites and TPs formed requires the use of more powerful techniques, able to unequivocally confirm the identity of the compound, which in some cases is more difficult owing to the absence of commercial reference standards. The analytical

techniques normally applied for elucidation of pesticide metabolites and TPs are ^{13}C -NMR spectroscopy and mass spectrometry (MS; both LC-MS and GC-MS) using a variety of mass analysers. Within the environmental field, some authors have identified the TPs of selected pesticides by using LC-MS/MS [2-4], GC-MS [5-7] or GC-MS/MS [4]. Regarding pesticide metabolism, different techniques have been employed for the identification of the metabolites formed, such as ^{13}C NMR spectroscopy [8,9] or LC-MS/MS [8].

A useful identification tool in the elucidation of TPs/metabolites is the time-of-flight (TOF) mass spectrometer owing to the increased resolution (more than 5000 full width at half maximum, FWHM) and high mass accuracy of this analyser (less than 5ppm), together with its inherent high sensitivity in full-scan mode. LC-TOF MS has been successfully applied in metabolism studies within the biomedical field for the identification of pharmaceuticals and their metabolites and for the characterization of new drug candidates at the discovery stage [10-13]. However, this technique has scarcely been applied to the elucidation of pesticide TPs in biological and environmental applications mainly owing to its high acquisition cost, as it demonstrated by the limited number of references published. Thus, some authors have used LC-TOF MS for the identification of photodegradation products of alachlor [2,14], acetochlor [14] and diuron [15]. In these cases, the LC-TOF full-scan data were completed with MS/MS experiments using triple quadrupole [2] or ion trap [15] analysers, and also in-source collision-induced dissociation (CID) [2,14], for structure elucidation of TPs.

Recently, a new powerful identification tool has become available: the hybrid quadrupole TOF (QTOF) mass spectrometer. In addition to the improved characteristics of TOF instruments, they offer the possibility of performing MS/MS acquisitions to obtain product ion spectra with accurate mass, which is sometimes necessary to differentiate between structural isomers. All these characteristics made QTOF an excellent analyser to study the degradation, transformation and metabolism of organic pollutants at relevant levels. In the environmental field, only a few authors have studied the potential of QTOF for the identification of pesticide TPs. Kouloumbos et al [3] identified photocatalytic degradation products of diazinon in TiO_2 aqueous suspensions using GC-ion trap MS and LC-QTOF MS after liquid-liquid extraction with dichlorometane. Recently, our research group has studied the potential of QTOF for the identification of TPs of triazine herbicides after UV exposure [4]. In the biological field, the use of QTOF for the elucidation of pesticides and metabolites is even more limited, and to our knowledge, it has not been reported yet.

The aim of this paper is to show the potential of LC-QTOF MS as a valuable tool for the detection, identification and confirmation of TPs and metabolites of pesticides in laboratory experiments that allow the estimation of degradation under environmental, in vivo and in vitro conditions. The organophosphorus insecticide, diazinon [O,O-diethyl O-(2-isopropyl-6-methylpyrimidin-4-yl) thiophosphate] has been used as a representative example, although the methodology employed in this work could be applied to many other pesticides, as the majority of TPs and/or metabolites are more properly determined by LC-MS techniques, rather than by GC-MS.

EXPERIMENTAL

Reagents and chemicals

Diazinon (97.5%) reference standard was purchased from Ehrenstorfer (Augsburg, Germany). The name, chemical structure, elemental composition and exact mass of the protonated molecule for the model compound are shown in Figure 1. Technical diazinon (60% w/v) used for *in vivo* studies was purchased from Novartis Agro (now Syngenta, Spain). High-performance LC (HPLC) grade methanol, HPLC-grade acetone, analytical-grade formic acid, and sodium hydroxide were obtained from Scharlab (Barcelona, Spain). Tetrabutylammonium acetate (TBA, 97%) and sesame oil were obtained from Sigma (St Louis, MO, USA). HPLC-grade water was obtained by purifying demineralized water in a Nanopure II system (Barnstead, Newton, MA).

Human liver microsomes were obtained from Advancell (Barcelona, Spain). NADP, D-glucose 6-phosphate sodium salt and glucose D-phosphate dehydrogenase were purchased from Sigma. Potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4) were obtained from Scharlab and magnesium chloride from Fluka (Buchs, Switzerland).

Diazinon stock standard solution was prepared dissolving approximately 50 mg of powder, accurately weighted, in 100 mL of acetone to obtain a final concentration of approximately 500 $\mu\text{g/mL}$. The solutions used for the photodegradation study and for the *in vitro* studies were prepared by diluting this solution with HPLC-grade water. The solution used for the *in vivo* studies was prepared by dissolving 2 mL of technical product solution (60% w/v) in 8 mL of sesame oil.

Instrumentation

A Waters Alliance 2695 LC system (Waters, Milford, PA, USA) was interfaced to a hybrid QTOF mass spectrometer (QTOFI, Micromass, Manchester, UK) using an orthogonal Z-spray-electrospray interface. The LC separation was performed using an X-Terra C_{18} 5 μm , 2.1 mm \times 250 mm (Waters) at a flow rate of 200 $\mu\text{L/min}$. The mobile phase used was an aqueous 0.01% HCOOH /methanol-0.01% HCOOH gradient where the percentage of organic solvent was changed linearly as follows: 0 min., 5%; 20 min., 95%; 23 min., 95%; 24 min., 5%; 29 min., 5%. The separations were completed within 26 minutes.

The drying gas as well as the nebulizing gas was nitrogen, generated from pressurized air in a nitrogen generator from Peak Scientific (Inchinnan, UK). The nebulizer gas flow was set to approximately 15 L/h and the desolvation gas flow to 500-600 L/h. TOF-MS resolution was approximately 5000 (FWHM). MS data were acquired over the m/z range 60-600. The multichannel plate detector potential was set to 2700 V and to 2800 V in positive and negative electrospray ionization (ESI) modes, respectively.

For operation in MS/MS mode, the collision gas was argon (99.995%, Carburas Metalicos, Valencia, Spain) with a pressure of approximately 2.5×10^{-5} mbar in the collision cell. A cone voltage of 25 V and a capillary voltage of 3.5 kV were used in both positive and negative ionization modes. The interface temperature was set to 350°C and the source temperature to 120°C. Dwell times of 1 s/scan were chosen. A solvent delay of 8 minutes was selected to perform a cleanup using the built-in divert valve monitored by time-controlled contact closures using the Masslynx NT version.4.0 software. A suitable MS profile was used.

Calibration experiments were performed daily using a Model 11 single-syringe pump (Harvard, Holliston, MA, USA), directly connected to the interface. Calibration in both positive and negative ESI modes from 60 to 600 m/z was conducted with a mixture of 0.1M NaOH—10% HCOOH (50:50) diluted (1:25) with acetonitrile-water (80:20) at a flow rate of 10 $\mu\text{L}/\text{min}$.

A 1 $\mu\text{g}/\text{mL}$ solution of 3,5-diiodo-L-tyrosine in methanol, used as lock mass, was introduced after the column using a model 2150 isocratic HPLC pump from LKB (Bromma, Sweden) at a flow rate of 100 $\mu\text{L}/\text{min}$ by means of a PEEK T-union [4].

Degradation/Metabolism studies

Photodegradation

The irradiation procedure applied in this work has been previously used in our laboratory [12]. Briefly, irradiation was performed using a mercury vapour lamp with its main output at 254 nm. The 500-mL surface water samples were kept in glass beakers. The distance from the lamp to the surface of the samples was approximately 15 cm. Irradiation was carried out in a fume hood at room temperature over a period of approximately 193 h under constant stirring of the samples. Prior to the photolysis experiment, a 500-mL surface water sample was spiked at 500 ng/mL with diazinon, by adding 1 mL of 500 $\mu\text{g}/\text{mL}$ standard solution. Additionally, a 500-mL non-spiked water sample was irradiated under the same conditions and used as a control sample for all experiences. Before irradiation ($t=0$), a 3-mL water aliquot was analysed, and during irradiation 3-mL water samples were taken from the beakers at different time intervals, centrifuged for 10

minutes (Angular 6 centrifuge, Selecta, Barcelona, Spain) to remove possible solid particulate matter, and analysed by direct injection of 50 μ L of the supernatant into the LC-QTOF mass spectrometer. Protective glasses for personal protection were used in order to avoid UV exposure.

Human liver microsomes incubation (in vitro studies)

Firstly, the NADPH-generating system was prepared, consisting of 1 M $MgCl_2$, 100 mM phosphate buffer (pH = 7.4), 100 mM NADP, 500 mM glucose 6-phosphate and 1.5 IU glucose D-phosphate dehydrogenase (final volume 0.5 mL). After 10-min prewarming at 37°C in presence of microsomes, phosphate buffer (100 mM, pH 7.4) and the substrate at a final concentration of 1 μ g/mL, the incubation was initiated by addition of the NADPH-generating system. After 15, 30, 45, 60, 90, 120 and 150 min the reaction was stopped by addition of 0.5 mL of acetonitrile. Finally, samples were centrifuged and 50 μ L of the supernatant was directly injected into the LC-QTOF mass spectrometer.

Additionally, and for each time, a control sample (without microsomes) and a blank sample (without substrate) were prepared in order to differentiate between metabolites originating by the microsomes and possible metabolites or TPs originating from the incubation procedure.

In vivo studies

Male Sprague-Dawley rats, weighting 300-350 g, were purchased from Janvier Spain (Barcelona, Spain). Upon receipt, the animals were examined and found to be in good health. Before use, the animals were acclimated to the laboratory environment for one week. The rats were housed individually in polypropylene cages at 22 \pm 2°C with 12-h light-and-dark cycles. Standard laboratory rodent chow and tap water were provided *ad libitum* throughout the study.

Diazinon, as a solution in sesame oil, was administered to rats via intraperitoneal administration, in target doses of 1250 mg/kg and 125 mg/kg of body weight (dose volume of 10 mL/kg). The control animals were given an equal volume of vehicle by the same method.

At the specified time after dose administration (3, 9 and 24 hours), the rats were killed by decapitation under anaesthesia. Trunk blood (1.5 mL) was collected in vials containing heparin. Samples were centrifuged at 7500 rpm (5031g) for 5 minutes and plasma was frozen at -80°C. All experimental procedures complied with the European Community Council Directive (86/609/ECC) for the use of laboratory animal subjects.

Before analysis, protein precipitation was carried out with acetonitrile. After centrifugation in a high-frequency induction motor centrifuge (Centronic B.L., Selecta), 50 μ L of the supernatant was directly injected in the LC-QTOF mass spectrometer.

Data processing

For the determination of accurate mass, the experiments were acquired in continuum in both positive and negative ionization modes and afterwards processed by the application manager MetaboLynx version 4.0 (Micromass). MetaboLynx compares two LC-MS data files (one corresponding to the analyte sample and the other corresponding to the blank sample) and highlight differences due to the presence of new compounds, which could be attributed to transformation processes. For all compounds detected by MetaboLynx, the exact mass of protonated/deprotonated molecules was determined on the basis of averaged spectra obtained in the survey scan. Where abundant peaks were observed (more than 400 counts/s), the digital dead time correction algorithm could not correct the deviations obtained, and therefore, data were taken from peak tail, i.e., at lower signal intensity.

On the basis of the accurate mass, possible elemental compositions of the peaks of interest were calculated using the elemental composition calculator, within the MassLynx software, with a maximum deviation of 5 mDa from the measured exact mass. The parameter settings were C 0-15, H 0-50, N 0-5, O 0-8, S 0-1 and P 0-1. The applied double-bond equivalent filter was set between -0.5 and 10. The maximum and minimum parameters were restricted as a function of the structure of diazinon.

The possibility of performing MS/MS experiments helped us to elucidate the structure of several transformation products as we obtained a product ion spectrum with the exact mass of fragments. In this case, the exact mass of the precursor ion was used as a lock mass.

RESULTS AND DISCUSSION

Metabolites and photodegradation products identified

The most important processes found to occur in the degradation/metabolism of diazinon were **hydrolysis** of the ester moiety, **hydroxylation** and **oxidation**, as can be seen in Figure 1. A combination of these processes was also observed.

The **hydrolysis** of the ester moiety gave 2-isopropyl-6-methyl-4-pyrimidin-4-ol (IMP, compound 6) and diethyl thiophosphate (DETP, compound 9). In the *in vitro* study, although IMP was detected, no DETP was found, possibly owing to the low sensitivity for alkylphosphates in electrospray interfaces. However both, IMP and DETP were detected in the *in vivo* study. Moreover, diethyl phosphate (DEP, compound 3) was also found, which could result from the hydrolysis of diazoxon (compound 2) or from the oxidation of DETP. In the photodegradation study, only IMP and DEP were detected, possibly owing to the oxidative conditions of these experiments, which favoured the oxidation previous to the hydrolysis.

As can be deduced from Figure 1, **hydroxylation** can occur in the aromatic ring or in one of the alkyl groups. In both *in vivo* and *in vitro* studies, the hydroxylation was only observed in the isopropyl group (1-hydroxy isopropyl diazinon, compound 7) meanwhile hydroxylation occurred in both the isopropyl group and the aromatic ring (compounds 5, 7 and 8) in the photodegradation study.

Finally, the **oxidation** of the sulfur atom on the P=S cleavage was observed. Although the oxidation products diazoxon and 1-hydroxy isopropyl diazoxon (compound 4) were only identified in the photodegradation study, the occurrence of DEP (compound 3) in the *in vivo* studies indicated that this metabolic pathway may also occur in rats.

A combination of several processes was observed in some cases, such as hydroxylation plus oxidation (1-hydroxy isopropyl diazoxon) or hydroxylation plus hydrolysis (compound 10).

Demethylation was only observed in the photodegradation study for 1-hydroxy isopropyl diazinon (compound 7) to give compound 8.

These results are consistent with others previously reported for environmental [3, 16-18] and biological [19-21] samples. However, to our knowledge, compounds 5 and 10 have not been previously reported in the bibliography.

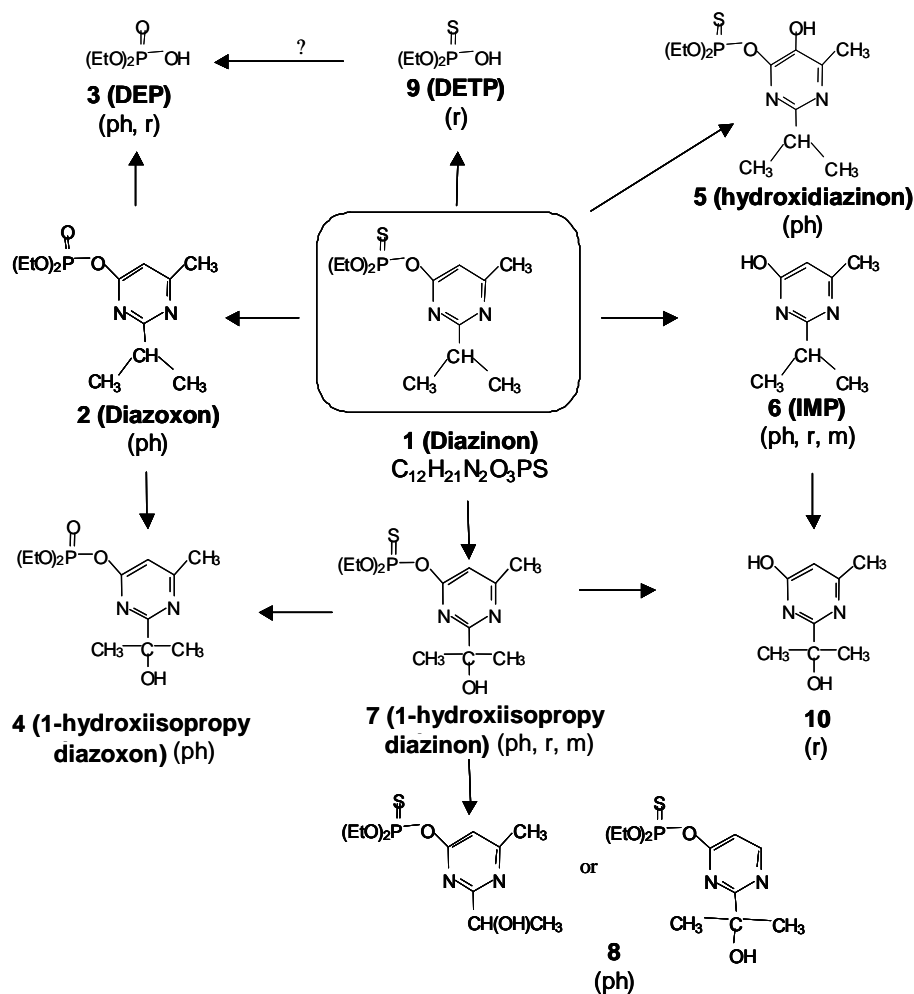


Figure 1. Transformation products (TPs)/metabolites identified for diazinon and proposed degradation pathways. (ph) TPs detected in the photodegradation experiment, (r) metabolites detected in *in vivo* experiments with rats and (m) metabolites detected in *in vitro* experiments with microsomes.

Photodegradation curves for diazinon and some of its TPs are shown in Figure 2, where separate signal normalization of each product to its maximum value was used and is represented as a function of irradiation time. This approach was used owing to the unavailability of standards for all TPs detected as well as because of the low linear dynamic range of QTOF instruments, which made difficult the correct quantitation even when reference standards were available. As depicted in Figure 2, simultaneously to the disappearance of diazinon the formation of several peaks is observed, giving oxidated and hydroxylated products mainly. Compounds 2, 5 and 7

were the main phototransformation products found. Under the experimental conditions of this work, parent diazinon was degraded in 1 week down to about 40%.

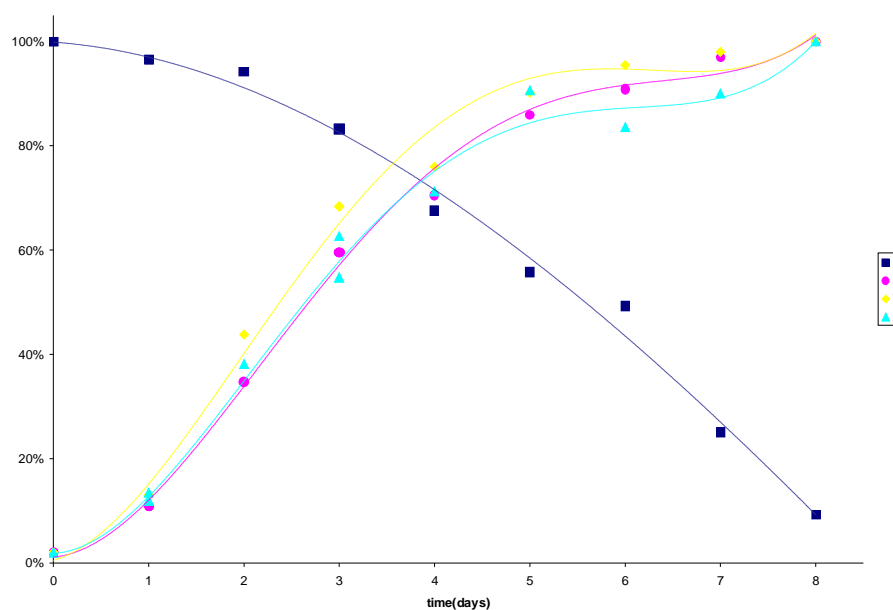


Figure 2. Degradation curves for diazinon (1) and some of its more relevant transformation products: (5, 7, 2)

LC considerations

Although GC has been used to determine pesticides and their TPs, LC is found to be more adequate as the majority of the TPs are more polar than their parent pesticides, less volatile and often also thermolabile. Besides, the feasibility of injecting directly an aqueous sample (such as water or plasma) into the LC column minimizes the risk of losses for TPs that could occur during the sample pretreatment, providing that sensitivity is sufficient for the objectives pursued. Otherwise, a preconcentration step would be required. Moreover, chromatographic conditions can be easily changed to obtain a better chromatography when required.

These advantages were observed in this work, where the inadequate chromatographic compounds detected by MetaboLynx at m/z 169.0 and 153.0 in the negative ionization mode (Figure 3a), prevented us from confirm that they were diazinon metabolites. Although the accurate masses obtained were in accordance with the exact masses of DEP and DETP, these types of broad peaks might also have been produced by the effect of the mobile-phase

gradient. These two alkylphosphates and other similar compounds were previously studied in our laboratory [22-23], and we proved the need to add an ion-pairing reagent into the mobile phase and/or the sample vial in order to obtain an adequate chromatographic behaviour. Thus, after adding TBA into the vial as the ion-pairing reagent, satisfactory chromatography was obtained (Figure 3b), confirming the presence of the metabolite DEP, although a decrease in sensitivity was observed. This example shows the potential of LC for the determination of polar metabolites, as the experimental conditions can be easily and efficiently modified depending on the analytical problem to be solved. Additionally, the possibility of injecting directly aqueous samples can be pointed out, which allowed us to determine metabolites as polar as alkylphosphates, which would have been lost by conventional sample pretreatment procedures such as solid-phase extraction or liquid-liquid extraction. In fact, these compounds were not reported by Koulombous et al [3] when studying the degradation of the diazinon by LC-MS, possibly owing to the loss of these polar compounds after extraction with dichloromethane. But, even if these metabolites were recovered during the sample treatment, their determination by GC-based methods would require a derivatization step, which would increase analysis time.

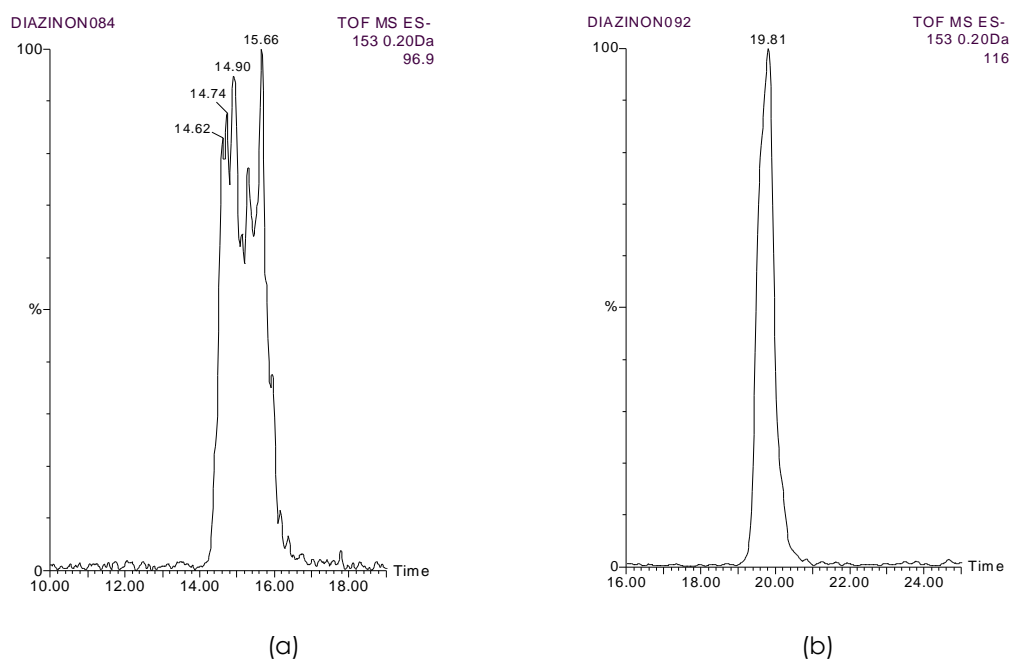


Figure 3. Liquid chromatography (LC)-mass spectrometry (MS) chromatograms (mass window 0.2 Da) for diethyl phosphate (DEP) (a) without tetrabutylammonium acetate (TBA) in the sample vial and (b) with 4mM TBA in the sample vial

TOF MS considerations

Undoubtedly, MS is one of the best techniques for the elucidation of metabolites owing to its sensitivity, universality and the sample information provided. Although quadrupole instruments can be a useful tool for the determination of the elemental composition, these instruments present an important drawback when dealing with isobaric compounds. The low resolution of these analysers avoids the differentiation of compounds with m/z differing less than 1 Da. Although this limitation could be minimized with low-resolution MS/MS instruments, the use of TOF analysers allows an easier and more confident estimation of their elemental composition owing to the high resolution and mass accuracy power. Moreover, the sensitivity achieved by TOF instruments in scan mode is superior than that of quadrupoles, facilitating the detection of less abundant metabolites.

In this work, the potential of TOF MS was proved in the differentiation of compounds 1 and 4. Both compounds have the same nominal mass (m/z 305) and, therefore, they were indistinguishable by quadrupole instruments. When studying the exact mass of both compounds (m/z 305.1266 for compound 4 and m/z 305.1089 for compound 1), we obtained a difference of 17.7 mDa, corresponding to the elemental composition $C_{12}H_{22}N_2O_5P$, for compound 4, and $C_{12}H_{22}N_2O_3PS$, for compound 1. This mass difference was found to be sufficient, as when applying the elemental composition calculator with the selected parameters (maximum deviation 5 mDa, see "Experimental" section) only one hit appeared, allowing us to assign the elemental composition to each of these compounds.

The results obtained in TOF experiments are summarized in Table 1. This table shows the parent compound and the nine TPs detected. When accurate mass was calculated by averaging the mass of all the products detected, deviations lower than 2 mDa were obtained for every compound except for compounds 1, 7 and 10, but still with deviations lower than 3 mDa. With these results, one can be confident about the elemental composition given for each analyte.

Table 1. TPs/metabolites identified for diazinon. Exact mass measurements and mass errors for the proposed compounds

Proposed compound	ret time (min)	Ionisation mode	Elemental composition [M+H] ⁺ /[M-H] ⁻	theoretical mass [M+H] ⁺ /[M-H] ⁻	Deviation (mDa)		
					Photodegradation ^a	In vitro ^b	In vivo ^a
1 (parent)	25.3	ESI+	C ₁₂ H ₂₂ N ₂ O ₃ PS	305.1089	-0.8	2.6	1.0
2	22.3	ESI+	C ₁₂ H ₂₂ N ₂ O ₄ P	289.1317	-0.9	-	-
3	11.9	ESI-	C ₄ H ₁₀ O ₄ P	153.0317	-0.3	-1.1	-
4	20.3.	ESI+	C ₁₂ H ₂₂ N ₂ O ₅ P	305.1266	0.1	-	-
5	23.1	ESI+	C ₁₂ H ₂₂ N ₂ O ₄ PS	321.1038	0.3	-	-
		ESI-	C ₁₂ H ₂₀ N ₂ O ₄ PS	319.0882	-1.0	-	-
6	13.7	ESI+	C ₈ H ₁₃ N ₂ O	153.1028	-0.2	-2.0	1.5
		ESI-	C ₈ H ₁₁ N ₂ O	151.0872	-	-1.0	-
7	23.7	ESI+	C ₁₂ H ₂₂ N ₂ O ₄ P	321.1038	2.7	0.4	-0.5
8	22.3	ESI+	C ₁₁ H ₂₀ N ₂ O ₄ PS	307.0881	-0.4	-	-
9	24.5	ESI-	C ₄ H ₁₀ O ₄ PS	169.0089	-	-1.0	-
10	12.9	ESI+	C ₈ H ₁₃ N ₂ O ₂	169.0977	-	2.9	-
		ESI-	C ₈ H ₁₁ N ₂ O ₂	167.0821	-	-0.6	-

^a Data obtained after 24 hours of exposition^b Data obtained after 2.5 hours of incubation

QTOF MS considerations

Despite the capability of TOF analysers to distinguish isobaric compounds (mass differences lower than 1Da), their usefulness is limited when dealing with isomers, as they present the same molecular composition and, consequently, the same exact mass. However, hybrid QTOF instruments give the possibility of performing MS/MS acquisitions to obtain product ion spectra with accurate mass, which can help to fully differentiate between isomeric analytes in a more confident way than when using low-resolution MS/MS instruments.

Thus, although the calculated composition for the compound 4 ($C_{12}H_{22}N_2O_5P$) suggested this compound to be a monohydroxylated product of diazoxon, with these data it is not feasible to know whether the TP corresponded to: a) hydroxylation of the aromatic methyl group (site 1 in Figure 4), b) hydroxylation of the ethyl group of the thiophosphoric part (site 2 in Figure 4), c) hydroxylation of the primary or secondary carbon atoms of the isopropyl group (sites 3 and 4 in Figure 4, respectively), or d) hydroxylation of the aromatic group (site 5 in Figure 4). In order to elucidate this metabolite, MS/MS experiments were carried out.

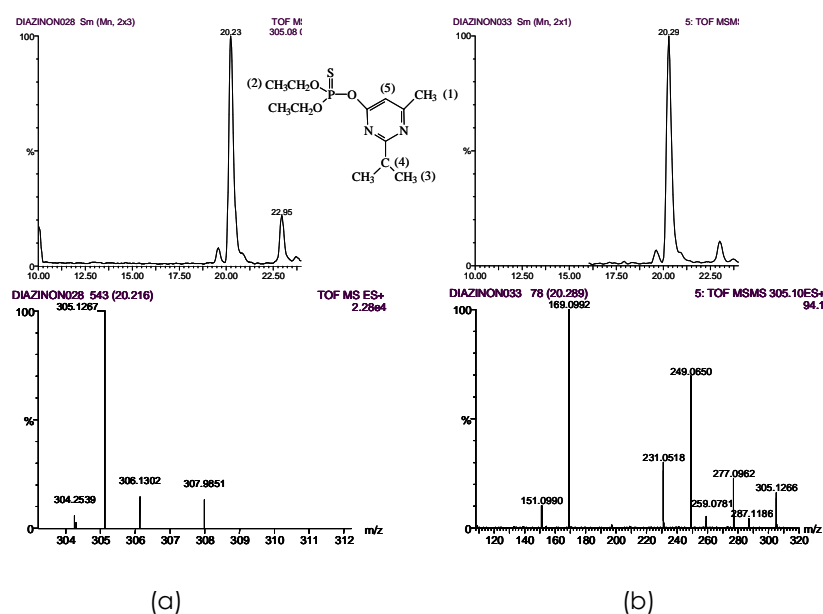


Figure 4. Elucidation of compound 4 (retention time 20.2-20.3 min): (a) LC-TOF MS chromatogram (top) and positive ion electrospray full scan mass spectrum (bottom). (b) LC-TOF MS/MS chromatogram (top) and positive ion electrospray product ion spectra (bottom). (1-5) Possible hydroxylation sites.

The product ion spectra of compound 4 (Figure 4b) showed a fragment at m/z 287.1186 ($\Delta mDa=2.5$), corresponding to the loss of water from the precursor ion m/z 305.1266 (Table 2. Note that the mass fragments shown in this table are theoretical masses), showing that the hydroxylation could not occur either in the aromatic or in the methyl group, owing to the absence of α hydrogens (sites 1 and 5 in Figure 4). This fact was corroborated in the MS/MS spectra of compound 5 (Table 2), where no loss of water was observed. The abundant fragment at m/z 169 might be obtained after the loss of the intact diethyl hydrogen phosphonate part (169.0992, $C_8H_{13}N_2O_2$), suggesting that oxidation took place on the isopropyl group of the pyridine moiety of the molecule (sites 3 and 4 in Figure 4), or after the loss of the alkylated aromatic ring (169.0266, $C_4H_{10}O_5P$), suggesting the possibility of hydroxylation in the phosphoric part (site 2 in Figure 4). Using a low-resolution MS/MS instrument it would not be feasible to distinguish where the hydroxylation took place. However, with the QTOF we observed a fragment at m/z 169.0977, that differed only 1.5 mDa from $C_8H_{13}N_2O_2$ (m/z 169.0992) and 76.6 mDa from $C_4H_{10}O_5P$ (169.0266), showing that the hydroxylation occurred in the isopropyl group. Nevertheless, in spite of these data we could not distinguish whether the hydroxylation had taken place in the primary or in the secondary carbon atoms of the isopropyl group (sites 3 or 4 in Figure 4, respectively). Kouloumbus et al [3] observed an ion of m/z 275 corresponding to the loss of a formaldehyde molecule from the protonated molecule in the MS/MS spectra of the 2-hydroxyisopropyl diazoxon. However, in our work this fragment was not observed, suggesting that hydroxylation occurred on the secondary carbon atom of the isopropyl group of diazinon. Compound 4 was identified as 1-hydroxyisopropyl diazoxon. The other fragments observed were m/z 277.0962 ($\Delta mDa=0.9$), corresponding to the loss of an ethylene group, m/z 249.0650 ($\Delta mDa= 1.0$) corresponding to the loss of two ethylene groups, and m/z 259.0781 ($\Delta mDa=-6.7$), 231.0518 ($\Delta mDa=-1.7$) and 151.0990 ($\Delta mDa=1.2$) corresponding to losses of water from fragments at m/z 277.0962, 249.0650 and m/z 169.0992, respectively. The identity of this compound could not be further validated, owing to the unavailability of a reference standard. In a similar way, all TPs of diazinon obtained under the different experimental conditions assayed were identified (Figure 1).

As can be seen in Table 1, both positive and negative ionization modes were used in this work. Acquisition in negative mode was required for detection of alkylphosphates (compounds 3 and 9), while for other compounds (compounds 5, 6 and 10) acquisition in both modes gave complementary information for a better assignment of the structure.

In spite of the evident advantages of QTOF-MS shown in this paper, there is a limitation that should be taken into account, and it is derived from the limited understanding of the fragmentation rules in MS/MS of (de)protonated molecules. In some cases, especially when dealing with poor MS/MS spectra, the unequivocal elucidation of the structure of a compound is

not possible. For example, we could not distinguish where the demethylation process had occurred in compound 8 (see Figure 1). In these cases, if standards are commercially available, both the retention time and MS/MS data can be used as a confirmatory tool. However, when reference standards are not available and their synthesis is not feasible, the combination of several techniques may help in this difficult task. For example, since the commercial introduction of LC-NMR systems, this hyphenated technique has been mainly applied to the structural elucidation of metabolites in drug development studies and to the identification of natural compounds in plant extracts, giving optimum structural information when both LC-MS and LC-NMR are used together. However, LC-NMR presents poor sensitivity, generally three orders of magnitude worse than LC-MS, which limits its applications in some cases. Another complementary tool that might be useful in the elucidation of the structure of potential TPs/metabolites is GC-electron impact (EI)-MS. In this case, the use of an EI source would allow the measurement of non-ESI ionizable compounds followed by an easier identification using spectra matching in commercial libraries, provided that TP/metabolite spectra are available.

Table 2. Transformation products/metabolites identified in the degradation studies of diazinon. Mass fragments and mass errors for the proposed compounds obtained by LC-ESI-QTOF MS

Compound	Ionisation mode	precursor ion-	theoretical mass [M+H] ⁺ /[M-H] ⁻	Deviation (mDa) photodegradation ^a	Deviation (mDa) <i>In vivo</i> ^a
1 (parent)	ESI+	305.1089	169.0799	1.9	-1.3
			153.1028	1.9	-0.8
			124.9826	2.4	0.1
			114.9619	0.4	0.3
2	ESI+	289.1317	261.1004	0.1	
			233.0691	-2.6	
			153.1028	1.2	
			135.0922	0.8	
3	ESI-	153.0317	125.0004	-0.4	-7.6
			78.9585	0.8	-1.1
4	ESI+	305.1266	287.1161	2.5	
			277.0953	0.9	
			259.0848	-6.7	
			249.0640	1.0	
			231.0535	-1.7	
			169.0977	1.5	
5	ESI+	321.1038	293.0725	-1.2	
			185.0749	2.0	
			169.0977	2.2	
			151.0871	1.2	
	ESI-	319.0882	291.0568	2.8	
			229.0412	-4.0	
			183.0592	-1.1	
			169.0088	1.4	
			167.0821	-0.1	

Table 2 (cont). Transformation products/metabolites identified in the degradation studies of diazinon. Mass fragments and mass errors for the proposed compounds obtained by LC-ESI-QTOF MS

Compound	Ionisation mode	precursor ion-	theoretical mass [M+H] ⁺ /[M-H] ⁻	Deviation (mDa) photodegradation ^a	Deviation (mDa) <i>In vivo</i> ^b
6	ESI+	153.1028	137.0715	1.6	0.3
			84.0575	-5.2	0.8
	ESI-	151.0872	135.0558		-0.7
			123.0558		1.8
			109.0402		0.6
			93.0092		0.3
		82.0293		0.2	
7	ESI+	321.1038	303.0932	0.5	-2.3
			275.0619	-1.0	-3.5
			247.0306	-1.8	-5.1
			185.0749	-2.3	-9.3
			169.0977	0.7	-0.5
			167.0643	-0.7	-6.5
			151.0871	0.5	-6.5
8	ESI+	307.0881	289.0776	0.9	
			261.0463	0.4	
			233.0150	-2.9	
			171.0592	1.6	
			153.0486	0.6	
			137.0715	1.0	
9	ESI-	169.0089	140.9775		0.5
			94.9357		-0.5
10	ESI+	169.0977	151.0871		0.7
			84.0449		6.1
	ESI-	167.0821	109.0402		-0.2

^a Data obtained after 24 hours of exposition

CONCLUSION

This work demonstrates that LC coupled to hybrid QTOF MS is a powerful analytical tool for the elucidation of metabolites and TPs of selected pesticides. The versatility of LC, together with the high mass accuracy, good sensitivity and possibility of performing MS/MS experiments with a QTOF instrument allows the detection and identification of TPs/metabolites after direct injection of aqueous sample extracts, without the need of laborious sample pretreatment, minimizing possible losses of analytes in the analytical procedure. However, this approach presents some limitations, as the interface selected restricts the application to those compounds ionizable with this interface, normally from medium polarity to ionic. Moreover, the achievement of information-rich product ion spectra becomes a relevant factor in the elucidation process on some occasions.

The main TPs identified in the photodegradation study of diazinon were IMP and two TPs produced by hydroxylation of the isopropyl group of both diazinon (1-hydroxyisopropyl diazinon) and its oxygen analogue diazoxon (1-hydroxyisopropyl diazoxon). As experiments were carried out under oxidative conditions, it would be interesting to extend the photodegradation experiments using a different source (e.g. xenon lamps) in order to better simulate sunlight conditions and to obtain more realistic results. Regarding *in vitro* studies, despite the use of different substrate concentrations and incubation times, only two metabolites, corresponding to hydrolysis (IMP) and hydroxylation (1-hydroxyisopropyl diazinon) processes were observed. Metabolism studies were completed by performing *in vivo* experiments, which led to the formation of metabolites corresponding to hydrolysis and hydroxylation, as well as oxidation of the diazinon.

Because diazinon metabolites/TPs are more polar than the parent compound, they must be more water-soluble and possibly more mobile and might have greater potential for leaching in the soil-water environment. Therefore, they might probably be found in surface and ground water at higher concentrations than the parent compound. It would be interesting to investigate within the water monitoring programmes the most relevant TPs found in the photodegradation study: diazoxon (compound 2), 1-hydroxyisopropyl diazinon (compound 7) and hydroxydiazinon (compound 5). In the case of human biofluids, IMP (compound 6) and 1-hydroxyisopropyl diazinon (compound 7) seem to be adequate biomarkers for evaluation of human exposure to this pesticide.

ACKNOWLEDGEMENTS

The authors are very grateful to Serveis Centrals d'Instrumentació Científica (SCIC) for use of the Q-TOF hybrid quadrupole time-of-flight mass spectrometer and to Marta Miquel and Carlos Aragonés from the Psicobiology Department, University Jaume I, for their excellent technical assistance in the *in vivo* studies.

This work forms part of the research project "Aplicación del acoplamiento cromatografía líquida-espectrometría de masas (LC-MS/MS y LC-QTOF MS) a la identificación de metabolitos de plaguicidas en muestras de interés ambiental y toxicológico"(Ref: BQU2003-02685) developed with financial support of the Ministerio de Ciencia y Tecnología, Spain.

M.I. is very grateful to Generalitat Valenciana for her pre-doctoral grant.

REFERENCES

- (1) Roberts T (1998) (ed) *Metabolic Pathways of Agrochemicals* (parts one and two). The Royal Society of Chemistry, Cambridge, UK
- (2) Hogenboom AC, Niessen WMA, Brinkman, UATH (2000) *Rapid Commun Mass Spectrom* 14: 1914-1924
- (3) Koulombos VN, Tsipi DF, Hiskia AE, Nikolic D, van Breemen RB (2003) *J Am Soc Mass Spectrom* 14: 803-817
- (4) Ibañez M, Sancho JV, Pozo OJ, Hernández F (2004) *Anal Chem* 76:1328-1335
- (5) Bandala ER, Gelover S, Leal MT, Arancibia-Bulnes C, Jiménez A, Estrada CA (2002) *Catal Today* 76: 189-199
- (6) Sakkas VA, Labropoulou DA, Sakellarides TM, Albanis TA (2002) *Anal Chim Acta* 467: 233-243
- (7) Konstantinou K, Sakkas VA, Albanis TA (2001) *Appl Catal B-Environ* 34: 227-239
- (8) Nagahori N, Yoshino H, Tomighara Y, Isobe N, Kaneko H, Nakatsuka I (2000) *J Agric Food Chem* 48: 5754-5759
- (9) Levsen K, Schiebel HM, Behnke B, Dotzer R, Dreher W, Elend M, Thiele H (2005) *J. Chromatogr. A* 1067: 55-72
- (10) Hongwei Z, Henion J, Yang Y, Spooner N (2000) *Anal Chem* 72: 3342-3348
- (11) Hopfgartner G, Chernushevich IV, Covey T, Plomley JB, Bonner R (1999) *J Am Soc Mass Spectrom* 10: 1305-1314
- (12) Corcoran O, Nicholson JK, Lenz EM, Abou-Shakra F, Castro-Perez J, Sage AB, Wilson ID (2000) *Rapid Commun Mass Spectrom* 14: 2377-2384
- (13) Zhang N, Fountain ST (2000) *Anal Chem* 72: 800-806
- (14) Thurman EM, Ferrer I, Parry R (2002) *J Chromatogr A* 957:3-9

- (15) Malato S, Albanis T, Piedra L, Agüera A, Hernando D, Fernandez-Alba A (2003) Chapter [5] in *Liquid Chromatography/Mass Spectrometry, MS/MS and Time of Flight MS: Analysis of Emerging Contaminants*; Ferrer I and Thurman M (eds), ACS Symposium series 850; American Chemical Society, Washington, DC 66-95
- (16) Sakkas VA, Dimou A, Pitarakis K, Mantis G, Albanis T (2005) *Environ. Chem. Lett.* DOI: 10.1007/s10311-004-0091-6
- (17) Bavcon M, Trebse P, Zupancic-Kralj (2003) *Chemosphere* 50: 595-601
- (18) Trebse P, Arcon I (2003) *Radiat. Phys. Chem.* 67: 527-530
- (19) Poet TS, Wu H, Kousba AA, Timchalk (2003) *Toxicol. Sci.* 72: 193-200
- (20) Abu-Qare A, Abou-Donia M.B. (2001) *Fresenius J Anal Chem* 370: 403-407
- (21) Sams C, Cocker J, Lennard MS (2004) *Toxicol. Lett. Teil SUPPL S.* s146
- (22) Hernández F, Sancho JV; Pozo OJ (2002) *Rapid Commun Mass Spectrom* 16:1766-1773
- (23) Hernández F, Sancho JV, Pozo OJ, Villaplana C, Ibáñez M, Grimalt S (2003) *J AOAC Int* 86: 832-838

2.3.3 Bibliografía

Barceló D, Hennion MC

Trace determination of pesticides and their transformation products in water
Techniques and Instrumentation in Analytical Chemistry, Vol 19, Elsevier (1997)

Bateman RH, Carruthers R, Hoyes JB, Jones C, Langridge JI, Millar A, Vissers JPC

A novel precursor ion discovery method on a hybrid quadrupole orthogonal acceleration time-of-flight (Q-TOF) mass spectrometer for studying protein phosphorylation
J. Am. Soc. Mass Spectrom. 13 (2002) 792-803.

Belfroid AC, van Drunen M, Beek MA, Schrap SM, van Gestel CAM, van Hattum B

Relative risks of transformation products of pesticides for aquatic ecosystems
Sci. Total Environ. 22 (1998) 167-183

Borchers C, Parker CE, Deterding LJ, Tomer KB

Preliminary comparison of precursor scans and liquid chromatography-tandem mass spectrometry on a hybrid quadrupole time-of-flight mass spectrometer.
J. Chromatogr. A 854 (1999) 119-130.

Detomaso A, Mascolo G, López A

Characterization of carbofuran photodegradation by-products by liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry
Rapid Commun. Mass Spectrom. 19 (2005) 2193-2202.

Drożdżewska K, Książczak A, Boniuk H

Thermal degradation of triazine herbicides substituted by Cl.
Thermochim. Acta 424 (2004) 123-129

Geerdink RB, Niessen WMA, Brinkman UATH

Trace-level determination of pesticides in water by means of liquid and gas chromatography.
J. Chromatogr. A 970 (2002) 65-93

Hogenboom AC, Niessen WMA, Little D, Brinkman UATH

Accurate mass determinations for the confirmation and identification of organic microcontaminants in surface water using on-line solid-phase extraction liquid chromatography electrospray orthogonal-acceleration time-of-flight mass spectrometry
Rapid Commun. Mass Spectrom. 13 (1999) 125-133

- Hogenboom AC, Niessen WMA, Little D, Brinkman UATH
Characterization of photodegradation products of alachlor in water by on-line solid-phase extraction liquid chromatography combined with tandem mass spectrometry and orthogonal-acceleration time-of-flight mass spectrometry
Rapid Commun. Mass Spectrom. 14 (2000) 1914-1929
- Kolpin DW, Thurman EM, Linhart SM
The environmental occurrence of herbicides: the importance of degradates in ground water
Arch. Environ. Contam. Toxicol. 35 (1998) 385-390
- Kolpin DW, Thurman EM, Linhart SM
Finding minimal herbicide concentrations in ground water? Try looking for their degradates
Sci. Total Environ. 248, (2000) 115-122
- Kolpin DW, Thurman EM, Linhart SM
Occurrence of cyanazine compounds in groundwater: Degradates more prevalent than the parent compound
Environ. Sci. Technol. 35 (2001) 1217-1222
- Kolpin DW, Schnoebelen D.J., Linhart SM
Degradates provide insight to spatial and temporal trends of herbicides in ground water
Ground Water 42 (2004) 601-608
- Kouloumbos VN, Tsiipi DF, Hiskia AE, Nikolic D, van Breemen RB
Identification of photocatalytic degradation products of diazinon in TiO₂ aqueous suspensions using GC/MS/MS and LC/MS with quadrupole time-of-flight mass spectrometry
J. Am. Soc. Mass Spectrom. 14 (2003) 803-817
- La Clair JJ, Bantle JA, Dumont J
Photoproducts and metabolites of a common insect growth regulator produce developmental deformities in Xenopus
Environ. Sci. Technol. 32 (1998) 1453-1461
- Lányi K, Dinya Z
Photodegradation study for assessing the environmental fate of some triazine-, urea- and thiocarbamate-type herbicides
Microchem. J. 80 (2005) 79-87

Lopes NP, Stark CBW, Hong H, Gates PJ, Staunton J

Fragmentation studies on monensin A by sequential electrospray mass spectrometry.
Rapid Commun. Mass Spectrom. 16 (2002) 414-420.

Malato S, Albanis T, Piedra L, Aguera A, Hernando D, Fernández-Alba A

LC-MS and LC-MS/MS strategies for the evaluation of pesticide intermediates formed by degradative processes: photo-fenton degradation of diuron in Liquid Chromatography/Mass Spectrometry, MS/MS and Time-of-Flight MS Analysis of Emerging Contaminants, I. Ferrer and E.M. Thurman eds., ACS Symposium series 850 (2003) pp 66-109

Maizels M, Budde WL

Exact mass measurements for confirmation of pesticides and herbicides determined by liquid chromatography/time of flight mass spectrometry
Anal. Chem. 73 (2001) 5436-5440

Nielen MWF, Vissers JPC, Fuchs REM, van Velde JW, Lommen A.

Screening for anabolic steroids and related compounds in illegal cocktails by LC/time-of-flight mass spectrometry and LC/quadrupole time-of-flight tandem MS with accurate mass measurement.
Rapid Commun. Mass Spectrom. 15 (2001), 1577-1585.

Parra S, Stanca SE, Guanaquillo I, Thampi KR

Photocatalytic degradation of atrazine using suspended and supported TiO₂
Applied Catalysis B: Environmental 51 (2004) 107-116

Rebich RA, Coupe RH, Thurman EM

Herbicide concentrations in Mississippi River Basin-the importance of chloroacetanilide herbicide degradates
Sci. Total Environ. 321 (2004) 189-199

Scribner EA, Thurman EM, Zimmerman LR

Analysis of selected herbicides metabolites in surface and ground water of the United States
Sci. Total Environ. 248 (2000) 157-167

Thurman EM, Ferrer I, Parry RJ

Accurate mass analysis of ethanesulfonic acid degradates of acetochlor and alachlor using high performance liquid chromatography and time-of-flight mass spectrometry
J. Chromatogr. A 957 (2002) 3-9

Thurman EM, Ferrer I, Furlong ET

TOF MS and Quadrupole ion-trap MS/MS for the discovery of herbicide degradates in groundwater in Liquid Chromatography/Mass Spectrometry, MS/MS and Time-of-Flight MS Analysis of Emerging Contaminants, I. Ferrer and E.M. Thurman eds., ACS Symposium series 850 (2003) pp 128-146

2.4 Identificación de compuestos desconocidos (*non target*) en muestras de agua

2.4.1 Introducción

En muchos países, el agua superficial es una de las principales fuentes en la producción de agua destinada al consumo humano. En la calidad de esta agua influyen muchos factores, como por ejemplo efluentes de procedencia agrícola o industrial, y por tanto ha de estar continuamente controlada. Dentro de los programas de control, se usan diferentes técnicas con el fin de detectar y cuantificar contaminantes preseleccionados.

En la actualidad existe un creciente interés por la determinación de los llamados "nuevos contaminantes", tales como fármacos, productos de cuidado personal o surfactantes, que actualmente no están cubiertos por la legislación vigente sobre la calidad del agua o que no han sido previamente estudiados y pueden ser una amenaza tanto para el medio ambiente como para la salud humana. Sin embargo, estos contaminantes son también seleccionados a priori, por lo que en el agua podrían seguir presentes otros muchos compuestos desconocidos, algunos de los cuales podrían ser un riesgo para la salud humana si no se eliminan en el tratamiento del agua.

Hasta hace unos pocos años, la técnica más usada en la determinación de compuestos desconocidos, lo que se conoce con las siglas GUS (*General Unknown Screening*) era la cromatografía de gases acoplada a espectrometría de masas. Esta técnica generalmente hace uso de la ionización por impacto electrónico (EI) en condiciones estándar (70eV) para la cual existen librerías de espectros de masas de miles de compuestos. La identificación de compuestos desconocidos se basa normalmente en la comparación de los espectros de masas de los picos de la muestra con los de la librería. Sin embargo, esta aproximación presenta algunas limitaciones como, por ejemplo, la necesidad de llevar a cabo procedimientos de extracción largos y tediosos. Por otro lado, esta técnica sólo es adecuada en el caso de compuestos poco polares, volátiles o semivolátiles, por lo que muchas veces se requieren reacciones de derivatización previas a la determinación.

En el caso de compuestos polares y semipolares, las técnicas tradicionalmente usadas para el análisis de plaguicidas, por ejemplo mediante analizadores de masa de triple cuadrupolo, se optimizan con el fin de obtener la mayor sensibilidad para ciertos compuestos previamente seleccionados. Sin embargo, en una situación de emergencia debida por ejemplo a una contaminación, esta aproximación sería lenta y susceptible de errores cuando el analito fuera desconocido y no hubiera sido preseleccionado. Así pues, la mayoría de las técnicas

analíticas actuales desarrolladas para el análisis de plaguicidas son demasiado específicas para la determinación de compuestos desconocidos (la mayoría de ellas hacen uso de MS/MS), no suficientemente sensibles (cuadruolos o trampas de iones) o demasiado lentas.

La espectrometría de masas con analizadores de tiempo de vuelo (TOF MS) ofrece las ventajas de máxima sensibilidad en modo de barrido (*full scan*) y elevada exactitud en la masa (menor de 5 ppm), lo que permite que todos los compuestos ionizables en la fuente electrospray sean detectados y a la vez confirmados, sin necesidad de ser previamente seleccionados. Sin embargo, y a pesar de las ventajas que presenta el QTOF, este equipo ha sido raramente usado en aplicaciones medioambientales, debido principalmente a su elevado coste. Así, se han encontrado en la bibliografía únicamente tres citas en cuanto a la elucidación de compuestos desconocidos mediante el uso del LC-(Q)TOF, que muestran la gran dificultad asociada a este tipo de análisis. Así, Nielen et al (2001) usaron el potencial de la LC-QTOF en la búsqueda de esteroides anabolizantes y compuestos relacionados en un *cocktail* ilegal. Para ello combinaron la información del tiempo de retención junto con el espectro de absorbancia UV y la masa exacta de los iones precursores, de los iones producto y de las pérdidas neutras con el fin de obtener composiciones elementales inequívocas. Estas fórmulas moleculares fueron posteriormente buscadas en bases de datos, como por ejemplo el índice Merck para comprobar si se correspondían con la estructura de algún esteroide comercial, para finalmente compararse con los datos obtenidos para un patrón de referencia. Por otra parte, también se identificó y elucidó la estructura de un aditivo no esteroide. Bobeldijk et al (2001) estudiaron la capacidad del QTOF-MS en la identificación de compuestos desconocidos en muestras de aguas en modo de ionización positivo. Para ello, en primer lugar se fortificaron varios extractos de agua superficial con 6 compuestos modelo a diferentes niveles de concentración y se exploró el potencia de esta técnica usando medidas de masa exacta. Posteriormente, aplicaron el método desarrollado a la elucidación de tres compuestos verdaderamente desconocidos en un agua superficial. La estructura de un cuarto compuesto desconocido no pudo ser elucidada. El mismo grupo de trabajo (Bobeldijk et al, 2002), combinó el uso del fraccionamiento LC con evaluación de la (geno)toxicidad e instrumentos de triple cuadruolo y de tiempo de vuelo en la identificación de 4 contaminantes toxicológicamente importantes en muestras de agua. En este caso los autores sólo pudieron proponer estructuras para dos de esos desconocidos ya que no fue posible una confirmación final con patrones, al no encontrarse comercialmente disponibles. Todos estos ejemplos muestran la gran dificultad asociada al análisis de compuestos desconocidos.

En la presente Tesis, una vez mostrado el potencial de este equipo en la elucidación estructural de TPs en un ambiente controlado, se investigó el potencial de LC-MS/MS con analizadores QTOF en la elucidación de compuestos totalmente desconocidos en muestras de agua de diversos orígenes. Para ello se desarrolló una estrategia de trabajo basada en la metodología anteriormente descrita para TPs y metabolitos (*artículo científico 3*).

Varias muestras de agua de diferentes orígenes fueron inyectadas en el equipo, trabajando en continuo en modo *scan*. En este caso, ante la imposibilidad de tener una muestra control, se inyectó una muestra de agua HPLC, con el fin de descartar todos aquellos picos derivados del gradiente o provocados por las características del sistema, como por ejemplo, picos debidos a los séptums, etc. Una vez procesados los datos con el MetaboLynx (en este caso, no se seleccionaba ningún compuesto como precursor) se calcularon las posibles composiciones elementales. La única diferencia consistió en el uso de valores mucho menos restrictivos en los parámetros. El posterior tratamiento de datos consistió en una estrategia analítica basada en el uso de las abundancias isotópicas como filtrado adicional para reducir el número de fórmulas empíricas plausibles para los compuestos desconocidos detectados en diferentes tipos de aguas. Con el fin de reducir aún más el número de posibles composiciones elementales, se realizaron experiencias en tándem, seleccionando el ión precursor de interés y obteniendo su espectro de iones producto en masa exacta, lo que posibilitaba la obtención de las fórmulas empíricas de algunas pérdidas neutras. Las composiciones elementales se buscaron entonces en las distintas bases de datos disponibles, en nuestro caso el índice Merck y la librería NIST. Finalmente, para discernir entre los posibles candidatos que podían satisfacer la/s fórmula/s empírica/s, se estudió el espectro de iones producto en masa exacta, lo que posibilitó la obtención de las fórmulas empíricas de los fragmentos y, por lo tanto, la elucidación final de la estructura del compuesto desconocido. Una vez desarrollada, se aplicó al análisis de compuestos totalmente desconocidos en diferentes tipos de aguas.

2.4.2 Resultados y discusión

En el artículo científico que viene a continuación se ha desarrollado una metodología analítica para la identificación de compuestos desconocidos en muestras de agua mediante el uso de SPE-LC-QTOF MS, basada en la medida de iones con masa exacta, tanto trabajando en MS como en MS/MS, en combinación con una estrategia basada en el uso de las abundancias isotópicas.

Esta aproximación fue aplicada con éxito al análisis de diferentes tipos de aguas, consiguiendo elucidar por completo la estructura de algunos compuestos desconocidos sin ningún tipo de preselección en muestras sobre los que no existía ninguna información sobre su composición. En aquellos casos en los que no fue posible la justificación del compuesto, se obtuvo la fórmula molecular y se detectó la presencia de algunos grupos funcionales en la molécula.

Obviamente esta aproximación presenta limitaciones derivadas de las características intrínsecas del LC/MS, tanto en lo que se refiere a la cromatografía como a la ionización, como ya se ha comentado en el capítulo anterior. Otra restricción viene dada por las bases de datos disponibles, ya que muchos compuestos pueden no aparecer en las mismas, así como por la necesidad de preseleccionar aquellos picos que consideramos relevantes, lo cual se suele realizar en función de su abundancia y no de, por ejemplo, su toxicidad.

Artículo científico 3

Rapid Commun. Mass Spectrom. (2005) 19: 1-10

USE OF QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY IN THE ELUCIDATION OF UNKNOWN COMPOUNDS PRESENT IN ENVIRONMENTAL WATER

María Ibáñez¹, Juan V. Sancho¹, Óscar J. Pozo¹, Wilfried Niessen² and Félix Hernández^{1*}¹Analytical Chemistry, Experimental Sciences Department, University Jaume I²Hyphen MassSpec Consultancy, de Wetstraat 8, 2332 XT Leiden, the Netherlands**ABSTRACT**

Target compound monitoring is often not sufficient to assess the quality of water, as many of the unknown micro-contaminants present might be a threat to the environment and human health. In this work, the potential of hybrid quadrupole time-of-flight mass spectrometry (QTOF MS) coupled to liquid chromatography in the elucidation of unknown compounds in environmental water samples has been explored. Based on accurate mass measurement, possible elemental compositions for the precursor ions were calculated. Using model compounds, a useful strategy was developed, enabling determination and evaluation of potential molecular formulae for the detected unknowns. The possibility of performing tandem mass spectrometric (MS/MS) acquisitions to obtain product ion spectra in accurate mass mode also helped to elucidate the structures of these unknowns or to detect some functional groups, to further evaluate potential formulae. The remaining formulae were searched against available databases as Merck Index and the NIST library. Where standards were commercially available, retention time and MS/MS data were both also used as confirmatory tools. The approach developed was applied for the identification of unknown compounds in different types of water. To improve sensitivity, environmental water samples were preconcentrated on-line in a polymeric cartridge by direct injection of 2 mL water in the SPE-LC-MS/MS system. For three unknowns, structures were proposed and confirmed with standards. Although other compounds could not be unequivocally identified based on the data available within this study, details about the possible structure of some are given.

INTRODUCTION

In recent years, liquid chromatography/ (tandem) mass spectrometry (LC/MS(/MS)) methods have been replacing gas chromatography/mass spectrometry (GC/MS) methods for the determination of organic micro-contaminants as they present obvious advantages, such as reduce sample pretreatment and the capability to determine very polar compounds and transformation products (TPs). However, monitoring methods are usually restricted to a few preselected compounds (target analysis), for which analytical methods, either single or multi-residue, have been developed, optimized and validated.

There is growing interest in the determination of emerging contaminants, i.e., pharmaceuticals, personal care products or surfactants that are not currently covered by existing regulations on water quality or have not been previously studied and are thought to be a threat to the environment and human health¹. However, these contaminants are also preselected and target analysis is performed.

Due to the high mass accuracy and inherent high sensitivity in full-scan mode, liquid chromatography/time-of-flight mass spectrometry (LC/TOF-MS) has been successfully used in the confirmation of pesticides as well as emerging contaminants in the environmental field¹⁻⁴.

LC/TOF-MS has been used to identify structures proposed for transformation products, e.g., photodegradation products of alachlor⁵ and diuron⁶, or alachlor and acetochlor degradates⁷. In these cases, LC/TOF has been combined with MS/MS experiments using a triple quadrupole mass spectrometer⁵, an ion trap mass spectrometer⁶, or in-source collision induced dissociation (CID),^{5,7} for final structure elucidation of "unknowns".

However, the use of hybrid quadrupole time-of-flight mass spectrometry (QTOF-MS) permits the acquisition of full scan and product ion spectra, with the accurate mass of the product ions for the potential unknowns with a single instrument.

For example, in previous work, exploring the potential of several MS techniques for confirmation of pesticides and TPs⁸, we found an additional peak in the accurate mass chromatogram of a "positive" environmental water sample. This peak was at the m/z corresponding to simazine (202.08), showing the presence of an isobaric unknown compound, identified as desethyl-terbutylazine (a terbutylazine degradate), by measuring its full scan and product ion spectra in accurate mass.

However, these instruments are rarely used in environmental applications mainly due to their high cost. Only very recently has the potential of QTOF-MS been explored for confirmation purposes in the environmental field; e.g., in the elucidation of transformation products of triazine

herbicides in water after UV exposure⁹. Bobeldijk et al.¹⁰ have explored the capability of QTOF-MS for the identification of unknowns in water samples. Using six model compounds added to surface water samples, the potential for identification using accurate mass measurements was tested, and the method subsequently applied to the elucidation of three real unknowns present in a surface water sample. The structure of a fourth unknown remained unresolved. The same research group has combined the use of LC fractionation and (geno) toxicity evaluation with tandem mass spectrometry (triple quadrupole and QTOF instruments) for the identification of four unknown toxicologically relevant water pollutants¹¹. In this case, the authors could only propose structures for two of these unknowns, but a final confirmation with standards was not possible, as they were not available.

These two examples demonstrate the difficulties associated with the identification of unknown compounds in environmental samples. In some cases, the combination of several techniques may help in this difficult task. Since the commercial introduction of liquid chromatography/nuclear magnetic resonance (LC/NMR) systems, this hyphenated technique has been mainly applied to the structural elucidation of metabolites in drug development studies¹² and to the identification of natural compounds in plant extracts¹³. However, recent studies have demonstrated its applicability to the analysis of unknown compounds in environmental samples as ¹H NMR spectra in general give more detailed structural information than mass spectra¹⁴. Optimum structural information is obtained when LC/MS and LC/NMR are used together to identify unknowns in complex mixtures¹⁵. However, trace analysis in environmental samples is not possible due to the poor sensitivity of LC/NMR, generally three orders of magnitude worse than LC/MS. Another limitation associated with this type of complex instruments is the high acquisition cost.

Grange et al.^{16,17} applied a time-consuming and laborious analytical approach to the identification of unknowns based on the use of gas chromatography/high resolution mass spectrometry (GC/HRMS). On the basis of repeated injections in the system under different MS conditions (full scan and SIM with different resolving powers), they obtained a unique empirical formula.

In this paper, the potential of LC/QTOF-MS in the identification of unknown compounds in several environmental water samples is explored. Additionally, a useful strategy is presented in order to evaluate potential molecular formulae for detected unknown compounds and to reduce their number.

EXPERIMENTAL

Instrumentation

A hybrid quadrupole time-of-flight (QTOF) mass spectrometer provided with an orthogonal Z-spray electrospray interface (Micromass, Manchester, UK) was interfaced to an LC system (Figure 1) based on a 233XL autosampler with a loop of 2000 μL (Gilson, Villiers-le-Bel, France) and two pumps: an Agilent 1100 (Agilent, Waldbron, Germany) binary pump used for conditioning and washing the cartridge (P-1) and a Waters Alliance 2695 (Waters, Milford, MA, USA) quaternary pump used for the chromatographic separation (P-2). The SPE pre-concentration (C-1 in Figure 1) was performed using a PRP-1 polymeric cartridge, 10 μm 10 x 2 mm i.d. (Teknokroma). For the LC separation (C-2 in Figure 1), a Kromasil column C_{18} , 5 μm 125 x 2.0 mm i.d. (Scharlab), was used. Mobile phases consisted in water and acetonitrile in P-1 and mixtures of 0.01% HCOOH in acetonitrile/0.01% HCOOH in water in P-2 (see Figure 1).

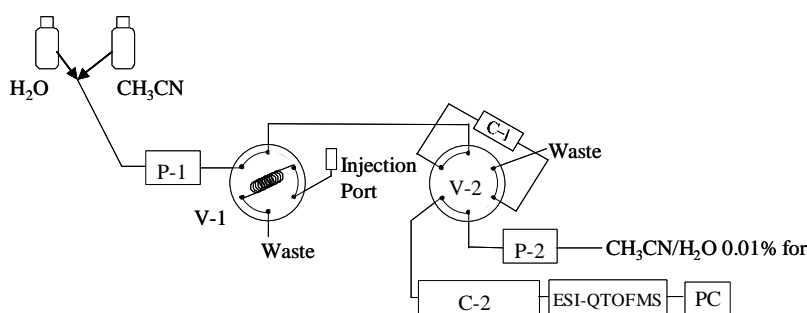


Figure 1. SPE-LC set up. V = six-port high-pressure valve; P-1 = binary LC pump; P-2 = quaternary LC pump; C-1 = SPE cartridge; C-2 = separation column; PC = data system; W = waste.

Drying gas as well as nebulizing gas was nitrogen, generated from pressurized air in a nitrogen generator from Peak Scientific (Inchinnan, UK). The nebulizer gas flow was set to approximately 15 L/h and the desolvation gas flow to 700-800 L/h. A cone voltage of 25 V and a capillary voltage of 3.5 kV were used in both positive and negative ionization modes. The nitrogen desolvation temperature was set to 350°C and the source temperature to 120°C. TOF-MS resolution was approximately 5000 (fwhm). MS and MS/MS spectra were acquired over an m/z range of 60-600. For MS/MS operation, argon (99.995%, Carbueros Metalicos, Valencia, Spain) was used as collision gas with a pressure of approximately 2.10^{-5} mbar in the collision cell. Product ion spectra were usually generated at 3 collision energies: 15, 25 and 30 eV. The MCP detector potential was set to 2700 V in positive ionization mode and to 2800 V in negative ionization mode.

Dwell times of 1 s/spectrum were chosen. A suitable MS profile was used. Data station operating software was MassLynx v4.0.

Mass calibration was performed daily using a Model 11 single syringe pump (Harvard instruments, Holliston, MA, USA), directly connected to the interface. Calibration in both positive and negative electrospray modes was conducted from m/z 60-600 with a 1:1 mixture of 0.1 M NaOH/10% HCOOH diluted (1:25) with acetonitrile/water (80:20), at a flow rate of 20 $\mu\text{L}/\text{min}$.

A 1 $\mu\text{g}/\text{mL}$ solution of 3,5-diiodo-L-tyrosine (Sigma, St. Louis, MO, USA) in methanol, used as lock mass, was introduced post-column using a model 2150 isocratic HPLC pump from LKB (Bromma, Sweden) at a flow rate of 100 $\mu\text{L}/\text{min}$ by means of a PEEK T-union[®].

SPE-LC procedure

The conditioning of the PRP-1 cartridge was performed with acetonitrile at a flow-rate of 1 mL/min for 3 min, following by 4 min with LC-grade water. A 2-mL water sample was preconcentrated (1 mL/min) into the cartridge and washed with 4 mL of LC-grade water. The sample was then transferred in backflush mode to the C-2 column (C_{18} analytical column) and a gradient using P-2 started. To perform the chromatographic separation, a gradient with water 0.01% HCOOH and acetonitrile 0.01% HCOOH was applied (Figure 1). The percentage of organic modifier was changed linearly as follows: 0 min., 5%; 2 min., 5%; 55 min., 90%; 58 min., 90 %; 59 min., 5%. The chromatographic separation was completed within 60 minutes.

Sample procedure

Several ground and surface water samples had been previously collected in selected sites from a Spanish Mediterranean area (Valencian region), with important agricultural activities. These samples were acidified with formic acid to 1%. Volumes of 2 mL of the acidified samples were loaded into the loop (2 mL) and directly injected into the SPE-LC/(ESI)-QTOF system. Other types of water samples were collected from the influent and the effluent of a wastewater treatment plant (WTP), as well as from landfill leachate from an urban solid waste plant. After centrifugation, these samples were directly injected (50 μL) in the LC/ESI-QTOF system without preconcentration.

Data processing

Data were acquired in continuum mode and afterwards processed by the MetaboLynx application manager (Micromass v4.0) to perform data centering and lock mass correction. To obtain accurate mass measurements, 15 spectra were combined and an Np value of 0.6 was used. The mass data was centered using peak areas at 80% of the centroid top. An HPLC water sample was used as control sample in the MetaboLynx application to eliminate possible peaks due to the gradient.

Based on the accurate mass that was obtained, all possible elemental compositions with a maximum deviation of 7 mDa from the measured mass were calculated, using the Elemental Composition program within the MassLynx software. Parameter settings for all calculations were C: 0-50, H: 0-100, N: 0-20, O: 0-20, F: 0-20 and P: 0-3. In addition, from the characteristic isotopic patterns of ^{37}Cl (31.98 % relative abundance), ^{81}Br (97.88 %) and ^{34}S (4.44 %), the appropriate number of Cl, Br and S atoms was evaluated and added. The number of Cl and Br atoms was easily adjusted. However, in the case of S atoms, the lower relative abundance of ^{34}S made their adjustment less precise, especially when halogens were present. In these cases, an interval was given. The double-bond equivalent (DBE) parameter was set from -0.5 to 50 and was not used as identification criteria, but information about aromaticity of the structure was obtained. Additionally, the option "even-electrons ions only" was selected for the precursor ion and "odd- and even- electrons ions" for the product ions.

RESULTS AND DISCUSSION

Methodology

Full scan experiments, in both positive and negative ionization modes, were carried out with the hybrid QTOF mass spectrometer, using the first quadrupole only as a RF-guide. In order to extract the chromatographic peaks present in the water samples and to accurately obtain mass-measured spectra, the experiments were acquired in continuum mode and afterwards processed by the MetaboLynx software. Accurate mass of protonated and deprotonated molecules were determined in positive and negative ionization modes, respectively. In this study, only chromatographic peaks with an area higher than 100 arbitrary units (a.u.) were taken into account. Accurate masses of these peaks were entered into the Elemental Composition program to calculate possible elemental compositions using the previously defined parameter settings.

The use of electrospray ionization (ESI), which is a soft ionization method, primarily leads to the formation of (de)protonated molecules. A constant amount of formic acid was maintained in the mobile phase, favoring the formation of the protonated molecule, $[M+H]^+$, and reducing the formation of various adducts (sodium, potassium, ammonium...) in positive ionization mode. Compared to the laborious procedures described by Grange et al.^{16,17} who experienced problems in assessing the correct molecular ion peak in GC/El-HRMS, the use of ESI greatly facilitated the reduction of the number of possible elemental compositions. First, this approach was tested using standards of several herbicides (terbutylazine, terbacil, terbumeton, simazine and bromacil) at different concentration levels. Extracted ion chromatograms with a 0.2 Da mass window at the exact m/z of the compound were obtained. From the chromatographic peak, spectra were combined and smoothed. Experimental percentages of M+1, M+2 isotope peaks, as well as M+3 or M+4... (if any), were calculated from the ion abundances obtained. These calculated percentages were then compared with the theoretical percentages of the known compound, obtaining experimental error of up to 50% when the abundance of an isotopic peak was lower than 60 counts; However, when the abundance of an isotopic peak was between 60 and 200 counts, the observed error was below 20%, and when the abundance was higher than 200 counts, the error decreased below 10%.

In the analysis of the real samples, theoretical isotopic percentages of all possible elemental compositions for the unknown compounds were calculated from the Isotope Model program within the MassLynx software, and compared with those obtained experimentally. The acceptable deviations between the experimental and the theoretical values were set up in accordance with those previously obtained from the model compounds.

This approach was applied to the identification of several unknowns found in the environmental and wastewater samples analyzed, leading to a considerable reduction in the number of potential elemental compositions to be evaluated.

Additionally, MS/MS experiments were performed of the unknown compounds. When different possible empirical formulae were obtained from the MS experiments, accurate masses of the various neutral losses observed in the product ion spectra were determined. Often, this information enabled a further reduction of the number of possible molecular formulae. Additionally, accurate masses of the product ions were obtained using the previously determined accurate mass of the precursor ions as lock masses. For the elemental composition calculation of product ions, the parameter settings were restricted by the calculated elemental composition of the selected precursor, while for neutral losses no restrictions were used. Finally, the resulting elemental compositions were searched against the Merck Index and NIST databases.

Structures found in databases were evaluated based on the fragmentation patterns observed in the experimental MS/MS spectra. Additionally, when the standard for a potential compound was commercially available, a LC/MS/MS experiment was carried out in order to confirm the elucidated structure of the unknown compound, using both the retention time under the experimental chromatography conditions and the acquired MS/MS spectra.

Selected examples

Unknown compound with MW 296 (surface water, El Clot)

The accurate mass of a protonated unknown compound with retention time 28.1 minutes was determined to be m/z 297.0567 (area = 363 a.u.) (Figures 2(a) and 2(c)). The ion abundances found in the smoothed combined spectrum (Figure 2(b)) as well as isotopic percentages and accepted deviations, are shown in Table 1. When abundances were between 60 and 200 counts, an error of $\pm 20\%$ between experimental and theoretical percentages was accepted; when the abundances were higher than 200 counts, an error of $\pm 10\%$ was considered. Taking account of the $M+2$ experimental percentage (67.8 %), we assumed the presence of two Cl atoms in the molecule, and nil to two S atoms, as deduced from the accepted tolerances in the $M+2$ percentage ($\pm 10\%$, i.e., 61.02-74.58%). Within the fixed limits, calculation of the possible elemental compositions resulted in 30 formulae. After the application of our filtering strategy based on a maximum error in isotope relative abundance depending on the abundance of each isotopic peak, only 2 compositions were plausible: $C_{14}H_{14}N_2OCl_2$ ($\Delta mDa = 0.6$ mDa) and $C_{16}H_{15}FCl_2$ ($\Delta mDa = -4.6$ mDa) (see Table 2)

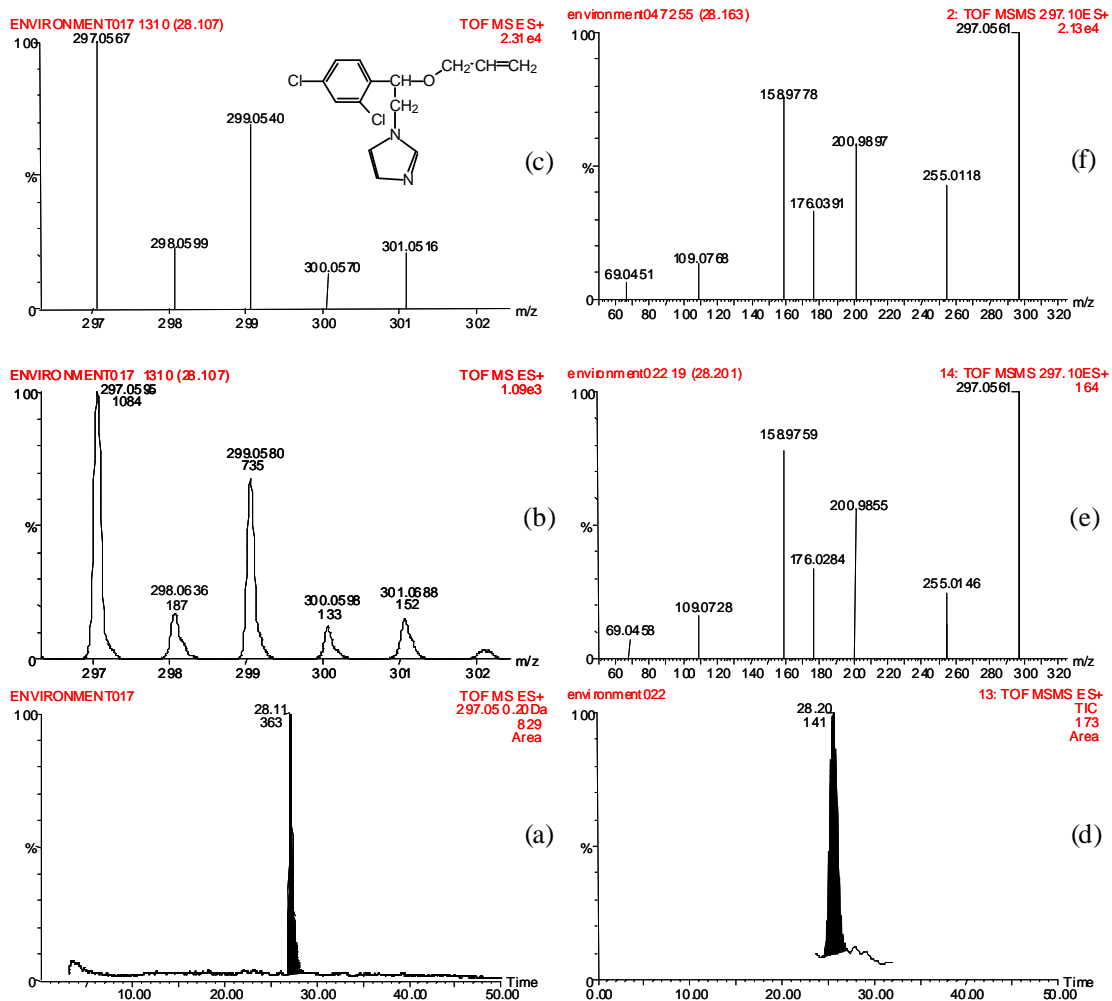


Figure 2. Unknown compound with MW 296 and retention time 28.1 min in a surface water: (a) XIC chromatogram at m/z 297.05, (b) combined smoothed spectrum and (c) centered mass-measured spectrum; MS/MS of the precursor ion 297: (d) TIC chromatogram, (e) product ion spectrum and (f) product ion spectrum of a standard

Table 1. Experimental ion abundances, isotopic percentages and accepted deviations for the unknown compounds found

Compound	Parameters	M	M+1	M+2	M+3	M+4	M+5	M+6
<i>Unknown</i>	Abundances (counts)	1084	187	735	133	152		
<i>MW 296</i>	Isotopic percentage		17.3	67.8	12.3	114.0		
	Tolerances		13.8-20.7	61.02-74.58	9.8-14.7	11.2-16.8		
<i>Unknown</i>	Abundances (counts)	1414	211	84				
<i>MW 241</i>	Isotopic percentage		14.9	5.9				
	Tolerances		13.41-16.39	4.8-7.1				
<i>Unknown</i>	Abundances (counts)	3039	339	1899	222	339		
<i>MW 232</i>	Isotopic percentage		11.2	62.5	7.3	11.2		
	Tolerances		10.08-12.32	56.25-68.75	6.6-8.0	10.08-		
<i>Unknown</i>	Abundances (counts)	1760	266	1874	286	752		
<i>MW 399</i>	Isotopic percentage		15.1	106.5	16.3	41.7		
	Tolerances		13.59-16.61	95.85-	14.67-	38.43-		
<i>Unknown</i>	Abundances (counts)	1334	217	38			113	
<i>MW 258</i>	Isotopic percentage		16.3	2.8			6.4	
	Tolerances		14.67-17.93	1.4-4.3			5.1-7.7	
<i>Unknown</i>	Abundances (counts)	2235	344					134
<i>MW 228</i>	Isotopic percentage		15.4					7.6
	Tolerances		13.86-16.94					6.1-9.1

Table 2. Possible elemental compositions for m/z 297.0567 \pm 7 mDa. Comparison between experimental and theoretical relative ion abundances.

	M	M+1	M+2	M+3	M+4	%M+1	%M+2	%M+3	%M+4
Experimental abundances	1084	187	735	133	152	17,3%	67,8%	12,3%	14,0%
Error accepted (%)		-20	-10	-20	-20:	13,8%	61,02%	9,8%	11,2%
Error accepted (%)		+20	+10	+20	+20:	20,7%	74,58%	14,7%	16,8%
[M+H] ⁺	error					%M+1	%M+2	%M+3	%M+4
Compositions	(mDa)								
C ₅ H ₁₇ N ₆ OFPCl ₂	0,4					8,1%	64,5%	5,2%	10,5%
C ₁₄ H ₁₅ N ₂ OCl ₂	0,6					16,6%	65,4%	10,7%	11,2%
C ₁₁ H ₁₆ N ₂ O ₂ FCl ₂	-0,6					13,7%	65,2%	8,9%	11,0%
C ₄ H ₁₃ N ₈ OF ₂ Cl ₂	1,0					7,6%	64,4%	4,9%	10,5%
C ₁₂ H ₂₀ O ₂ PCl ₂	-1,1					10,0%	65,0%	6,5%	10,9%
C ₄ H ₁₆ N ₈ FSCl ₂	-1,3					8,4%	68,7%	5,7%	13,3%
C ₁₀ H ₁₉ F ₃ PCl ₂	1,3					13,7%	69,3%	9,3%	13,7%
C ₈ H ₁₆ N ₆ PCl ₂	1,6					11,3%	64,5%	7,3%	10,6%
C ₈ H ₁₇ N ₂ O ₃ F ₂ Cl ₂	-1,7					10,0%	65,0%	6,5%	10,9%
C ₉ H ₁₅ N ₂ F ₄ Cl ₂	1,9					11,0%	64,5%	7,0%	10,6%
C ₇ H ₁₂ N ₈ FCl ₂	2,1					10,9%	64,5%	7,0%	10,6%
C ₃ H ₁₅ N ₈ O ₄ Cl ₂	-2,6					6,7%	65,0%	4,3%	10,9%
C ₁₁ H ₁₉ N ₂ O ₅ Cl ₂	-2,8					14,1%	69,5%	9,6%	13,8%
C ₆ H ₁₆ N ₄ O ₄ FCl ₂	3,4					8,5%	65,1%	5,5%	11,0%
C ₂ H ₁₅ N ₁₀ O ₅ Cl ₂	3,9					6,2%	64,7%	4,0%	10,7%
C ₄ H ₁₁ N ₁₂ Cl ₂	-4,0					9,0%	64,3%	5,8%	10,5%
C ₆ H ₁₄ N ₆ F ₃ Cl ₂	-4,2					9,1%	64,3%	5,8%	10,5%
C ₉ H ₁₃ N ₄ O ₃ Cl ₂	4,6					11,0%	64,5%	7,0%	10,6%
C ₁₆ H ₁₆ FCl ₂	-4,6					18,0%	65,5%	11,6%	11,2%
C ₇ H ₁₈ N ₄ F ₂ PCl ₂	-4,7					9,5%	64,4%	6,1%	10,5%
C ₇ H ₁₇ N ₄ F ₂ S ₂ Cl ₂	4,8					9,6%	64,8%	6,2%	10,8%
CH ₁₂ N ₁₂ OFPCl ₂	-5,1					5,7%	64,3%	3,7%	10,4%
C ₃ H ₁₆ N ₈ O ₂ PCl ₂	5,6					6,6%	64,5%	4,2%	10,6%
C ₂ H ₁₆ N ₁₀ OPCl ₂	-5,6					6,2%	64,3%	4,0%	10,5%
C ₁₃ H ₁₇ OF ₂ Cl ₂	-5,8					14,8%	65,2%	9,5%	11,0%
C ₂ H ₁₂ N ₁₀ O ₂ FCl ₂	6,1					6,2%	64,5%	4,0%	10,6%
C ₈ H ₁₈ N ₂ OF ₂ PCl ₂	6,5					9,9%	64,6%	6,4%	10,6%
C ₈ H ₁₅ N ₆ O ₂ Cl ₂	-6,7					11,4%	65,0%	7,3%	10,9%
C ₁₀ H ₁₈ O ₂ F ₃ Cl ₂	-6,9					11,5%	65,0%	7,4%	10,9%
C ₇ H ₁₄ N ₄ OF ₃ Cl ₂	7,0					9,5%	64,6%	6,1%	10,6%

"x" indicates that the experimental values fall inside the maximum error limits for that elemental composition

The MS/MS spectrum of the m/z 297 ion is shown in Figure 2(e). The product ion at m/z 255.0146 was formed by the loss of a propene group (C_3H_6 , 42.0415, $\Delta mDa = -5.5$ mDa), which matched both elemental compositions.

A database search was performed and only the elemental composition $C_{14}H_{14}N_2OCl_2$ resulted in one structure (Merck Index), corresponding to the veterinary fungicide enilconazole (m/z $[M+H]^+ = 297.0561$). The presence of a 2-propenyl substituent in the structure of enilconazole (see structure in Figure 2) agrees with the propene loss observed in the product ion spectra of the unknown. The MS/MS spectrum of a commercially available standard was obtained (Figure 2(f)) and compared with the MS/MS spectrum of the unknown (Figure 2(e)), showing that the unknown was present at a concentration of approximately 50 $\mu g/L$. The standard showed similar retention time (28.16 min) and the MS/MS spectra showed a good agreement. Table 3 shows the product ions as well as their corresponding mass errors when comparing with the theoretical ions, assuming enilconazole to be the compound investigated. With all data available within this study, the unknown compound was unequivocally identified as the fungicide, enilconazole.

This compound was found and confirmed in two other samples, the influent (Figures 3(a)-3(c)) and the effluent (Figures 3(d)-3(f)) of an urban wastewater treatment plant (WTP) located within an agricultural area (see Figure 3). As can be seen in this figure, the concentration levels found in the influent and effluent, were similar (approximately 85 and 70 $\mu g/L$, respectively) showing little, or no treatment of this compound. In order to explain the relatively high levels of the pollutant observed in this urban water, other possible applications of this compound were investigated, with the discovery of its use as a post-harvest fungicide in citrus, the predominant crop in the area (in this application it is called imazalil). The presence of many companies involved in commercial orange activities in the area close to the WTP, could explain the high pollution degree in the water especially if discharges of contaminated waters to the public sewage took place without any previous treatment.

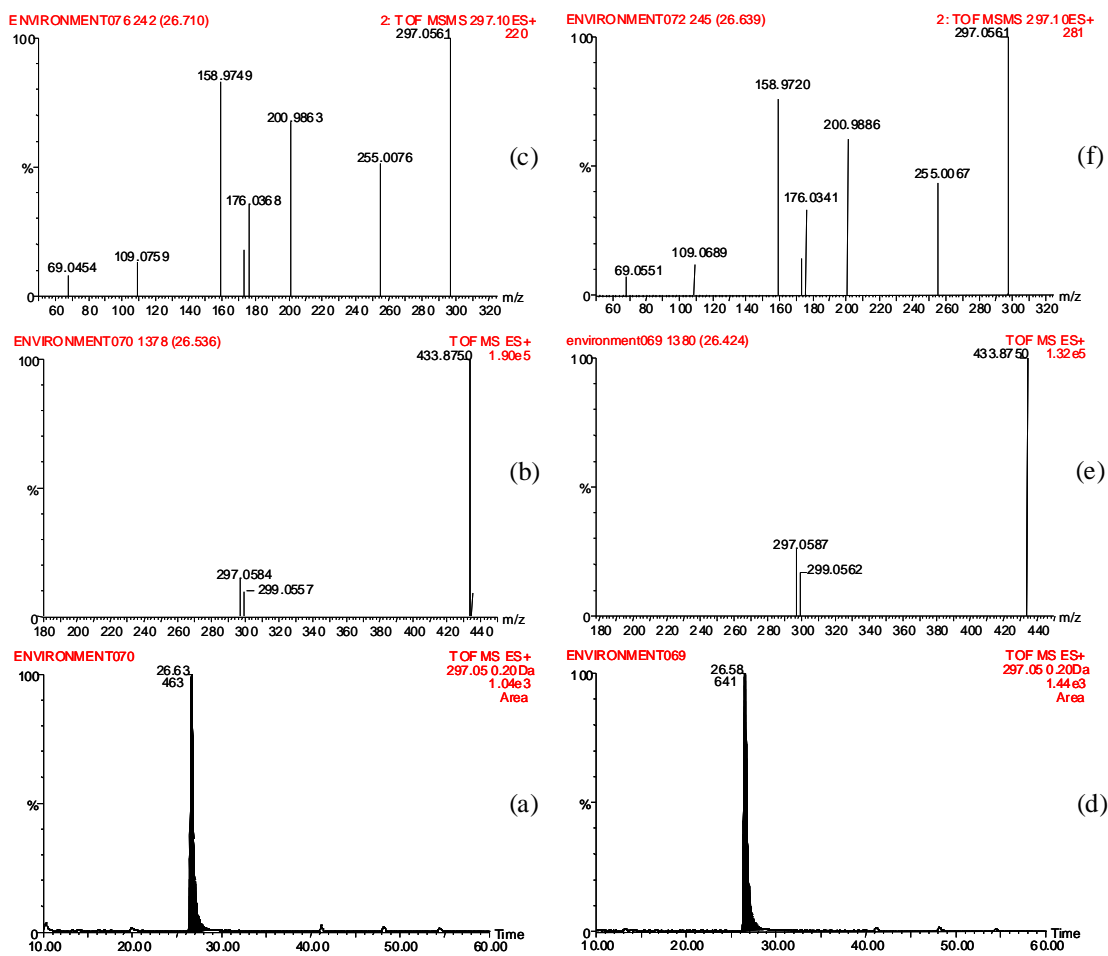


Figure 3. MS and MS/MS data of an unknown compound with MW 296 and retention time 26.6 min in (a-c) an influent and (d-f) an effluent of an urban wastewater treatment plant; (a, d) mass chromatogram of m/z 297.05, (b, e) full scan spectrum and (c, f) product ion spectrum of m/z 297.

Unknown compound with MW 241 (Landfill leachate)

The m/z value of a protonated unknown compound with retention time 32.60 min was found to be 242.1448 (area 582 a.u.). Twelve elemental compositions were possible using the parameter settings described above and assuming the presence of an S atom (5.9% M+2, see Table 1). After applying the approach that has been developed, there were 3 possible molecular formulae: $C_{12}H_{21}N_2OS$ ($\Delta mDa = -0.5$), $C_{10}H_{19}N_5S$ ($\Delta mDa = 0.8$), and $C_{13}H_{20}NFS$ ($\Delta mDa = 6.9$). Table 3 shows the product ions in the MS/MS spectrum as well as their corresponding mass errors. The predominant product ion at m/z 186.0808 is formed by the loss of a butene (C_4H_8 , 56.0631, $\Delta mDa = 0.5$ mDa). As this neutral loss did not enable us to reduce the number of empirical formulae, the product ion at the lowest m/z (91.0326, $\Delta mDa = -0.4$, $C_2H_7N_2S$) was studied. This ion formula allowed us to discard the two molecular formulae without two N atoms and one S atom. After a database search, only the elemental composition $C_{10}H_{19}N_5S$ was found, resulting in two possible structures: prometryn and terbutryn, both used as herbicides in the area under study. The loss of C_4H_8 , i.e., methylpropene is typical of the terbutyl group that was present in only one of these two candidates (terbutryn). The commercial availability of terbutryn standard allowed the confirmation of the identity of this unknown, using both retention time and MS/MS spectra data. Concentration estimated in the sample was around 20 $\mu g/L$.

Unknown compound with MW 232 (Surface water, Les Coves)

In a similar way, the m/z value of a protonated unknown compound with retention time 32.95 min was 233.0251. Ten elemental compositions were possible using the parameter settings described above and assuming the presence of two Cl atoms (no S atoms were considered, 62.5% M+2, see Table 1). After applying the developed approach, only one possibility resulted: $C_9H_{10}N_2OCl_2$ ($\Delta mDa = 0.3$). The MS/MS spectrum only a single product ion at m/z 72.0480 ($\Delta m = 3.1$ mmu) with an elemental composition of C_3H_6NO , which was in accordance with the remaining elemental composition. This elemental composition was found in the database, resulting in two possible structures: 3,4-dichloro-N,N-dimethylphenylurea (the herbicide diuron) and 3,5-dichloro-N,N-dimethylphenylurea (a diuron isomer). The only available product ion could be obtained from both structures, making it therefore not feasible to differentiate between these two compounds. As diuron was commercially available, the MS and MS/MS spectra of a standard were obtained showing a retention time and MS/MS spectra similar to those of the unknown compound. This result, combined with the wide use of this herbicide in the area under investigation, and the fact that no applications of the 3,5-dichloro have been described, led us to

conclude the identity of the unknown compound to be diuron. In this case, the estimated concentration was around 10 µg/L.

Unknown compound with MW 400 (Ground water, Carcaixent)

Based on the negative ion mode full scan spectrum, the exact mass of a deprotonated unknown compound with retention time of 32.8 minutes was found to be m/z 398.8762 (area = 510 a.u., see Figures 4(a), 4(c)). Experimental isotope percentages were calculated from the spectrum, considering an error of $\pm 20\%$ when signal abundances were between 60 and 200 counts, and an error of $\pm 10\%$ when signal abundances were higher than 60 counts (see Table 1). In this case, the $M+2$ and $M+4$ experimental percentages (106.5 % and 42.7%, respectively), indicated the presence of three Cl atoms and a maximum of four S atoms due to the tolerances permitted. Additionally, the possible presence of iodine, zinc and selenium in the molecule was also studied based on the mass defect of the unknown molecule. However, the theoretical isotopic percentages did not match the experimental ones. Finally, the calculation of the elemental compositions yielded 367 solutions, although, after applying the proposed strategy, only four were considered: $C_{12}H_5N_2OF_2S_2Cl_3$ ($\Delta mDa = -3.7$), $C_{12}H_7O_5S_2Cl_3$ ($\Delta mDa = 4.0$), $C_{13}H_9OFPS_2Cl_3$ ($\Delta mDa = -4.2$) and $C_{10}H_9O_2F_2S_2Cl_3$ ($\Delta mDa = 4.2$).

The MS/MS spectrum of this unknown was obtained at high collision energy, as little fragmentation was observed with the default collision energies used (Table 3). As the spectrum showed one loss of SO_2 (63.9645, $\Delta mDa = 2.6$), the two formulae without a minimum of one S atom and two O atoms were discarded (see Figure 4(d)). Two elemental compositions still remained plausible: $C_{12}H_7O_5S_2Cl_3$ ($\Delta mDa = 4.0$) and $C_{10}H_9O_2F_2S_2Cl_3$ ($\Delta mDa = 4.2$). However, neither of these possible two elemental compositions was found in the available databases.

MS/MS spectra were also obtained for precursor ions at m/z 400.8 ($M+2$) (Figure 4(e)) and 402.8 ($M+4$) (Figure 4(f)), in order to elucidate which fragments retained Cl atoms and in which number, trying to explain the fragmentation pathway observed. All this information was still not sufficient to elucidate the structure of the unknown compound, although we deduced that the unknown molecule probably contain sulfone moieties.

Unknown compound with MW 258 (Landfill leachate)

The negative ion mode scan mass spectrum of a deprotonated unknown compound with retention time 34.7 min provided a value for the m/z of the $[M-H]^-$ ion of 257.1729. Calculation of the possible elemental composition provided 24 hits within the set limits (no sulfur or halogen atoms were considered, see Table 1). Three possible elemental compositions were obtained after applying the developed approach: $C_{14}H_{26}O_4$ ($\Delta mDa = -2.4$ mDa), $C_{14}H_{27}O_2P$ ($\Delta mDa = 5.8$ mDa) and $C_{13}H_{23}N_2O_2F$ ($\Delta mDa = 6.3$ mDa).

The MS/MS spectrum showed only two important fragments corresponding to $[M-H_2O]^-$ and $[M-H_2O-CO_2]^-$, even at higher collision energies. Therefore all elemental compositions with fewer than three oxygen atoms in their molecule were discarded. Thus, the only possible elemental composition was $C_{14}H_{26}O_4$ ($\Delta mDa = -2.4$ mDa). This elemental composition resulted in 30 plausible structures after database searching, although none of these structures explained the fragmentation pathway observed.

The MS/MS spectrum indicated the probable presence of a carboxylic group and an alcohol group in the molecule. According to previous research^{18,19}, losses of H_2O and CO_2 , are characteristic of fulvic and humic acids, although other hydroxy carboxylic acids could also be possible.

Unknown compound with MW 228 (Surface water, Vilanova d'Alcolea)

The positive ion mass spectrum of a protonated unknown compound with retention time 13.46 min gave a mass for the $[M+H]^+$ ion of 229.0985. Calculation of the possible elemental composition provided 42 hits within the set limits (no sulfur or halogen atom was considered, see Table 1). This number was reduced to two possibilities after applying the developed approach: $C_{13}H_{12}N_2O_2$ ($\Delta mDa = 0.8$) and $C_{15}H_{13}OF$ ($\Delta mDa = -4.4$).

The MS/MS spectrum showed a $HCOOH$ loss (46.0075, $\Delta mDa = 2.0$), thus excluding the structure without at least two oxygen atoms. The remaining elemental composition was searched in the available databases, resulting in 34 possible structures.

Although this formic acid loss could be related to the presence of a carboxylic moiety, the full scan mass spectrum in negative ion mode did not show a peak at the m/z expected ($[M-H]^- = 227.0821$), causing us to discard this option. However, none of the structures found in the databases could explain the fragmentation pathway observed.

Under these circumstances, the best option seemed to be to elucidate the potential structure, by interpretation of the full scan product ion spectra in exact mass. This task is normally very complicated, especially when dealing with poor MS/MS spectra, as in this case, where only the predominant fragment due to the formic acid loss was observed.

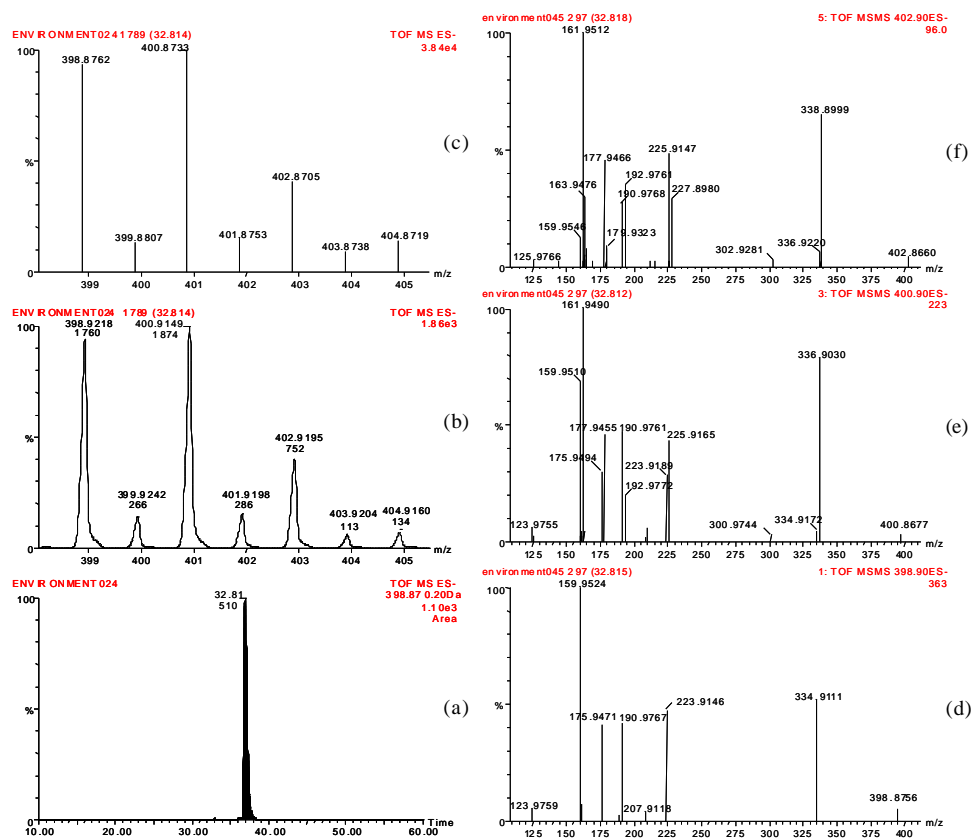


Figure 4. Unknown compound with MW 399.9 and retention time 32.8 min in a ground water : (a) mass chromatogram of m/z 398.87, (b) combined smoothed spectrum and (c) centered mass-measured spectrum of the unknown.(d) MS/MS spectrum of precursor ion m/z 398, (e) MS/MS spectrum of precursor ion m/z 400 and (f) MS/MS spectrum of precursor ion m/z 402.

Evaluation

This work has shown the interesting possibilities of quadrupole time-of-flight mass spectrometry in combination with an elimination strategy based on the isotopic abundances for the identification of unknown compounds present in environmental water. The information on accurate mass for the precursor and product ions provided by QTOF instruments, together with the developed strategy, has provided a rapid and useful procedure with important applicability in this field.

However, there are some limitations that should be taken into account. Due to the need of pre-selecting "relevant" peaks (in our case ions with area greater than 100 a.u.), information on other ions, which could correspond to other relevant contaminants, is missing. For example, the presence of imazalil in urban wastewater, led us to suspect the possible presence of other post-harvest fungicides used on citrus, such as thiabendazole (m/z $[M+H]^+$ 202.0439). Thus, an extracted ion chromatogram with a 0.2 Th mass window at the exact m/z corresponding to thiabendazole was carried out, showing an $[M+H]^+$ ion (area lower than 100 a.u.) at m/z 202.0441 ($\Delta mDa = 0.2$). The product ion spectrum was recorded obtaining two important product ions, at m/z 175.0341 ($\Delta mDa = 1.1$) and at m/z 131.0629 ($\Delta mDa = 2.0$), also present in the MS/MS spectrum of the standard, in this way confirming the presence of thiabendazol in these water samples at a concentration level of around 50 $\mu g/L$. The presence of these two compounds helps us to suspect the origin of these fungicides to be the warehouses dedicated to citrus fruit operations. Due to the different response of analytes in a LC-MS system, higher areas do not necessary imply higher concentrations. Thus, pre-selecting relevant peaks based on their areas does not seem to be a sufficient criterion to apply. However, a compromise has to be reached between appropriate preselection filtering and the time and effort involved in research. An additional criterion based on toxicological information appears to be one of the most interesting approaches.

Moreover, the interface used restricts the application to those compounds easily ionizable with this interface, normally from medium polar to ionic. Thus, the use of other interfaces, as APCI or APPI, or the use of a GC/TOF system with electron ionization, would be an ideal complementary tool to widen the scope of the determination of pollutants in water samples towards the less polar ones.

CONCLUSION

A data-processing and interpretation approach to help in the identification of unknown compounds in water based on the use of (SPE)-LC/QTOF-MS in combination with data processing has been developed and successfully applied to different types of water samples.

After acquisition in full scan mode, data were processed by MetaboLynx software application. Accurate mass measurement of selected peaks based on their MS response was performed and possible elemental compositions were calculated, using appropriate parameter settings. One of the main problems associated with the limited resolution of TOF instruments (5000 fwhm with our instrument) is the large number of possible elemental compositions obtained for a mass measured with a mass error of below 10 mDa. Therefore, an approach based on the experimental isotopic abundances was tested using model compounds and subsequently applied to unknowns in order to reduce the number of possible compositions. Additionally, QTOF-MS/MS experiments were performed in order to further reduce the number of possible empirical formulae. In this way, the accurate masses of small neutral losses and product ions were both used. The remaining elemental compositions were searched in the available compound databases. The success of the procedure described depends in some way on the availability of compound databases where the search can be performed. However, extending these databases to include a larger number of potential structures to a particular elemental composition could result in some cases in even more ambiguity in the interpretation of the data, simply because without standards one cannot really and straightforwardly discriminate between various isomeric compounds from the MS/MS spectrum. One of the major limitations comes from the limited understanding of the fragmentation rules in MS/MS of (de)protonated molecules.

By applying the developed approach, the structures of several unknown compounds were elucidated while, in other cases, when we did not succeed in obtaining the structure of the unknown, molecular formulae and some functional groups could be highlighted. Obviously, limitations in this approach result from the intrinsic characteristics and requirements of LC/MS, both in relation to the chromatographic, and to the ionization and fragmentation processes. Further restrictions came from the need to preselect relevant peaks based on ion abundance in order to reach a good compromise between time and effort invested and information gathered.

The availability of accurate masses for precursor and product ions, leading to feasible, possible elemental compositions, in combination with the developed approach and the fragmentation pathway, has been shown to be a useful tool that can solve the identification of unknown compounds. In some cases, however, the MS expertise, as well as the acquisition of information-rich product ion spectra, became relevant factors in the elucidation process,

especially when no hits were found in the databases. The availability of standards is a factor of outstanding importance in order to unequivocally confirm the identity of the compound.

ACKNOWLEDGEMENTS

The authors are very grateful to Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of the triple quadrupole mass spectrometer (Quattro LC) and the Q-TOF hybrid quadrupole time of flight mass spectrometer. Both instruments have been bought thanks to European FEDER Funds.

This work forms a part of the research project "Aplicación del acoplamiento cromatografía líquida-espectrometría de masas (LC-MS/MS y LC-QTOF MS) a la identificación de metabolitos de plaguicidas en muestras de interés ambiental y toxicológico"(Ref: BQU2003-02685) developed under financial support of the Ministry of Science and Technology, Spain.

M.I. is very grateful to Generalitat Valenciana for her pre-doctoral grant.

REFERENCES

- (1) Ferrer I., Thurman E.M. *Trends Anal. Chem.* 2003; **22**: 750-756
- (2) Ferrer I., Thurman E.M. *Liquid chromatography Mass Spectrometry, MS/MS and Time-of-Flight MS: Analysis of Emerging Contaminants.* 2003, American Chemical Society Symposium Series 850, New York
- (3) Maizels M., Budde W.L.; *Anal. Chem.* 2001; **73**: 5436-5440
- (4) Hogenboom A.C., Niessen W.M.A., Little D., Brinkman U.A.T. *Rapid Commun Mass Spectrom* 1999; **13**: 125-133
- (5) Hogenboom A.C., Niessen W.M.A., Brinkman U.A.T. *Rapid Commun Mass Spectrom* 2000; **14**: 1914-1924
- (6) Malato S., Albanis T., Piedra L., Aguera A., Hernando D, Fernandez-Alba A.; in [2], Chapter 8
- (7) Ferrer I., Thurman E.M., Parry R. *J. Chromatogr A* 2002; **957**: 3-9
- (8) Hernández F., Ibañez, M., Sancho J.V., Pozo O.J. *Anal. Chem.*, in press
- (9) Ibañez M., Sancho J.V., Pozo O.J., Hernández F. *Anal. Chem.* 2004; **76**: 1328- 1335
- (10) Bobeldijk I., Vissers J.P.C., Kearney G., Major H., van Leerdam J.A. *J. Chromatogr. A* 2001; **929**: 63-74
- (11) Bobeldijk I., Stoks P.G.M., Vissers J.P.C., Emke E., van Leerdam J.A. Mulwijk B., Berbee R., Noij Th.H.M. *J. Chromatogr. A* 2002; **97**: 167-181
- (12) Corcoran O., Spraul M. *Drug Discovery Today* 2003; **8**: 624-631
- (13) Wolfender J.L., Ndjoko K., Hostettmann K. *Phytochem. Anal.* 2001; **12**: 2-22
- (14) Kim H., Feng W., Chan T.M., Ringden D., Bryant M., Cox K.A., Xia Y., Reichard G., Paliwai S., Hesk D., Palamanda J., Whit R.E., Nomeir A.A. *J. Sep. Sci* 2002; **25**: 877-882
- (15) Levsen K., Preiss A., Godejohann M. *Trends Anal. Chem.* 2000; **19**: 27-48
- (16) Grange A.H., Donnelly J.R., Sovocool G.W., Brumley W.C. *Anal. Chem.* 1996; **68**: 553-560
- (17) Grange A. H., Sovocool G. W., Donnelly J. R., Genicola F. A., Gorka, D. F. *Rapid Commun. Mass Spectrom* 1998; **12**: 1161-1169
- (18) Planque G., Amekraz B., Moulin V., Toulhoat P., Moulin C. *Rapid Commun. Mass Spectrom* 2001; **15**: 827-835

- (19) Leenheer J., Rostad C.E., Gates P.M., Furlong E.T., Ferrer, I. *Anal. Chem.* 2001; **73**: 1461-1471

Identificación de interferencias reveladas en etapas de confirmación.

Al tratar de confirmar la presencia del herbicida diurón en una muestra de agua subterránea (Serratella, Burriana, 12/06/2003), al extraer el ion correspondiente a su masa (m/z 233.02) observamos un pico cromatográfico a un tiempo de retención próximo al del diurón cuya masa exacta difería alrededor de 100 mDa de la del analito. Al observar el cromatograma atentamente (Figura 2.11 (a)), comprobamos que en realidad no había un pico sino dos. Uno de los picos presentaba un *pattern* isotópico característico que revelaba la presencia de dos átomos de cloro, concordando por tanto con la estructura del diurón (Figura 2.11 (b) abajo), mientras que el primer pico no presentaba este *pattern*, confirmando que se trataba de una interferencia (Figura 2.11 (b) arriba).

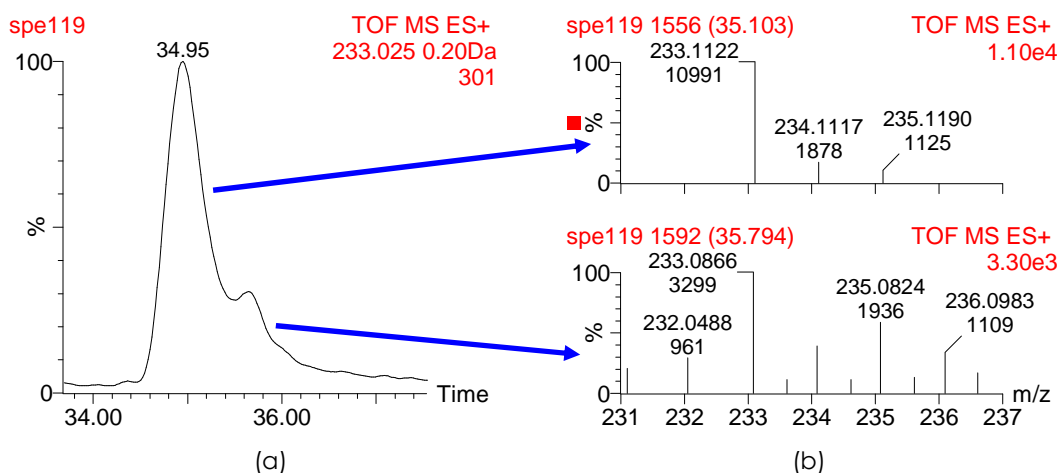


Figura 2.11 (a) XIC a la masa correspondiente al diuron (m/z 233.02) con una ventana de 0.2 Da, (b) espectros correspondientes a cada uno de los picos: (arriba) interferencia y (abajo) diurón

Para elucidar la estructura de este interferente, aplicamos la metodología desarrollada anteriormente en la elucidación de compuestos desconocidos basada en el uso de las abundancias isotópicas relativas. En la Figura 2.12(a) se muestra el espectro combinado y posteriormente suavizado, a partir del cual se calculan las relaciones isotópicas, y en la 2.12(b) el espectro centrado, a partir del cual se obtiene la masa exacta del compuesto (m/z 233.1122).

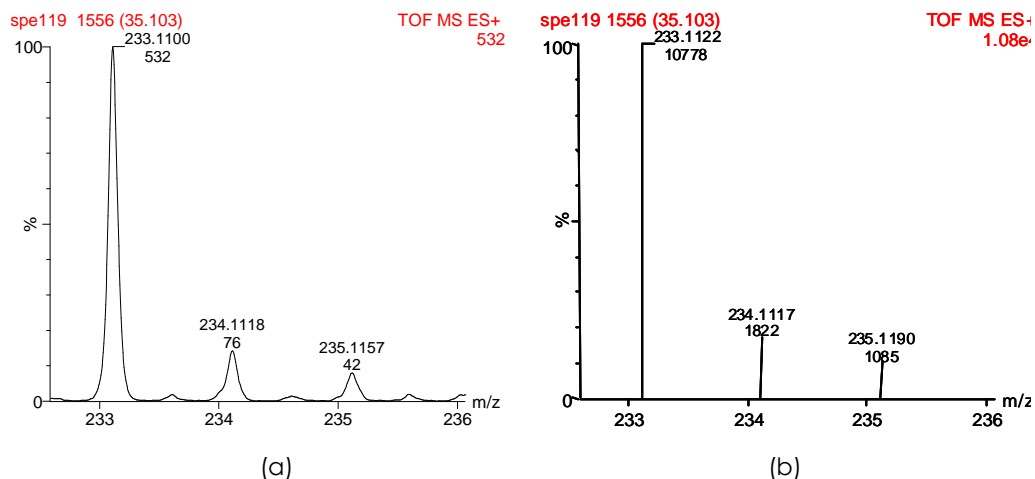


Figura 2.12 (a) espectro combinado y posteriormente suavizado, (b) espectro centrado, correspondientes al compuesto interferente de la Figura 2.11

Se calcularon los porcentajes isotópicos experimentales ($M+1$ y $M+2$) considerando un error experimental de $\pm 20\%$ cuando la abundancia se encontraba entre 60 y 200 cuentas, y un error de $\pm 50\%$ cuando la abundancia se encontraba por debajo de 60 cuentas (ver tabla 2.9). En este caso, en base al porcentaje de $M+2$ (7.9 %) y debido a las tolerancias permitidas, se asumió la presencia de entre 1 y 2 átomos de azufre. Se calcularon todas las posibles composiciones elementales, teniendo en cuenta los siguientes parámetros C: 0-50, H: 0-100, N: 0-20, O: 0-20, F: 0-20, P: 0-3 y S: 1-2, y aceptándose una desviación máxima de 10 mDa. Las abundancias isotópicas relativas teóricas se calcularon a partir del *Isotope model program*. Tras aplicar nuestra estrategia basada en un filtrado dependiendo de las abundancias isotópicas, resultaron 8 composiciones posibles (en negrita, Tabla 2.9).

Tabla 2.9 Composiciones elementales resultantes tras aplicar la estrategia basada en el uso de abundancias isotópicas

	M	M+1	M+2	60<area<200	Area <60
				(+/-20%)	(+/-50%)
				%M+1	%M+2
Espectro combinado	532	76	42	14,2%	7,9%
+ Error				17,0%	11,8%
- Error				11,4%	3,9%
	M	M+1	M+2	%M+1	%M+2
C₁₀H₁₇N₂OFS	8402	1088	454	12,9%	5,4%
C₁₁H₂₁OPS	8366	1120	456	13,4%	5,5%
C₁₃H₁₆N₂S	8148	1323	461	16,2%	5,7%
C₉H₁₄N₆F₂S	8906	649	415	7,3%	4,7%
C₁₀H₂₀N₂S₂	7999	1099	779	13,7%	9,7%
H₁₃N₁₂FS	9074	491	414	5,4%	4,6%
C₈H₁₆N₄O₂S	8508	977	462	11,5%	5,4%
C₁₂H₁₈F₂S	8297	1196	447	14,4%	5,4%
C₇H₁₆N₆OS	8560	947	444	11,1%	5,2%
CH₁₃N₁₀OFS	9019	525	431	5,8%	4,8%
C₆H₁₅N₄F₃S	8741	802	420	9,2%	4,8%
C₁₄H₁₂N₁₀S	8746	796	421	9,1%	4,8%
C₁₁H₂₀OS₂	7950	1126	794	14,2%	10,0%
C₁₁H₂₀O₃S	8327	1120	488	13,5%	5,9%

El espectro de MS/MS de este ion m/z 233 se muestra en la figura 2.13. El ion producto a m/z 151.0336 estaba formado por la pérdida de un grupo C₆H₁₀ ($\Delta mDa = 0.1$ mDa) mientras que el ion producto a 124.0207 era debido a la pérdida de un grupo HCN respecto al fragmento anterior ($\Delta mDa = 2$ mDa). Esto nos permitió descartar todas aquellas composiciones elementales sin ningún átomo de N (resaltados en negrita y tachados en Tabla 2.9), resultando posibles sólo 4 de ellas. Tras buscarlas en la librería NIST y en el Índice Merck, sólo se encontraron compuestos para dos composiciones: C₁₃H₁₆N₂S ($\Delta mDa = 0.8$ mDa), que resultó en 9 estructuras posibles, y C₁₀H₂₀N₂S₂ ($\Delta mDa = 2.4$ mDa) que resultó en una única estructura posible. Algunas de estas estructuras pudieron ser descartadas ya que no podían ser ionizadas en modo de electrospray positivo. Tras observar el resto de las estructuras posibles, observamos que dos de ellas, correspondientes al N-Ciclohexil-2-BenzotiazolAmina (NCBA) y 2-amino-3-ciano-4,4a,5,6,7,8,8a,9-octahidronaftol [2,3b]tiofene, concordaban perfectamente tanto con la posibilidad de ser ionizadas en modo positivo como con el espectro de impacto electrónico (GC-MS) que se muestra en la Figura 2.14. Los espectros de ambos compuestos presentaban el ion molecular

(M⁺ 232) así como un fragmento (M⁺ 150). Sin embargo, debido a la no disponibilidad de patrones comerciales, la identidad del compuesto no pudo ser confirmada con total seguridad.

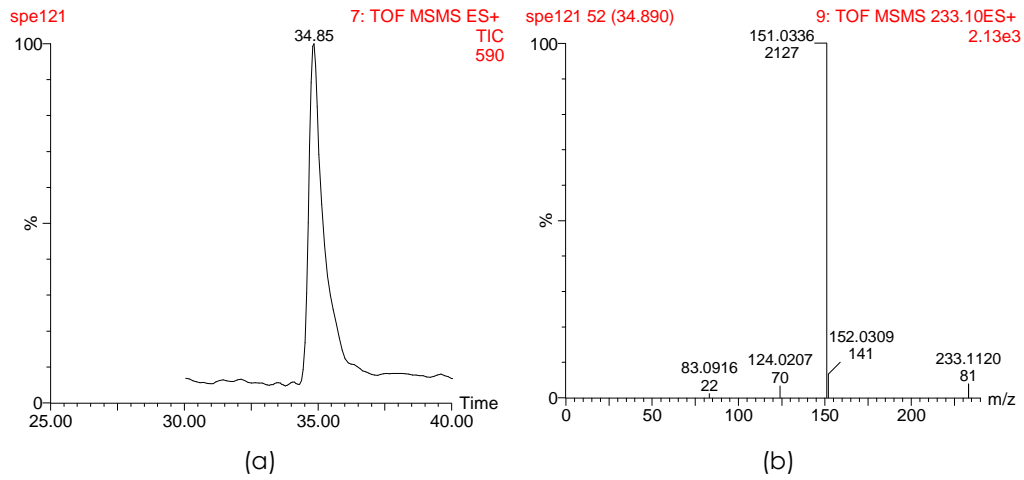


Figura 2.13 (a) Cromatograma LC-MS/MS, (b) Espectro MS/MS

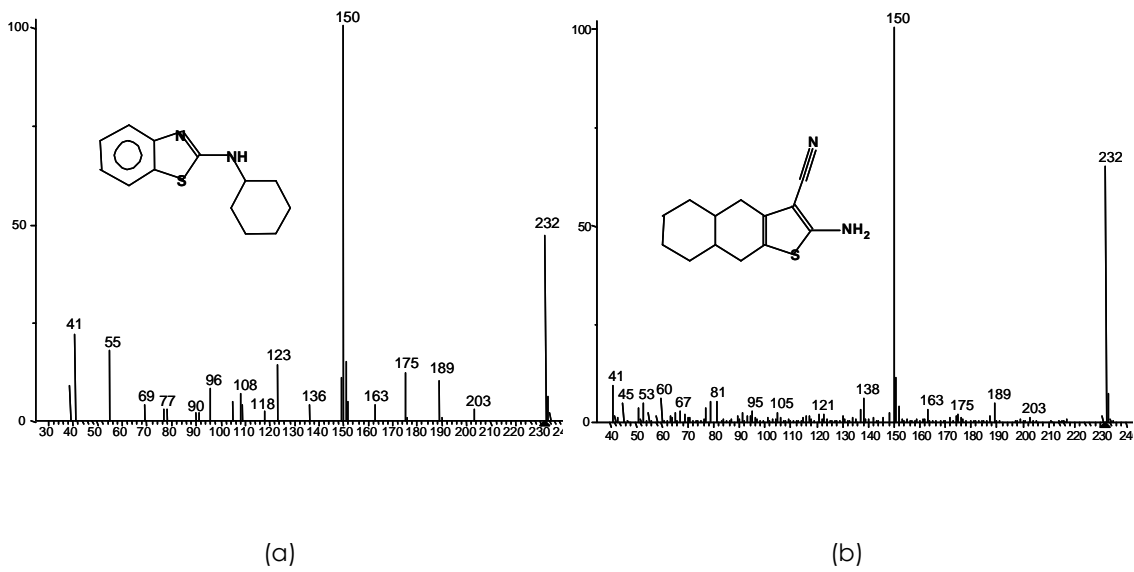


Figura 2.14 Espectros GC-MS del (a) 2-amino-3-ciano-4,4a,5,6,7,8,8a,9-octahidronaftol[2,3b]tiefene (b) N-ciclohexil-2-benzotiazolamina

Tras una búsqueda bibliográfica, encontramos que el NCBA forma parte del acelerador de vulcanización CBS, usado en la goma de los neumáticos. El NCBA ha sido encontrado en diversas matrices ambientales (Kumata et al, 2000), y ha sido propuesto como un posible marcador de los residuos de neumáticos. Por otra parte, la EPA lo ha clasificado como un peligroso constituyente de la basura. Por lo que se refiere al otro compuesto, no encontramos información.

Método de cribado (screening) para plaguicidas. Búsqueda de plaguicidas “desconocidos” (non target)

El objetivo principal del trabajo llevado a cabo durante la estancia de trabajo en Waters (Manchester) consistió en el desarrollo de un método UPLC-ToF MS para el análisis del orden de 100 plaguicidas en agua destinada al consumo humano, que nos permitiera tanto la identificación y confirmación de pesticidas preseleccionados, como la detección e identificación de pesticidas “desconocidos” (*non target*). Este trabajo formaba parte de una serie de iniciativas desarrolladas por Waters para el análisis multiresiduo de estos mismos plaguicidas en muestras vegetales mediante diversas técnicas como HPLC-MS/MS o UPLC-MS/MS.

Como ya hemos comentado, la espectrometría de masas con analizadores de TOF ofrece las ventajas de máxima sensibilidad en modo de barrido (*full scan*) y elevada exactitud de masa (menor de 5 ppm), lo que permite que todos los compuestos ionizables en la fuente electrospray sean detectados y a la vez confirmados, sin necesidad de ser previamente seleccionados. Este aspecto es de especial importancia en el caso de LC-MS, donde no se dispone de librerías de espectros MS.

Así pues resultaba interesante desarrollar un método que permitiera identificar y confirmar compuestos previamente seleccionados de una manera automática (por comparación con una librería de espectro de masas, por ejemplo), pero que también permitiera la detección e identificación de compuestos desconocidos.

Experimental

Hoy en día, la calidad de la separación en HPLC viene limitada por la columna analítica utilizada, y principalmente por el tamaño de partícula, ya que a medida que éste disminuye aumenta la eficacia de la separación. Obsevando la curva de Van Deemter (Figura 2.15), vemos que a tamaños de partícula muy pequeños, esta eficacia se extiende sobre un rango más amplio de velocidad lineal, consiguiendo por lo tanto mayor resolución y mayor rapidez en la separación.

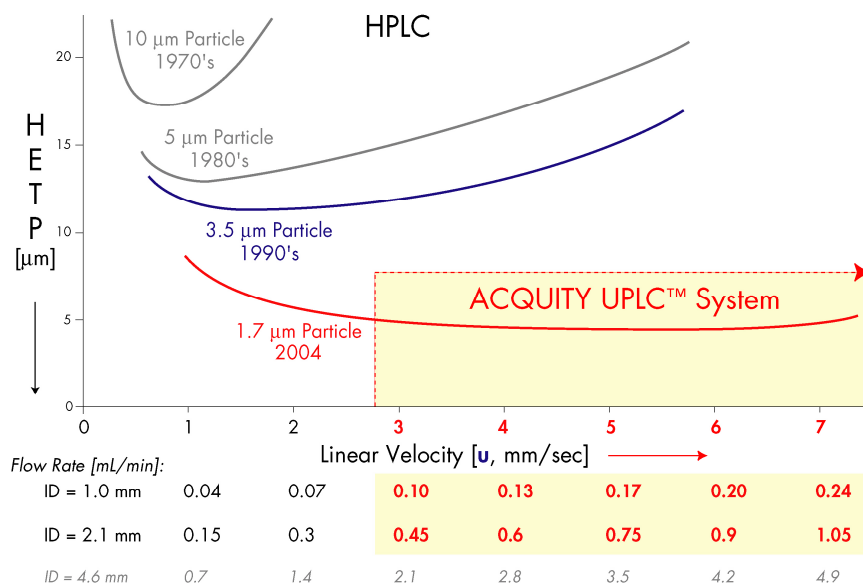


Figura 2.15 Curva de Van Deemter, ilustrando la evolución del tamaño de partícula a lo largo de las últimas décadas

La ACQUITY UPLC™ es una nueva categoría en las técnicas de separación que hace uso de estas premisas y que incorpora pequeños tamaños de partícula, volúmenes muertos muy bajos y una rápida detección. Estos sistemas han sido diseñados para controlar y optimizar todos los parámetros requeridos para aprovechar los beneficios de la UPLC en el laboratorio.

Por lo que se refiere al espectrómetro de masas, se utilizó un analizador de alta resolución de tiempo de vuelo ortogonal (LCT Premier). Este equipo dispone de la tecnología V-optics y W-optics, ya comentada en el capítulo de Introducción, con la que se consigue una mayor resolución (FWHM 5000 y 10000, respectivamente), al aumentar el camino que los iones han de recorrer en su vuelo. Esta resolución es vital para obtener medidas de masa exacta de modo automático en la identificación de componentes en muestras complejas, ya que minimiza

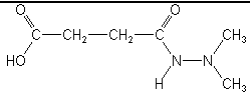
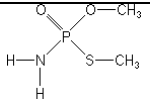
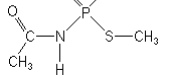
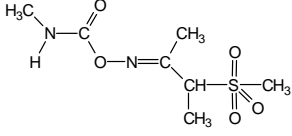
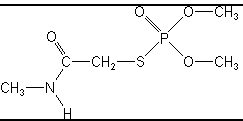
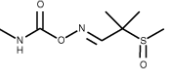
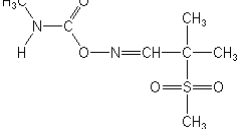
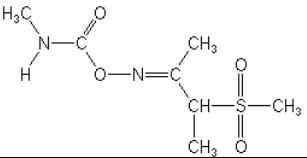
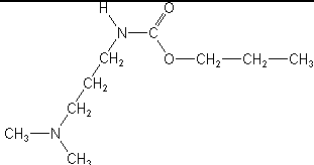
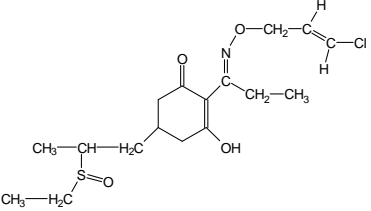
las potenciales interferencias en el pico del analito. Eliminado el efecto matriz, se puede obtener una medida más exacta de la relación m/z . La inherente alta resolución de un TOF ortogonal, junto con su capacidad de masa exacta, posibilita que los compuestos sean más fácilmente identificados y ayuda a la determinación de composiciones elementales. Por otra parte, la rápida adquisición del espectro total repercute en una excelente sensibilidad si se compara con otros instrumentos tradicionales adquiriendo en modo *scan*. Finalmente, el uso de la disociación inducida por colisión en la propia fuente permite obtener información estructural.

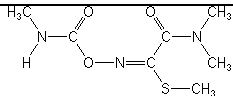
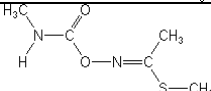
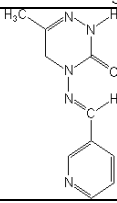
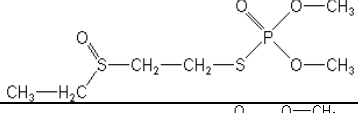
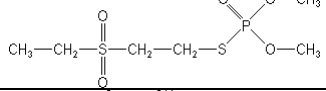
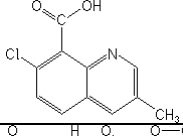
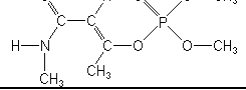
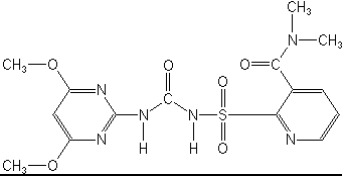
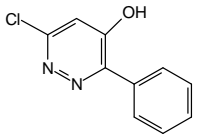
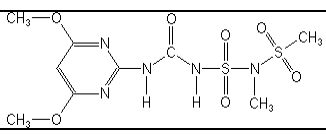
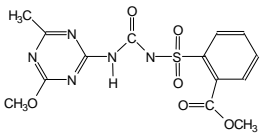
Este equipo disponía de una fuente dual de electrospray (*lock spray*) de modo que la adquisición paralela de un compuesto de referencia (*lock mass*) corrige cualquier cambio en las condiciones experimentales durante el análisis, manteniendo una exactitud de masa con errores inferiores a 5 ppm. De esta manera, ya no resulta necesario introducir el *lock mass* post-columna mediante una T, con las consiguientes ventajas, siendo la principal de ellas la no competencia en la ionización entre los analitos y el *lock mass*.

Compuestos estudiados

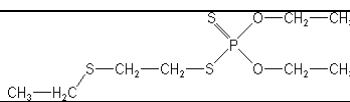
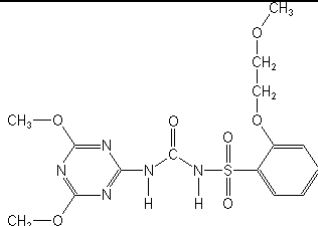
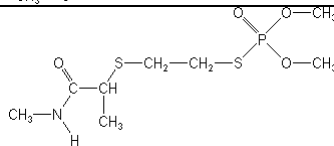
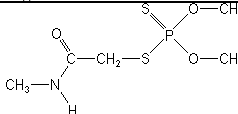
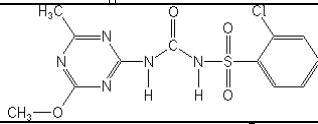
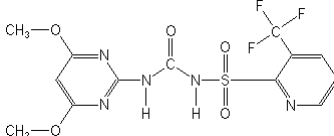
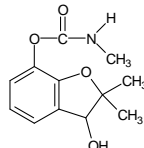
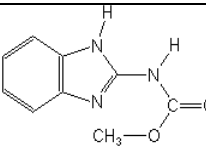
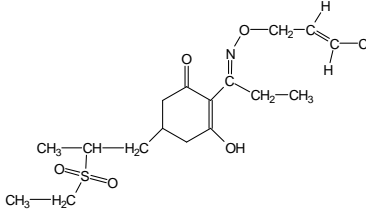
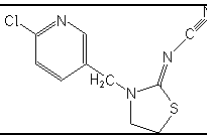
En la tabla 2.10 se muestran los compuestos estudiados (alrededor de 100 plaguicidas), entre los cuales se encontraban todos aquellos considerados importantes por la Agencia para la Protección del Medioambiente (EPA). Junto a la estructura de cada compuesto se muestra la composición elemental y la masa exacta del ion precursor. La masa exacta de cada ion precursor fue calculada usando la calculadora de pesos moleculares, incluida en el software. Además se estudió en cada caso la posible formación de aductos, con sodio o amonio, o posibles rupturas en la misma fuente (*in source CID*).

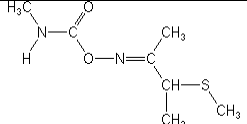
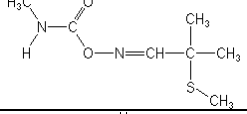
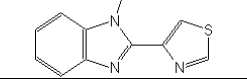
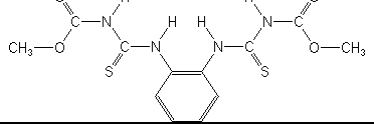
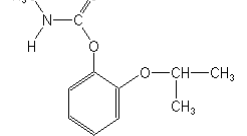
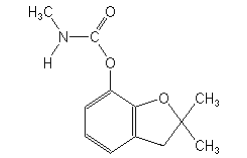
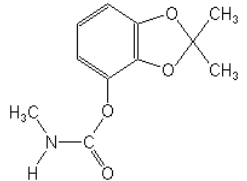
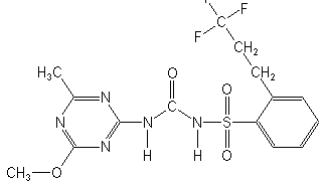
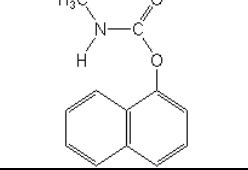
Tabla 2.10 Compuestos estudiados por UPLC-TOF MS

Compuesto	Estructura	t _{ret}	Fórmula Molecular	Ion molecular
Daminozide		0.6	C ₆ H ₁₂ N ₂ O ₃	161.0926(MH ⁺)
Methamidophos		0.8	C ₂ H ₈ NO ₂ PS	142.0092(MH ⁺)
Acephate		0.9	C ₄ H ₁₀ NO ₃ PS	184.0197(MH ⁺)
Butoxycarboxim-sulfoxid		1.0	C ₇ H ₁₄ N ₂ O ₅ S	207.0803 (frag cono C ₇ H ₁₄ N ₂ O ₃ S)
Omethoate		1.0	C ₅ H ₁₂ NO ₄ PS	214.0303 (MH ⁺)
Aldicarb-sulfoxid		1.1	C ₇ H ₁₄ N ₂ O ₃ S	207.0803 (MH ⁺) 224.1069 (MNH ₄ ⁺)
Aldoxicarb		1.2	C ₇ H ₁₄ N ₂ O ₄ S	223.0753 (MH ⁺) 240.1018
Butoxycarboxym		1.2	C ₇ H ₁₄ N ₂ O ₄ S	223.0753 (MH ⁺) 240.1018 (MNH ₄ ⁺)
Propamocarb		1.3	C ₉ H ₂₀ N ₂ O ₂	189.1603 (MH ⁺)
Clethodim-sulfoxid		1.4	C ₁₇ H ₂₆ NO ₄ SCl	376.1349 (MH ⁺)

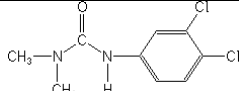
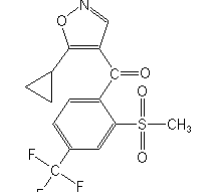
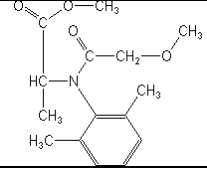
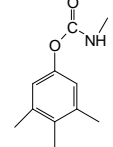
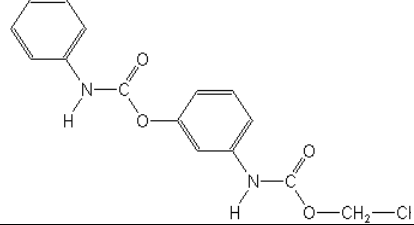
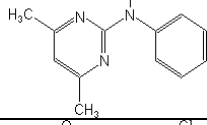
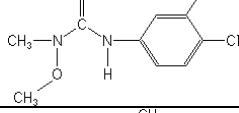
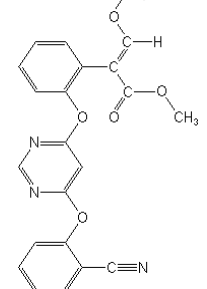
Oxamyl		1.4	C ₇ H ₁₃ N ₃ O ₃ S	220.0756 (MH ⁺) 237.1021 (MNH ₄ ⁺)
Methomyl		1.5	C ₅ H ₁₀ N ₂ O ₂ S	163.0541 (MH ⁺)
Pymetrozin		1.5	C ₁₀ H ₁₁ N ₅ O	218.1042 (MH ⁺)
Oxydemeton-methyl		1.5	C ₆ H ₁₅ O ₄ PS ₂	247.0228 (MH ⁺)
Demethon-S-methyl-sulfon		1.6	C ₆ H ₁₅ O ₅ PS ₂	263.0177 (MH ⁺) 280.0442 (MNH ₄ ⁺)
Quinmerac		1.7	C ₁₁ H ₈ ClNO ₂	222.0322 (MH ⁺)
monocrotophos		1.8	C ₇ H ₁₄ NO ₅ P	224.0688 (MH ⁺) 241.0953 (MNH ₄ ⁺)
Nicosulfuron		1.8	C ₁₅ H ₁₈ N ₆ O ₆ S	411.1087 (MH ⁺)
6-chloro-4-hydroxy-3-phenyl-pyridazin		2.0	C ₁₀ H ₇ N ₂ OCl	207.0325 (MH ⁺)
Amidosulfuron		2.0	C ₉ H ₁₅ N ₅ O ₇ S ₂	370.0491 (MH ⁺) 392.0311 (MNa ⁺)
Metsulfuron-methyl		2.1	C ₁₄ H ₁₅ N ₅ O ₆ S	382.0821 (MH ⁺)

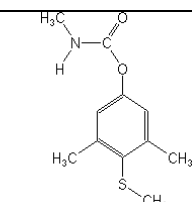
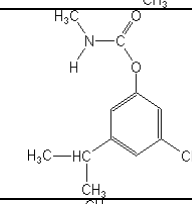
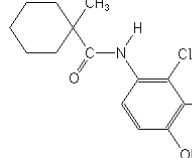
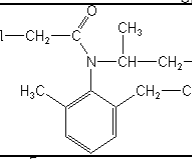
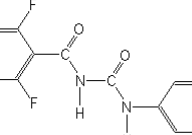
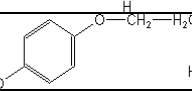
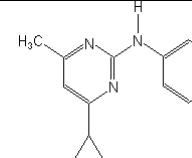
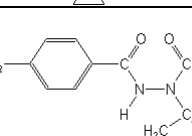
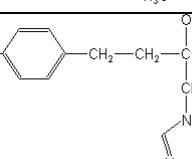
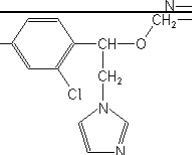
Ethiofencarbsulfon		2.1	C ₁₁ H ₁₅ NO ₄ S	258.0800 (MH ⁺) 275.1066 (MNH ₄ ⁺)
Thifensulfuron-methyl		2.1	C ₁₂ H ₁₃ N ₅ O ₆ S ₂	388.0386 (MH ⁺)
Triasulfuron		2.1	C ₁₄ H ₁₆ ClN ₅ O ₅ S	402.0639 (MH ⁺)
Imidacloprid		2.2	C ₉ H ₁₀ ClN ₅ O ₂	256.0601 (MH ⁺)
Thiofanox-sulfoxide		2.2	C ₉ H ₁₈ N ₂ O ₃ S	235.1116 (MH ⁺) 252.1382 (MNH ₄ ⁺)
5-OH-clethodim-sulfon		2.4	C ₁₇ H ₂₆ NO ₆ SCl	408.1248 (MH ⁺)
Clethodim-imin-sulfon		2.4	C ₁₄ H ₂₃ NO ₄ S	302.1426 (MH ⁺)
Thiofanox-sulfon		2.4	C ₉ H ₁₈ N ₂ O ₄ S	251.1066 (MH ⁺) 268.1331 (MNH ₄ ⁺)
Clethodim-imin-sulfoxide		2.5	C ₁₄ H ₂₃ NO ₃ S	286.1477 (MH ⁺)

Metamitron		2.5	C ₁₀ H ₁₀ N ₄ O	203.0933 (MH ⁺)
Cinosulfuron		2.5	C ₁₅ H ₁₉ N ₅ O ₇ S	414.1083 (MH ⁺)
Vamidothion		2.6	C ₈ H ₁₈ NO ₄ PS ₂	288.0493 (MH ⁺)
Dimethoat		2.6	C ₅ H ₁₂ NO ₃ PS ₂	230.0075 (MH ⁺)
Chlorsulfuron		2.6	C ₁₂ H ₁₂ N ₅ O ₄ SCl	358.0377 (MH ⁺) 375.0642 (MNH ₄ ⁺)
Flazasulfuron		2.7	C ₁₃ H ₁₂ N ₅ O ₅ S	408.0590 (MH ⁺)
Carbofuran-3-OH		2.7	C ₁₂ H ₁₅ NO ₄	238.1079 (MH ⁺) 255.1345 (MNH ₄ ⁺)
Carbendazim		3	C ₉ H ₉ N ₃ O ₂	192.0773 (MH ⁺)
Clethodim-sulfon		3.1	C ₁₇ H ₂₆ NO ₅ SCl	392.1298 (MH ⁺)
Thiacloprid		3.1	C ₁₀ H ₉ ClN ₄ S	253.0315 (MH ⁺)

Butocarboxim		3.4	C ₇ H ₁₄ N ₂ O ₂ S	191.0854 (MH ⁺) 213.0674 (MNH ⁺)
Aldicarb		3.6	C ₇ H ₁₄ N ₂ O ₂ S	191.0854 (MH ⁺) 208.1120 (MNH ⁺)
Thiabendazol		3.7	C ₁₀ H ₇ N ₃ S	202.0439 (MH ⁺)
Thiophanate-methyl		4.4	C ₁₂ H ₁₄ N ₄ O ₄ S ₂	343.0535 (MH ⁺)
Propoxur		4.4	C ₁₁ H ₁₅ NO ₃	210.1130 (MH ⁺)
Carbofuran		4.5	C ₁₂ H ₁₅ NO ₃	222.1130 (MH ⁺)
Bendiocarb		4.5	C ₁₁ H ₁₃ NO ₄	224.0923 (MH ⁺) 241.1188 (MNH ⁺)
Prosulfuron		4.8	C ₁₅ H ₁₆ F ₃ N ₅ O ₄ S	420.0953 (MH ⁺)
Carbaril		4.9	C ₁₂ H ₁₁ NO ₂	202.0868 (MH ⁺) 219.1134 (MNH ⁺)

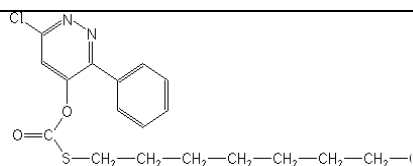
Bensulfuron-methyl		5.0	C ₁₆ H ₁₈ N ₄ O ₇ S	411.0974 (MH ⁺)
Ethiofencarb		5.1	C ₁₁ H ₁₅ NO ₂ S	226.0902 (MH ⁺) 243.1167 (MNH ₄ ⁺)
Thiofanox		5.2	C ₉ H ₁₈ N ₂ O ₂ S	219.1167 (MH ⁺) 241.0986 (MNa ⁺)
Trisulfuron-methyl		5.2	C ₁₇ H ₁₉ F ₃ N ₆ O ₆ S	493.1117 (MH ⁺) 515.0937 (MNa ⁺)
Pirimicarb		5.3	C ₁₁ H ₁₈ N ₄ O ₂	239.1508 (MH ⁺)
Thiodicarb		5.3	C ₁₀ H ₁₈ N ₄ O ₄ S ₃	355.0568 (MH ⁺)
Clethodim		5.3	C ₁₇ H ₂₆ NO ₃ SCI	360.1400 (MH ⁺)
Atrazine		5.4	C ₈ H ₁₄ N ₅ Cl	216.1016 (MH ⁺)
Isoproturon		5.7	C ₁₂ H ₁₈ N ₂ O	207.1497 (MH ⁺)

Diuron		5.7	C ₉ H ₁₀ N ₂ OCl ₂	233.0248 (MH ⁺)
Isoxaflutole		5.8	C ₁₅ H ₁₂ F ₃ NO ₄ S	360.0517 (MH ⁺) 377.0783 (MNH ₄ ⁺)
Metalaxyl		5.8	C ₁₅ H ₂₁ NO ₄	280.1549 (MH ⁺)
3,4,5-trimethacarb		5.9	C ₁₁ N ₁₅ NO ₂	194.1181 (MH ⁺)
Desmedipham		6.1	C ₁₆ H ₁₆ N ₂ O ₄ (NH ₄)	301.1188 (MH ⁺) 318.1454 (MNH ₄ ⁺)
Pyrimethanil		6.4	C ₁₂ H ₁₃ N ₃	200.1188 (MH ⁺)
Linuron		6.4	C ₉ H ₁₀ Cl ₂ N ₂ O ₂	249.0198 (MH ⁺)
Azoxystrobin		6.6	C ₂₂ H ₁₇ N ₃ O ₅	404.1246 (MH ⁺)

Methiocarb		6.6	C ₁₁ H ₁₃ NO ₂ S	226.0902 (MH ⁺) 243.1167 (MNH ₄ ⁺)
Promecarb		6.8	C ₁₂ H ₁₇ NO ₂	208.1338 (MH ⁺) 225.1603 (MNH ₄ ⁺)
Fenhexamid		7.3	C ₁₄ H ₁₇ Cl ₂ NO ₂	302.0715 (MH ⁺)
Metolachlor		7.5	C ₁₅ H ₂₂ ClNO ₂	284.1417 (MH ⁺)
Diflubenzuron		7.6	C ₁₄ H ₉ ClF ₂ N ₂ O ₂	311.0399 (MH ⁺) 333.0218 (MNa ⁺)
Fenoxycarb		7.7	C ₁₇ H ₁₉ NO ₄	302.1392 (MH ⁺)
Cyprodinil		7.9	C ₁₄ H ₁₅ N ₃	226.1344 (MH ⁺)
Tebufenozide		7.9	C ₂₂ H ₂₈ N ₂ O ₂	353.2229 (MH ⁺) 375.2048 (MNa ⁺)
Tebuconazole		8.0	C ₁₆ H ₂₂ ClN ₃ O	308.1530 (MH ⁺)
Imazalil		8.2	C ₁₄ H ₁₄ Cl ₂ N ₂ O	297.0561 (MH ⁺)

Triflumuron		8.3	C ₁₅ H ₁₀ ClF ₃ N ₂ O ₃	359.0410 (MH ⁺) 381.0230 (MNa ⁺)
Haloxifop-methyl		8.6	C ₁₆ H ₁₃ ClF ₃ NO ₄	376.0563 (MH ⁺) 393.0829 (MNH ⁺)
Spiroxamine		9.0	C ₁₈ H ₃₅ NO ₂	298.2746 (MH ⁺)
Quizalofop-ethyl		9.0	C ₁₉ H ₁₇ ClN ₂ O ₄	373.0955 (MH ⁺)
Haloxifop-ethoxyethyl		9.1	C ₁₉ H ₁₉ ClF ₃ NO ₅	434.0982 (MH ⁺) 456.0802 (MNa ⁺)
Furathiocarb		9.1	C ₁₈ H ₂₆ N ₂ O ₅ S	383.1641 (MH ⁺)
Fluazifop-P-butyl		9.8	C ₁₉ H ₂₀ F ₃ NO ₄	384.1423 (MH ⁺)
Flufenoxuron		9.9	C ₂₁ H ₁₁ ClF ₆ N ₂ O ₃	489.0441 (MH ⁺) 511.0260 (MNa ⁺)
Fenpropimorph		11.5	C ₂₀ H ₃₃ NO	304.2640 (MH ⁺)

Pyridate



11.6

C₁₉H₂₃ClN₂O₂S379.1247 (MH⁺)

Instrumentación

Se utilizó una bomba HPLC Acquity (Waters, Milford) acoplada a un espectrómetro de masas de tiempo de vuelo (LCT Premier, Micromass, Manchester, UK) usando una interfase electrospray ortogonal de Z-spray. Como gas de desolvatación se utilizó nitrógeno, generado a partir de aire presurizado en un generador de nitrógeno, a un flujo de 600 L/h. Se seleccionó un cono de 50 V y un capilar de 1.0 kV tanto en modo de ionización positivo como negativo. Los voltajes de apertura fueron 1, 10, 15, 20 y 25 V. La temperatura de la interfase fue de 120°C y la temperatura de desolvatación 450°C.

El equipo se calibró semanalmente usando una bomba de jeringa directamente conectada a la interfase, en el mismo rango con el que después se iba a trabajar, normalmente de m/z 50 a m/z 1000. La calibración se llevó a cabo en ambos modos de ionización utilizando una mezcla formada por hidróxido sódico 0.1M:ácido fórmico 10% (50:50) diluida con agua:isopropanol (en modo de ionización positivo) o agua:acetonitrilo (en modos de ionización negativo), en el rango m/z 50-1000 Da.

Como *lock mass* se estudiaron diversos compuestos con el fin de obtener el más adecuado, ya que debido al bajo potencial del capilar empleado no todos los compuestos se ionizaban bien. Así, se estudió el verapamil, el cloranfenicol y la leucina enkefalina, seleccionando finalmente este último compuesto para ambos modos de ionización. Su estructura se muestra en la Figura 2.16.

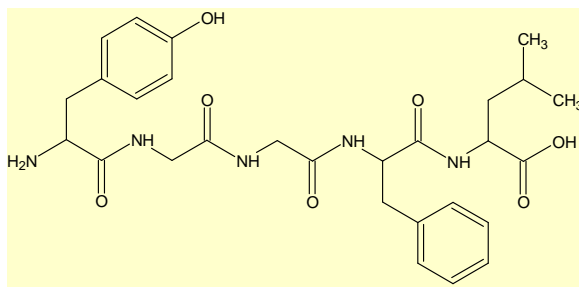


Figura 2.16 Estructura de la leucina enkefalina

Para la separación cromatográfica se utilizó una columna analítica C₁₈ Waters ACQUITY UPLC BEH (Waters) con unas dimensiones de 2.1 mm de diámetro interno x 100 mm de longitud con un tamaño de partícula de 1.7 µm, a un flujo de 450 µL/min. La fase móvil estaba formada por A = MeOH/H₂O (1:19 v/v) + acetato amónico 2mM y B = MeOH/H₂O (19:1 v/v) + acetato amónico 2mM. El porcentaje de B varió linealmente del siguiente modo: 0 min.,0%; 8.5 min.,100%; 11 min.,100%; 11.1 min.,0%; 13.5 min.,0%. El volumen de inyección fue 50 µL.

Tratamiento de muestra

No fue necesario ningún pretratamiento de las muestras, ya que se inyectaron directamente en el sistema UPLC-TOF MS.

Resultados y discusión

Creación de la librería

Tal y como se ha comentado anteriormente, uno de los objetivos de este trabajo era desarrollar un método que permitiera la identificación y confirmación de un buen número de plaguicidas previamente seleccionados. Para ello era necesario aplicar algún tipo de software que permitiera la deconvolución de los cromatogramas, con el fin de asignar a cada componente su identidad por comparación con una librería previamente creada. Dado que disponíamos de un software apropiado para tal propósito (ChromaLynx), el siguiente paso, una vez optimizadas las condiciones experimentales, era la creación de nuestra propia librería.

Para ello, se cromatografiaron todos los compuestos objeto de estudio, intentando obtener espectros lo más puros posibles. Para cada uno de ellos se completó una ficha como la que se muestra a continuación (Figura 2.17), en donde se indicaba el nombre del compuesto, la masa molecular nominal, el tiempo de retención, la masa molecular exacta, el modo de ionización en el que se había adquirido y el voltaje del cono. Estas experiencias se repitieron a diversas energías de apertura 1, con el fin de incluir en la librería los espectros de los compuestos tras ser fragmentados.

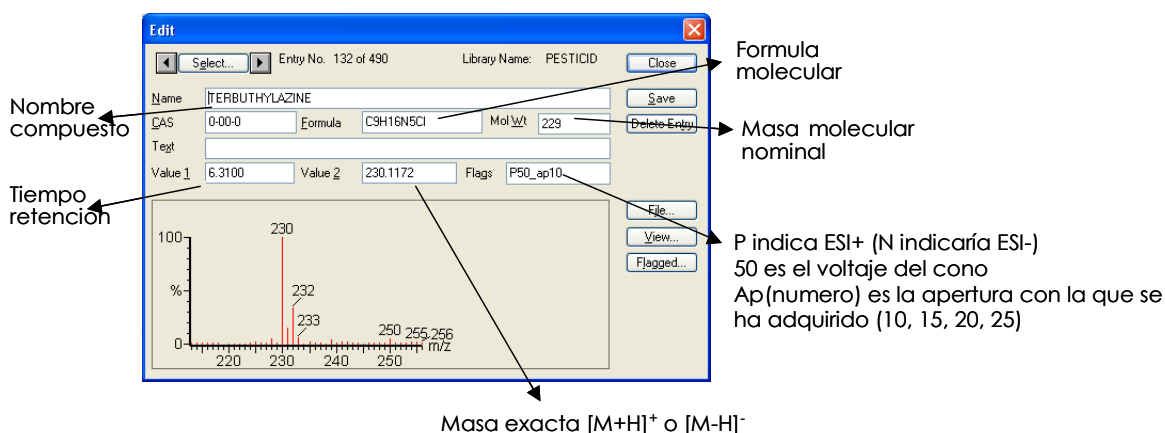


Figura 2.17 Información incluida en la librería para cada compuesto

Detección e identificación de compuestos preseleccionados

La revisión manual de cromatogramas complejos, con el fin de localizar potenciales componentes suele ser una tarea muy lenta, y a menudo no es capaz de detectar componentes que coeluyen. La automatización de esta tarea haciendo uso de un software específicamente diseñado para ello (ChromaLynx), permite una rápida detección, identificación y determinación semicuantitativa de todos los componentes con bastante seguridad.

Tras procesar los datos con el ChromaLynx, y haciendo uso de la librería que previamente habíamos creado, este software era capaz de detectar todos los picos presentes en el cromatograma, agruparlos por componentes y asignarles una posible identidad con una cierta probabilidad, como se muestra en la Figura 4 de la *Nota de Aplicación 1*.

Confirmación

Como ya hemos comentado anteriormente, este instrumento permite obtener medidas de masa exacta, lo cual puede utilizarse no sólo para la identificación de los compuestos sino también para su confirmación.

Identificación de compuestos desconocidos

Otra de las posibilidades del CromaLynx es la detección de todos los componentes presentes en el cromatograma, sin tener en cuenta si están o no en la librería. Si se detectara algún compuesto desconocido, se podría hacer uso de las herramientas vistas (masa exacta en MS, posibilidad de realizar fragmentación en la misma fuente, cálculo de composiciones elementales con ajuste automático del *pattern isotópico*) con el fin de identificar o elucidar su estructura. De este modo, no solo se conseguiría determinar y confirmar compuestos preseleccionados, sino también identificar otros compuestos desconocidos también presentes.

Cuantificación

Como ya es sabido, una de las principales limitaciones de los instrumentos con analizadores TOF es el análisis cuantitativo, debido al bajo rango lineal que suelen poseer (normalmente dos órdenes de magnitud). Sin embargo, gracias a las mejoras introducidas en este instrumento, el rango dinámico lineal ha sido ampliado posibilitando la cuantificación de muchos de los compuestos estudiados, llegando a alcanzar 3 y 4 órdenes de magnitud. Para ello, usamos el software QuanLynx (ver Figura 5 de la *Nota de aplicación 1*).

Así pues se ha desarrollado un método UPLC-ToF MS que permite el análisis de aproximadamente 100 compuestos en tan sólo 13 minutos, aplicable tanto a muestras simples como complejas. Por una parte, el sistema UPLC utilizado proporciona una cromatografía rápida con buena resolución, minimizando las posibles interferencias de compuestos que coeluyan con el analito. Por otra parte, la elevada exactitud de masa proporcionada por el LCT Premier permite la confirmación de los compuestos preseleccionados y ayuda en la identificación de compuestos desconocidos. El software CromaLynx permite la deconvolución de cromatogramas complejos, permitiendo asignar a cada compuesto su identidad por comparación con una librería de MS previamente creada. Por otra parte y dado que se adquiere en modo *full scan*, también pueden ser identificados los compuestos no incluidos en la librería en función de la masa exacta del ion precursor y de los fragmentos formados por disociación inducida en la propia fuente. Esta capacidad de masa exacta puede ser utilizada también con fines confirmatorios. Finalmente, debido a la mejora en el rango dinámico es posible la cuantificación de los compuestos. En un futuro cercano está previsto ampliar la librería para que contenga el mayor número posible de contaminantes.

Nota de aplicación 1

Waters Application Note (2006)

UPLC-ToF MS FOR RAPID SCREENING OF MULTIPLE PESTICIDE RESIDUES

Daniel McMillan¹ and María Ibañez Martínez²

¹Waters Corp. MS Technologies Centre, Atlas Park, Simonsway, Manchester, UK

²Research Institute for Pesticides and Water, Universitat Jaume I, Castellon, Spain

INTRODUCTION

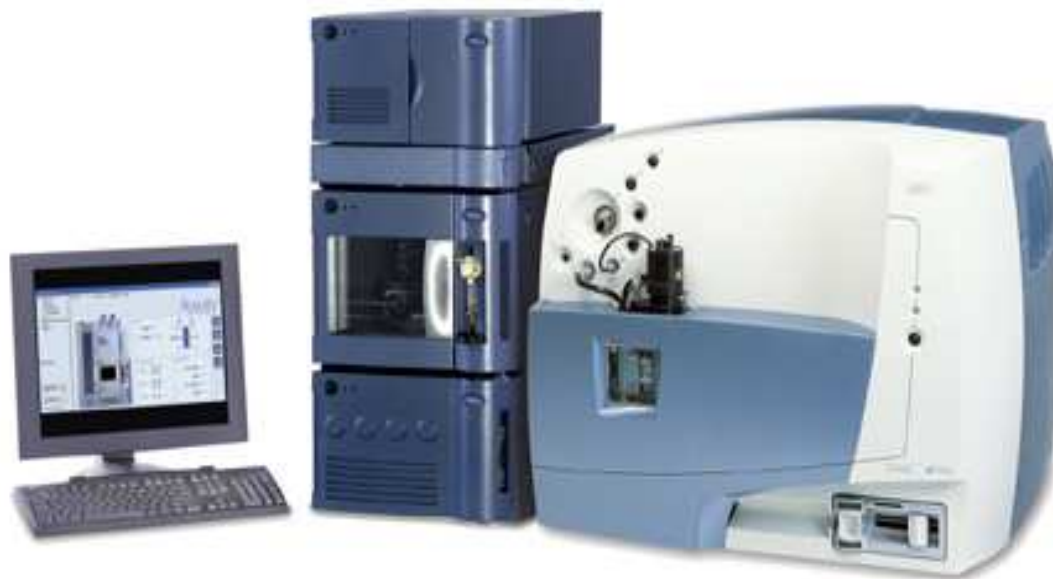
Pesticides Analysis Background

New pesticides are continually being developed and introduced for use in agricultural production. Recent advances in analytical techniques, as well as changes in the types of pesticide compounds and the way in which they are used have led to a requirement for a rapid and sensitive yet generic pesticide residue screening method.

Traditionally, GC and LC coupled to either single quadrupole MS or other detectors have been the most commonly employed analytical techniques. However, due to their relatively low sensitivity and selectivity, they have required large injection volumes or more rigorous sample preparation to reach the required limits of detection. More recently, tandem quadrupole MS/MS analysers have been used to attain low detection levels and the increased selectivity provided by multiple reaction monitoring (MRM) experiments has enabled the analysis of complex matrices without extensive clean-up. These advantages have made MS/MS the method of choice for low level quantitation and confirmation for a large number of targeted compounds.

Setting up MRM methods for the selected analytes is time consuming and the analysis is inherently targeted towards a limited number of compounds. This has led analysts to consider what other potentially harmful, non-targeted analytes may be in the samples and has resulted in the demand for an analytical method that is sensitive and selective, but not specific.

The Waters® Micromass® LCT Premier™, a new time of flight mass spectrometer, provides a solution for this dilemma. The LCT Premier couples very high full-spectral sensitivity with high resolution mass spectra allowing any ionisable component in a sample to be exact mass-measured and its elemental composition calculated or confirmed to <3ppm.



Waters ACQUITY UPLC with Waters Micromass LCT Premier Mass Spectrometer

The Waters® ACQUITY UPLC™ is a novel ultra-performance liquid chromatograph utilising 1.7 µm stationary phase particles in a high pressure system. This provides a fast, high resolution separation which increases LC/MS sensitivity and mitigates matrix interference arising from minimal sample preparation.

Outline of Work

The aims of the work presented here are principally to demonstrate the applicability of ToF MS analysis for pesticide screening and the potential advantages of such a technique in a controlled laboratory environment.

Drinking water has recently become the subject of much discussion due to homeland security concerns, and here the analysis of unexpected contaminants is a particularly high priority. Although water is a relatively simple medium, the techniques used in such an analysis are applicable to a wide range of different matrices. In order to illustrate this, quantitative results from tomato extracts are also presented.

Data generated by the UPLC/MS system is processed with Waters® ChromaLynx™ Application Manager software which de-convolutes the chromatograms and displays the mass-measured spectra from each peak. The spectra can be compared against a library of target analytes or used to help determine the identity of an unknown compound.

METHODS

Extraction Procedure – developed by Jeannette Klein and Lutz Alder at FIRA, Berlin.

- 10g sample weighed. 5g for dry sample materials.
- Water is added to obtain 10ml as a sum of natural and added water.
- After addition of 20ml methanol the sample is blended for 2min.
- 6ml of extract is mixed with 2ml of NaCl solution
- 5ml is transferred to a ChemElut column.
- The column is eluted with 16ml of dichloromethane and evaporated.
- The dry residue is dissolved in 250ml methanol and 1000ml water.
- Extract is filtered through 0.45mm syringe filter.
- Matrix equivalent of 1g/ml for normal produce or 0.5g/ml for dry produce.

LC Conditions

LC System: Waters ACQUITY UPLC
Mobile Ph. A: 5% aqueous MeOH + 2mM CH₃CO₂NH₄
Mobile Ph. B: 95% aqueous MeOH + 2mM CH₃CO₂NH₄
Column: ACQUITY UPLC BEH C18 1.7 µm 2.1 x 100 mm
Flow Rate: 0.45 mL/min
Injection Vol.: 20 µL
Column Temp: 40 °C
Gradient: t = 0 min, 0% B ; t = 8.5 min, 100% B; t = 11 min, 100% B; t = 11.1 min, 0% B ; t = 13.5 min, 0% B

MS Conditions

Instrument: Waters Micromass LCT Premier
Ion Mode: Electrospray +/-
Capillary V: 1000 V
Source T: 120 °C
Desolvation T: 400°C
Gas Flow: 600 L/hr
Mass Range: 50—1000 Da
Acq. Time: 0.25 s/function
Calibration: NaCH₂O₂ in pos. and neg. modes
LockSpray™: Leucine Enkephalin
reference [M+H]⁺ = 556.2771 Da [M-H]⁻ = 554.2615 Da

RESULTS & DISCUSSION

Figure 1 shows the base peak intensity chromatograms from the analysis of drinking water spiked with 92 pesticide residues at a concentration of 100 ppb. Six of the components ionise exclusively in negative mode, and both traces are shown to illustrate the simultaneous acquisition of all spiked pesticides in positive and negative mode. The chromatographic peaks here are typically ~5s wide at base.

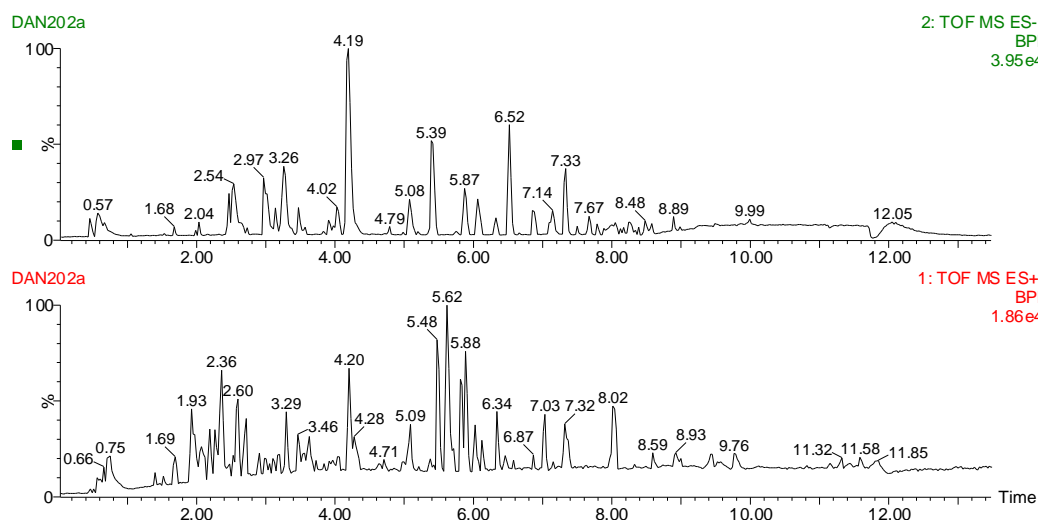


Figure 1. Positive and negative ion BPI chromatograms of drinking water spiked at 100 ppb

Figure 2 shows a typical combined spectrum of Diuron, together with its elemental composition calculation. It is clear that there are a number of possibilities within 5 ppm of the measured mass, so the observed isotope pattern is compared to a theoretical model using i-FIT™ software. In this case, although not the closest match by exact mass ($\Delta M = 1.3$ ppm) the correct formula is displayed as the highest rank, since its isotope pattern is the closest match.

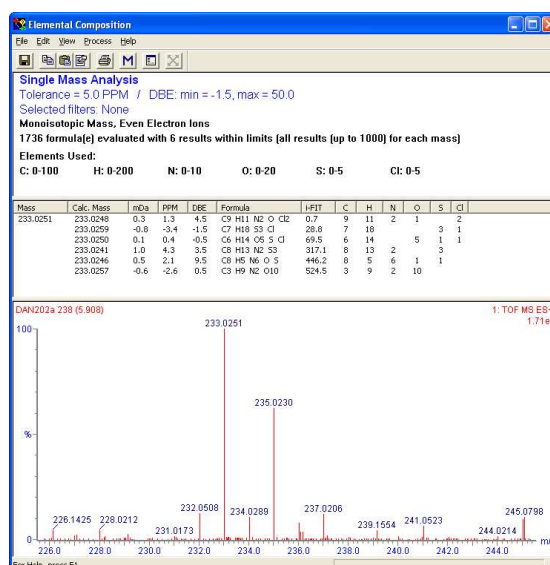


Figure 2. Spectrum and EleComp report for diuron, ranked by closest isotope ratio fit.

When targeting a specific compound, it is a simple operation to plot an extracted ion chromatogram (XIC) of its exact mass. Because of the high mass accuracy of the ToF data, the selectivity of this technique is greatly enhanced, and good signal to noise ratios are obtained as illustrated in Figure 3. However, if multiple residues are to be investigated, it becomes time consuming to target them all individually. In this case, it is useful to process data using the powerful chromatographic deconvolution software provided by the ChromaLynx application manager. ChromaLynx automatically plots the XICs of up to the eight most intense ions at any point in the chromatogram, and if a peak is found to satisfy user defined parameters, displays its deconvoluted exact mass spectrum. This can then be analysed to elucidate an elemental composition or compared against a library of spectra obtained from standards. Each library entry will include mass, formula, retention time and polarity/cone voltage information, all of which can be used to filter the 'hit list' and effectively minimise the occurrence of false positive results. In Figure 4 below, the ChromaLynx browser indicates the presence of various pesticides in the spiked drinking water sample. Peaks found to match with a high degree of confidence to the library entry are highlighted green; tentative matches in yellow and low 'fits' in red.

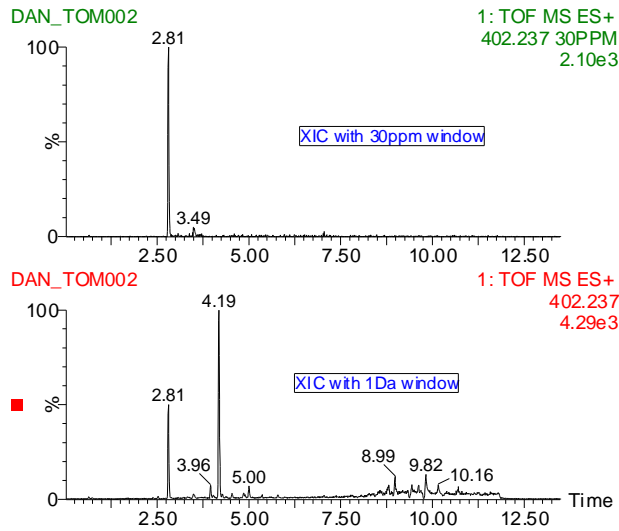


Figure 3. Comparison of different mass extraction windows

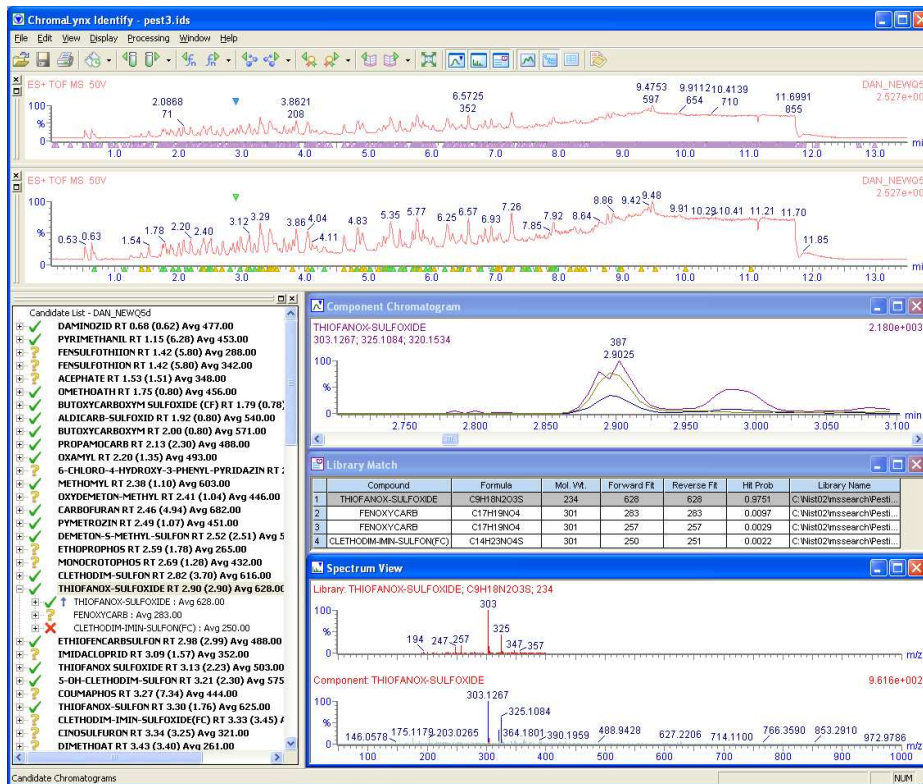


Figure 4. ChromaLynx browser window showing screening results

Traditional ToF MS instruments suffer from detector saturation at relatively low concentration levels, thus limiting the quantitative capability of an analysis. The LCT Premier utilises novel ion optics to provide the Dynamic Range Enhancement (DRE) function which enables precise and easy quantification comparable to that of more conventional mass analysers. Figure 5 shows an example of the quantification of loxynil performed in positive/negative switching mode with DRE.

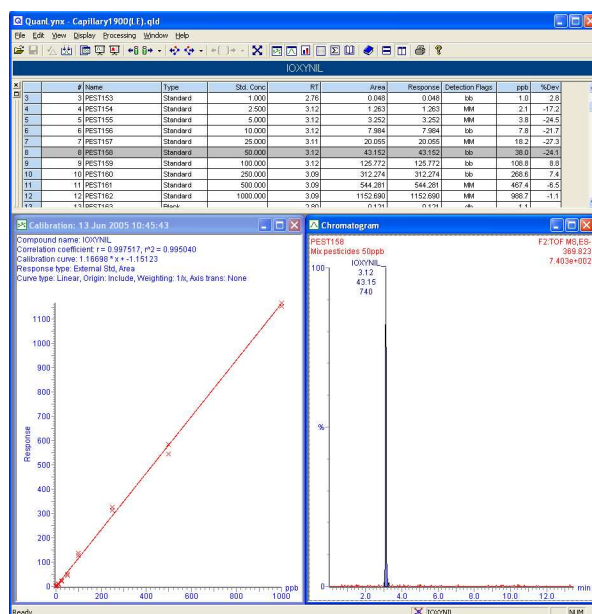
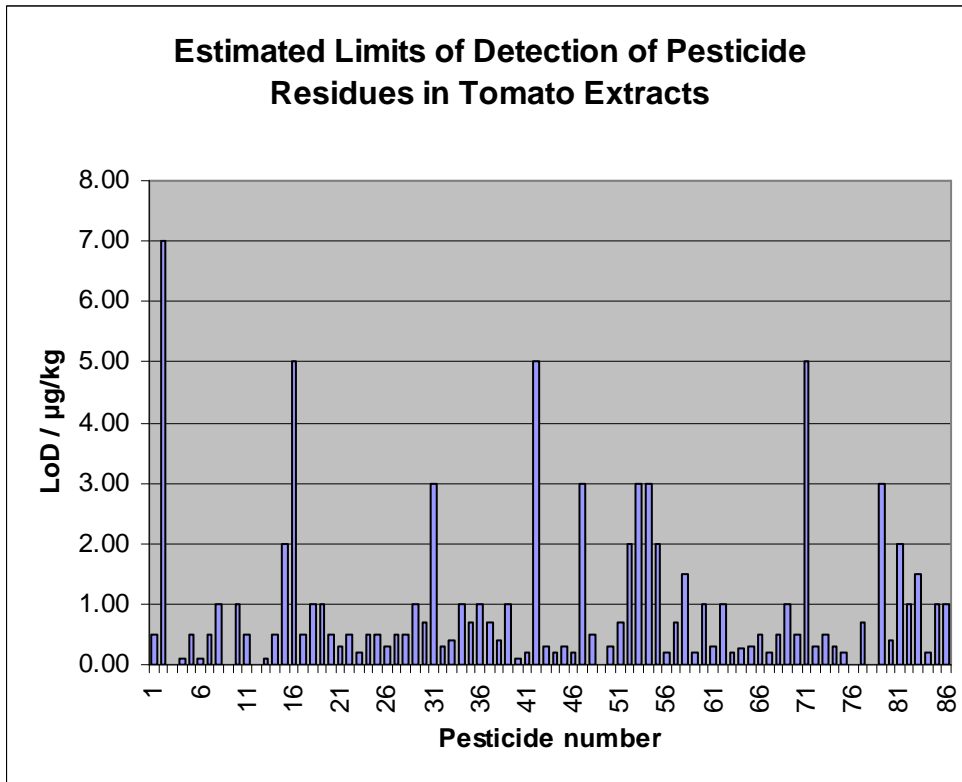


Figure 5. Quantification of loxynil in +/- switching mode with Dynamic Range Enhancement.

Table 1. (a) Graph of estimated LoDs and (b) list of pesticides analysed in tomato extracts



(a)

Number	Compound	Number	Compound
1	Acephate	44	Metolachlor
2	Aldicarb	45	Pymetrozin
3	Amidosulfuron	46	Pyrimethanil
4	Azoxystrobin	47	Thiofanox
5	Bendiocarb	48	Thiofanox-sulfoxide
6	butoxycarboxym	49	Butocarboxim
7	Carbaril	50	Clethodim-imin-sulfoxide
8	Carbendazim	51	Ethiofencarb
9	pyridazin	52	Fenhexamid
10	Chlorsulfuron	53	Imidacloprid
11	Clethodim	54	Triflurumuron
12	Daminozide	55	Isoproturon
13	Fenoxycarb	56	Metamitron
14	Fenpropimorph	57	Methamidophos
15	Flufenoxuron	58	Methiocarb
16	Pyridate	59	Metsulfuron-methyl
17	Aldoxicarb	60	monocrotophos
18	Atrazine	61	Oxydemeton-methyl
19	Butoxycarboxim-sulfoxid	62	Quizalofop-ethyl
20	Carbofuran	63	Tebuconazole
21	Cinosulfuron	64	Thiacloprid
22	Clethodim-sulfon	65	Trisulfuron-methyl
23	Demethon-S-methyl-sulfon	66	Fluazifop-P-butyl
24	Desmedipham	67	Omethoate
25	Ethiofencarbsulfon	68	Pirimicarb
26	Imazail	69	Propamocarb
27	Linuron	70	Quinmerac
28	Oxamyl	71	Tebufenozide
29	Aldicarb-sulfoxid	72	Thifensulfuron-methyl
30	Bensulfuron-methyl	73	Thiofanox-sulfon
31	Carbofuran-3-OH	74	Thiophanate-methyl
32	Clethodim-sulfoxid	75	triasulfuron
33	Diuron	76	Dimethoat
34	Furathiocarb	77	Nicosulfuron
35	Methomyl	78	Thiodicarb
36	Promecarb	79	3,4,5-trimethacarb
37	propoxur	80	5-OH-clethodim-sulfon
38	Thiabendazol	81	Haloxifop-ethoxyethyl
39	diflubenzuron	82	Flazasulfuron
40	Clethodim-imin-sulfon	83	Prosulfuron
41	Cyprodinil	84	Vamidotion
42	Haloxifop-methyl	85	Isoxafutole
43	Metalaxyl	86	Spiroxamine

(b)

In order to verify the method on samples in a more complex matrix, organic tomatoes were purchased locally, spiked at various concentrations around the typical MRL and extracted as outlined in the procedure above. Limits of detection as shown in table 1 were estimated by extrapolating the calibration curve to the concentration which would give a signal-to-noise ratio of 3:1. It should be noted that the lowest MRL currently standing for tomatoes is 20 µg/kg in the cases of acephate, daminozide, oxydemeton-methyl, dimethoate and prosulfuron. All others, where set, are 50 µg/kg or higher. Figure 6 overlaid shows an example of the quantification of triasulfuron in tomato.

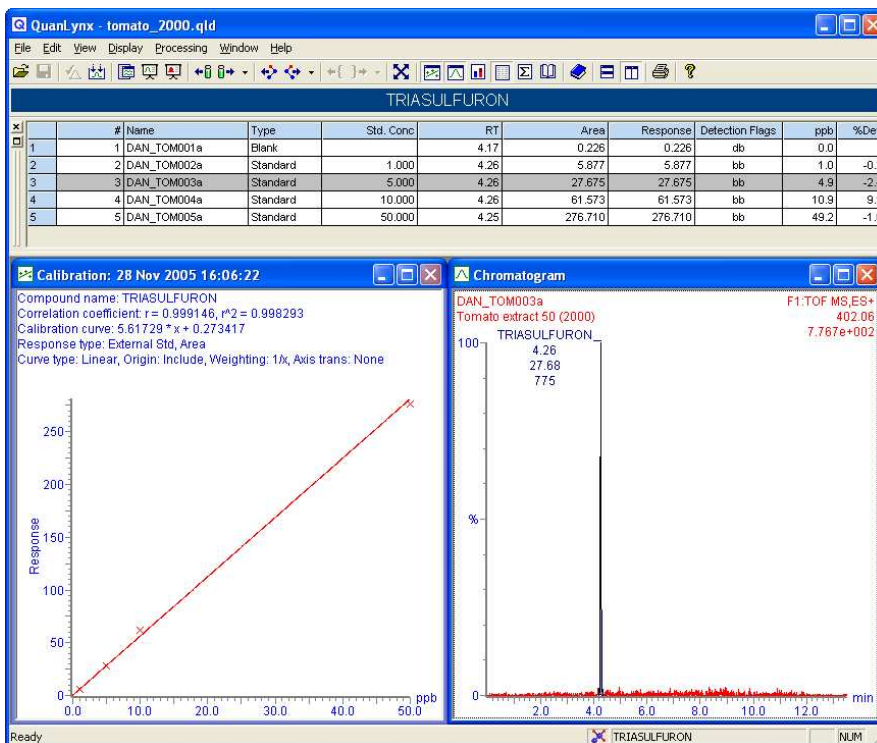


Figure 6. Quantification of triasulfuron in tomato extract. The MRL for this compound in tomatoes is 50 µg/kg

CONCLUSIONS

- The method is shown to provide a rapid and sensitive, automated screening analysis which is applicable to both simple and complex matrices.
- ACQUITY UPLC provides a fast chromatographic run with good resolution so as to minimise interference from co-eluting peaks.
- High mass-accuracy MS spectra provided by the LCT Premier mass spectrometer allow confirmation of targeted compounds and help identify unknowns.
- ChromaLynx Application Manager software performs automated deconvolution of complex chromatographic data to provide simplified results.
- Further work should extend the library to contain as many contaminants as possible, and investigate the use of exact-mass fragments formed by in-source CID for confirmation purposes.

2.4.3 Bibliografía

76/464/CEE Directiva del consejo, de 4 de mayo de 1976, relativa a la contaminación causada por determinadas sustancias peligrosas vertidas en el medio acuático de la comunidad. Diario oficial nº I 129 de 18/05/1976 pp 0023 - 0029

Bobeldijk I, Vissers JPC, Kearney G, Major H, van Leerdam JA

Screening and identification of unknown contaminants in water with liquid chromatography and quadrupole-orthogonal acceleration-time-of-flight tandem mass spectrometry.

J. Chromatogr. A 929 (2001) 63-74

Bobeldijk I, Stoks PGM, Vissers JPC, Emke E, van Leerdam JA, Muilwijk B, Berbee R, Noij ThHM

Surface and wastewater quality monitoring: combination of liquid chromatography with (geno)toxicity detection, diode array detection and tandem mass spectrometry for identification of pollutants

J. Chromatogr. A 97 (2002) 167-181

Nielen MWF, Vissers JPC, Fuchs REM, van Velde JW, Lommen A

Screening for anabolic steroids and related compounds in illegal cocktails by LC/time-of-flight mass spectrometry and LC/quadrupole time-of-flight tandem MS with accurate mass measurement.

Rapid Commun. Mass Spectrom. 15 (2001) 1577-1585

Kumata H, Yukihsa S, Hideshige T, Takashi U.

Historical Trends of N-Cyclohexyl-2-benzothiazolamine, 2-(4-Morpholinyl)benzothiazole, and Other Anthropogenic Contaminants in the Urban Reservoir Sediment Core

Environ. Sci. Technol. (2000) 34 246 -253

Capítulo 3.

Determinación de plaguicidas y productos de transformación mediante LC-MS/MS con analizador de triple cuadrupolo (QqQ)

- 3.1 Introducción
- 3.2 Experimental
- 3.3 Determinación multiresiduo de plaguicidas y productos de transformación en aguas mediante preconcentración en línea (SPE-LC)
 - 3.3.1 Introducción
 - 3.3.2 Desarrollo y validación de un método multiresiduo en aguas
 - 3.3.3 Resultados y discusión
 - Artículo científico 4
 - Liquid chromatography-mass spectrometry role in the elucidation and determination of transformation products of pesticides in water*
(En preparación)
 - 3.3.4 Bibliografía
- 3.4 Determinación individual de plaguicidas y productos de transformación en aguas
 - 3.4.1 Introducción
 - 3.4.2 Resultados y discusión
 - Artículo científico 5
 - Residue determination of glyphosate, glufosinate and aminomethylphosphonic acid in water and soil samples by liquid chromatography coupled to electrospray tandem mass spectrometry*
J. Chromatogr. A (2005) 1081: 145-155
 - Artículo científico 6
 - Re-evaluation of glyphosate determination in water by liquid chromatography coupled to electrospray tandem mass spectrometry*
J. Chromatogr. A (2006) 1134: 51-55
 - 3.4.3 Bibliografía

3.1 Introducción

Como se ha visto en el Capítulo 2, la elevada sensibilidad del QTOF en modo *scan* así como su capacidad para realizar medidas con masa exacta hace que estos instrumentos sean ideales tanto para la elucidación estructural como para la búsqueda de moléculas desconocidas. Sin embargo, este analizador presenta algunas limitaciones como son su bajo rango de respuesta lineal (apenas dos órdenes de magnitud) y su menor sensibilidad con respecto a instrumentos cuadrupolares en modo SRM, lo que limita sus aplicaciones en el análisis cuantitativo y de residuos.

Estas limitaciones en el análisis cuantitativo son suplidas con creces por los espectrómetros de masas de triple cuadrupolo ya que, debido a sus características en cuanto al principio de separación y detección de los iones, estos instrumentos presentan una gran sensibilidad trabajando en modo SRM. El primer cuadrupolo aísla un ion e invierte todo el tiempo de medida en determinar ese ion aumentando así su sensibilidad; sensibilidad que a su vez es aumentada al invertir todo el tiempo en la medida en un sólo ion en el tercer cuadrupolo. Por ello los triples cuadrupolos son muy adecuados para el análisis cuantitativo, especialmente a bajos niveles de concentración. Sin embargo, en instrumentos de triple cuadrupolo, al hacer experimentos de barrido (*full scan*, pérdidas neutras o barrido de iones producto) se puede llegar a perder hasta 3 órdenes de magnitud en la sensibilidad, siendo ésta insuficiente en la determinación de residuos. Por otra parte, la utilización de estos equipos en modo MS presenta la limitación de su baja resolución, lo que dificulta considerablemente la identificación de compuestos desconocidos debido al elevado número de moléculas que pueden compartir una misma masa nominal.

Por estas razones, la configuración más útil para trabajar con instrumentos de triple cuadrupolo en el análisis de residuos es la determinación de moléculas objetivo previamente seleccionadas (*target analytes*). En este caso se trata de cuantificar un grupo de moléculas ya conocidas sin tener en cuenta el resto de los contaminantes que pueda contener la muestra.

Para ello, se requiere conocer los potenciales contaminantes a los que puede estar expuesta la muestra, aunque muchas veces la selección de los analitos se hace en base a listas prioritarias establecidas por diferentes organismos. Muchas veces, estas listas, no tienen en cuenta los contaminantes que realmente pueden estar presentes en las aguas, sino que más bien se elaboran bajo criterios de peligrosidad. Uno de los aspectos a tener en cuenta a la hora de trabajar con un instrumento de triple cuadrupolo en modo SRM es la necesidad de determinar a priori las transiciones que se van a monitorizar. Para ello, es necesario optimizar las condiciones MS/MS del compuesto objeto de estudio (iones producto, voltaje de cono, energía de colisión...). La adquisición del espectro de masas se suele realizar mediante la introducción directa de un patrón (0.5-10 mg/L) en el equipo. Sin embargo, dado que la mayoría de los compuestos presentan varias fragmentaciones, resulta necesario seleccionar la transición que se va a monitorizar, es decir, efectuar lo que normalmente se denomina *ion shopping* usando la terminología anglosajona. Esto puede acarrear problemas, como se verá más adelante en el capítulo de confirmación, si la transición es poco selectiva ya que aumenta la probabilidad de reportar falsos positivos. En general, basta seleccionar dos transiciones para cada compuesto, siempre que éstas sean específicas, aunque la mejor solución sería adquirir todas las transiciones disponibles para un compuesto pues, de este modo, se podría confirmar inequívocamente su presencia a la vez que se cuantifica. Sin embargo, esto no es fácil cuando se pretende desarrollar un método multiresiduo. Podría parecer que el hecho de determinar un número mayor de compuestos por LC-MS/MS no supone ningún problema ya que bastaría con añadir más transiciones en el método creado. Sin embargo, tal y como se muestra en la Figura 3.1, el número de transiciones que contiene un método tiene una relación directa sobre la sensibilidad y la definición del pico cromatográfico y, por tanto, sobre la calidad de la determinación, ya que la sensibilidad en equipos de cuadrupolo está muy relacionada con el tiempo que se emplea en monitorizar un determinado ion (*dwell time*). Cuanto más iones se necesiten medir, menos tiempo se estará monitorizando cada ion y por tanto menos sensible será la técnica. Otro aspecto importante es el número de puntos por pico, el cual se reduce al añadir más transiciones al método. Como los analizadores cuadrupolares miden los iones de manera secuencial, cuantas más transiciones contenga el método más tiempo habrá entre puntos correspondientes a una misma transición, pudiendo hacer que el número de puntos contenidos en un pico sea inferior al necesario para obtener una buena definición de pico.

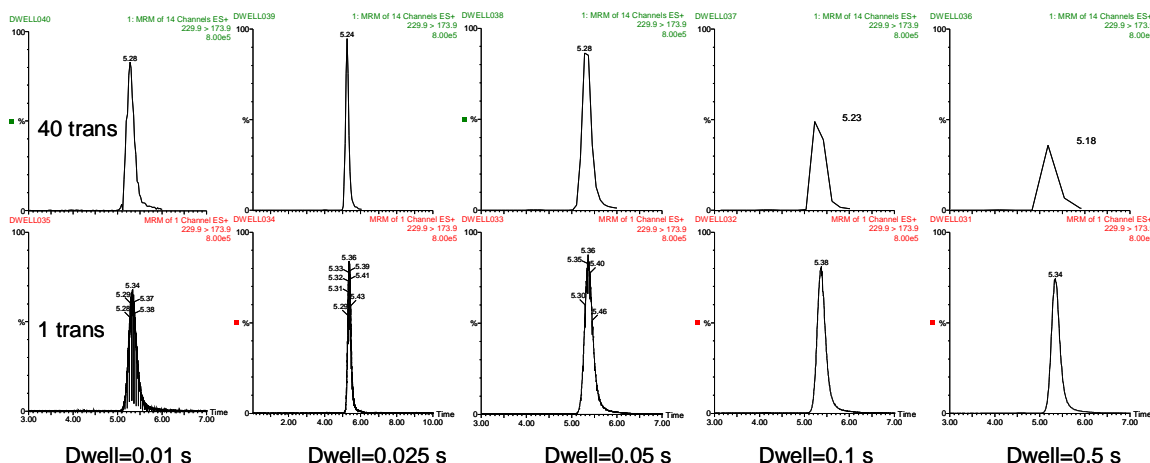


Figura 3.1 Efecto del *dwell time* y del aumento del número de transiciones sobre la señal y la definición del pico cromatográfico

Las características de este tipo de analizadores hacen que el número de transiciones monitorizadas no sea ilimitado. Así en el equipo utilizado en la presente Tesis no resulta conveniente adquirir más de 15 transiciones simultáneamente. Ésta es la principal razón (junto con el efecto matriz) por la que es necesario optimizar la cromatografía con el fin de tener grupos cromatográficos diferenciados de modo que no se supere este número de compuestos en una ventana de tiempo determinado. Para ello, cuando se realiza una determinación multiresiduo de un número elevado de analitos, normalmente se debe elegir entre realizar un análisis confirmatorio de un número menor de compuestos, incluyendo varias transiciones para un mismo analito, o bien realizar un método de *screening* rápido y de amplio rango incluyendo una única transición, lo cual permitiría aumentar el número de compuestos diana. Esta última opción suele ser la más habitual cuando se pretenden hacer controles medioambientales.

Recientemente, han aparecido en el mercado nuevos diseños de analizadores de triple cuadrupolo que intentan minimizar la problemática anterior. Las celdas de colisión se han rediseñado permitiendo reducir el tiempo de monitorización de un ion sin merma aparente en la sensibilidad. Por lo tanto, los métodos de *screening* desarrollados se podrían ampliar fácilmente a métodos confirmatorios mediante el empleo de estos nuevos equipos añadiendo simplemente una transición adicional adecuada para cada analito.

En los dos apartados siguientes se explora la capacidad analítica de la LC-MS/MS con analizadores QqQ en el análisis cuantitativo medioambiental. En el primero de ellos se desarrolla un método multiresiduo para plaguicidas habitualmente usados en la Comunidad Valenciana, junto con sus TPs más importantes (*artículo científico 4*). En el segundo, se desarrolla un método específico para un herbicida muy utilizado y altamente polar como es el glifosato y su principal TP (AMPA), ante la imposibilidad de incluirlos en el método multiresiduo anterior dadas sus especiales características y la conocida problemática analítica que supone su determinación a niveles de sub-ppb (*artículos científicos 5 y 6*).

3.2 Experimental

En la Figura 3.2 se muestra el esquema del equipo instrumental utilizado, donde el inyector automático carga el *loop*. Una vez cargado, la bomba binaria P-1 arrastra el contenido del *loop* hacia el cartucho, en el cual se queda retenido el compuesto de interés. Tras un lavado del cartucho, la bomba P-2 transfiere el analito, junto con otros componentes de la muestra, en contracorriente desde el cartucho hasta la columna analítica C-2 en la que son separados. La bomba P-1, además de cargar el *loop*, es la encargada de acondicionar el cartucho y de realizar un lavado con agua, fundamentalmente para la eliminación de sales y otros compuestos muy polares y poco volátiles que pudieran precipitar en el cono del instrumento.

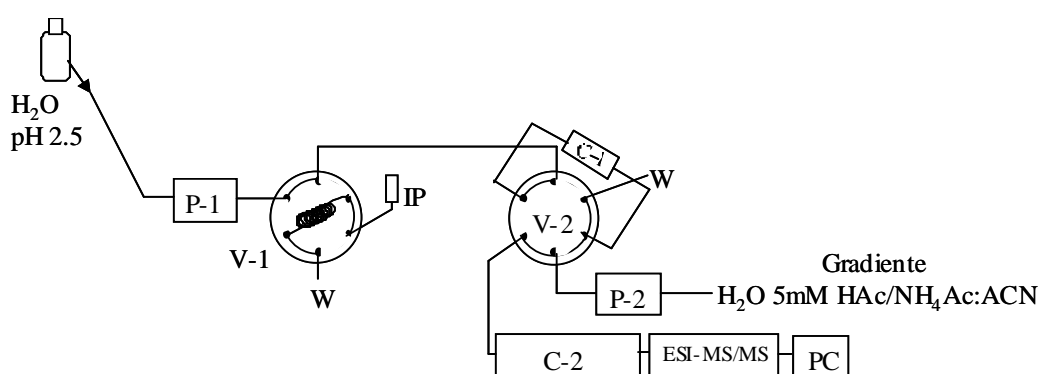


Figura 3.2 Esquema del equipo instrumental utilizado. IP= inyector automático con un *loop* de 4300 μL ; V= válvula de 6 vías; P-1= Bomba binaria HPLC; P-2= bomba cuaternaria HPLC; C-1= cartucho SPE; C-2= columna analítica; MS/MS= detector de masas en tándem; PC= adquisición de datos; W= desecho

Se utilizó una bomba HPLC Waters Alliance 2695 (Waters, Milford, USA) acoplada a un espectrómetro de masas de triple cuadrupolo, modelo Quattro LC (Micromass, Manchester, UK), equipado con una interfase ortogonal Z-spray. Este sistema se encontraba acoplado en línea con un sistema de extracción en fase sólida, compuesto por un autoinyector 233XL (Gilson, Villiers-le-Bel, France) y una bomba binaria Agilent 1100 (Agilent, Waldbron, Alemania), encargada de cargar la muestra y de acondicionar y lavar el cartucho SPE.

Como gas de nebulización y de desolvatación se utilizó nitrógeno, obtenido a partir de aire presurizado en un generador de nitrógeno NG-7 (Aquila, Eetten-Leur, NL). El flujo de gas de nebulización fue ajustado a aproximadamente 80 L/h y el de desolvatación a 800-900 L/h. Para trabajar en modo MS/MS, se utilizó argón como gas de colisión (99.995%, Praxair, Madrid, Spain)

con una presión de $1 \cdot 10^{-3}$ mbar en la celda de colisión. El voltaje del capilar usado fue 3 kV y 3.5 kV en modo de ionización negativo y positivo, respectivamente. Se seleccionó una temperatura de la interfase de 350 °C, mientras que la temperatura de la fuente fue ajustada a 120 °C. Se seleccionó un *solvent delay* de 2 minutos y un tiempo de monitorización de 0.1 s/scan. El software utilizado fue Masslynx NT v.4.0.

El equipo se calibró mensualmente usando una bomba de jeringa (Model 11, Harvard, Holliston, MA) directamente conectada a la interfase, en un rango de m/z 50 a m/z 2000. La calibración se llevó a cabo usando una mezcla de PEGs.

La columna analítica utilizada así como la fase móvil y el gradiente dependió del método a desarrollar en cada caso. El cartucho de SPE empleado en todos los casos fue un OASIS HLB de 20 x 2.1 mm i.d. y 25 μ m de tamaño de partícula (Waters).

3.3 Determinación multiresiduo de herbicidas y productos de transformación en aguas mediante preconcentración en línea (SPE-LC)

3.3.1 Introducción

La posible presencia de plaguicidas en muestras medioambientales, como aguas y suelos, genera una gran preocupación social, sobretodo en el caso de contaminación de aguas subterráneas o superficiales que se utilizan para el suministro de agua potable o de parajes de interés ecológico cercanos a entornos agrícolas. Una de las familias de plaguicidas más problemáticas es la de los herbicidas, ya que son aplicados directamente al suelo. Además, la elevada polaridad de la mayoría de ellos junto con su alta movilidad en los suelos los hace susceptibles de contaminar las aguas subterráneas. Por esta razón, en nuestro laboratorio se vienen realizando estudios desde hace varios años, con el fin de determinar los niveles de herbicidas presentes en aguas de la Comunidad Valenciana (Sancho et al, 2004), habiéndose encontrado niveles significativos de varios compuestos, destacando las triazinas, y también algunos insecticidas organofosforados relativamente polares.

Aprovechando que en nuestro grupo de investigación se iba a desarrollar un método multiresiduo para la determinación de plaguicidas en aguas de la Comunidad Valenciana, se decidió incluir en el mismo los TPs de las triazinas (de los que se disponían patrones comerciales) identificados en el capítulo 2.2, para así comprobar la utilidad de las experiencias de fotodegradación. Se analizaron cerca de 140 muestras de aguas superficiales y subterráneas, tomadas en 20 puntos de la Comunidad Valenciana (Marín et al, 2006). A modo de resumen, la Tabla 3.1 muestra el número de muestras positivas para las triazinas y compuestos relacionados, así como el porcentaje de detecciones positivas y de aquellas muestras que superan el valor de 0.1 µg/L. Además, se indica la concentración máxima encontrada para cada plaguicida.

Como se puede ver, tanto en el caso de la terbutilazina como en el de la terbumetona, el porcentaje de muestras en las que se detectaron TPs es mayor que el de muestras en las que se detectó el plaguicida original. Así, en el caso de la terbumetona, el plaguicida intacto fue detectado en un 28% de las muestras, mientras que su TP lo fue en un 69%.

Tabla 3.1 Resumen de los resultados obtenidos en la determinación de triazinas y sus productos de transformación en aguas subterráneas y superficiales de la Comunidad Valenciana (2003).
Número total de muestras analizadas 147

Compuesto	Muestras positivas	% detecciones positivas	Nº total muestras >0.1µg/L	% detecciones >0.1µg/L	Conc. máx. (µg/L)
Terbutilazina	72	49	7	5	0.46
2-OH-terbutilazina	111	76	15	10	0.53
Desetil-2-OH-tbza	107	73	16	11	0.21
Desetilterbutilazina	87	59	10	7	1.42
Simazina	98	67	6	4	0.52
2-OH-simazina	13	9	1	1	0.15
DIA	35	24	7	5	0.25
Terbumetona	41	28	7	5	1.07
Desetilterbumetona	101	69	13	9	1.62
Terbutrina	22	15	0	0	0.10

En la tabla 3.2, se muestran a modo de ejemplo los resultados obtenidos para triazinas y sus TPs en un agua subterránea procedente de un pozo de riego rodeado de campos de cítricos (Carcaixent). En todas las muestras de Carcaixent analizadas se detectaron los herbicidas terbutilazina y terbumetona, así como la mayoría de sus TPs. En este caso cabe destacar la mayor concentración de compuestos desetilados frente a los herbicidas de partida.

Otros plaguicidas como carbendazima, diuron, imazalil, bentazona, terbacilo o bromacilo fueron detectados en un gran número de muestras, destacando especialmente la carbendazima y el diuron, que fueron detectados en un 40% de las muestras, o el bromacilo, que lo fue en un 30%. Cabe señalar, por otra parte, las altas concentraciones, especialmente en el caso del bromacilo, donde más del 50% de las detecciones superaron el nivel de 0.1µg/L. Así pues, resultaría también interesante estudiar la degradación de estos compuestos con el fin de incluir sus TPs en posteriores métodos multiresiduo.

Tabla 3.2 Triazinas y productos de transformación detectados en los muestreos procedentes de Carcaixent ($\mu\text{g/L}$)

Compuesto	12/06	25/06	09/07	30/07	09/09	28/10	17/12
Terbutilazina	0.31	0.40	0.39	0.46	0.36	0.36	0.12
2-OH-terbutilazina	0.53	0.07	0.06	0.05	0.05	0.04	0.03
Desetil-2-OH-tbza	0.14	0.18	0.17	0.13	0.12	0.13	0.12
Desetilterbutilazina	0.49	0.54	0.63	0.64	0.66	1.42	0.43
Simazina	0.07	0.07	0.07	0.10	0.10	0.07	0.04
2-OH-simazina	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
DIA	0.19	0.22	0.18	0.11	0.05	0.12	0.08
Terbumetona	0.96	0.90	0.89	0.96	1.07	0.82	0.32
Desetilterbumetona	1.37	1.47	1.62	1.10	1.27	1.30	0.47
Terbutrina	0.02	0.01	0.02	0.02	0.02	0.01	0.01

A la vista de estos resultados, en esta Tesis se decidió desarrollar un método que permitiera efectuar un programa de control de aguas subterráneas y superficiales procedentes de distintos puntos del litoral mediterráneo, que incluyera tanto los plaguicidas más frecuentemente detectados (principalmente herbicidas) como sus principales TPs (identificados en las experiencias realizadas en el capítulo 2 de la presente Tesis), con el fin de obtener una visión más amplia de la contaminación por plaguicidas en esta zona, ya que hasta la fecha la mayoría de los métodos desarrollados se habían centrado casi exclusivamente en los plaguicidas de partida, sin tener en cuenta sus posibles TPs.

3.3.2 Desarrollo y validación de un método multiresiduo en aguas.

La elevada polaridad de algunos plaguicidas, y especialmente de sus productos de transformación, ha hecho que en los últimos años los equipos de LC-MS hayan empezado a competir e incluso a aventajar a los de GC-MS en el campo medioambiental. Sin embargo, los instrumentos LC-MS presentan una menor sensibilidad y selectividad que los GC-MS. En este aspecto, la implementación de equipos LC-MS/MS (triple cuadrupolo, trampa de iones, cuadrupolo tiempo de vuelo) ha supuesto un gran avance, ya que a su mayor selectividad se les une también su mejor sensibilidad.

Como ya se ha indicado, las características del acoplamiento LC-MS/MS empleando analizadores de triple cuadrupolo lo hacen idóneo para la determinación de moléculas objetivo en muestras de interés ambiental, cuya finalidad es cuantificar un grupo de moléculas conocidas a priori, sin tener en cuenta el resto de los contaminantes de la muestra. En el caso de las aguas, la elevada sensibilidad y selectividad de la técnica y la naturaleza de la muestra, pueden permitir la inyección directa de la misma. Este hecho dependerá de la sensibilidad que presente el compuesto objetivo, así como del efecto matriz observado.

Debido a la gran cantidad de compuestos distintos utilizados en un mismo cultivo y a la gran variedad de cultivos normalmente presentes en una misma zona, el número de plaguicidas que potencialmente pueden alcanzar las aguas es elevado, y por tanto se necesitan métodos que puedan determinar un número considerable de compuestos (multiresiduo). Sin embargo, esta ampliación en el número de compuestos presenta una serie de problemas. En primer lugar, la gran diversidad de propiedades físico-químicas dificulta la optimización de las condiciones experimentales para la determinación simultánea de todos los analitos. Así, es difícil encontrar un tratamiento de muestra que pueda purificar y preconcentrar a todos los analitos sin que varios de ellos se vean afectados. De la misma manera, las condiciones cromatográficas óptimas para un compuesto pueden no ser adecuadas para otro en cuanto a retención o forma de pico. Esto hace que un buen número de los métodos multiresiduo publicados se hayan desarrollado para compuestos de una misma familia química. Así, por ejemplo, se han utilizado reductores como el NaBH_4 para evitar interferencias de los ácidos húmicos en la determinación de fenilureas (Di Corcia et al, 1999), se han utilizado columnas específicas para compuestos muy polares (Ingelse et al, 2001), o se han formado pares iónicos previos a la preconcentración para la determinación de plaguicidas que contienen un amonio cuaternario (Castro et al, 2001). Estas aproximaciones, sin embargo, no serían válidas si el método multiresiduo pretende la determinación de un elevado número de compuestos con diferentes características físico-químicas, pertenecientes a distintas familias químicas (*multiresidue-multiclass analysis*).

Otro problema que presenta la determinación multiresiduo de compuestos de distinta naturaleza, es la imposibilidad de corregir la cuantificación mediante un patrón interno adecuado. El efecto matriz depende en gran medida de la naturaleza del compuesto investigado; por tanto, si se incluye un número elevado de compuestos con propiedades distintas, se necesitarían numerosos patrones internos con el fin de corregir todas las posibles desviaciones. Esta aproximación es inviable en la mayoría de los casos. En este sentido, se obtuvieron resultados poco satisfactorios cuando se intentó corregir las desviaciones de más de 50 compuestos utilizando un solo patrón interno (Kienhuis et al, 2000).

Finalmente, en lo referente a la técnica, todas las desventajas derivadas del análisis multiresiduo ya han sido descritas en la introducción del presente capítulo. A modo de recordatorio, cabe comentar que el número de transiciones que contiene el método tiene una relación directa sobre la sensibilidad y sobre la forma de pico y, por tanto, sobre la calidad de la determinación.

El objetivo de nuestro trabajo era la realización de un programa de control que permitiera la determinación simultánea de los plaguicidas más detectados con anterioridad en la Comunidad Valenciana (principalmente herbicidas) y sus principales TPs, en aguas subterráneas y superficiales procedentes de distintos puntos del litoral mediterráneo.

Se pretendía aprovechar la versatilidad del acoplamiento LC-MS/MS para desarrollar un método rápido, sensible y robusto que permitiera la determinación simultánea de todos los compuestos seleccionados. Haciendo uso de esta técnica se puede minimizar el tratamiento de la muestra facilitando la realización de programas de control en los que es necesario el análisis de un elevado número de muestras.

En primer lugar se estudiaron las condiciones óptimas de MS a partir de los espectros de barrido (*full scan*) en modo MS y barrido de iones producto en modo MS/MS. Para aquellos plaguicidas y TPs que se encontraban comercialmente disponibles estas condiciones se establecieron por infusión de los patrones individuales; en el caso de los TPs no comercialmente disponibles, se optimizaron por cromatografía, utilizando los viales obtenidos en las experiencias de fotodegradación. En ambos casos, se optimizó la separación cromatográfica, probando diferentes fases móviles y columnas. Con el fin de preconcentrar la muestra para alcanzar la sensibilidad objetivo ($LOQ \leq 0.1 \mu\text{g/L}$), se optimizó un método SPE en línea con cromatografía líquida, minimizando el tiempo empleado por muestra. En este caso, se probaron diferentes precolumnas y tiempos de lavado. Los resultados obtenidos y el propio desarrollo de las

investigaciones nos llevaron a poner a punto dos métodos multiresiduo para la determinación de los compuestos seleccionados, ya que debido a la elevada polaridad de algunos compuestos, y especialmente de algunos productos de transformación, era muy difícil el análisis de todos ellos en un mismo método.

Una vez validado el método, se aplicó a muestras reales tanto de aguas superficiales como subterráneas, procedentes de diferentes puntos de la Comunidad Valenciana, así como a aguas de lixiviado procedentes de parcelas experimentales en las que se habían aplicado plaguicidas seleccionados.

Los compuestos seleccionados presentan diversos usos y pertenecen a distintas familias, predominando los herbicidas y fungicidas. A pesar de que el método desarrollado incluye metabolitos muy polares de herbicidas ampliamente utilizados, como bromacilo y terbutilazina, todavía sufre algunas limitaciones al no incluir algunos plaguicidas iónicos muy usados, como el glifosato. Aún así, la información que aportará este método sobre la presencia de plaguicidas en aguas es mucho más completa que la mayoría de metodologías aplicadas hasta la fecha para el control de plaguicidas en aguas. En la tabla 3.3 se muestran todos los compuestos seleccionados. Para todos aquellos TPs no disponibles comercialmente, se ha utilizado la siguiente nomenclatura $M_i = A_b$, donde A indica el compuesto de partida y b es la masa molecular (de)protonada del compuesto.

Tabla 3.3 Compuestos seleccionados en el método multiresiduo

Compuesto	Estructura química	Familia
Terbutilazina		Triazina (herbicida)
Desetil-terbutilazina		Producto de transformación
2-hidroxi-terbutilazina		Producto de transformación
Desetil-2-hidroxi-terbutilazina		Producto de transformación
M1=terbutilazina_226		Producto de transformación

Tabla 3.3 (cont) Compuestos seleccionados en el método multiresiduo

Compuesto	Estructura química	Familia
Simazina		Triazina (herbicida)
2-Hidroxi-simazina		Producto de transformación
Desisopropil-atrazine		Producto de transformación
M2=simazina_184		Producto de transformación
Terbutrina		Triazina (herbicida)
M3=terbutrina_196		Producto de transformación
M4=terbutrina_168		Producto de transformación

Tabla 3.3 (cont) Compuestos seleccionados en el método multiresiduo

Compuesto	Estructura química	Familia
Terbumetona		Triazina (herbicida)
Desetil-terbumetona		Producto de transformación
M5=terbumetona_240		Producto de transformación
M6=terbumetona_256		Producto de transformación
M7=terbumetona_240		Producto de transformación

Tabla 3.3 (cont) Compuestos seleccionados en el método multiresiduo

Compuesto	Estructura química	Familia
Bentazona		No clasificado (herbicida)
Bentazona-metil		Producto de transformación
Acido antranílico isopropilamida		Producto de transformación
Bromacilo		Uracilo (herbicida)
M8=bromacilo_117		Producto de transformación
Terbacilo		Uracilo (herbicida)

Tabla 3.3 (cont) Compuestos seleccionados en el método multiresiduo

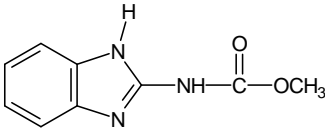
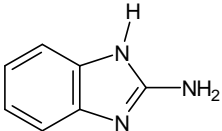
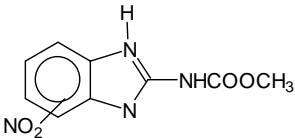
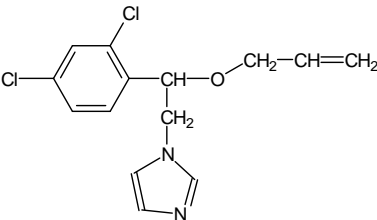
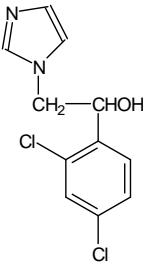
Compuesto	Estructura	Familia
Carbendazim		Bencimidazol (Fungicida)
2-Aminobenzimidazol		Producto de transformación
M9=carbendazim_237		Producto de transformación
Imazalil		Conazol (fungicida)
1-(2,4)-diclorofenil-2-imidazol-1-il-etanol		Producto de transformación

Tabla 3.3 (cont) Compuestos seleccionados en el método multiresiduo

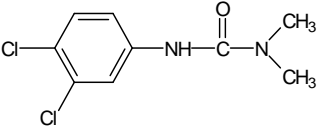
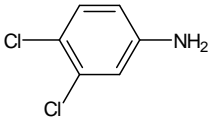
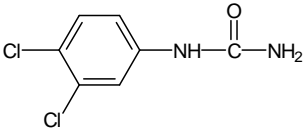
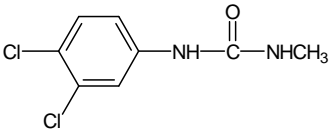
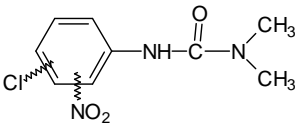
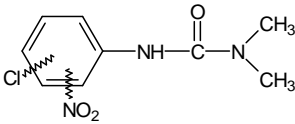
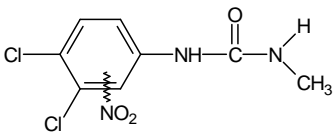
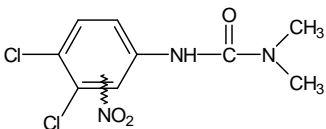
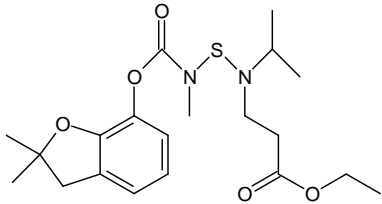
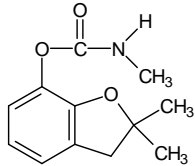
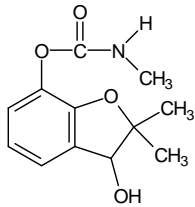
Compuesto	Estructura química	Familia
Diuron		Fenilurea (herbicida)
3,4-dicloroanilina		Producto de transformación
1-(3,4)-diclorofenilurea		Producto de transformación
1-(3,4)-diclorofenil-3-metilurea		Producto de transformación
M10=diuron_244		Producto de transformación
M11=diuron_264		Producto de transformación
M12=diuron_258		Producto de transformación
M13=diuron_278		Producto de transformación

Tabla 3.3 (cont) Compuestos seleccionados en el método multiresiduo

Compuesto	Estructura química	Familia
Benfuracarb		Carbamato (insecticida)
Carbofuran		Producto de transformación (insecticida)
Carbofuran-3-OH		Producto de transformación

3.3.3 Resultados y discusión

- **Optimización de las condiciones MS/MS**

La mayor parte de los compuestos fueron determinados en modo de ionización positiva a excepción de 4 analitos (terbacilo, bromacilo, bentazona y M13 (TP del diuron)) que presentaron ionización en modo negativo. En todos los casos, se seleccionó como ion precursor $[M+H]^+$ o $[M-H]^-$. Para cada compuesto se seleccionaron dos transiciones con el fin de confirmar su presencia, excepto en el caso del M13 dónde sólo fue posible seleccionar una transición debido a la presencia de un único ion producto. En la medida de lo posible, se evitó seleccionar transiciones poco específicas como, por ejemplo, pérdidas de agua, con el fin de disminuir la posibilidad de reportar falsos positivos.

En la Tabla 1 del *artículo científico 4* pueden verse los parámetros MS/MS seleccionados para la determinación de todos los plaguicidas y sus TPs. En ella se muestran todos los compuestos seleccionados, así como el ion precursor, los iones producto, el cono y la energía de colisión utilizados.

- **Optimización cromatográfica/etapa de extracción**

Como ya se ha comentado en la introducción, la extracción en fase sólida (SPE) en modo *on line* presenta varias ventajas frente a la modalidad *off line*, entre las que destacan la posibilidad de utilizar un menor volumen de muestra y sobre todo el mayor grado de automatización del proceso. De este modo, la utilización de esta técnica en línea con un sistema de LC reduce al máximo el tratamiento de muestra, minimizando de esta manera errores típicos asociados a la manipulación de la muestra. Por otra parte, la extracción en fase sólida combina la preconcentración con la purificación de la muestra. Estas razones, junto con la compatibilidad y fácil acoplamiento al sistema de LC-MS/MS, hacen esta técnica ideal para la determinación de contaminantes orgánicos en aguas, permitiendo su cuantificación a los niveles de concentración requeridos sin necesidad de ningún tratamiento adicional de la muestra. En nuestro trabajo el mínimo tratamiento de muestra no sólo redujo errores sino que además facilitó la sencilla incorporación de los métodos optimizados a la realización de programas de control ya que permitió analizar cerca de 30 muestras por día, incluyendo los blancos, los calibrados y los QCs.

Para realizar la extracción en fase sólida se probaron diversos cartuchos de fase polimérica (PRP1, OASIS), con el fin de evitar la ruptura temprana (*breakthrough*) de los compuestos más polares, pudiendo de esta manera incluir compuestos tan polares como la desetil-2-OH-terbutilazina. Como columna analítica también se probaron dos columnas, la Discovery C18 (Supelco) y la X-Bridge (Waters). Los mejores resultados se obtuvieron con el cartucho OASIS y la columna X-Bridge.

Cuando una muestra se pasa a través de un cartucho de SPE con el fin de preconcentrarla, también se concentran muchos de los interferentes que pueda contener, y que pueden afectar a la ionización de los analitos en el sistema MS impidiendo su correcta cuantificación. Por ello, se suele incluir un lavado del cartucho después de la carga de la muestra. Cuando se determina un único analito, este lavado puede ser altamente específico, pudiéndose incluso hacer lavados con un apreciable contenido en disolvente orgánico sin perder el compuesto de interés. Sin embargo, en la determinación multiresiduo de plaguicidas, debido a la diversidad en sus propiedades físico-químicas, el lavado tiene que ser genérico con el fin de eliminar el mayor número de interferentes posibles sin perder ninguno de los analitos. Debido a que, en el caso de aguas, los componentes que principalmente pueden afectar a la ionización son sales, se seleccionó un lavado simplemente con agua HPLC.

A pesar del bajo poder eluotrópico de este lavado, los compuestos más polares pueden sufrir pérdidas si la cantidad de agua utilizada es importante. Por esta razón se optimizó el volumen de agua que permitía el mejor lavado posible de sales sin presentar pérdida de señal debida al *breakthrough*. En el sistema SPE-LC en línea utilizado en este trabajo, la carga de la muestra la realiza una bomba HPLC que la transfiere desde el *loop* hasta el cartucho. Para llenar este *loop* en su totalidad, se toma un pequeño volumen de aire antes y después de la aspiración de la muestra. Este aire desplaza al líquido presente en el *loop* haciendo que no haya mezcla entre éste y la muestra, permitiendo su llenado completo sin necesidad de aspirar 3 veces su volumen. La fase móvil utilizada para la carga es la misma que se utiliza en el lavado. Así, la cantidad de solvente de lavado utilizado se puede controlar en función del tiempo que se está bombeando este solvente antes de que los compuestos pasen a la columna analítica. La Figura 3.3 presenta el efecto del lavado sobre la recuperación de dos compuestos de polaridad distinta, el terbacilo y la 2-OH-terbutilazina, en muestras de aguas subterráneas fortificadas a 0.2 µg/L. Se indican las recuperaciones respecto de un patrón en solvente sometido al mismo proceso que la muestra, con lo que estas experiencias muestran cómo se puede reducir el efecto matriz con el lavado. Como puede verse, el efecto del lavado sobre la recuperación de los analitos depende de la naturaleza de los mismos. Así, mientras en la mayoría de compuestos menos polares (como el terbacilo) no se observaron diferencias significativas en función del

volumen/tiempo de lavado, compuestos muy polares, como la 2-OH-terbutilazina, presentaron recuperaciones crecientes en matrices reales de agua al aumentar el tiempo de lavado. Este aumento de la recuperación es debido a la eliminación de compuestos muy polares presentes en la muestra que inhiben la ionización de los analitos, impidiendo su correcta cuantificación. En el caso de las aguas estos compuestos son principalmente sales que además de interferir en la cuantificación pueden precipitar en el cono de muestreo disminuyendo la sensibilidad.

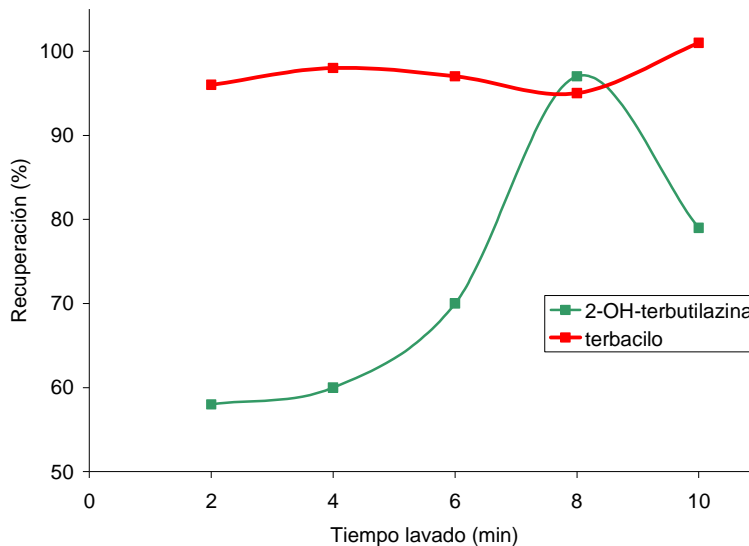


Figura 3.3 Efecto del tiempo de lavado sobre la recuperación en aguas subterráneas para 2-OH-terbutilazina y terbacilo

A pesar de que en los procedimientos SPE-LC en línea la cuantificación se lleva a cabo con patrones sometidos al mismo proceso de SPE que las muestras, es evidente que prefiere trabajarse, en general, en condiciones tales que no se supere el volumen de ruptura o, al menos, en condiciones de máxima respuesta de los analitos, con el fin de mejorar la sensibilidad de los métodos. El compromiso general para todos los analitos se obtuvo utilizando un tiempo de lavado de 8 minutos.

- **Optimización del método**

El amplio rango de características físico-químicas existentes de los plaguicidas seleccionados y de sus TPs aconsejaba encontrar un compromiso entre todos ellos, en lo referente a SPE/retención cromatográfica y su respuesta en MS/MS. Aunque en algunos casos fue necesaria la adición de ácido con el fin de protonar compuestos ácidos y así aumentar su retención en el cartucho, este contenido en ácido también fue capaz de protonar algunos compuestos básicos, como los productos de transformación de las triazinas, perjudicando su retención. Por otro lado, el reactivo formador de pares iónico necesario para la retención de los TPs catiónicos, producía una fuerte disminución en la sensibilidad de los compuestos medidos en modo de ionización negativo. Esta situación ya había sido estudiada por nuestro grupo de investigación, por lo que, en base a nuestra experiencia, se decidió desarrollar dos métodos complementarios: 1) uno para los compuestos ácidos, y en el cual se utilizaría HCOOH para la preconcentración, y 2) otro para los compuestos catiónicos, en el cual la preconcentración se llevaría a cabo usando HFBA como formador de pares iónicos. El resto de analitos se distribuiría entre ambos métodos dependiendo de su tiempo de retención con el fin de minimizar el número de compuestos con tiempos de retención semejantes, es decir, con el fin de monitorizar el máximo número de transiciones sin pérdida de sensibilidad. Así pues, finalmente, cada muestra de agua se dividió en dos alícuotas previamente a los análisis, una fue tratada con HCOOH (método 1) y la otra con HFBA (método 2).

- **Validación del método**

Se estudió la linealidad, la exactitud, la precisión, la especificidad, el límite de cuantificación (LOQ) y el límite de detección (LOD). El estudio de la linealidad se llevó a cabo mediante la inyección por triplicado de 6 disoluciones patrón en el rango comprendido entre 0.025 y 1 µg/L (0.025, 0.050, 0.1, 0.25, 0.5 y 1 µg/L). Los resultados obtenidos se muestran en la Tabla 2 del *artículo científico* 4. Ambos métodos mostraron una linealidad satisfactoria para todos los compuestos en el rango estudiado, con $r^2 > 0.99$ y residuales inferiores al 30%. Para una correcta cuantificación, se consideró que la respuesta de la muestra a cuantificar debía ser como mínimo cinco veces mayor que la ordenada en el origen.

Los métodos fueron validados mediante ensayos de recuperación en dos tipos de aguas (subterránea y superficial) fortificadas a dos niveles (0.05 y 0.5 µg/L) obteniendo los valores correspondientes a la exactitud (% recuperación) y precisión (desviación standard relativa) en cada caso (Tabla 2 del *artículo científico* 4). Puede observarse cómo todos los valores de

recuperación están dentro del rango 70-110%, mientras que las RSD para todos los niveles fueron inferiores al 15%. Queda patente el amplio rango de aplicación del método, capaz de determinar de modo fiable un considerable número de compuestos de diversas familias químicas y con características físico-químicas bien diferentes. Ya que el nivel de fortificación más bajo ensayado (0.05 µg/L) también cumplió estos requisitos se estableció este valor como LOQ del método.

Para la realización de los estudios de especificidad, se analizó un blanco de procedimiento (una alícuota de agua HPLC sometida al mismo tratamiento que la muestra), una muestra blanco de agua y una muestra blanco fortificada al nivel del LOQ. Se considera que el método tiene una adecuada especificidad si los valores obtenidos para las muestras blanco no exceden en un 30% el LOQ. Se observó la ausencia de picos en ambos blancos para todos los compuestos estudiados.

A partir del patrón de 25 ng/L, usado en el calibrado, se estimó el límite de detección para cada compuesto como aquella concentración cuya señal era tres veces la del ruido de fondo. Los límites de detección, que se muestran en la Tabla 2 del *artículo 4*, se encontraron entre 2-25 ng/L, que se consideran adecuados para la determinación de plaguicidas tanto en aguas subterráneas como superficiales.

La metodología descrita en los apartados anteriores se aplicó a muestras de aguas superficiales y subterráneas, previamente analizadas en nuestro laboratorio, y que habían resultado positivas para algunos plaguicidas, principalmente triazinas. El objetivo de este segundo análisis fue, por un lado, confirmar las detecciones previas, y por otro, investigar la presencia de TPs en dichas aguas.

Por otra parte, y con el fin de estudiar la transformación de plaguicidas en el medioambiente suelo/agua se utilizaron parcelas experimentales equipadas con lisímetros, en colaboración con el Dr. Ignacio Morell, director de la Unidad de Hidrogeología de la Universitat Jaume I de Castellón. En estas parcelas experimentales, situadas en el término municipal de Castellón, se aplicaron 3 plaguicidas (terbutilazina, simazina y diuron) a las dosis habitualmente utilizadas en la zona. Cada cierto tiempo se tomaron muestras de agua de lixiviado, abordando de un modo real la degradación/transformación de los plaguicidas aplicados en el campo, obteniendo información sobre aquellos TPs que presentan mayor movilidad y que por tanto pueden amenazar a las aguas subterráneas.

Los resultados de este estudio se recogen en el *artículo científico 4*, que se presenta a continuación.

Artículo científico 4

(En preparación)

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY ROLE IN THE ELUCIDATION AND DETERMINATION OF PESTICIDE TRANSFORMATION PRODUCTS

María Ibáñez, Óscar J. Pozo, Juan V. Sancho and Félix Hernández

Research Institute for Pesticides and Water, University Jaume I, E-12071, Castellón, Spain

ABSTRACT

The high occurrence of polar pesticides and derivatives in environmental water makes necessary the development of reliable analytical methodology for identification of their transformation products and subsequent determination at the sub- $\mu\text{g/L}$ levels required by the legislation. In this paper, photodegradation of several pesticides frequently detected in water of the Mediterranean Spanish area (benfuracarb, bentazone, bromacil, carbendazim, diuron, imazalil and terbacil) was studied utilising the high resolution and exact mass capabilities of hybrid quadrupole time-of-flight mass spectrometry (QTOF MS) hyphenated to liquid chromatography (LC). Once the main photodegradation products formed in aqueous media were identified by LC-QTOF MS, analytical methodology for their determination in water was developed based on LC hyphenated to tandem MS using a triple quadrupole (QqQ) analyzer. Two methods based on on-line solid trace enrichment (SPE)-LC-MS/MS were optimized for the simultaneous quantification and confirmation of all selected pesticides and the most important transformation products (TPs) identified. The methodology was validated in both ground and surface water samples spiked at 50 ng/L and 500 ng/L, obtaining quantitative recoveries (70-110%) for all compounds. It was applied to ground and surface samples collected from the Mediterranean area of Spain, pointing out the high amount of TPs findings in relation to their parent pesticides. Additionally, several water samples collected from the unsaturated zone were analyzed to explore the degradation/transformation of selected pesticides in field, performed in experimental plots equipped with lisimeters. This paper shows the high potential of LC-MS(/MS) in the determination of pesticide TPs. The complementary use of QTOF and triple quadrupole analyzers allows to take advantage of their excellent analytical characteristics for qualitative/structural and quantitative analysis, respectively, offering to the analytical chemists one of the most powerful analytical tools at present.

INTRODUCTION

Pesticides are among the most investigated priority pollutants in water due to their extensive worldwide use and toxicity. The high occurrence of these compounds in environmental waters makes necessary the development of adequate analytical methodology for their monitoring at the low levels required by the European Union regulations [1] (sub- $\mu\text{g/L}$ levels).

The majority of methods applied until now are focused on the determination of unchanged (parent) pesticides; however, when monitoring environmental waters, the occurrence of pesticide transformation products (TPs) can be often higher than of unchanged pesticides [2-4]. Although normally TPs are less toxic than the parent compound, in some cases they could present similar toxicity or even higher [5]. As a consequence of their polarity, they have higher mobility in the soil-water environment, and can reach more easily groundwater than parent compounds. Present regulations on drinking water also include TPs within the group of compounds to be monitored [1]. Therefore, the most relevant TPs should be incorporated into current analytical methods in order to have a wider knowledge of the water quality regarding pesticide contamination. The inclusion of TPs in multiresidue methods is an analytical challenge for several reasons: the general lack of knowledge about them, the high number of potential analytes to be investigated in water, their higher polarity that can affect the efficiency of the preconcentration step, and the limited commercial availability of reference standards.

Thus, modern analytical methodology is required for the determination of TPs to properly evaluate the presence and effects of pesticides in environmental samples. Obviously, the accurate quantification and unequivocal confirmation of the target TPs included in a multiresidue method are needed, but before developing a method realistic information of the TPs to be included as target analytes is also required.

Although gas chromatography (GC)-mass spectrometry (MS) or liquid chromatography (LC) with ultraviolet or fluorescence detection have been traditionally used for the determination of organic pollutants in water, LC-MS has gained increased popularity for this purpose in the last decade [6-9]. Even more attractive is the use of tandem mass spectrometry (MS/MS), which notably increases the sensitivity and specificity of the methods. For this reason, applications based on LC-MS/MS have drastically increased in the last years [10-20].

In our previous papers [21, 22], applications of LC-MS/MS with different analyzers, triple quadrupole (QqQ) and hybrid quadrupole time-of-flight (QTOF), in pesticide analysis have been discussed, and the performances of these analyzers have been compared for screening purposes, including the quantification and confirmation of positive water samples. The most

common and simple approach for the multiresidue screening and quantification of medium polar compounds was found to be the use of QqQ instruments. The high sensitivity achieved in Selected Reaction Monitoring (SRM) mode makes this instrument ideal for screening of target analytes, due to the excellent characteristics of the LC-MS/MS methods (improved sensitivity and selectivity, little sample manipulation and rapid analysis).

Regarding QTOF MS, this instrument has become a powerful identification tool in the elucidation of pesticide TPs due to its increased resolution (>5000 FWHM), inherent high sensitivity in full-scan mode, mass accuracy (< 5ppm) and the possibility of performing both MS and MS/MS modes. However, although LC-QTOF MS has been successfully applied in the biomedical field, this technique has been scarcely used in the environmental field, as it is shown by the limited number of references published [23-27].

The usefulness of photodegradation experiments on aqueous samples has been formerly demonstrated, as a way to elucidate pesticide TPs formed in water [28-30]. As an example, the most important triazine photodegradation products identified under laboratory controlled conditions [23] were included in a multiresidue LC-MS/MS method developed at our laboratory [31]. An interesting fact was pointed out when real water samples were analyzed and the findings for unchanged triazines were compared with those for their related TPs. Around 75% of the triazines detected at concentrations higher than 0.1 µg/L corresponded to TPs, meanwhile only a 25% of positive findings came from unchanged triazines. Moreover, in several water samples, concentrations levels for some parent triazines as terbuthylazine and terbumeton were lower than for their main TPs (desethylterbuthylazine and desethylterbumeton, respectively) [31].

Given the interest of combining photodegradation studies (using LC-QTOF MS) with developing analytical methodology for the determination of pesticides and their most relevant TPs in water (using LC-QqQ MS), this methodology has been extended to other pesticides frequently detected in the Mediterranean Spanish area, as for example the herbicides bromacil and diuron, or the fungicides carbendazim and imazalil, among others.

The goal of this paper is to explore this analytical strategy for the elucidation of TPs of selected pesticides and for the subsequent determination (quantification and confirmation) of these compounds in environmental water samples at sub µg/L levels.

EXPERIMENTAL

Reagents and chemicals

Pesticides and commercially available TPs reference standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), Riedel de Haën (Seelze, Germany) and Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol, reagent-grade formic acid (HCOOH, content >98%), and sodium hydroxide (NaOH) were purchased from Scharlab (Barcelona, Spain). LC-grade water was obtained by purifying demineralised water in a Nanopure II system (Barnstead Newton, MA, USA). Heptafluorobutyric acid (HFBA) was supplied by Fluka (Buchs, Switzerland). 3,5-Diiodo-L-tyrosine, used as lock mass, was purchased from Sigma.

Stock standard solutions of solid commercially available pesticides and TPs were individually prepared dissolving approximately 50 mg, accurately weighed, in 100 mL of HPLC-grade acetonitrile obtaining a final concentration of 500 mg/L. When pesticides and TPs were purchased as solutions, standard solutions were prepared by dissolving the stock solution in acetonitrile. For LC-MS analysis, stock solutions were mixed and diluted with acetonitrile to 5 mg/L and subsequently with HPLC-grade water to obtain working solutions of the pesticide mixture.

Standard stock solution of 3,5-diiodo-L-tyrosine was prepared by dissolving approximately 50 mg powder in 100 mL of HPLC-grade water obtaining a final concentration of 500 µg/mL. 1 µg/mL solution was prepared by dilution of the stock solution with methanol.

Photodegradation experiments

Instrumentation

A LC system Alliance 2695 (Waters, Milford, MA, USA) was interfaced to a hybrid QTOF mass spectrometer (Micromass, Manchester, UK) using an orthogonal Z-spray-electrospray interface. The LC separation was performed using a X-Terra C₁₈ 5µm, 2.1 x 250 mm (Waters) at a flow rate of 200 µL/min. The mobile phase used was a time-programmed gradient using water and methanol, both with 0.01% HCOOH. The percentage of methanol was changed linearly as follows: 0 min., 5%; 0.1 min., 5%; 20 min., 95%; 23 min., 95%; 24 min., 5%; 29 min., 5%. The injection volume was 50 µL.

Drying gas as well as nebulising gas was nitrogen, generated from pressurized air in a nitrogen generator from Peak Scientific (Inchinnan, Scotland). The nebuliser gas flow was set to approximately 15 L/h. and the desolvation gas flow to 500-600 L/h. TOF-MS resolution was approximately 5000 (FWHM). MS data were acquired over a m/z range of 50-600. The MCP detector potential was set to 2900 V.

For operation in MS/MS mode, collision gas was Argon 99.995% (Carbueros Metalicos, Valencia, Spain) with a pressure of approximately 2.10^{-5} mbar in the collision cell. A cone voltage of 25 V and a capillary voltage of 3.5 kV were used in both positive and negative ionization modes. The interface temperature was set to 350°C and the source temperature to 120°C. Dwell times of 1 s/scan were chosen. A solvent delay of 3 minutes was selected to avoid the entrance of salts using the built-in divert valve controlled by time-controlled contact closures using the Masslynx NT v.4.0 software. A suitable MS profile was used.

Calibration experiments were performed daily using a Model 11 single-syringe pump (Harvard, Holliston, MA), directly connected to the interface. Calibration in both positive and negative electrospray modes from 50 to 600 m/z was conducted with a mixture of NaOH 0.1M-HCOOH 10% (50:50) diluted (1:50) with acetonitrile-water (80:20) at a flow rate of 10 $\mu\text{L}/\text{min}$.

The lock mass was introduced postcolumn using a model 2150 isocratic HPLC pump from LKB (Bromma, Sweden) at a flow rate of 100 $\mu\text{L}/\text{min}$ by means of a PEEK T-union.

Samples and irradiation procedures

Following the methodology employed in our previous paper [23], several herbicides, two fungicides and one insecticide widely detected in the Valencian region environmental waters were irradiated on spiked water samples using a mercury vapor-lamp (254 nm), over a period of 15 days. 500-mL surface water was spiked at 500 ng/mL with diuron, bromacil, terbacil, benfuracarb, bentazone, carbendazime, and imazalil. Additionally, a 500-mL non-spiked water sample was irradiated under the same conditions and used as a control. Along irradiation experiments, 3-mL sample aliquots were taken from the glass beakers at different intervals and analyzed by LC-QTOF MS.

Analysis and data processing

1 mg/L solution of 3,5-Diido-L-tyrosine [(M+H)⁺ m/z 433.8750/(M-H)⁻ m/z 431.8594] was used as lock mass. Data were processed using the MetaboLynx application (Micromass v 4.0), a postacquisition data processing package that compares two LC-MS data files and highlight differences in the presence of compounds. These differences when comparing spiked and control samples can be attributed to transformation processes of pesticides added to samples.

Based on accurate mass measurements, possible elemental compositions of the relevant peaks were calculated using the elemental composition calculator, within the MassLynx software, accepting a maximum deviation of 5 mDa between the experimental measured exact mass and the theoretical one. Parameter settings for all compounds were C 0-40, H 0-80, N 0-10, O 0-10, F:0-10, P:0-10, S:0-5. Additionally, for those compounds with halogen atoms in their molecule (diuron, bromacil, terbacil and imazalil), the appropriate number of Cl or Br was determined from the isotopic pattern and added if required. The double-bond equivalent (DBE) filter was set between 0 and 10. In addition to full scan data acquired in MS mode, the possibility of performing MS/MS experiments helped us to elucidate the structure of TPs from the product ion spectra measured with high mass accuracy.

Multiresidue quantitative/confirmative method

Instrumentation

A Quattro LC (quadrupole-hexapole-quadrupole) mass spectrometer with an orthogonal Z-spray-electrospray interface (Micromass) was coupled to a HPLC system based on a 233XL autosampler with a sample loop of 2.2 mL (Gilson, Villiers-le-Bel, France) and 2 pumps: an Agilent 1100 (Agilent, Waldbron, Germany) binary pump (P-1) for loading the sample, conditioning and washing the SPE cartridge (P-1), and a Waters Alliance 2695 (Waters) quaternary pump used for the chromatographic separation (P-2). The SPE preconcentration was performed using an OASIS HLB cartridge 20 x 2.1 mm i.d. (Waters). For the LC separation, a X-Bridge C₁₈, 5µm 80 x 2.0 mm i.d. analytical column (Waters) was used.

Two complementary methods were applied for the determination of all targeted analytes. For the determination of acidic and neutral pesticides (method 1), mobile phases consisting in mixtures 1mM aqueous HCOOH:acetonitrile were used in P-1 and 0.01% aqueous HCOOH: acetonitrile in P-2. Regarding the determination of basic and some other neutral analytes (method 2), 1mM aqueous HFBA:acetonitrile and water: acetonitrile were used for P-1 and P-2, respectively.

The chromatographic gradient was common for both methods and it was performed changing the organic modifier as follows: 0 min, 10 %; 2 min, 10 %; 20 min, 40 %; 24 min, 90 %; 25 min., 90 %; 26 min., 10 %, at a flow rate of 200 $\mu\text{L}/\text{min}$.

Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a NG-7 nitrogen generator (Aquila, Etten-Leur, NL). The nebuliser gas flow was set to approximately 80 L/h and the desolvation gas flow to 800-900 L/h. Infusion experiments were performed using a Model 11 single syringe pump (Harvard Instruments, Holliston, MA, USA), directly connected to the interface.

For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Madrid, Spain) with a pressure of 1.10^{-3} mbar in the collision cell. Capillary voltages of -3 kV and 3.5 kV were used in negative and positive ionization mode, respectively. The interface temperature was set to 350°C and the source temperature to 120°C. Dwell times of 0.1 s/scan were selected. Masslynx NT v 4.0 (Micromass, Manchester, UK) software was used to process the quantitative data obtained from calibration standards and from water samples.

Recommended procedure

Water samples (around 20 mL) were centrifuged at 3500 rpm for 10 min (only in presence of suspended particulate matter) and divided into two aliquots. One of them was acidified to 0.5% with formic acid to perform the determination of acidic and neutral pesticides, and the other one to HFBA 25 mM for the determination of basic and the rest of neutral analytes. These acidified samples were loaded into the loop and directly injected into the SPE-LC-(ESI)-MS/MS system.

Validation study

Linearity of the methods was studied by analyzing in triplicate standard solutions at six concentration values ranging from 0.025 to 1 $\mu\text{g}/\text{L}$. The method was found to be linear if correlation coefficient (r) higher than 0.99 and residuals lower than 30% were obtained.

Accuracy (expressed as % recovery) and precision (expressed as relative standard deviation, in %) were evaluated by analyzing a ground and a surface water sample spiked at two concentration levels each (50 and 500 ng/L). These experiments were performed in quintuplicate ($n=5$).

The limit of quantification objective (LOQ) was established as the lowest concentration level that was fully validated, from spiked samples subjected to the overall analytical procedure, with satisfactory recovery (between 70 and 110%) and precision (RSD < 15%). The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was estimated for a signal-to-noise ratio of 3 from the chromatograms of samples spiked at the lowest analyte concentration assayed.

Application to real samples

Several ground and surface water samples from the Spanish Mediterranean area, that had been previously analyzed by LC-MS/MS QqQ for a number of parent pesticides, were reanalyzed using the methodology developed in this paper with two aims i) to confirm the positive previously detected and ii) to investigate the presence of their TPs.

Additionally, to obtain a more realistic overview of the TPs formed in the water/soil environment, experimental plots equipped with lysimeters were used (1-2 metres depth). In these plots, located nearby Castellon, three herbicides (terbuthylazine, simazine and diuron) were applied at the applications rates usually employed in this citric crop area. Every three/four days, water samples from the unsaturated zone were collected and analyzed to obtain information about the TPs formed that were able to lixiviate through the soil and the unsaturated zone.

Data evaluation

Within each batch of samples, a calibration curve consisting of standards prepared in HPLC-grade water at concentrations between 25 and 1000 ng/L was injected before and after the samples. To ensure the quality of the analysis when processing real-world samples, blank ground water and surface water samples fortified at the LOQ and 10 x LOQ concentration levels (i.e, 50 and 500 ng/L, respectively) were used as quality controls (QC) injected every three-four samples. QCs were prepared only for those compounds whose reference standards were commercially available (see Table 2). These blank samples were previously analyzed to confirm the absence of the target analytes. Quantification of a sample batch was considered satisfactory if QC recoveries for all analytes were in the range of 70-110%. Confirmation of compounds detected was carried out by independently quantifying positive samples by using the quantification and confirmation transitions selected for each compound (see MS Optimisation). It was accepted a deviation of $\pm 20\%$ in the concentrations obtained with the two transitions (Q/q conc) was accepted as criterion for confirmation. On the other hand, a mixture

of the non-commercially TPs was prepared by using the vials obtained from photodegradation experiments and injected in each sample batch to check retention times of all these analytes.

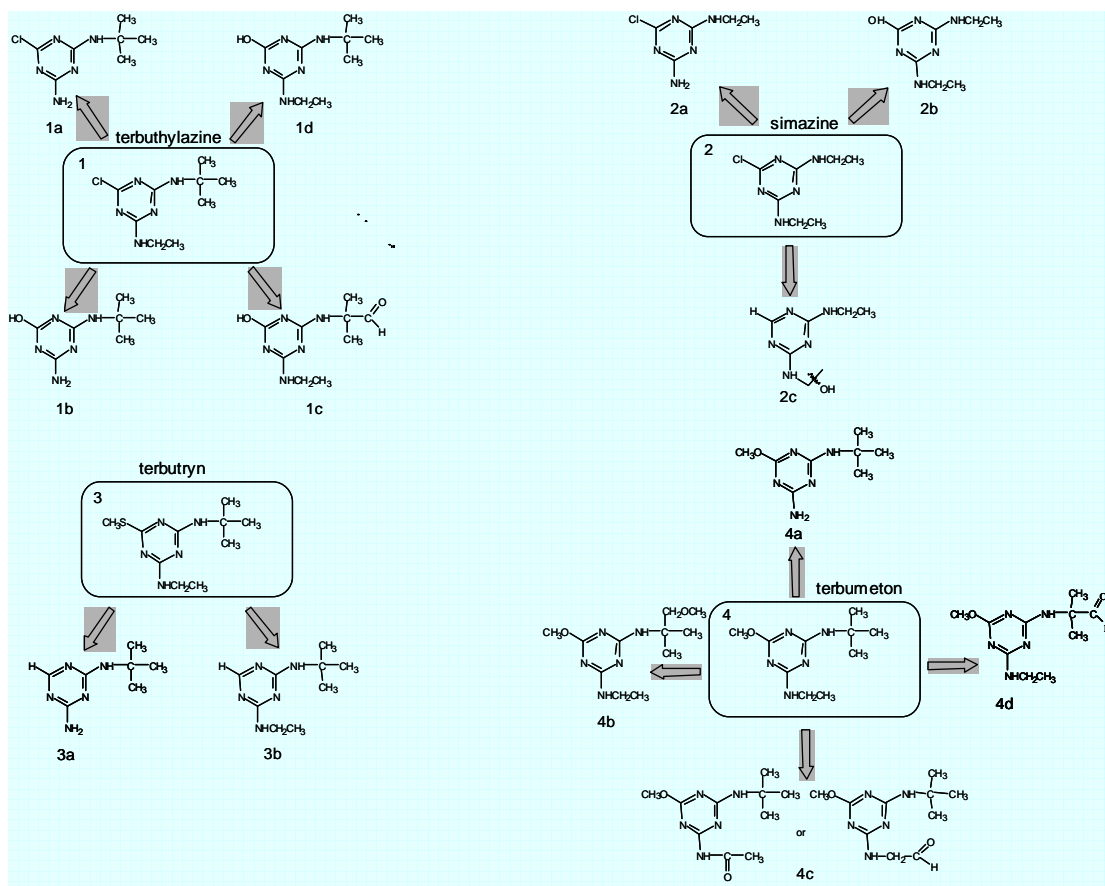
Table 2. Method validation for ground water (GW) and surface water (SW). Limits of detection (LOD), percentage recovery (in %) and relative standard deviation (in brackets) for five replicates

Compound	Recovery (RSD)						r
	LOD (ng/L)		50 ng/L		500 ng/L		
	SW	GW	SW	GW	SW	GW	
Terbutylazine (1)	2	2	106(3)	96(5)	96(8)	76(4)	0.9936
<i>Desethyl-terbutylazine (1a)</i>	3	3	99(3)	100(4)	92(9)	84(4)	0.9917
<i>Desethyl-2-hydroxy-terbutylazine (1b)</i>	17	4	72(2)	85(3)	85(2)	77(7)	0.9998
<i>2-hydroxy-terbutylazine (1c)</i>	3	2	97(5)	106(6)	103(3)	104(3)	0.9981
Simazine (2)	8	2	97(3)	90(3)	93(9)	85(5)	0.9956
<i>2-hydroxy-simazine (2a)</i>	5	2	95(5)	100(4)	98(3)	88(7)	0.9985
<i>Desisopropyl-atrazine (2b)</i>	25	25	104(12)	94(5)	89(7)	80(4)	0.9940
Terbutryne (3)	6	3	89(5)	89(5)	107(3)	93(10)	0.9957
Terbumeton (4)	7	3	92(9)	91(3)	102(5)	98(4)	0.9920
<i>Desethylterbumeton (4a)</i>	5	7	97(5)	93(4)	95(3)	91(2)	0.9978
Bentazone (5)	11	20	79(15)	102(7)	110(3)	107(4)	0.9940
<i>Bentazone-methyl (5a)</i>	25	25	91(13)	89(8)	98(9)	85(5)	0.9958
<i>Acid antranilic isopropylamide (5b)</i>	6	4	94(4)	89(6)	89(5)	80(3)	0.9922
Imazalil (6)	9	9	108(2)	107(4)	99(10)	96(6)	0.9988
<i>1-(2,4) Dichlorophenylimidazol (6e)</i>	7	4	71(4)	70(3)	97(3)	104(5)	0.9965
Bromacil (7)	23	25	102(5)	105(2)	97(3)	90(4)	0.9945
Terbacil (8)	12	12	91(4)	96(4)	97(4)	94(3)	0.9962
Carbendazim (9)	10	7	83(8)	84(3)	109(1)	100(2)	0.9903
<i>2-aminobenzimidazol (9a)</i>	13	10	91(5)	91(7)	96(7)	78(11)	0.9962
Diuron (10)	2	7	93(4)	86(8)	101(2)	92(6)	0.9940
<i>3,4-dichloroaniline (10a)</i>	18	9	76(8)	71(12)	100(5)	92(9)	0.9925
<i>3,4 Dichlorophenyl methylurea (10b)</i>	21	25	96(3)	94(4)	95(4)	93(3)	0.9988
<i>1-(3,4) Dichlorophenylurea (10c)</i>	10	8	87(12)	71(7)	105(9)	94(8)	0.9907
Benfuracarb (11)	-	-					
<i>Carbofuran (11b)</i>	3	6	93(4)	94(3)	94(4)	97(2)	0.9990
<i>Carbofuran-3-hydroxy (11c)</i>	22	15	97(9)	101(10)	93(7)	109(11)	0.9996

RESULTS AND DISCUSSION

Photodegradation

Following the methodology employed in our previous paper [23], 14 TPs were elucidated, some of them had not been previously reported. Figure 1 shows the parent compounds and the TPs identified in water, detected under the experimental conditions applied in this paper. The majority of them were determined in positive ionization mode, meanwhile only two TPs (10f, 11a) presented ionization in negative mode. Some compounds, as for example compound 10b, could be measured in both ionization modes. It is also interesting to remark that benfuracarb (compound 11) was immediately degraded in water to carbofuran.



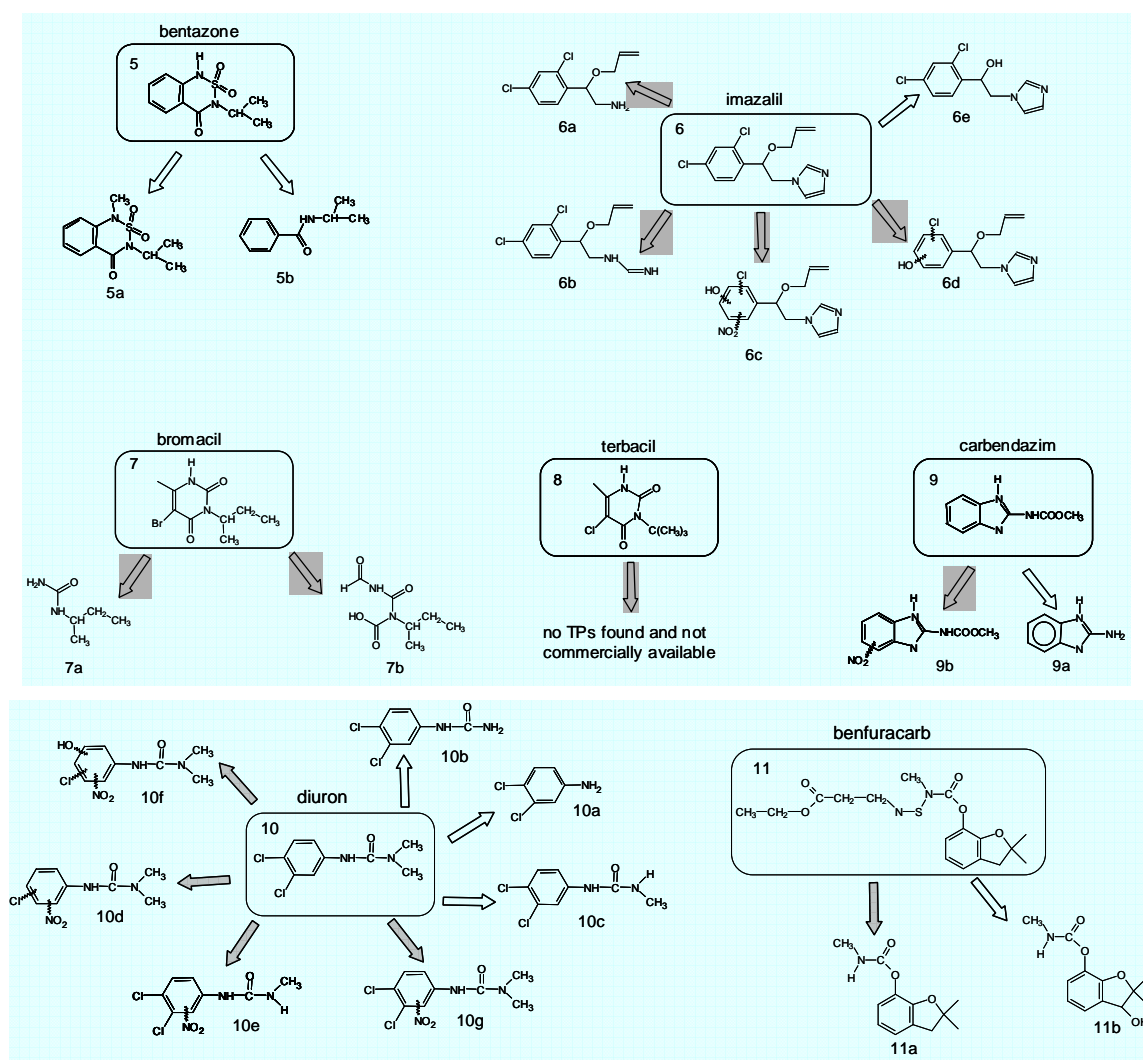




Figure 1. Structures of parent compounds and TPs

-  TPs obtained in photodegradation experiments (both commercially and non commercially available)
 TPs non obtained in the photodegradation experiment but commercially available

The high resolution and mass accuracy provided by the TOF analyzer allowed the assignment of a highly probable empirical formula for each compound. For example, the nominal mass of a diuron TP, compound 10c (m/z 219), could be attributed to the elemental compositions $C_8H_9N_2OCl_2$ (m/z 219.0092) or $C_9H_{13}N_2Cl_2$ (m/z 219.0456). When studying the measured accurate mass of this compound (m/z 219.0135), differences of 4.3 and 32.1 mDa were obtained, respectively. Therefore, in this case, the exact mass was needed to obtain the correct

elemental composition of the compound ($C_8H_9N_2OCl_2$), and the use of a TOF instrument was compulsory. This situation would be indistinguishable by quadrupole instruments.

In some cases, the possibility offered by hybrid QTOF instruments to perform MS/MS spectra with accurate mass measurements was used for the reliable elucidation of a TP and for the differentiation between isomeric compounds. For example, although the calculated elemental composition of the compound 6c ($C_{14}H_{14}N_3O_4Cl$) suggested this compound to be a nitrated product of imazalil ($C_{14}H_{15}N_2OCl_2$), data from full scan experiments did not allow to know where the group was located. In order to elucidate this TP, MS/MS experiments were carried out. As can be seen in Figure 2b, the product ion spectra of the TP showed a fragment at m/z 185.9958 ($C_7H_5NO_3Cl$, $\Delta mDa=0$), showing that the $-NO_2$ group was located in the phenyl group and not in the imidazol group, when comparing this product ion with the parent pesticide imazalil (m/z 297.0561) (Figure 2a).

However, some limitations were also observed. For example, in the study of terbacil and bentazone, despite the progressive disappearance of the parent compound, no TPs were found. This might be due to the source used (ESI), that restricts its application to those compounds easily ionisable with this interface. Therefore, the use of other interfaces, as APCI or APPI, or the use of GC-TOF with EI, for example, would be useful for a wider coverage of TPs. Another drawback comes from the limited understanding of the fragmentation rules in MS/MS of (de)protonated molecules so that, in some cases, an unequivocal structure could not be obtained. This was the case of diuron, where several nitrated TPs (10d-10g) were found, but the position of the NO_2 group was not definitively determined. To our knowledge, these compounds had not been previously reported, so diuron photodegradation experiments were carried out again using drinking water. In this case, nitrated compounds were not found, showing the possible matrix influence on the degradation of the parent compound, as nitrate concentration in the surface water used for experiments was notably higher than in drinking water. Imazalil as well as carbendazim also produced nitrated TPs when using surface water.

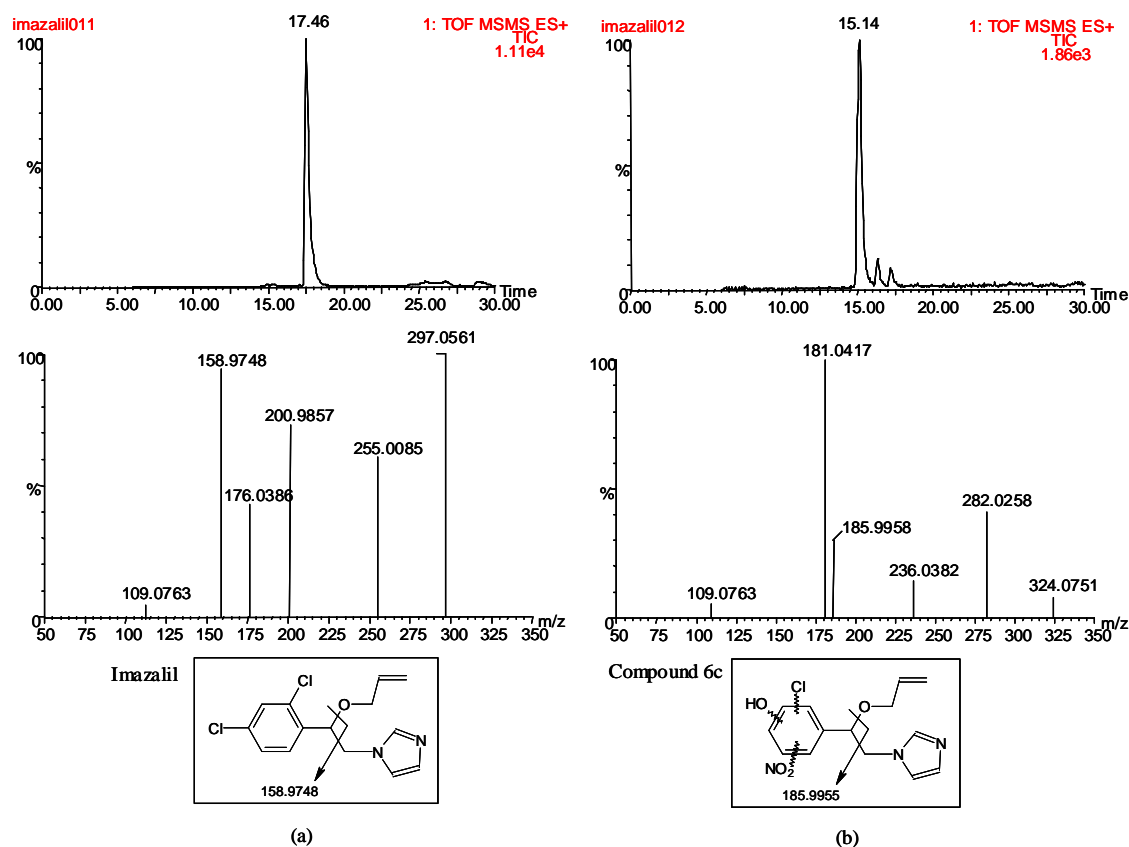


Figure 2. LC-MS/MS chromatogram and spectra of (a) imazalil (b) an imazalil TP (compound 6c)

Multiresidue Method

The developed multiresidue method included: 1) all the parent compounds studied in this paper (except benfuracarb), 2) all their commercially available TPs, (3) the most abundant TPs elucidated in this paper, and 4) those triazines and related TPs studied in our previous paper [23].

MS optimisation

Full-scan and MS/MS spectra of parent pesticides and commercially available TPs were obtained from infusion of 5 mg/L acetonitrile: water (50:50) individual solutions of each compound, at a flow rate of 10 μ L/min. In those cases where TPs were not commercially available, the corresponding vial obtained from the photodegradation experiment, with the highest concentration of analyte, was used for MS and MS/MS experiments.

All compounds presented an abundant $[M+H]^+$ or $[M-H]^-$ ion, and they were selected as precursor ions. The majority of the analytes were determined by positive ionization mode, meanwhile only four (bentazone (5), bromacil (7), terbacil (8) and a diuron TP (10f)) presented more abundant ionization in negative mode.

The development of confirmative methods made necessary to obtain two specific transitions for each compound. In some cases, analyte fragmentation occurred following a dominant pathway, leading to a product ion much more abundant than the rest, difficulting in this way the confirmation of the identity of the analyte at low concentration levels. However, the presence of halogenated atoms (typically Cl or Br) in some molecules allowed us to obtain two sensitive transitions for these analytes.

Mass spectrometric parameters selected, as precursor and product ions, cone voltage and collision cell energy for each analyte are shown in Table 1.

LC optimisation

The very distinct physico-chemical characteristics for selected pesticides and TPs made advisable to find a compromise between all compounds regarding SPE/chromatographic retention and MS response. Thus, the presence of acid was necessary in order to protonate acidic compounds such as terbacil or bromacil, favouring the retention in the cartridge. However, the added acid also protonated some basic analytes such as most of triazine metabolites difficulting their chromatographic retention. Additionally, the ion-pairing reagent needed for retention of these cationic TPs (a long-chain carboxylic acid) produced strong ion suppression for those compounds monitored in negative ionization mode. This situation was studied in depth in a previous paper [22]. We considered as the best option the development of two complementary methods: one using HFBA as ion-pairing reagent for cationic compounds and the other using HCOOH for the preconcentration and determination of acidic compounds. Neutral compounds were distributed in both methods depending on their retention time, trying to minimise the number of compounds at the same retention window and allowing the monitoring of the maximum number of transitions without significant loss in sensitivity.

Table 1. MS/MS optimized conditions for determining target analytes by triple quadrupole mass analyzer

Compound	Polarity	Precursor Ion (m/z)	Cone (V)	Collision (eV)	Product ion (m/z) ^a	Method ^b
Terbutylazine (1)	ES+	229.9	30	15	173.9	1
		231.9	30	15	175.9	
Desethyl-terbutylazine (1a)	ES+	202.1	25	15	145.9	1
		204.1	25	15	147.9	
Desethyl-2-hydroxy-terbutylazine (1b)	ES+	184.0	30	15	128.0	2
		184.0	30	25	86.1	
2-hydroxy-terbutylazine (1c)	ES+	212.2	30	15	155.9	2
		212.2	30	25	86.0	
M_1 =Tbza_226 (1d)	ES+	225.9	20	20	207.8	2
		225.9	20	30	114.1	
Simazine (2)	ES+	201.9	30	20	124.0	1
		201.9	30	15	131.9	
2-hydroxy-simazine (2a)	ES+	183.9	35	20	114.0	2
		183.9	35	30	69.1	
Desisopropyl-atrazine (2b)	ES+	173.9	30	15	68.1	2
		173.9	30	10	96.0	
M_2 =Simazine_184 (2c)	ES+	184.1	15	25	113.96	1
		184.1	15	15	156.1	
Terbutryn (3)	ES+	242.0	30	20	185.9	2
		242.0	30	30	71.1	
M_3 = Terbutryn_196 (3a)	ES+	196.0	15	15	139.9	1
		196.0	15	25	45.3	
M_4 = Terbutryn_168 (3b)	ES+	168.1	15	15	111.9	1
		168.1	15	30	70.1	
Terbumeton (4)	ES+	226.0	25	20	169.9	2
		226.0	25	25	114.0	
Desethylterbumeton (4a)	ES+	197.9	30	15	141.9	1
		197.9	30	25	86.0	
M_5 = Terbumeton_240 (4b)	ES+	240.1	30	20	170.1	2
		240.1	30	25	137.9	
M_6 =Terbumeton_256 (4d)	ES+	256.1	15	20	170.1	1
		256.1	15	15	226.1	
M_7 = Terbumeton_240 (4c)	ES+	240.1	30	20	141.9	1
		240.1	30	15	184.1	
Bentazone (5)	ES-	238.9	40	25	131.9	1
		238.9	40	15	196.8	
Bentazone-methyl (5a)	ES+	254.9	15	10	212.9	1
		254.9	15	20	148.9	
Antranillic acid isoproylamide (5b)	ES+	179.0	15	15	119.9	1
		179.0	15	35	92.0	

^a Top transition used for quantification (Q); bottom transition used for confirmation (q)

^b Method with HCOOH = 1; Method with HFBA = 2

Table 1 (cont). MS/MS optimized conditions for determining target analytes by triple quadrupole mass analyzer

Compound	Polarity	Precursor ion (m/z)	Cone (V)	Collision (eV)	Product ion (m/z) ^a	Method
Imazalil (6)	ES+	296.9	35	30	41.3	1
		296.9	35	30	158.8	
1-(2,4)-dichloro-phenyl-2-imidazol-1-yl-ethanol (6e)	ES+	256.9	20	20	69.1	2
		256.9	20	30	124.9	
Bromacil (7)	ES-	259.1	40	20	203	1
		261.9	40	20	205	
<i>M</i> ₈ =Bromacil_117 (7a)	ES+	117.1	15	10	61.2	1
		117.1	15	15	57.2	
Terbacil (8)	ES-	215.0	25	15	159.1	1
		217.0	25	15	161.1	
Carbendazim (9)	ES+	192.1	25	15	160.0	2
		192.1	25	25	132.0	
2-aminobenzimidazol (9a)	ES+	133.9	45	25	92.0	2
		133.9	45	35	65.1	
<i>M</i> ₉ = Carbendazim_237 (9b)	ES+	236.9	15	15	204.9	1
		236.9	15	30	158.8	
Diuron (10)	ES+	232.8	30	15	72.1	2
		232.8	30	10	46.3	
3,4-dichloroaniline (10a)	ES+	161.8	50	15	126.9	2
		163.8	50	15	128.9	
1-(3,4)-dichlorophenylurea (10b)	ES+	204.8	20	20	126.9	1
		206.8	20	20	128.9	
1-(3,4)-dichlorophenyl-3-methylurea (10c)	ES+	219.0	20	30	126.9	1
		219.0	20	30	161.8	
<i>M</i> ₁₀ =Diuron_244 (10d)	ES+	244.0	25	25	72.1	1
		244.0	25	25	46.3	
<i>M</i> ₁₁ =Diuron_264 (10e)	ES+	264.0	15	10	206.9	2
		264.0	15	15	159.0	
<i>M</i> ₁₂ =Diuron_258 (10f)	ES-	258.0	25	15	212.8	2
		258.0	25	25	167.7	
<i>M</i> ₁₃ =Diuron_278 (10g)	ES+	278.0	25	15	72.0	2
Benfuracarb (11)						
Carbofuran (11a)	ES+	222.0	25	10	164.9	2
		222.0	25	20	122.9	
Carbofuran-3-OH (11b)	ES+	219.9	20	15	162.9	2
		219.9	20	30	107.0	

^a Top transition used for quantification (Q); bottom transition used for confirmation (q)

^b Method with HCOOH = 1; Method with HFBA = 2

Method validation

Linearity of the methods was studied in the range from 25 to 1000 ng/L for all compounds with commercially available reference standards, showing satisfactory results as can be seen in Table 2, with correlation coefficients higher than 0.99.

Precision and accuracy of the overall analytical procedures were evaluated in ground and surface water samples spiked at 50 and 500 ng/L by analyzing each one in quintuplicate (Table 2). In both groundwater and surface water samples, recoveries were satisfactory (between 70 and 110%) for all compounds at the two fortification levels. Both methods showed satisfactory precision (RSD<15%).

As an example, Figure 3 shows the chromatograms obtained for a surface water and a groundwater spiked at the LOQ level (50 ng/L). As can be seen in the figure, the high sensitivity of the developed methods allowed the correct quantification of the selected pesticides and transformation products at these low levels independently of their class (cationic, anionic or neutral). We set-up the LOQ objective at 50 ng/L, but it was quite evident that lower values could have been reported for most of compounds if required.

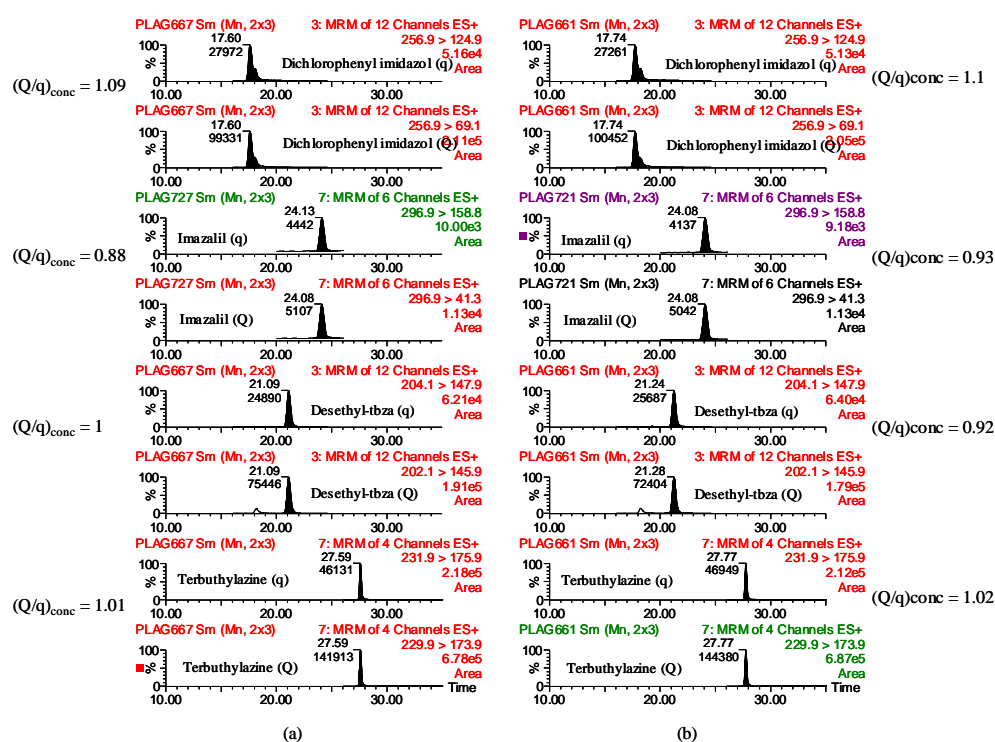


Figure 3. LC-MS/MS chromatograms corresponding to (a) surface water and (b) groundwater, both spiked at 50 ng/L. (Q) quantitative transition, (q) qualitative transition.

Monitoring of pesticides in environmental samples

In order to assure the quality of data along sample analysis, several QCs were prepared and analyzed during the sample batch. Recoveries for QCs varied from 70% to 110% for all selected analytes. Moreover, positive findings were simultaneously quantified and confirmed by the acquisition of two SRM transitions for each compound. As a summary, the majority of positive findings obtained by measurement of the quantification transition were confirmed by the confirmation transition with concentration ratios deviations always lower than 20%.

Triazines and related TPs and bromacil were the most frequently detected compounds. For example, desethyl-2-hydroxy-terbutylazine and desethylterbumeton were found in more than 80% of the samples. It is pointed out the lower number of findings for unchanged triazines when compared to those obtained for their related TPs. As an example, Figure 4 shows the chromatograms obtained for two real samples.

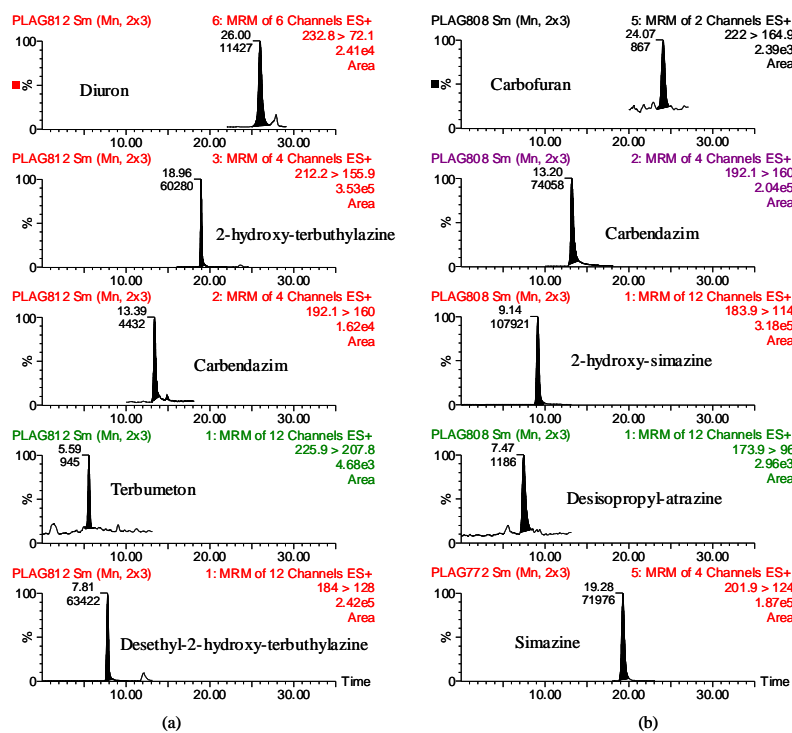


Figure 4. LC-MS/MS chromatograms for positive groundwater samples containing (a) diuron, 2-hydroxy-terbutylazine, carbendazim, terbumeton and desethyl-2-hydroxy-terbutylazine (b) carbofuran, carbendazim, 2-hydroxy-simazine, desisopropyl-atrazine and simazine

Most of detected TPs corresponded to compounds that are commercially available as reference standard. This allowed us to adequately quantify them in samples (Table 3). Several of the TPs found in water samples were not obtained in the photodegradation experiments, as for example bentazone-methyl. This compound was detected mainly in ground water, and it might be formed by a degradation process promoted by micro organisms, which can not be properly simulated by the irradiation system used in the laboratory. Some TPs not commercially available but elucidated in the photodegradation experiments were also detected, as for example, a diuron TP (10e) and a terbumeton TP (4b).

These results indicate that the methodology based on irradiation of spiked water samples and subsequent investigation of the compounds formed is useful, although using different light sources, as for example, xenon lamps, would be also helpful in order to simulate the sunlight conditions.

In order to obtain a wider knowledge on TPs formed in the soil-water environment, field studies were performed by applying three selected pesticides in an experimental plot equipped with lisimeters. With this experiment we tried to obtain a more realistic approach of the pesticide TPs that can reach the groundwater.

The analysis of water samples collected from the unsaturated zone showed the gradual disappearance of the parent compound. After application of simazine and terbuthylazine, those TPs corresponding to dechlorination plus hydroxylation (1c, 2a), dealkylations (1a, 2b) or a combination of both processes (1b) were observed in the water from the unsaturated zone. These TPs were also detected in the ground and surface water samples analyzed (see Table 3). Oppositely to ground and surface water compounds (1d) and (2c) were detected. After application of diuron in the experimental plot, the main TPs detected were 3,4-dichloroaniline (10a), 3,4-dichlorophenylurea (10b) and 3,4-dichlorophenylmethylurea (10c). None of these TPs were, however, found in ground or surface water. All detections were confirmed by means of the two transitions used.

Table 3. Pesticides and related TPs detected in several selected water samples from the Valencian region of Spain. Concentration data in ng/L.

Compound	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
	GW	GW	SW	SW	GW	GW
Terbutylazine (1)		422			344	323
<i>Desethyl-terbutylazine (1a)</i>		854		(41.5)	936	770
<i>Desethyl-2-hydroxy-terbutylazine (1b)</i>	(46)	1257	469	193	1191	945
<i>2-hydroxy-terbutylazine (1c)</i>		142	77		132	106
Simazine (2)	618	91			108	58
<i>2-hydroxy-simazine (2a)</i>	236					
<i>Desisopropyl-atrazine (2b)</i>	815	130	(41)		164	143
Terbumeton (4)		919			950	811
<i>Desethylterbumeton (4a)</i>		2134		89	2223	1578
<i>Bentazone-methyl (5a)</i>		71				
Bromacil (7)		399			686	440
Terbacil (8)		(31)	(28)		56	62
Carbendazim (9)	123					
<i>2-aminobenzimidazol(9a)</i>	280	(45)	76			
Diuron (10)		(36)			(42.4)	(34)
<i>Carbofuran (11b)</i>		51				
<i>Carbofuran-3-hydroxy (11c)</i>	(30.9)					

() data in brackets are estimated, as they are below the LOQ objective of the method (50 ng/L)

CONCLUSION

The methodology followed in this paper, based on the combination of LC with QTOF and QqQ instruments, has allowed the identification, quantification and confirmation of several selected pesticides and their TPs.

On the one hand, the high resolution and exact mass capabilities of TOF MS instruments was applied to the identification of pesticide TPs in water after photodegradation laboratory experiments. In some cases, the possibility of performing MS/MS experiments helped us to elucidate the structure of the compound, from the product ion spectra with the exact mass of the fragments.

On the other hand, the development of two SPE-LC-MS/MS methods by QqQ MS allowed the simultaneous determination and confirmation of a total number of 40 pesticides and TPs in surface and groundwater samples. After injection of 2.2 mL of sample with the only treatment of acidification (either with HFBA or HCOOH), quantitative recoveries were obtained in both types of samples at 50 ng/L and 500 ng/L levels with LODs normally lower than 15 ng/L.

The application of both methods to real samples pointed out the higher amount of TPs findings in relation to unchanged pesticides. These results advise the inclusion of TPs in pesticide multiresidue methods applied in water. However, although TPs are apparently required in water legislation, it seems reasonable to evaluate their toxicity to set-up the most "relevant" compounds to be included in the analytical method. This is important to have a realistic scenario that could justify the analytical efforts required considering the added difficulties that it means the inclusion of TPs.

ACKNOWLEDGEMENTS

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for using the Quattro LC triple quadrupole mass spectrometer and the Q-TOF hybrid quadrupole time-of-flight mass spectrometer.

This work forms a part of the research project "Aplicación del acoplamiento cromatografía líquida-espectrometría de masas (LC-MS/MS y LC-QTOF MS) a la identificación de metabolitos de plaguicidas en muestras de interés ambiental y toxicológico" developed under financial support of the Ministry of Science and Technology (Ref: BQU2003-02685), Spain

M.I. is very grateful to Generalitat Valenciana for her pre-doctoral grant.

REFERENCES

- (1) ECC Council Directive 80/778/ECC, Off. J. European Communities, No. L 229, August 30, 1980, p 11
- (2) D. W. Kolpin, E. M. Thurman, S. M. Linhart, *Sci. Total Environ.* 248 (2000) 115
- (3) D. W. Kolpin, E. M. Thurman, S. M. Linhart, *Environ. Sci. Technol.* 35 (2001) 1217
- (4) R. A. Rebich, R. H. Coupe, E. M. Thurman, *Sci. Total Environ.* 321 (2004) 189
- (5) A. C. Belfroid, M. van Drunen, M. A. Beek, S. M. Schrap, C. A. M. van Gestel, B. Van Hattum, *Sci. Total Environ.* 222 (1998) 167
- (6) T. Reemtsma, *J. Chromatograph. A* 1000 (2003) 477
- (7) Y. Picó, C. Blasco, G. Font, *Mass Spectrom. Rev.* 23 (2004) 45
- (8) R. B. Geerdink, W. M. A. Niessen, U. A. Th. Brinkman, *J. Chromatogr. A.* 970 (2002) 65
- (9) A. C. Hogenboom, W. M. A. Niessen, U. A. Th. Brinkman, *J. Sep. Sci.* 24 (2001) 331
- (10) J. C. Marr, J. B. King, *Rapid Commun. Mass Spectrom.* 11 (1997) 479
- (11) C. Hogenboom, W. M. A. Niessen, U. A. Th. Brinkman, *J. Chromatogr. A.* 794 (1998) 201
- (12) Koppen, N. H. Spliid, *J. Chromatogr. A.* 803 (1998) 157
- (13) R. J. C. A. Steen, A. C. Hogenboom, P. E. G. Leonards, R. A. L. Peerboom, W. P. Cofino, U. A. Th. Brinkman, *J. Chromatogr. A.* 857 (1999) 157
- (14) E. Dijkman, D. Mooibroek, R. Hoogerbrugge, E. Hogendoorn, J. V. Sancho, O. Pozo, F. Hernandez., *J. Chromatogr. A.* 926 (2001) 113
- (15) A. Ingelse, R. C. J. van Dam, R. J. Vreeken, H. G. J. Mol, O. M. Steujger, *J. Chromatogr. A.* 918 (2001) 67
- (16) F. Hernández, J. V. Sancho, O. Pozo, A. Lara, E. Pitarch, *J. Chromatogr. A.* 939 (2001) 1
- (17) R. Castro, E. Moyano, M. T. Galceran, *J. Chromatogr. A.* 914 (2001) 111
- (18) R. Bossi, K. V. Vejrup, B. B. Mogensen, W. A. H. Asman, *J. Chromatogr. A.* 957 (2002) 27
- (19) J. V. Sancho, O. J. Pozo, F. Hernández, *Analyst* 129 (2004) 38
- (20) A. C. Borba da Cunha, M. J. Lopez de Alda, D. Barcelò, T. M. Pizzolato, J. H. Z. Dos Santos, *Anal Bioanal Chem* 378 (2004) 940
- (21) F. Hernández, M. Ibáñez, J. V. Sancho, O. J. Pozo, *Anal. Chem.* 76 (2004) 4349

- (22) F. Hernández, O.J. Pozo, J.V. Sancho, F.J. López, J.M. Marín, M. Ibáñez, *Trac-Trends Anal. Chem.* 24 (2005) 596
- (23) M. Ibañez, J.V. Sancho, O.J. Pozo, F. Hernandez *Anal. Chem.* 76 (2004) 1328
- (24) Bobeldijk, I.; Vissers, J.P.C.; Kearney, G.; Major, H.; van Leerdam, J.A. *J Chromatogr. A.* 929 (2001) 63
- (25) Ibáñez M., Sancho J.V., Pozo O.J. , Niessen W., Hernández F. *Rapid Commun. in Mass Spectrom.* 19 (2005) 1
- (26) Ibáñez M., Sancho J.V., Pozo O.J., Hernández F. *Anal and Bioanal Chem.* 384 (2006) 448
- (27) Sancho J.V., Pozo O.J., Ibáñez M., Hernández F *Anal and Bioanal Chem.* 2006, DOI: 10.1007/s00216-006-0532-0
- (28) Detomaso A., Mascolo G, López A. *Rapid Commun in Mass Spectrom* 19 (2005) 2193
- (29) Hogenboom A.C., Niessen W.M.A, Brinkman U.A.Th *Rapid Commun in Mass Spectrom* 14 (2000) 1914
- (30) Kouloumbus V.N., Tsiipi D.F., Hiskia A.E., Nikolic D., van Breemen R.B. *J. Am Soc Mass Spectrom* 14 (2003) 803
- (31) Marín J.M., Sancho J.V., Pozo O.J., López F.J., Hernández F. Submitted

3.3.4 Bibliografía

Castro R, Moyano E, Galcerán MT

Determination of quaternary ammonium pesticides by liquid chromatography-electrospray tandem mass spectrometry

J. Chromatogr. A 914 (2001) 111-121

Di Corcia A, Constantino A, Crescenzi C, Samperi R

Quantification of phenylurea herbicides and their free and humic acid-associated metabolites in natural waters

J. Chromatogr. A 852 (1999) 465-474

Ingelse BA, van Dam RCJ, Vreeken RJ, Mol HGJ, Setijger OM

Determination of polar organophosphorus pesticides in aqueous samples by direct injection using liquid chromatography-tandem mass spectrometry

J. Chromatogr. A 918 (2001) 67-78

Kienhuis PGM, Geerdink RB

Liquid chromatography-tandem mass spectrometric analysis of surface and waste water with atmospheric pressure chemical ionisation-II. Applications.

Trends Anal. Chem. 19 (2000) 460-474

Marín JM, Sancho JV, Pozo OJ, López FJ, Hernández F

Quantification and Confirmation of Anionic, Cationic and Neutral Pesticides and Transformation Products in Water by on-line Solid Phase Extraction - Liquid Chromatography - Tandem Mass Spectrometry.

J. Chromatogr. A, en prensa (2006)

Sancho JV, Pozo OJ, Hernández F

Liquid chromatography and tandem mass spectrometry: a powerful approach for the sensitive and rapid multiclass determination of pesticides and transformation products in water

Analyst 129 (2004) 38-44

3.4 Determinación individual de plaguicidas y productos de transformación en aguas

3.4.1 Introducción

Como ya se ha comentado en el capítulo anterior, una de las familias de plaguicidas más problemáticas desde el punto de vista de contaminación medioambiental es la de los herbicidas. El glifosato es el herbicida no selectivo más utilizado en todo el mundo para el control de malas hierbas y malezas. Dado su elevado uso en la Comunidad Valenciana, resultaba interesante desarrollar un método analítico para la determinación de este compuesto en muestras medioambientales. Sin embargo, debido a sus características físico-químicas, era necesario desarrollar un método específico, al no poderse incluir en el método multiresiduo anteriormente descrito. Por similitud, tanto de estructura como de función, se decidió incluir también el herbicida glufosinato.

Estos dos plaguicidas interfieren en la capacidad de la planta para formar aminoácidos además de afectar negativamente tanto a la fotosíntesis como a la respiración. Tras la muerte de las plantas, estos herbicidas pueden ser adsorbidos por el suelo y entrar en el medio acuático debido a su alta solubilidad en agua. Estudios realizados en agua indican una degradación aparentemente inexistente en condiciones ambientales. Este hecho junto con su elevado uso hace que sea bastante alta la probabilidad de encontrar el plaguicida intacto. Sin embargo, su movilidad en suelos depende de la composición del suelo, por lo que factores como el contenido en arcillas o en materia orgánica pueden hacer que aumente su retención, aumentando también la posible degradación a su principal producto de transformación. En el caso del glifosato, su principal producto de transformación es el ácido aminometilfosfórico (AMPA), por lo que se decidió incluirlo también en el método analítico.

En la Figura 3.4 se muestran las estructuras químicas de los tres compuestos organofosforados estudiados:

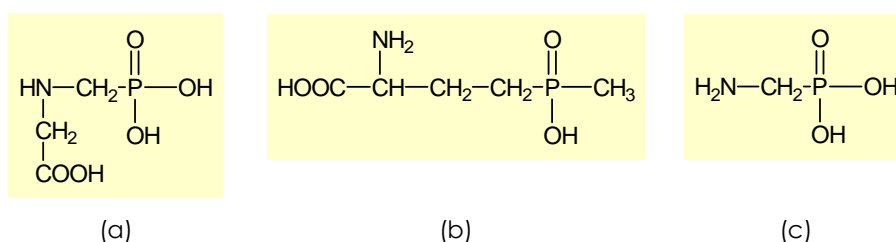


Figura 3.4 Estructuras del (a) glifosato, (b) glufosinato y (c) AMPA

La aplicación de métodos para la determinación individual de analitos conlleva algunas ventajas frente a los métodos multiresiduo, ya que se puede desarrollar un procedimiento específico para el analito objetivo. Así, se puede optimizar el tratamiento de muestra para aislar y/o preconcentrar el compuesto de interés teniendo en cuenta sus características físico-químicas. De la misma manera, la optimización cromatográfica es más sencilla si sólo se tiene que determinar un compuesto ya que se pueden utilizar las fases móviles óptimas para su determinación, independientemente del resto de compuestos de la muestra.

Como ya hemos visto anteriormente, a la hora de determinar una molécula objetivo en muestras ambientales, las dos características más importantes que tiene que cumplir el método son la especificidad, ya que se desea conocer con certeza la presencia de dicho analito en la muestra minimizando el riesgo de reportar falsos positivos o falsos negativos, así como la elevada sensibilidad requerida, debido a los bajos límites exigidos por la legislación (0.1 µg/L en el caso de plaguicidas en aguas potables).

En este capítulo se estudia el potencial del acoplamiento LC-MS/MS mediante analizadores de triple cuadrupolo para la determinación de glifosato, glufosinato y AMPA en muestras de aguas y suelos.

Debido a las características físico-químicas de estos compuestos (carácter iónico, baja volatilidad, bajo peso molecular y falta de grupos cromóforos), la mayoría de los métodos desarrollados hasta la fecha, tanto por LC como por GC, han requerido algún tipo de derivatización previa con el fin de posibilitar su análisis. Así, en el caso de GC se han utilizado diferentes reactivos derivatizantes, como por ejemplo mezclas de trifluoroetanol y anhídrido trifluoroacético, con el fin de aumentar la volatilidad. Sin embargo, estos procesos requieren mucha manipulación de muestra. Por otra parte, aunque las características físico-químicas de estos compuestos los hacen potenciales candidatos para su determinación por métodos basados en LC, la falta de grupos químicos adecuados, como grupos cromóforos, fluorogénicos o que absorban en el UV, ha limitado su análisis mediante detectores convencionales. En este caso también se han llevado a cabo derivatizaciones con el fin de formar derivados fluorescentes y así mejorar la detección, y/o reducir el carácter polar de los analitos, facilitando su retención en la columna cromatográfica. La mayoría de las derivatizaciones han hecho uso del FMOC como agente derivatizante.

En nuestro trabajo, en primer lugar se intentó determinar estos compuestos directamente, es decir, sin una derivatización previa, usando una columna HILIC (Waters). Ante la imposibilidad de alcanzar la sensibilidad requerida, se procedió a la determinación de estos compuestos previa derivatización con FMOC. Se comprobó el efecto de añadir diferentes

concentraciones de FMOc así como el tiempo o la temperatura de reacción. Por último, se optimizaron tanto las condiciones MS/MS como la cromatografía y la extracción en fase sólida. Una vez validado, el método fue aplicado al análisis de muestras reales de aguas y suelos. Por otra parte, y dada la tendencia de los fosfatos y fosfonatos a adsorberse en vidrio no silicizado, se llevó a cabo un pequeño estudio con el fin de evaluar este efecto.

Finalmente, se participó en un ejercicio de intercomparación organizado por Monsanto Europa en el cual se analizaron diferentes muestras de aguas superficiales y subterráneas, lo cual nos permitió re-evaluar el método desarrollado.

3.4.2 Resultados y discusión

a. Optimización del método 1

En la Figura 3.5 se muestran los espectros MS y MS/MS del glifosato en ambos modos de ionización, obtenidos tras infundir un patrón del compuesto a una concentración de aproximadamente 10 µg/mL. Inicialmente se intentó determinar los tres compuestos directamente, es decir, sin una derivatización previa. Para ello, se usó la Cromatografía de Interacción Hidrofílica con una columna Atlantis™ HILIC (100 x 2.1 mm i.d., 5 µm, Waters). Esta columna ofrece una mayor retención para compuestos polares, que no son retenidos adecuadamente bajo condiciones de cromatografía reversa.

A pesar de que la retención con esta columna era satisfactoria, la sensibilidad obtenida era muy baja, siendo por tanto necesaria una importante preconcentración, difícil de realizar a niveles tan bajos. Por otra parte, las condiciones necesarias para obtener una buena retención y una buena forma de pico eran muy específicas, cambiando drásticamente con las condiciones de pH de la muestra o de la fase móvil, y disminuyendo por tanto la robustez del método. Finalmente, se llevó a cabo un proceso de derivatización con el fin de aumentar la retención de los analitos, y así poder trabajar con columnas en fase reversa y en condiciones no tan estrictas.

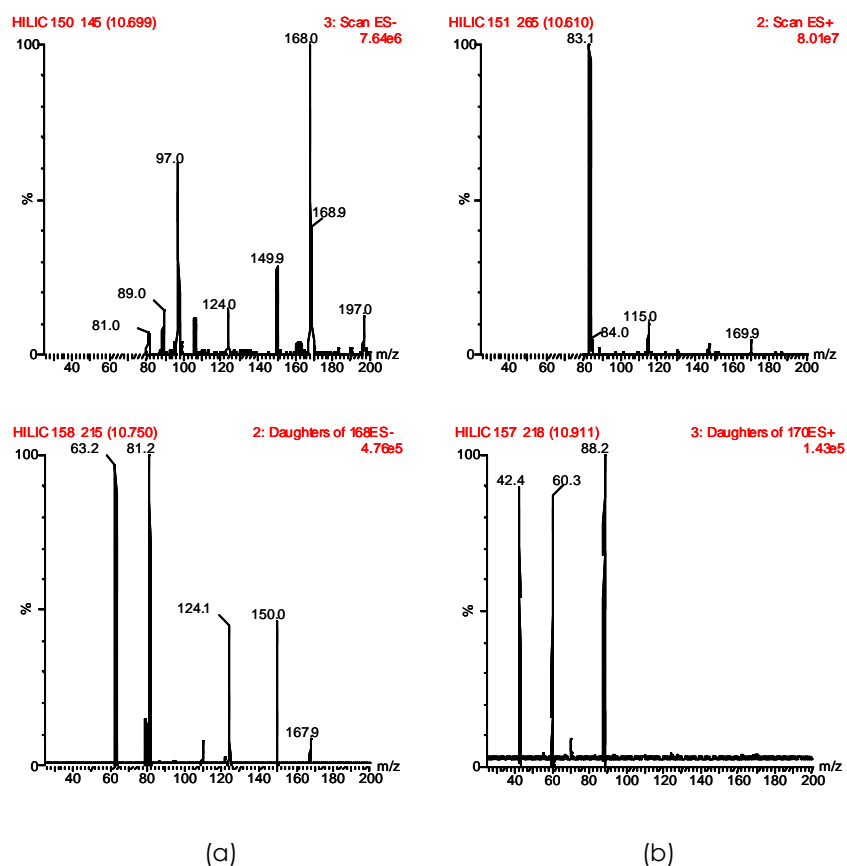


Figura 3.5 Espectros MS (arriba) y MS/MS (abajo) del glifosato en (a) modo de ionización negativo y (b) modo de ionización positivo.

Con el fin de determinar los parámetros MS/MS para cada compuesto, se cromatografió una disolución patrón mezcla de los tres compuestos, de aproximadamente 10 $\mu\text{g/mL}$, previamente derivatizada según un método ya desarrollado en nuestro laboratorio (Sancho et al, 1994, 1996).

Los tres compuestos presentan características ácidas por lo que la interfase electrospray en modo de ionización negativo (ES-) podría considerarse a priori la más adecuada para su determinación por LC-MS/MS. De hecho, éste ha sido el modo de ionización más utilizado hasta la fecha. Los espectros MS y MS/MS del glifosato, glufosinato y AMPA obtenidos en modo de ionización negativo se muestran en la Figura 3.6. Como se observa, los espectros MS/MS sólo presentaban picos para las pérdidas neutras a m/z $[\text{M}-\text{H}-222]^-$ y $[\text{M}-\text{H}-196]^-$. Estos fragmentos eran debidos a pérdidas poco selectivas procedentes del agente derivatizante, tal como se muestra en las Figuras 3.7-3.9.

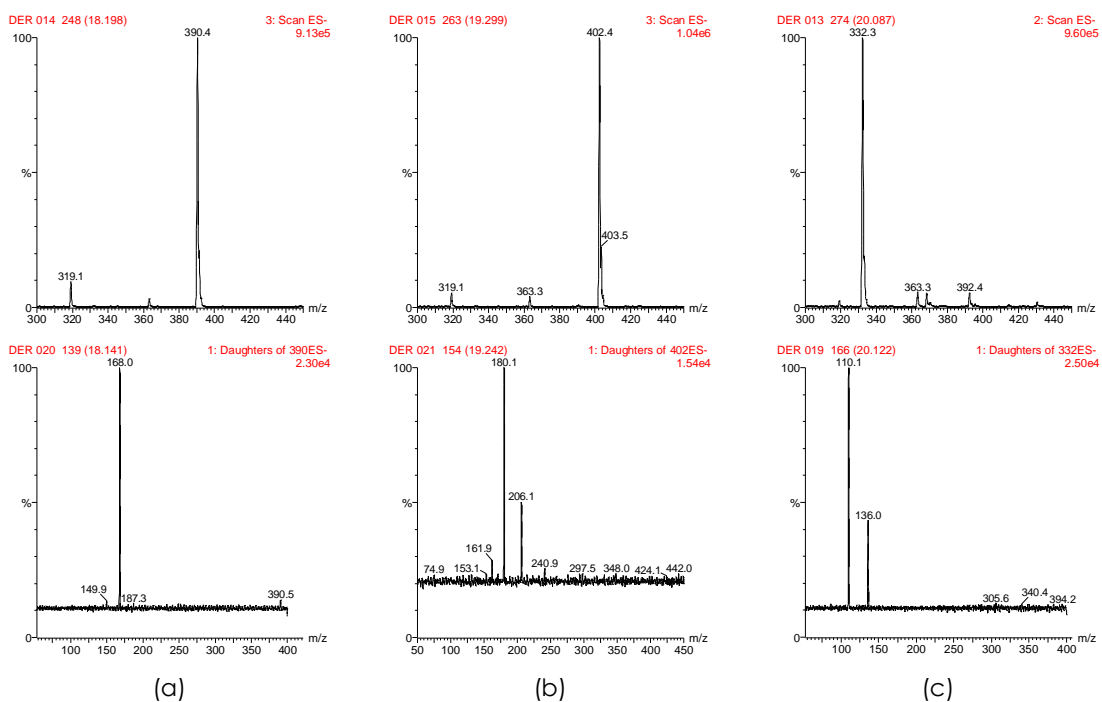


Figura 3.6 Espectros MS (arriba) y MS/MS (abajo) en modo de ionización negativo obtenidos tras cromatografiar un patrón de (a) glifosato, (b) glufosinato y (c) AMPA a una concentración de 10 µg/mL

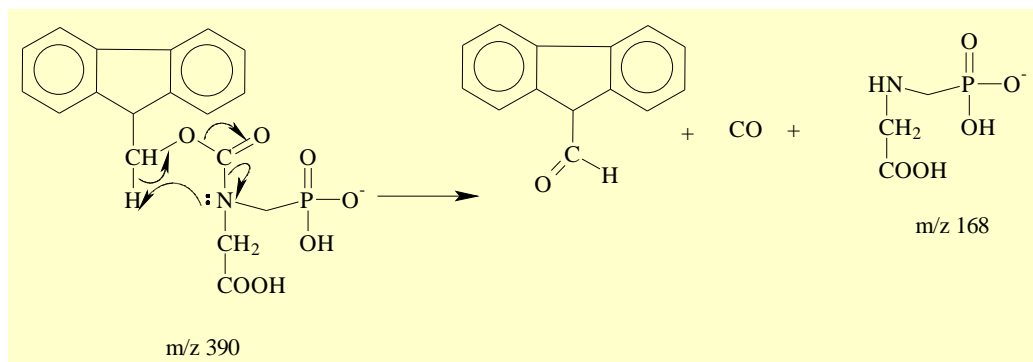


Figura 3.7 Ruta de fragmentación MS/MS en ESI negativo propuesta para el glifosato bajo las condiciones ensayadas

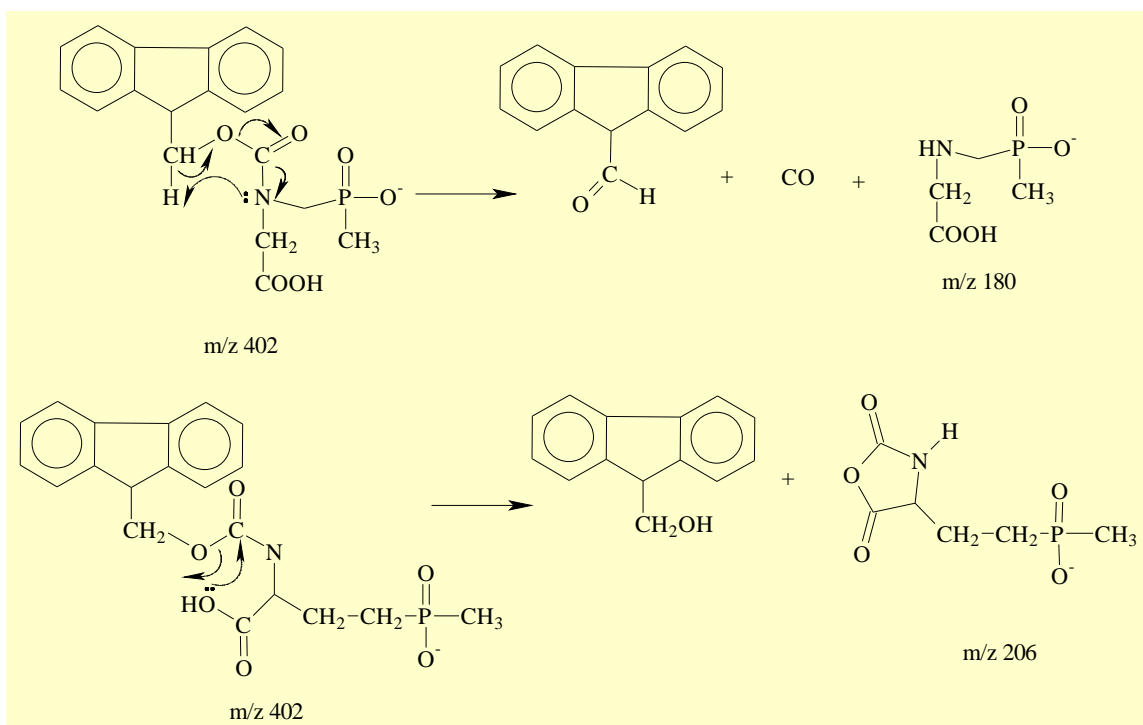


Figura 3.8 Ruta de fragmentación MS/MS en ESI negativo propuesta para el glufosinato bajo las condiciones ensayadas

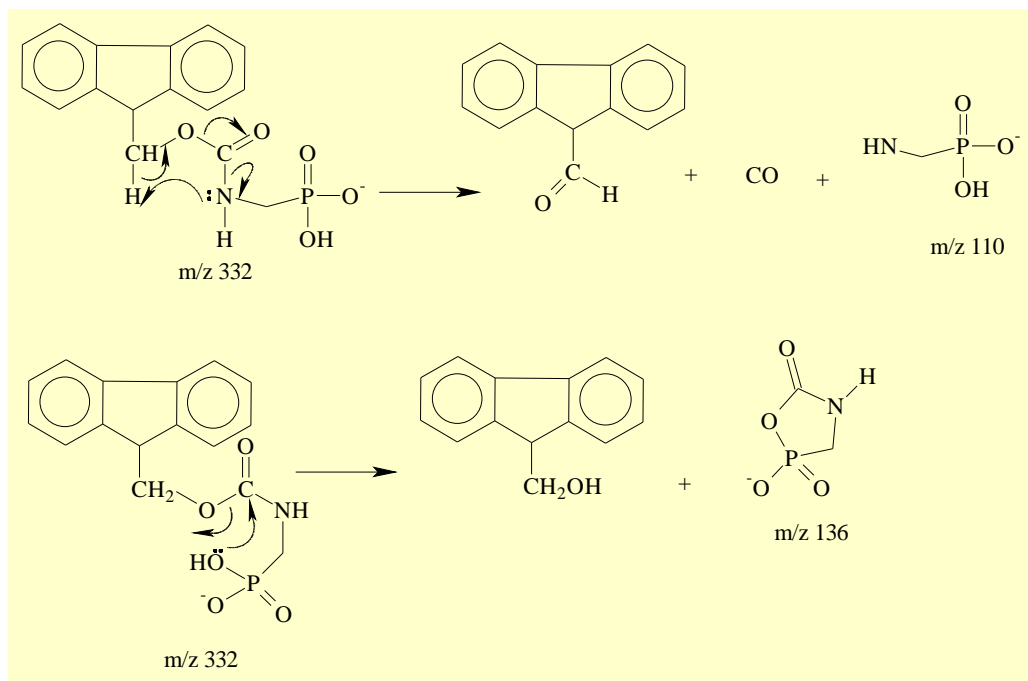


Figura 3.9 Ruta de fragmentación MS/MS en ESI negativo propuesta para el AMPA bajo las condiciones optimizadas

Los espectros MS y MS/MS de estos tres compuestos obtenidos en modo de ionización positivo se muestran en la Figura 3.10. Los fragmentos obtenidos en este modo de ionización eran debidos a pérdidas más selectivas, cuya justificación se muestra en las Figuras 3.11-3.13. Además, la sensibilidad obtenida fue mayor a la observada en modo negativo.

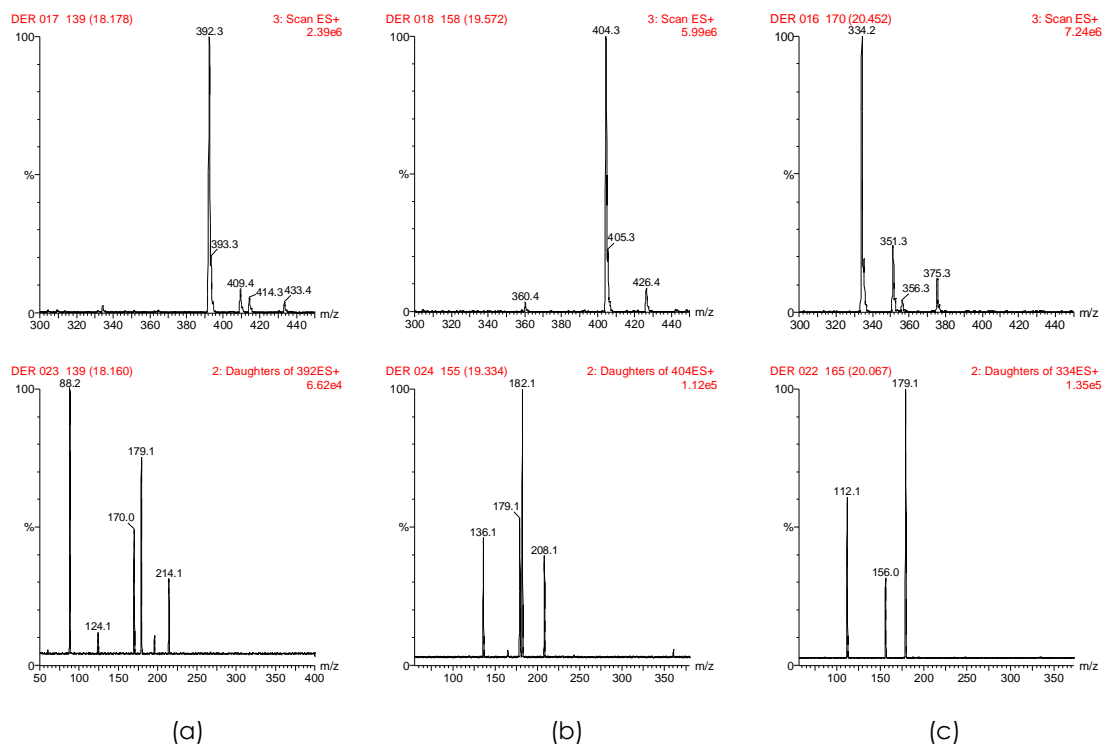


Figura 3.10 Espectros MS (arriba) y MS/MS (abajo) en modo de ionización positivo obtenidos tras cromatografiar un patrón de (a) glifosato, (b) glufosinato y (c) AMPA a una concentración de 10 µg/mL

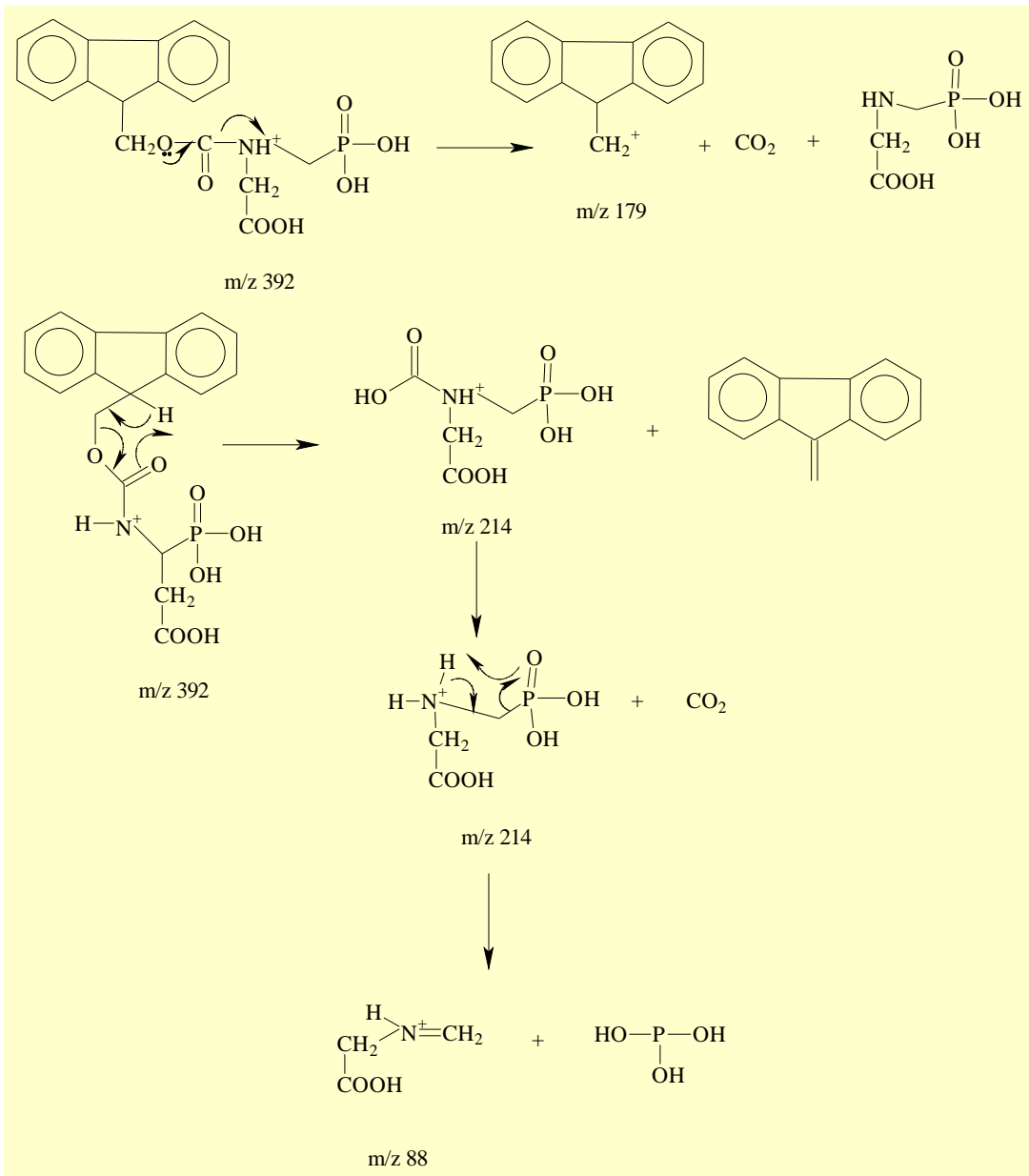


Figura 3.11 Ruta de fragmentación MS/MS en ESI positivo propuesta para el glifosato bajo las condiciones ensayadas

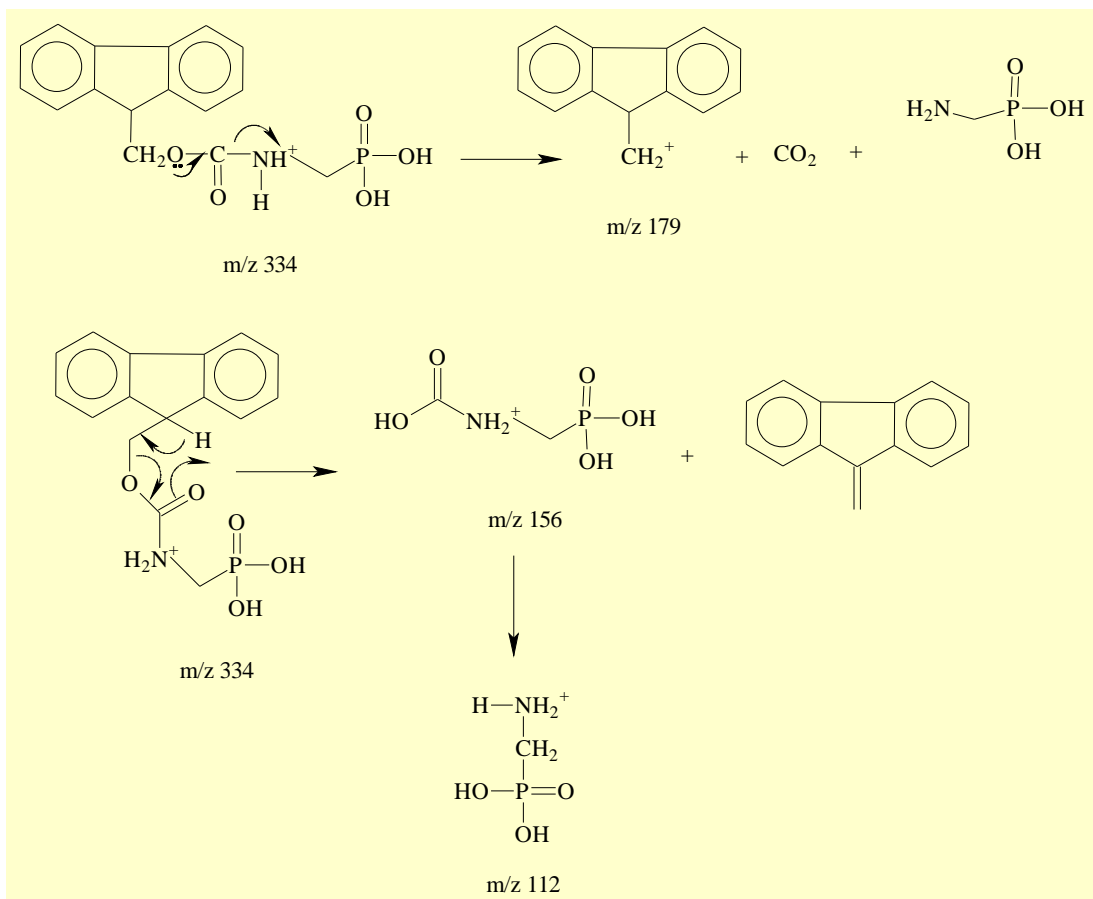


Figura 3.12 Ruta de fragmentación MS/MS en ESI positivo propuesta para el AMPA bajo las condiciones ensayadas

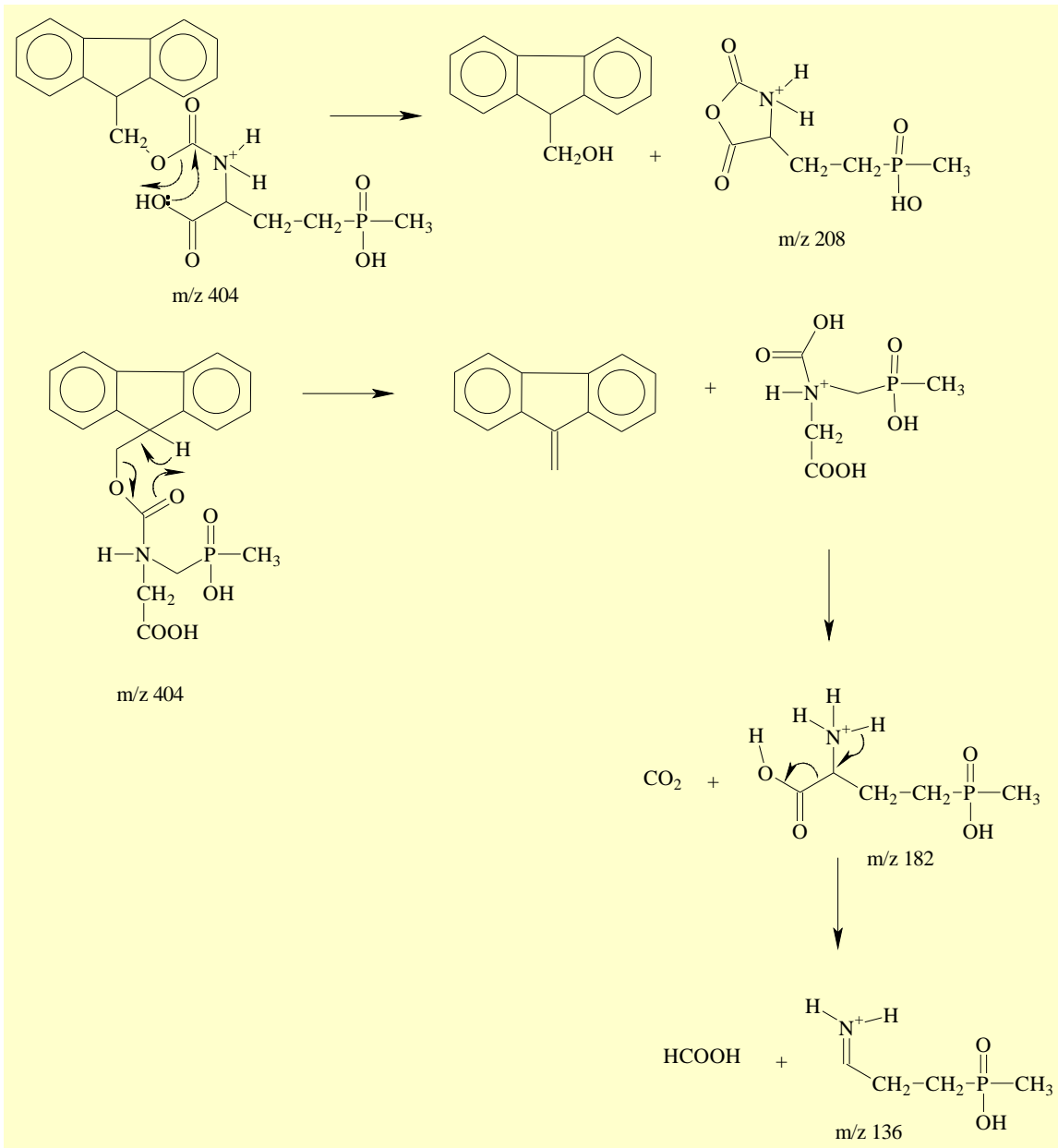


Figura 3.13 Ruta de fragmentación MS/MS en ESI positivo propuesta para el glufosinato bajo las condiciones ensayadas

Con estos resultados se seleccionó el modo de ionización positivo y las transiciones más selectivas posibles para cada compuesto. En el caso del AMPA las transiciones seleccionadas corresponden, sin embargo, a pérdidas poco selectivas, debido a la imposibilidad de elegir otras. Las transiciones usadas, así como el voltaje de cono y las energías de colisión se muestran en la Tabla 1 del *artículo científico 5*.

Como se ha comentado anteriormente, se utilizó una derivatización con FMOC previamente aplicada en nuestro laboratorio, pero ligeramente modificada con el fin de aumentar la sensibilidad. Como es sabido, debido a la baja solubilidad y estabilidad del FMOC en agua, este reactivo es preparado normalmente en acetonitrilo. Por ello, las altas concentraciones de FMOC requeridas para la derivatización hacen que la muestra derivatizada presente un alto contenido orgánico, siendo necesaria una dilución del extracto con el fin de retener estos compuestos en la columna. Esto provoca una importante pérdida de sensibilidad. Con el fin de minimizar este proceso de dilución, se añadió un volumen menor de FMOC pero de mayor concentración. Por una parte, se estudió el efecto de añadir diferentes concentraciones de FMOC (12000 y 48000 ppm). Por otra parte, se estudiaron diferentes tiempos de reacción así como el efecto de la temperatura. El hecho de aumentar demasiado la concentración de FMOC provocaba una disminución de la sensibilidad del glifosato, debido posiblemente al efecto matriz. Los otros dos compuestos, en cambio, no se veían afectados. La mayor sensibilidad para el glifosato se obtenía tras 21 horas de reacción (se comprobó que no seguía aumentando a pesar de aumentar el tiempo de reacción), mientras que para el glufosinato y el AMPA era suficiente una hora. Por lo que se refiere a la temperatura de la reacción, se probaron distintas temperaturas (entre 18-40°C) observando que la sensibilidad máxima en el caso del glifosato se obtenía trabajando a temperatura ambiente, mientras que para glufosinato y AMPA la sensibilidad no cambiaba apreciablemente. Así pues se dejó la reacción a temperatura ambiente durante una noche con una concentración de 12000 mg/L de FMOC.

Por lo que se refiere a la cromatografía, se estudió el efecto del eluyente orgánico de la fase móvil (acetonitrilo y metanol) así como la adición de varios modificadores a la fase acuosa de la fase móvil (HAc/NH₄Ac y ácido fórmico). Finalmente, se seleccionaron agua 5mM NH₄Ac/HAc:acetonitrilo como fases móviles. La columna utilizada fue una Discovery C18 (5 µm, 50x2.0 mm id).

Dado que en el caso de aguas, el objetivo final era obtener un límite de cuantificación de 0.05 µg/L, resultaba necesaria una etapa de preconcentración. Como ya hemos visto en el capítulo anterior, el acoplamiento en línea SPE-LC parece ideal para la determinación de contaminantes orgánicos en aguas, ya que permite alcanzar los niveles de concentración

requeridos sin necesidad de ningún tratamiento adicional de la muestra. Se barajó la posibilidad de utilizar un cartucho polimérico (PRP-1), con un mayor poder de retención, o un C₁₈, ya que el hecho de utilizar acetonitrilo en la fase móvil no nos limitaba el uso de uno u otro cartucho. Se estudió, así mismo, el efecto del pH de la muestra, obteniendo los mejores resultados a un pH de 1.5 (Figura 3.14). Por lo que se refiere al lavado del cartucho, debido a las características polares de estos compuestos, se utilizó agua a pH ácido.

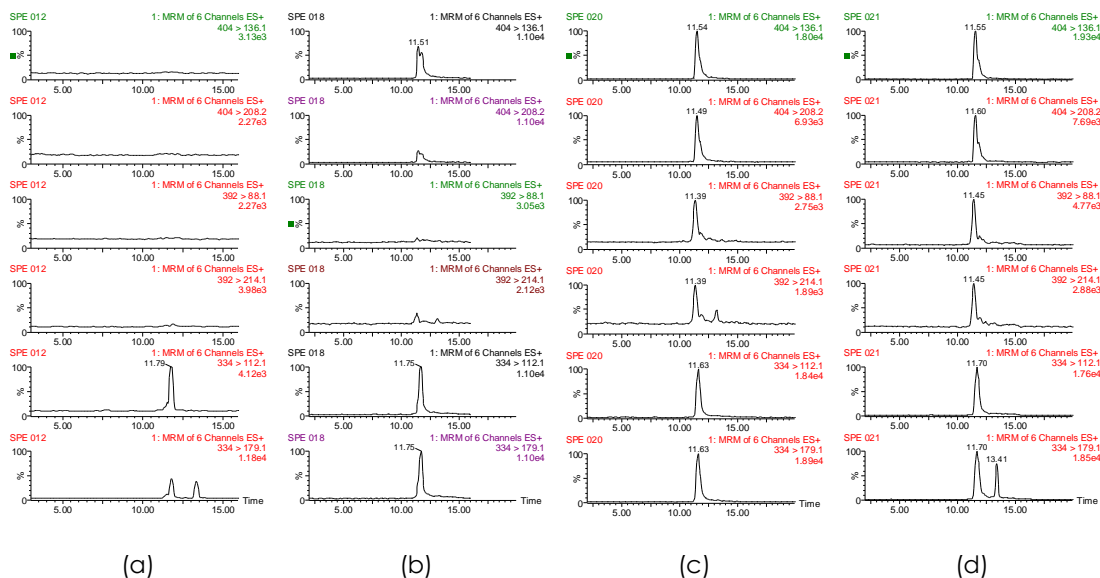


Figura 3.14 Efecto del pH en la sensibilidad y forma de pico del glifosato, glufosinato y AMPA

(a) pH de la muestra = 6, (b) pH de la muestra =3, (c) pH de la muestra =1.5 y (d) pH de la muestra=1.5 y pH de la fase móvil =2.5

• Muestras de agua

Por lo que se refiere a muestras de agua, tras inyectar directamente la muestra derivatizada, se obtuvieron recuperaciones alrededor del 60% para glifosato y AMPA, y alrededor del 75% para glufosinato, mostrando un importante efecto matriz sobre la derivatización y/o sobre la ionización en LC-MS. Como ya se ha comentado en la introducción, existen diversas soluciones para este problema. Dado que en los programas de control se requieren métodos rápidos, el aumento del tratamiento de muestra no fue considerada una buena opción. Por otro lado, el uso de calibrado en matriz no es una aproximación adecuada cuando se analizan muestras medioambientales, debido a la diferente composición de cada una de ellas, que hace muy difícil la selección de una muestra blanco representativa. Así pues,

se probó el uso de patrones internos marcados isotópicamente. En este caso, sólo se encontraba comercialmente disponible el glifosato marcado. El uso del IS mejoró los resultados para el glifosato y para el AMPA. Sin embargo, en el caso del glufosinato se obtuvieron resultados inaceptables (alrededor del 140%), por lo que, finalmente, tanto el glifosato como el AMPA fueron cuantificados usando IS mientras que el glufosinato fue cuantificado sin él. Una situación similar ya ha sido previamente descrita en la bibliografía al usar IS análogos, demostrando la dificultad de elegir un buen IS cuando el mismo analito marcado no se encuentra disponible (Sancho et al. 2002; Kienhuis et Geerdink, 2000; Lagerwerf et al, 2000)

• Muestras de suelo

En las muestras de suelo, tras inyectar directamente 50 µL del extracto previamente derivatizado y acidificado, se obtuvieron recuperaciones de alrededor del 25% con RSD de hasta el 80% para los tres compuestos, mostrando al igual que antes un importante efecto matriz en el instrumento y/o en el proceso de derivatización. De nuevo, el uso del IS mejoró la exactitud y precisión del glifosato. Sin embargo, todavía presentaba inhibición en la ionización, disminuyendo la sensibilidad del método. En el caso del AMPA y el glufosinato, aunque se obtuvieron mejores recuperaciones, las RSD obtenidas seguían siendo inaceptables. Así pues, se procedió a diluir los extractos de suelo con agua HPLC como una manera rápida y simple de minimizar las interferencias de la matriz. Con este fin, cinco suelos de diferentes orígenes fueron fortificados y sus extractos diluidos 10 y 20 veces, antes de ser derivatizados. Tanto la dilución por 10 como por 20 eran adecuadas para una correcta cuantificación incluso sin el uso del IS, tal como se muestra en la Tabla 2 del *artículo científico 5*. Sin embargo, el uso del IS mejoraba las RSDs, especialmente en el caso del glifosato. Al igual que antes, en el caso del glufosinato, la cuantificación con IS no mejoró los resultados. Así pues, glifosato y AMPA fueron cuantificados usando IS mientras que el glufosinato fue cuantificado sin él. Se eligió una dilución por 10 con el fin de obtener los mejores LODs.

Los procedimientos propuestos se esquematizan en la Figura 3.15.

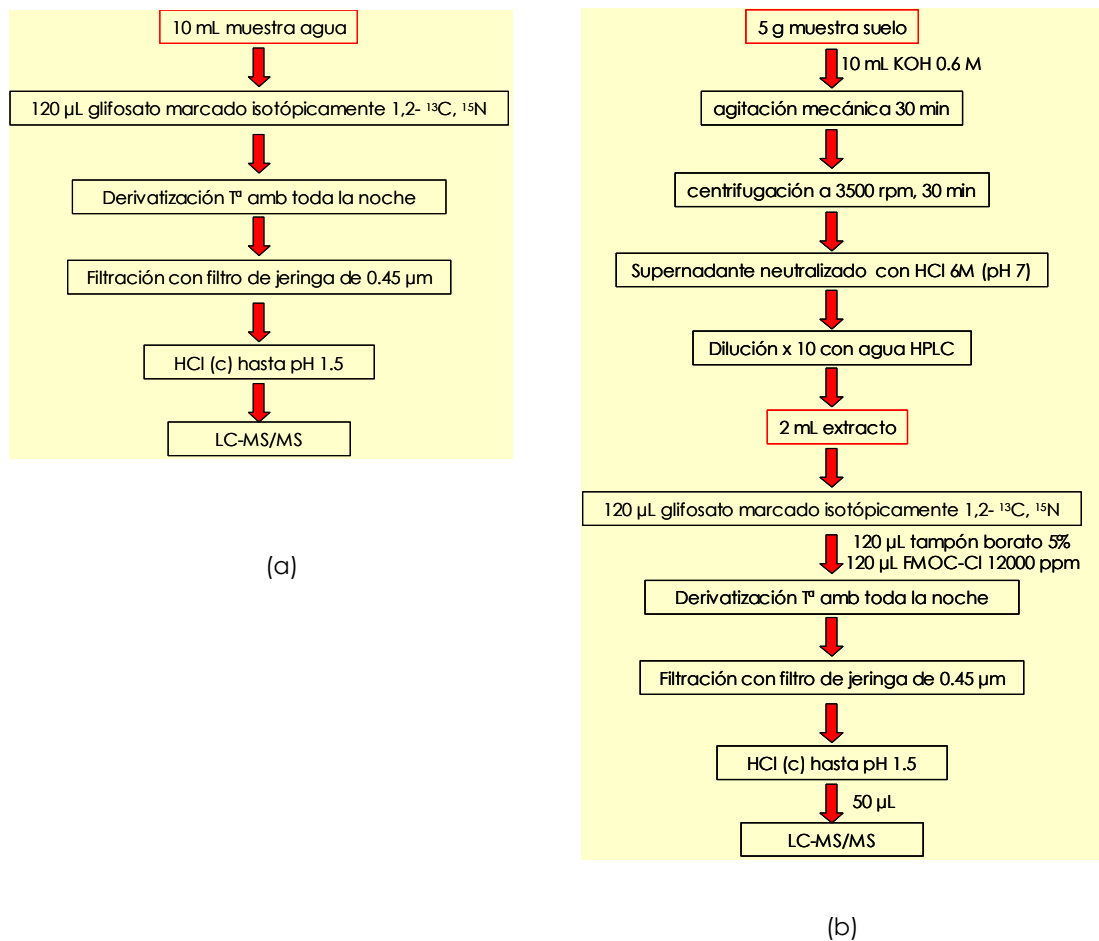


Figura 3.15 Diagrama de flujo de la etapa de derivatización propuesta para la determinación de glifosato, glifosinato y AMPA en (a) muestras de agua y (b) muestras de suelo

Dado que los fosfatos y fosfonatos son compuestos que tienden a adsorberse en vidrio no silinizado, especialmente cuando están en contacto con disolventes libres de metales, realizamos un sencillo estudio con el fin de evaluar este efecto. Para ello se usaron disoluciones patrón y muestras de agua fortificadas. Tras dejar las muestras en los tubos de cristal durante 24 horas antes de ser derivatizadas, no se observó una adsorción significativa, con recuperaciones entre 70-110%.

Los métodos fueron validados tanto en muestras de agua (superficial y subterránea) como de suelo, obteniéndose los resultados mostrados en la Tabla 3 del *artículo científico 5*. Los métodos mostraron una excelente linealidad en el rango estudiado (entre 0.025 µg/L y 5 µg/L en el caso de aguas, y entre 1 µg/L y 500 µg/L en suelos) con $r^2 > 0.994$ para todos los compuestos estudiados. Todos los residuales fueron menores del 30% cumpliendo por tanto los requisitos de aceptación de la linealidad. Por lo que se refiere a la recuperación y RSD, se realizaron cinco réplicas de fortificaciones a dos niveles distintos (0.05 µg/L y 0.5 µg/L en el caso de aguas, y 0.05 y 0.5 mg/kg en suelos). Todos los valores se encontraron dentro del rango 70-110%, con RSD inferiores al 15%.

• **Análisis de muestras reales. Resultados en aguas superficiales/ subterráneas**

El método descrito fue aplicado a unas 50 muestras de agua, tanto superficial como subterránea. El objetivo principal del estudio era tener una idea global del grado de contaminación por el herbicida glifosato en la Comunidad Valenciana. Para ello, se analizaron muestras de 20 puntos distintos de esta Comunidad. Concretamente se realizaron tres muestreos a lo largo del año 2003, en abril, julio y octubre. Estos puntos, que se muestran en la Figura 3.16, fueron seleccionados básicamente en función de las actividades agrícolas. La toma de las muestras se realizó en botellas de plástico de 60 mL, previamente enjuagadas con agua de la misma muestra. En el caso de aguas superficiales se evitó la presencia de materia sólida en suspensión. Las muestras se mantuvieron unas horas en nevera hasta la llegada al laboratorio y entonces se congelaron a -18°C hasta el momento de ser analizadas. Antes de su análisis, se procedió a su descongelación durante una noche en frigorífico a aproximadamente 4°C . Una vez descongeladas, se dejaron atemperar y se procedió al análisis.

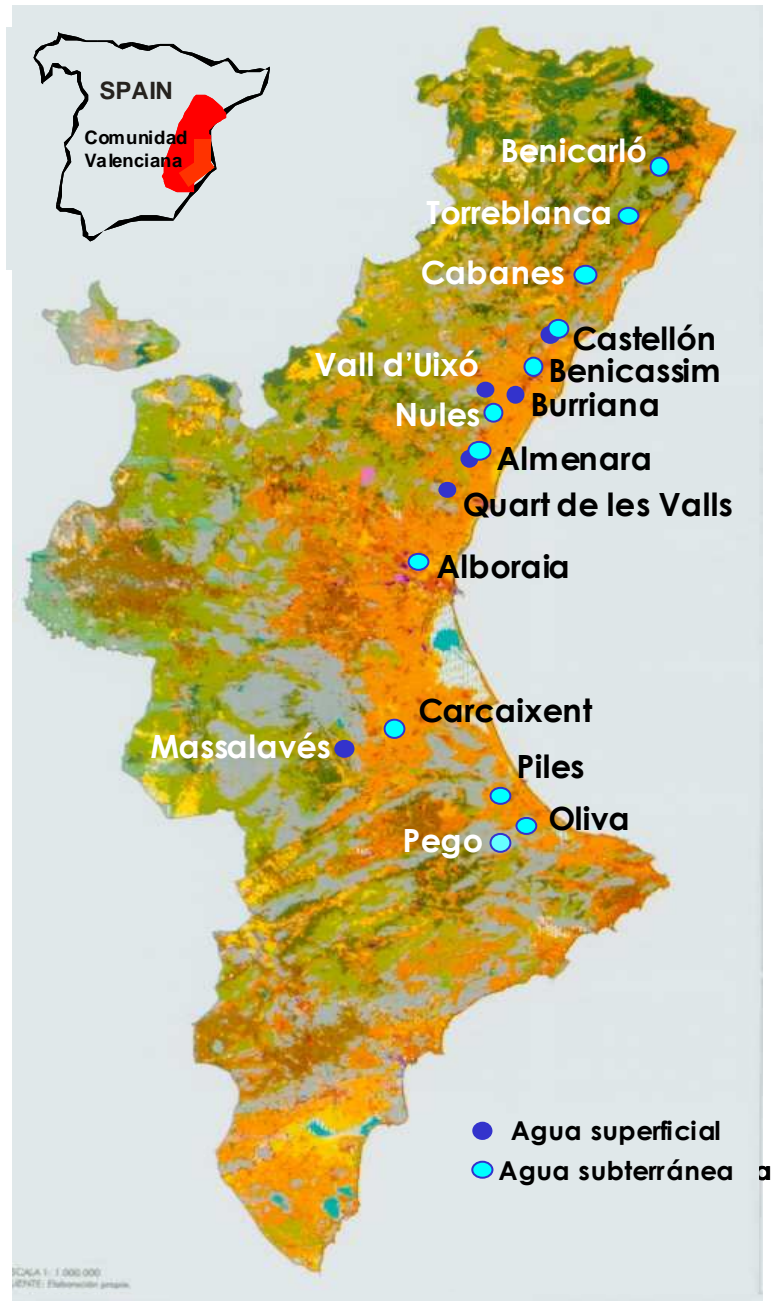


Figura 3.16 Puntos de muestreo. Determinación de glifosato en aguas de la Comunidad Valenciana (2003)

El glifosato fue detectado en el 22% de las muestras de agua analizadas, con concentraciones entre 55 y 484 ng/L, mientras que el AMPA fue encontrado en el 38% de las muestras, con niveles de concentración entre 51 y 175 ng/L. El glufosinato, por su parte, no fue detectado en ninguna de las muestras analizadas. Cabe destacar la altas concentraciones de AMPA encontradas en el Clot de Borriana, la Marjal de Pegó o el Molí La Font de Castellón, todas ellas por encima de 100 ng/L. Por lo que se refiere al glifosato, las concentraciones encontradas fueron en general superiores a las de AMPA, destacando especialmente Massalavés, la Marjal de Pegó o la Ribera de Cabanes. En este último punto, la concentraciones de glifosato encontradas fueron superiores a 100 ng/L en los tres muestreos, indicando un uso frecuente y continuado de este herbicida. Los resultados obtenidos se muestran en la Tabla 3.4

Tabla 3.4 Resultados obtenidos en el análisis de aguas de la Comunidad Valenciana

Lugar	Tipo de agua	Conc AMPA (ng/L)			Conc glifosato (ng/L)		
		abr-03	jul-03	oct-03	abr-03	jul-03	oct-03
Clot(Borriana)	Superficial	175	65	36	58	n.d.	102
Estany Almenara	Superficial	n.d.	n.d.	20	n.d.	17	196
Marjal de Pegó	Superficial	135	32	19	393	64	42
Massalavés	Superficial	n.d.	29	n.d.	12	484	14
Moli La Font(Castellón)	Superficial	n.d.	n.d.	106	13	n.d.	109
Quart de les Valls	Superficial	82	n.d.	n.d.	13	n.d.	12
Vall d'Uixó	Superficial	40	84	68	41	77	28
Alboraia	Subterránea	n.d.	n.d.	n.d.	n.d.	n.d.	15
Benicarló	Subterránea	18	14	17	28	105	n.d.
Benicassim	Subterránea	n.d.	3,7	10	n.d.	10	n.d.
Carcaixent	Subterránea	48	29	14	n.d.	114	11
La Marjal(Castellón)	Subterránea	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Nules	Subterránea	n.d.	n.d.	24	n.d.	n.d.	62
Oliva	Subterránea	11	n.d.	n.d.	105	55	n.d.
Piles	Subterránea	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pozo Almenara	Subterránea	12	n.d.	n.d.	10,91	n.d.	n.d.
Pozo Borriana	Subterránea	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ribera de Cabanes	Subterránea	12	46	87	156	177	304
Serratella(Borriana)	Subterránea	nd	n.d.	n.d.	n.d.	n.d.	n.d.
Torreblanca-Alcalá	Subterránea	10	17	n.d.	39	44	38

• Análisis de muestras reales. Resultados en suelos

Por otra parte se analizaron seis muestras de suelo procedentes de un jardín urbano de Mallorca, que se sospechaba podían presentar contaminación por glifosato. En este caso, el glufosinato tampoco fue detectado en ninguna de las muestras analizadas. El glifosato fue detectado en 4 de las 6 muestras, con concentraciones que oscilaban entre 0.17 mg/kg y 0.73 mg/kg, mientras que el AMPA fue detectado en 3 muestras, con niveles de concentración entre 0.06 y 5.61 mg/kg. Los resultados obtenidos se muestran en la Tabla 3.5. Como puede verse, la muestra 6 es la que presentaba una mayor contaminación tanto por glifosato como por AMPA.

Muestra	Conc AMPA (mg/kg)	Conc glifosato (mg/kg)
Muestra G02_2003_1	<i>n.d.</i>	<i>n.d.</i>
Muestra G02_2003_2	0.06	0.17
Muestra G02_2003_3	<i>n.d.</i>	<i>n.d.</i>
Muestra G02_2003_4	0.38	0.28
Muestra G02_2003_5	<i>n.d.</i>	0.24
Muestra G02_2003_6	5.61	0.73

▪ Análisis de muestras reales. Resultados en vegetales

Aunque el método no fue validado en vegetales, debido a una petición de análisis recibida en nuestro laboratorio, éste se aplicó también a la determinación de glifosato en 5 plantas decorativas que se sospechaba podían haber sido contaminadas por este herbicida. En este caso, tras un proceso previo de extracción utilizando agua HPLC, los extractos fueron derivatizados del mismo modo que los extractos de suelo. Los resultados se muestran en la Tabla 3.6

Muestra	Conc glifosato (mg/kg)
1	0.36
2	0.37
3	2.29
4	0.14
5	< 0.05

El glifosato fue detectado en 4 de las 5 muestras analizadas, con concentraciones entre 0.14 y 2.29 mg/kg. Como se observa en la Tabla 3.6, la muestra 3 presentaba una elevada concentración de glifosato (2.29 mg/kg). En la Figura 3.17 se muestran los cromatogramas correspondientes a dos muestras .

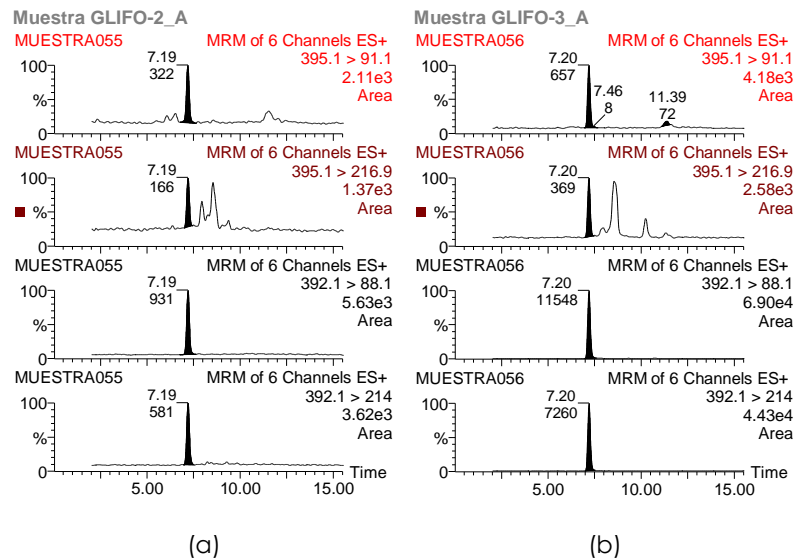


Figura 3.17 Cromatogramas LC-MS/MS obtenidos para la determinación de glifosato en muestras reales de plantas decorativas: (a) patron de 5 µg/L, (b) muestra de planta conteniendo 2.29 mg/kg

En el análisis de muestras reales, se fortificaron diversas muestras blanco a los niveles de concentración del LOQ y 10 x LOQ (es decir, 50 y 500 ng/L para aguas, y 0.05 y 0.5 mg/kg para suelos). Estas muestras, usadas como controles de calidad (QC), fueron distribuidas a lo largo de la secuencia de muestras, cada 3 o 4 inyecciones. La cuantificación se consideró correcta si las recuperaciones de los QCs se encontraban en el rango de 70-110%. Por otra parte, los valores encontrados en las muestras reales se confirmaron mediante el uso de las dos transiciones seleccionadas para cada compuesto. Así, la cuantificación fue llevada a cabo independientemente con cada transición, aceptándose una desviación del $\pm 20\%$ en las concentraciones obtenidas con ambas transiciones.

Artículo científico 5

J. Chromatogr. A (2005) 1081: 145-155

RESIDUE DETERMINATION OF GLYPHOSATE, GLUFOSINATE AND AMINOMETHYLPHOSPHONIC ACID IN WATER AND SOIL SAMPLES BY LIQUID CHROMATOGRAPHY COUPLED TO ELECTROSPRAY TANDEM MASS SPECTROMETRY

María Ibáñez, Óscar J. Pozo, Juan V. Sancho, Francisco J. López, Félix Hernández
Research Institute for Pesticides and Water, University Jaume I, E-12071, Castellón, Spain

ABSTRACT

This paper describes a method for the sensitive and selective determination of glyphosate, glufosinate and aminomethylphosphonic acid (AMPA) residues in water and soil samples. The method involves a derivatization step with 9-fluorenmethylchloroformate (FMOC) in borate buffer and detection based on liquid chromatography coupled to electrospray tandem mass spectrometry (LC-ESI-MS/MS). In the case of water samples a volume of 10 mL with was derivatized and then 4.3 mL of the derivatized mixture was directly injected in an on-line solid phase extraction (SPE)-LC-MS/MS system using an OASIS HLB cartridge column and a Discovery chromatographic column. Soil samples were firstly extracted with potassium hydroxide. After that, the aqueous extract was ten-fold diluted with water and 2 mL were derivatized. Then, 50 µL of the derivatized ten-fold diluted extract were injected into the LC-MS/MS system without pre-concentration into the SPE cartridge. The method has been validated in both ground and surface water by recovery studies with samples spiked at 50 and 500 ng/L, and also in soil samples, spiked at 0.05 and 0.5 mg/kg. In water samples, the mean recovery values ranged from 89% to 106% for glyphosate (RSD<9%), from 97% to 116% for AMPA (RSD<10%), and from 72% to 88% in the case of glufosinate (RSD<12%). Regarding soil samples, the mean recovery values ranged from 90% to 92% for glyphosate (RSD<7%), from 88% to 89% for AMPA (RSD<5%) and from 83% to 86% for glufosinate (RSD<6%). Limits of quantification for all the three compounds were 50 ng/L and 0.05 mg/kg in water and soil, respectively, with limits of detection as low as 5 ng/L, in water, and 5 µg/kg, in soil. The use of labelled glyphosate as internal standard allowed improving the recovery and precision for glyphosate and AMPA, while it was not efficient for glufosinate, that was quantified by external standards calibration. The method developed has been applied to the determination of these compounds in real water and soil samples from different areas. All the detections were confirmed by acquiring two transitions for each compound.

INTRODUCTION

Glyphosate [N-(phosphonomethyl)glycine] and glufosinate [ammonium DL-homosalanin-4-(methyl) phosphinate] are broad spectrum, nonselective, post-emergence herbicides extensively used in various applications for weed control in aquatic systems and vegetation control in non-crop areas. Aminomethylphosphonic acid (AMPA) is the major degradation product of glyphosate found in plants, water and soil [1]. Chemical structures of these phosphorus-containing herbicides are given in Figure 1.

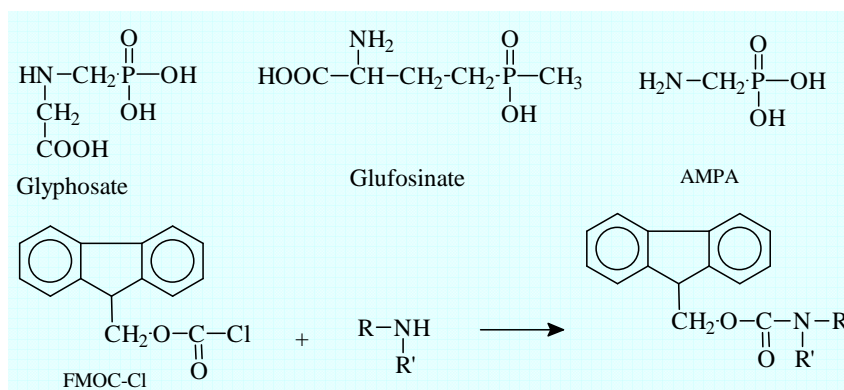


Figure 1. Chemical structures of glyphosate, AMPA and glufosinate, and derivatization reaction with FMOC. R: H or alkyl group.

Due to the extensive worldwide use of these compounds and the restrictive regulations for water in the European Union, very sensitive methods for the determination of pesticide residues are required. However, the determination of these two herbicides at the sub $\mu\text{g/L}$ level is difficult due to their ionic character, low volatility, low mass and lack of chemical groups that could facilitate their detection. Even more difficult can result the residue determination in soil at low concentration levels (e.g. below 0.1 mg/kg), due to the complexity of this matrix sample. Most methods developed until now require derivatization procedures to enable analysis by gas chromatography (GC) or high-performance liquid chromatography (HPLC). GC/MS methods involved derivatization with different reagents [2-8] to confer volatility to the analytes. Normally, there is quite a lot of sample manipulation, and the methods are time-consuming and tedious.

Physicochemical characteristics of these compounds fit better with LC analysis, although the lack of adequate chemical groups (e.g. chromophores, UV absorption, fluorogenics) hamper their measurement by conventional detectors. For these reasons, both pre-column and post-

column derivatization procedures have been employed. Pre-column procedures are based mainly on derivatization with 9-fluorenylmethyl chloroformate (FMOC) [9-15] to form fluorescent derivatives (improve detection) and/or to reduce the polar character of the analytes facilitating the chromatographic retention. In post-column procedures, the most common reaction is with o-phthalaldehyde (OPA) and mercaptoethanol [16] or with OPA and N,N-dimethyl-2-mercaptoethylamine [17]. Normally, HPLC has been used in combination with fluorescence detection after derivatization [11-17], although in a few cases glyphosate has been determined directly by ion chromatography (IC) with UV detection [18] or suppressed conductivity detection [19], but with limited sensitivity. The potential of capillary electrophoresis combined with mass spectrometry [20] and with indirect fluorescence detection [21] has also been explored, although the lack of sensitivity and/or selectivity of these techniques together with the difficulty for preconcentrating the analytes, limited their application in the field of residues.

In our research group, we have developed efficient and selective methods based on the use of coupled-column liquid chromatography (LC-LC), which was proved to be an excellent way of minimizing sample treatment and improving sensitivity in a variety of sample matrices, as water, soil, fruit and vegetables [11,13-15, 22]. However, the use of conventional fluorescent detection limited the sensitivity required in pesticide residue analysis, and also hampered the unequivocal confirmation of the residues detected, which nowadays is widely accepted that has to be reached by MS techniques. Searching a method that could satisfy the requirements of sensitivity and selectivity, and unequivocal confirmation of glyphosate in water, the use of MS spectrometric techniques in combination with LC has been investigated by several groups. Thus, IC has been applied, due the ionic character of this analyte, coupled to MS with electrospray interface [23], while RPLC has been used in combination with ICP-MS with P detection [24]. However, the sensitivity reached with these techniques was not sufficient. Lee et al [9] obtained better results with the combination LC-MS. In this case, the molecular ions of the derivatized glyphosate, AMPA and glufosinate, as well as a fragment ion of each compound, were monitored in negative ionisation mode obtaining detection limits around 0.1 µg/L. The use of isotope-labelled glyphosate as internal standard minimised derivatization variations and matrix effects. However, although MS based methods could be considered as highly selective methods, the occurrence of false positives might be still possible mainly in the analysis of relatively dirty samples, as some interferences can share the same MS properties as the analyte. This may also occur in water sample analysis as it has been reported in some papers, producing constructive discussions on this subject [25].

The improved sensitivity and selectivity of tandem MS make this technique ideal for the trace level determination of polar and/or ionic pesticides in water by LC-MS/MS methods, as it has been proved in our laboratory [26-27]. This technique was also applied several years ago to the determination of glyphosate and AMPA in water [10], although considerable variation was observed caused by irreproducibility in derivatization and fragmentation. 4-mL volume was passed through the SPE cartridge, claiming detection limits for glyphosate and AMPA around 0.03 µg/L.

When dealing with more complex matrices, such as soil samples, an important loss in the sensitivity can occur as a consequence of the ionisation suppression from the co-extracted components of the matrix, hampering correct quantification. This matrix-effect depends on the analyte-sample combination. Different approaches have been used either to minimize or to correct the matrix effect, such as increasing the sample pretreatment, performing matrix-matched calibration, using an isotope labelled standard or simply diluting the sample [28]. Thus, the labeled glyphosate has been used as internal standard for the LC-MS determination of this herbicide [9].

Confirmation of the identity of residues in unknown samples is of utmost importance in order to avoid reporting false positives. Recently, the European Union has adopted the concept of identification points (IPs) as quality criterium for the confirmation of contaminant residues [29]. For compounds with an established MRL, a minimum of three IPs is required for satisfactory confirmation of the compound identity. When LC-MS/MS technique is used, the monitoring of two MS/MS transitions, e.g. using one precursor ion and two product ions, allows to earn four IPs, fulfilling the requirements of these criterium [25].

The aim of this paper is to develop a rapid and robust method for the determination of low concentrations of glyphosate, its principal degradation product, AMPA, and glufosinate in water and soil by (SPE-)LC-ESI-MS/MS, that fulfil the requirements of excellent sensitivity and unequivocal confirmation of the residues detected according to the European Union guidelines. Following the most widely accepted criteria, four IPs will be achieved, thus avoiding the possibility of reporting false positives.

EXPERIMENTAL

Chemicals

Glyphosate (98%), glufosinate (99%) and AMPA (99%) reference standards were purchased from Dr Ehrenstorfer (Augsburg, Germany), Riedel-de-Häen (Seelze, Germany) and Sigma (St Louis, MO, USA), respectively. Isotope-labeled glyphosate (1,2-¹³C, ¹⁵N), used as surrogate internal standard (IS), was purchased from Dr Ehrenstorfer. Analytical reagent-grade disodium tetraborate decahydrate was obtained from Scharlab (Barcelona, Spain) and 9-fluorenmethylchloroformate (FMOC-Cl) was purchased from Sigma. Reagent-grade hydrochloric acid, formic acid, potassium hydroxide (KOH), acetic acid (HAc) and ammonium acetate (NH₄Ac) as well as LC-grade acetonitrile were purchased from Scharlab. LC-grade water was obtained by purifying demineralised water in a Nanopure II system (Barnstead Newton, MA, USA).

Standard stock solutions were prepared dissolving approximately 50 mg powder, accurately weighted, in 100 mL of water obtaining a final concentration of approximately 500 mg/L. A 50-mg/L composite standard was prepared in water by mixing and diluting the individual standard stock solutions. Standard working solutions for the LC-MS/MS analysis and for fortification of samples were prepared by dilution of the 50-mg/L composite standard with water. All standard solutions were stored in nonsilanized glass.

The isotope-labeled glyphosate was purchased as 1.1 mL of 100- μ g/mL stock solution in water. An 11- μ g/mL standard solution was prepared by dissolving 1.1 mL of the stock solution in 10 mL of water. Standard working solutions were prepared by diluting the intermediate standard solution with water.

Solutions of 5% borate buffer (pH approximately 9) in HPLC-grade water and solutions containing 12000 mg/L of FMOC-Cl in acetonitrile were used for the derivatization step prior to the analysis.

Instrumentation

For the analysis of water samples, the mass spectrometer was interfaced to a LC system based on a 233XL autosampler with a loop of 4325 μL (Gilson, Villiers-le-Bel, France) and 2 pumps: an Agilent 1100 (Agilent, Waldbron, Germany) binary pump used to condition and wash the cartridge (P-1) and a Waters Alliance 2695 (Waters, Milford, MA, USA) quaternary pump used for the chromatographic separation (P-2), as can be seen elsewhere [24]. The SPE preconcentration was performed using an Oasis HLB cartridge, 20 x 2.1 mm i.d. (Waters), as C-1. For the LC separation, a Discovery column C₁₈, 5 μm 50 x 2.0 mm i.d. (Supelco, Bellefonte, PA, USA), was used as C-2. Mobile phase consisted of water pH 2.5 (adjusted with formic acid) in P-1, and mixtures of 5mM acetic acid/ammonium acetate (pH 4.8) water and acetonitrile in P-2.

For the analysis of soil samples, the mass spectrometer was directly interfaced to the Waters Alliance 2695 (Waters) quaternary pump. The mobile phases and the column used were the same as in the case of water samples.

A Quattro LC (quadrupole-hexapole-quadrupole) mass spectrometer (Micromass, Manchester, UK) with an orthogonal Z-spray-electrospray interface was used. Drying gas as well as nebulising gas was nitrogen, generated from pressurized air in a NG-7 nitrogen generator (Aquila, Etten-Leur, NL). The nebuliser gas flow was set to approximately 80 L/h. and the desolvation gas flow to 800-900 L/h. Datastation operating software was MassLynx v4.0.

For operation in MS/MS mode, collision gas was Argon 99.995% (Carburros Metalicos, Valencia, Spain) with a pressure of approximately 1.10^{-3} mbar in the collision cell. Capillary voltage of 3.5 kV was used in positive ionization mode. The interface temperature was set to 350°C and the source temperature to 120°C. Dwell times of 0.17 s/scan were chosen.

SPE procedure

The conditioning of the Oasis cartridge was performed with LC-grade water at pH 2.5 at a flow-rate of 1 mL/min for 7 min. An aliquot of 4.3 mL of water sample was preconcentrated (1 mL/min) into the cartridge and washed with acidified LC-grade water during 4 minutes. After washing, the sample was transferred in backflush mode to the C-2 column and a gradient in P-2 started.

LC procedure

To perform the chromatographic separation, the gradient used in P-2 was water 5mM HAc/NH₄Ac (pH 4.8) - acetonitrile, where the percentage of organic modifier was changed as follows: 0 min., 10%; 5 min., 10%; 5.1 min., 90%; 9 min., 90%; 9.1 min., 10%; 14 min, 10%. The chromatographic separations were completed within 20 minutes.

Sample procedure

The derivatization procedure was based on Sancho *et al.* [14,15] (see Figure 1), with slight modifications.

Water samples: Ground and surface water samples were collected in plastic bottles from different sites of the Valencian Mediterranean region and stored in a freezer at -18°C until analysis. 10 mL of water sample was introduced into a glass tube together with 100 µL of isotope-labeled glyphosate standard (110 µg/L). Samples were derivatised by adding 0.6 mL of 5% borate buffer (pH 9) followed by 0.6 mL of FMOC-Cl reagent (12000 mg/L), and allowing the reaction to take place overnight at room temperature. After that, samples were filtered through a 0.45 µm syringe filter and acidified with hydrochloric acid until pH 1.5. Finally, 4.3 mL of the acidified derivatized samples were directly injected into the SPE-LC-ESI-MS/MS system.

Fortification of surface or ground waters for recovery experiments was performed by adding 1 mL of 5 ng/mL or 50 ng/mL mixture solutions to 100 mL of blank water sample in order to yield fortification levels of 50 ng/L or 500 ng/L, respectively.

Soil samples: Soil was sampled from a public garden, suspected to have been contaminated by glyphosate. Air-dried soil samples were homogenized and 5.0 g subsamples were transferred to centrifuge tubes (50 mL). Samples were extracted by shaking with 0.6 M KOH (10 mL) on a mechanical shaker for 30 min, and then centrifuged at 3500 rpm for 30 min. The alkaline sample extracted was separated and neutralized by adding drops of HCl 6M and 0.6M until pH 7, approximately. After that, the neutralized supernatant was 10-fold diluted with HPLC-grade water. The derivatization step was performed as follows: 2-mL of the 10-fold diluted supernatant was pipetted into a glass tube together with 120 µL of the labelled internal standard (1.10 mg/L), 120 µL of 5% borate buffer (pH 9) and 120 µL of FMOC-Cl reagent (12000 mg/L). The tube was swirled and left overnight at room temperature. After that, samples were filtered through a 0.45 µm syringe filter and acidified with hydrochloric acid until pH 1.5. Finally, 50 µL of the acidified derivatized extract was directly injected into the LC-ESI-MS/MS system.

Fortification of soil samples for recovery experiments was performed by adding 1 mL of 250 ng/mL or 2500 ng/mL mixture solutions to 5.0 g of blank soil sample in order to yield fortification levels of 0.05 mg/kg or 0.5 mg/kg, respectively. Samples were equilibrated for 1 h prior to extraction.

AMPA and glyphosate were quantified using isotope labelled glyphosate as internal standard, in both water and soil samples. In the case of glufosinate, quantification was performed with external calibration.

Validation study

Linearity of the method was evaluated analysing eight standard solutions by duplicate, in the range 25-5000 ng/L for water samples, and in the range 1-500 µg/L for soil extracts.

Precision (repeatability, expressed as relative standard deviation, in %) and recoveries were determined within day by analysing fortified blank samples in quintuplicate. This experiment was performed at two spiking levels: 50 and 500 ng/L in water, and 0.05 and 0.5 mg/kg in soil.

The limits of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, were obtained when the signal was three times the average of background noise in the chromatogram at the lowest analyte concentration assayed. The limits of quantification (LOQ) were established as the lowest concentration assayed and validated, which gave satisfactory recovery (70-120%) and precision (<15% RSD).

The specificity of the method was evaluated by analysing a blank procedure, a processed blank sample, and a blank sample spiked at the lowest fortification level assayed (LOQ), i.e. 50 ng/L in water and 0.05 mg/kg in soil. Under these conditions, the response obtained for both the blank procedure and the blank samples should not exceed 30% of the response corresponding to the LOQ

Data evaluation

To ensure the quality of the analysis when processing real-world samples, blank samples fortified at the LOQ and 10 x LOQ concentration levels (50 and 500 ng/L for waters, and 0.05 and 0.5 mg/kg for soils) were used as quality controls (QC) distributed along the batch of samples every three-four injections. The quantification of the sample batch was considered satisfactory if the QC recoveries were in the range of 70-120%. The values found in real samples were confirmed

by means of the two transitions selected for each compound. In this way, quantification was carried out independently with each transition (see MS Optimisation), accepting a deviation of $\pm 20\%$ in the concentrations obtained with both transitions.

RESULTS AND DISCUSSION

MS optimisation

Full-scan MS spectra and product-ion MS/MS spectra of the FMOOC derivatives of glyphosate, glufosinate and AMPA were recorded in both positive and negative ionisation modes. Spectra were obtained from the chromatographic peak of 10 mg/L standard solution of each compound, previously derivatized.

Although these compounds have been traditionally recorded in negative ion mode [9, 10], in our work the sensitivity in positive ion mode was found to be approximately two times higher. Moreover, the product ions observed in negative ion mode were due to neutral unspecific losses of FMOOC, or FMOOC plus water. Thus, any isobaric compound that could have been derivatized with FMOOC and also presented a water loss, would show the same product ions in its MS/MS spectra, being therefore not very selective. For all these reasons, positive ion mode was selected.

The positive-ion electrospray full scan spectrum of AMPA-FMOOC at a cone of 30 V showed a base peak at m/z 334 corresponding to the protonated derivatized molecule $[M+H]^+$. The MS/MS spectra showed three abundant fragments at m/z 179, 156 and 112 (Figure 2a). As can be seen in Figure 3a, fragments at m/z 179, m/z 156 ($M-178$) and m/z 112 ($M-222$) would appear in any isobaric amine that could have been derivatized with FMOOC. As there were not significant differences in the selectivity of these transitions, the criterium applied for their selection was the sensitivity, choosing the two most sensitive ones.

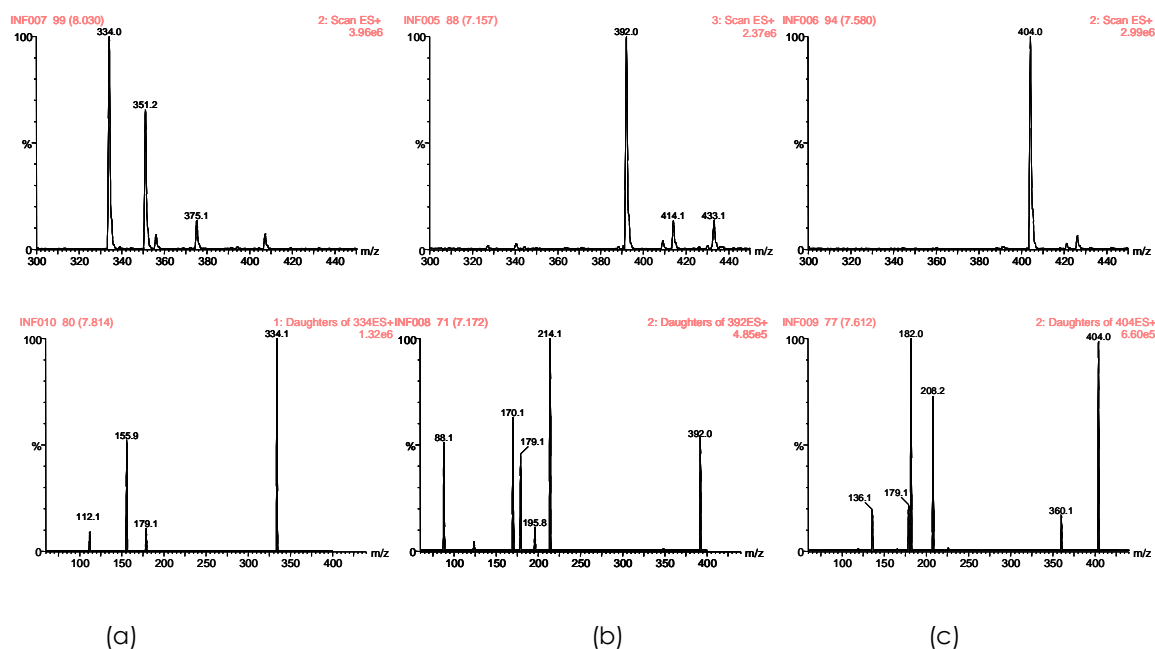


Figure 2. The positive ion electrospray full scan mass spectrum (top) and product ion spectra (bottom) of (a) AMPA-FMOC, (b) glyphosate-FMOC and (c) glufosinate-FMOC, obtained from the chromatographic peak of 10 mg/L standard solution of each compound, previously derivatized.

The positive-ion electrospray full scan spectrum of glyphosate-FMOC at a cone of 30 V showed a peak at m/z 392 corresponding to the protonated derivatized molecule $[M+H]^+$. The MS/MS spectra showed abundant fragments at m/z 214, 179, 170 and 88 (Figure 2b). The fragments at m/z 179 and the fragments at m/z 214 ($M-178$) and m/z 170 ($M-222$) would appear in any isobaric amine that could have been derivatized with FMOC (Figure 3a). Thus, the selected reaction monitoring (SRM) transitions chosen were $392 \rightarrow 88$ for quantification as the most selective (see Figure 3b) and $392 \rightarrow 214$ for confirmation as it was the most sensitive among the less selective.

In the case of glufosinate, the positive-ion electrospray full scan spectrum showed a peak at m/z 404 corresponding to the protonated molecule of glufosinate-FMOC. The MS/MS spectrum showed four abundant fragments at m/z 208, 182 ($M-222$), 179 and m/z 136 (Figure 2c). We choose the most selective transitions: $404 \rightarrow 208$ and $404 \rightarrow 136$ (see Figure 3b) despite their lower sensitivity.

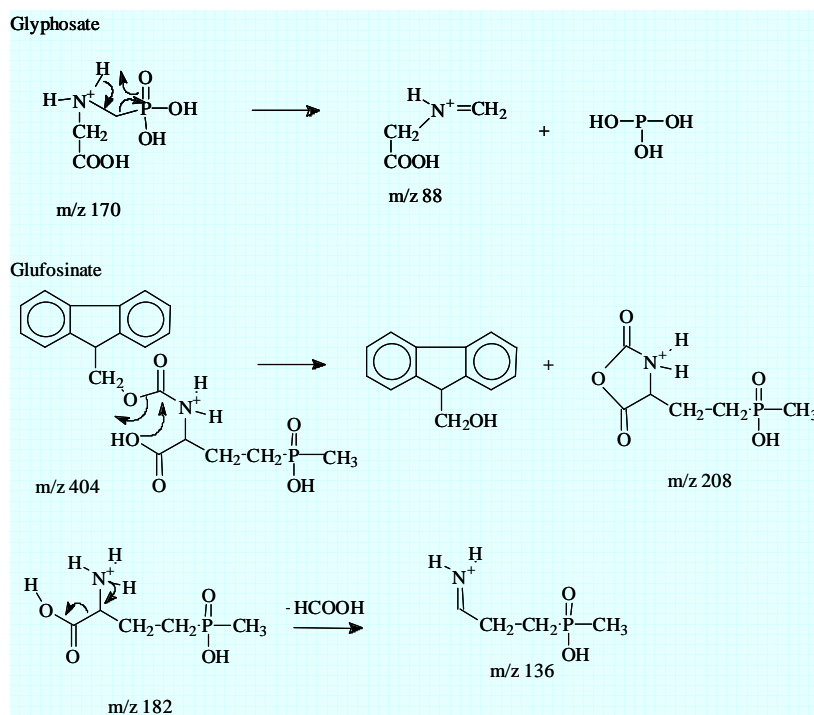


Figure 3. (a) Common fragmentation pathway for the three derivatised-compounds

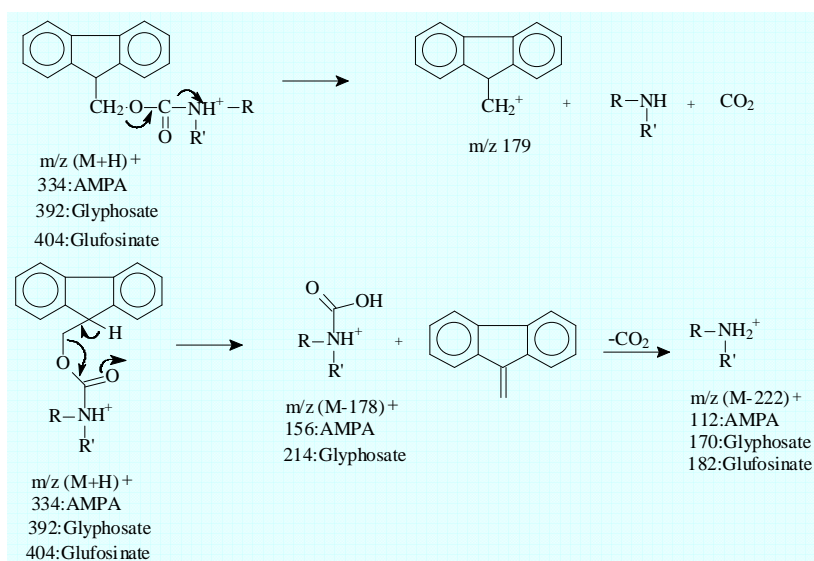


Figure 3. (b) Specific fragmentation pathway for glyphosate and glufosinate.

The selected reaction monitoring (SRM) transitions chosen for the residue determination of the three compounds, as well as the optimised MS/MS parameters, are shown in Table 1.

Table 1. Optimised MS/MS parameters for the FMOC derivatives of glyphosate, AMPA, glufosinate and internal standard, selected for the residue analysis of water and soil.

Compound	Cone Voltage (V)	Precursor ion (m/z)	Product ion (m/z) ^a	Collision Energy (eV)
Glyphosate-FMOC	30	392.0	Q 88.1 q 214.1	20 10
Glufosinate-FMOC	30	404.0	Q 136.1 q 208.2	25 10
AMPA-FMOC	30	334.0	Q 112.1 q 179.1	15 20
Isotope-labeled glyphosate-FMOC	30	395.0	Q 91.1 q 217.1	20 10

^a Q, Transition used for quantification; q; Transition used for confirmation

Method optimisation

Firstly, several attempts were carried out in order to determine these compounds directly, i.e., without any previous derivatization. For this purpose we checked Hydrophilic Interaction Chromatography using an Atlantis TM HILIC 5 µm Silica Column (100 x 2.1 mm i.d., Waters). This column offers superior retention for very polar compounds that are not well retained under reversed-phase conditions. Although the retention obtained with this column at acidic pH was satisfactory, we observed poor sensitivity, making necessary a preconcentration step. We did not try to perform such a preconcentration because this step is difficult for sub-ppb levels of glyphosate and forces one to a higher sample manipulation. Additionally, the conditions to obtain satisfactory retention and peak shape were very specific and changed drastically when changing either pH of the sample or modifier concentration in the mobile phase, decreasing the

robustness of the method. For these reasons, a derivatisation procedure was carried out in order to increase the retention of analytes in the most common RPLC cartridges and to work under no so strict conditions.

Derivatization procedures with FMOC-Cl have already been reported in the literature [9-15]. Due to the low solubility and stability of FMOC-Cl in water, this reagent is usually prepared in acetonitrile. Normally the high concentration of FMOC required for the derivatization, makes that the derivatized sample presents a high percentage of acetonitrile. Thus, a dilution step with water is necessary to reduce the organic percentage [14], with the subsequent loss of sensitivity, to retain glyphosate, glufosinate and AMPA in the cartridge due to the high polar character of these compounds, even derivatized. In this paper, we decreased the volume of the FMOC solution used but increasing its concentration and also the volume of water sample derivatized with the aim of minimizing the dilution factor. The effect of adding different FMOC concentrations with different reaction times was studied. The best results for both, water and soil samples, were obtained after performing the reaction overnight with a FMOC concentration of 12000 mg/L.

On the other hand, as the borate solution could not buffer properly the alkaline sample extract, a neutralizing step was necessary before the derivatization. Any attempt of fixing the volume of HCl necessary to neutralize the KOH excess failed due to the different nature of the soils. Therefore, this step was done manually adding drops of HCl 6M and 0.6 M until pH around 7.

Once the derivatization reaction took place overnight, hydrochloric acid was added to stop the reaction, by lowering the pH.

In soil samples, after direct injection of 50 μ L of the derivatized acidified extract, recoveries around 25% with RSD up to 80% were obtained for the three analytes, showing a severe matrix effect in both the MS instrument and/or the derivatization procedure. Among the solutions described to solve this problem (see Introduction), the increase of the sample treatment was not considered as the best strategy for monitoring programs where rapid methods are preferred. Moreover, the use of matrix-matched standards calibration is not a robust approach when environmental samples are analysed, due to their different origin and composition, making the selection of a blank matrix difficult. Thus, the use of internal standards (IS) was tested, but only isotope-labelled glyphosate was commercially available

As expected, the use of this IS improved accuracy and precision for glyphosate as it compensated the matrix effects, due to the similar chemical behaviour of analyte and IS. However, still ionization inhibition occurred lowering the sensitivity of the overall analytical

procedure. In the case of AMPA and glufosinate, although better recoveries were obtained (around 116-127%), the RSDs were still unacceptable (higher than 15%).

Therefore, the dilution of soil extracts with LC grade water was assayed as a fast and simple way to minimize matrix interferences. Thus, 5 soil samples of different origins were fortified at the 0.5 mg/kg and their extracts derivatized and, 10-fold and 20-fold diluted with water. According to our results (see Table 2), 10- and 20-fold dilution would be adequate for accurate quantification, even without internal standard. However, the use of internal standard improved the RSDs, especially for glyphosate. In the case of glufosinate, quantification with labelled glyphosate IS did not improve the results. A similar situation has been previously reported in literature, when using analogues IS, demonstrating the difficulty of selecting an adequate IS when the labelled analyte is not available [28]. Finally, glyphosate and AMPA were quantified using internal standard meanwhile glufosinate was quantified with external standard calibration. A 10-fold dilution of the extract was chosen as it led to the best LODs.

In regard to water samples, after injection of 4.3 mL of the derivatized samples into the SPE-LC-MS/MS, recoveries around 60% were obtained for glyphosate and AMPA and around 75% for glufosinate, showing an important matrix effect. The use of IS improved the results for glyphosate and AMPA, while unsatisfactory recoveries were obtained (around 140%) for glufosinate. Thus, glyphosate and AMPA were quantified using IS, but not glufosinate, similarly to the analysis of soil. In this case, a dilution of the water sample was not assayed due to the high sensitivity required.

Phosphate and phosphonate compounds might be prone to adsorption onto nonsilanized glass especially in not metal-free solvents. Therefore, a simple adsorption study was carried out in order to evaluate this effect for the three analytes, using standard solutions and fortified surface and groundwater samples. After letting stand 24 h before derivatization, no significant adsorption was observed, as the recoveries were within the normal accepted values, i.e. 70-110%. Therefore, the unsatisfactory recoveries obtained in the preliminary experiments were found to be related to matrix effects rather than to adsorption processes onto the glass material.

Table 2. Effect of dilution of soil extracts previously to the derivatization step on the recovery and reproducibility of the method (n=5)^a.

Compound	Without dilution		10-fold dilution		20-fold dilution	
	% Recovery ^b	% Recovery ^c	% Recovery ^b	% Recovery ^c	% Recovery ^b	% Recovery ^c
	(% RSD)	(% RSD)	(% RSD)	(% RSD)	(% RSD)	(% RSD)
Glyphosate	25 (79)	97 (6)	83 (24)	98 (3)	83 (23)	91 (11)
AMPA	28 (46)	127 (27)	87 (9)	98 (11)	89 (8)	98 (10)
Glufosinate	27 (56)	116 (18)	94 (8)	118 (19)	92 (8)	107 (9)

^a Five different soil samples, spiked at 0.5 mg/kg each

^b Quantification without internal standard

^c Quantification with internal standard

Method validation

Quadratic calibration curves were obtained for all three compounds, in the range 25-5000 ng/L for water analysis, and in the range 1-500 µg/L for soil extracts, with correlation coefficients (r^2) higher than 0.995 in all cases. The method was found to be precise (RSD < 12%) and accurate, with satisfactory recoveries, between 72% and 116% in water, and between 83% and 92% in soil. The slightly higher recoveries for AMPA in water samples could be explained by a partial correction when using the labelled glyphosate as IS. Notwithstanding, the results were considered satisfactory at the low concentration levels assayed.

Limits of quantification (LOQ) were taken as the lowest fortification level successfully validated, i.e. 50 ng/L for all compounds in water samples and 0.05 mg/kg in soil. Limits of detection were calculated from the most diluted standard analysed (25 ng/L for water samples and 1 µg/L for soil samples) and were estimated to be 5 ng/L for all compounds in water samples, and 5 µg/kg in soil. Table 3 summarizes all data obtained during method validation.

Table 3. Validation of the developed LC-MS/MS procedure for the determination of FMOc derivatives of glyphosate, aminomethylphosphonic acid (AMPA) and glufosinate in water and soil samples.

Compound	Groundwater		Surface water		Soil samples		LOD	
	(ng/L)		(ng/L)		(mg/kg)		Water ^a (ng/L)	Soil ^b (µg/kg)
	50	500	50	500	0.05	0.5		
Glyphosate ^c	89 (9)	96 (3)	106 (3)	102 (2)	90 (7)	92 (4)	5	5
AMPA ^c	97 (10)	116 (9)	111 (8)	106 (9)	89 (5)	88 (1)	5	5
Glufosinate ^d	72 (7)	75 (12)	84 (9)	88 (7)	83 (6)	86 (5)	5	5

Detection limits, mean recoveries (%) and relative standard deviations (%) of the overall analytical procedure (n=5).

^aEstimated from a LC-MS/MS chromatogram corresponding to a 25 ng/L standard

^bEstimated from a LC-MS/MS chromatogram corresponding to a 1 µg/L standard

^cRelative recovery, using labeled glyphosate as IS

^dAbsolute recovery

As an example of the excellent sensitivity and selectivity of the method, Figure 4 shows typical SPE-LC-MS/MS chromatograms for a standard solution (50 ng/L) and a groundwater sample spiked at 50 ng/L. This figure also shows the benefit of selecting selective transitions. As can be seen, chromatograms corresponding to the less selective transitions, i.e. those chosen for AMPA (Q, q) and glyphosate (q), show the presence of additional peaks.

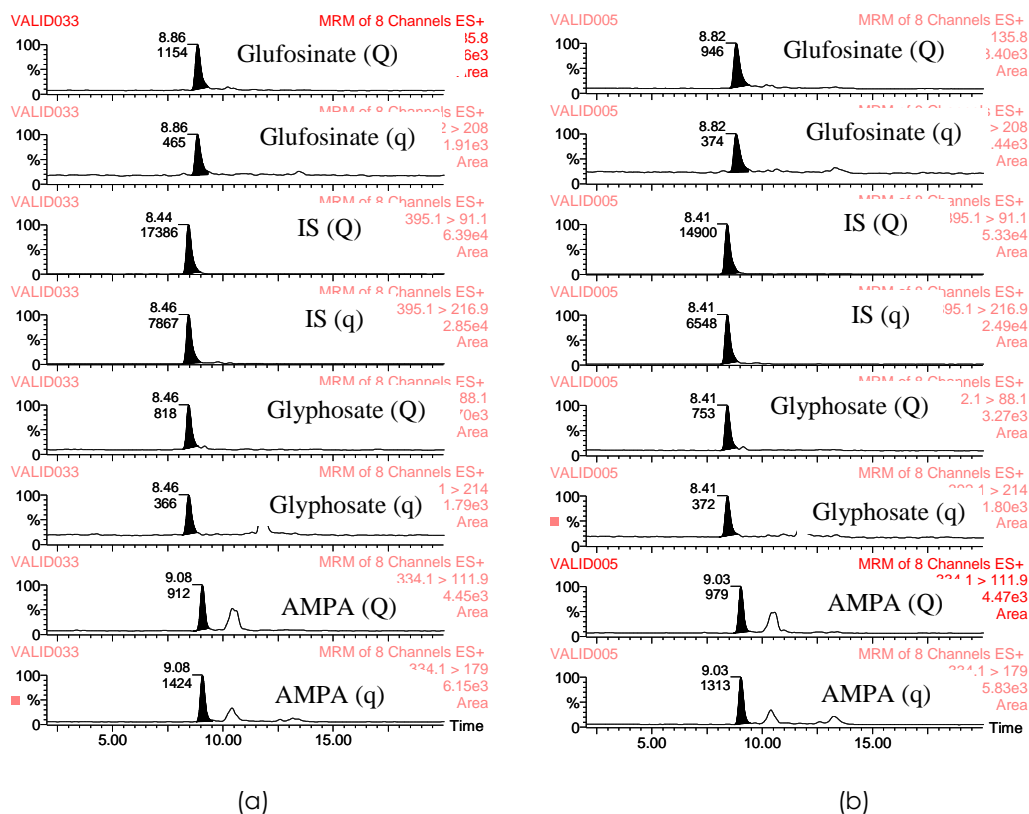


Figure 4. LC-MS/MS chromatograms for (a) standard solution of glyphosate, glufosinate and AMPA (50 ng/L) and (b) groundwater sample spiked at 50 ng/L. (IS) Internal standard, (Q) quantitative transition, (q) qualitative transition.

Analysis of real-world samples

The developed SPE-LC-MS/MS method was applied to the analysis of both ground and surface water (approximately 50 samples) collected in selected sites from the Spanish Mediterranean region, an important agricultural area where glyphosate is widely used. Moreover, the developed LC-MS/MS method was applied to the analysis of 6 soil samples.

Within each batch of 8-10 samples, a calibration curve at concentrations between 25 and 1000 ng/L, in the case of water, and between 1 and 500 µg/L, in the case of soil, was injected before and after the samples. Every three samples, QC prepared at the LOQ level and at the 10xLOQ level were alternately inserted. Quality control (QC) consisted on blank groundwater, surface water or soil, which were spiked with the analytes. These blank samples were previously analysed to confirm the absence of the analytes. Satisfactory QC recoveries

were obtained for all the compounds (between 70-120%) demonstrating the robustness of the method along the period of time of the analysis.

Glufosinate was not detected in any of the samples analysed. However, glyphosate was found in 20% of the water samples, at concentration levels between 55 and 484 ng/L, whereas AMPA was detected in 38% of the water samples, at concentrations between 51 and 175 ng/L. Figure 5b shows chromatograms corresponding to a groundwater sample, that contained glyphosate and AMPA (304 and 87 ng/L, respectively).

In relation to soil, glyphosate was detected in four of the six samples analysed, at concentration levels between 0.17 and 0.73 mg/kg, and AMPA was found in 3 of these samples, at concentrations between 0.04 and 5.61 mg/kg. Figure 5c shows chromatograms corresponding to a soil sample, containing glyphosate and AMPA (0.28 and 0.38 mg/kg, respectively).

The developed procedure for water has also been applied for the analysis of twelve surface water and twelve ground water samples as well as one standard solution within a Round-robin study organized by Monsanto Europe during 2004. The results were highly satisfactory in all cases, except from some ground water samples where the reported values for glyphosate were lower than expected. This deviation has also been observed for the majority of participants, being a possible reason the presence of some metal ions that acted as chelating agents, mainly for glyphosate and in less extension for AMPA, making glyphosate unavailable for direct analysis in only some samples.

All the detections were confirmed by the qualification transition (q) selected, obtaining a deviation in the calculated concentration (using both Q and q transitions) within the accepted tolerance, in all cases $<\pm 20\%$.

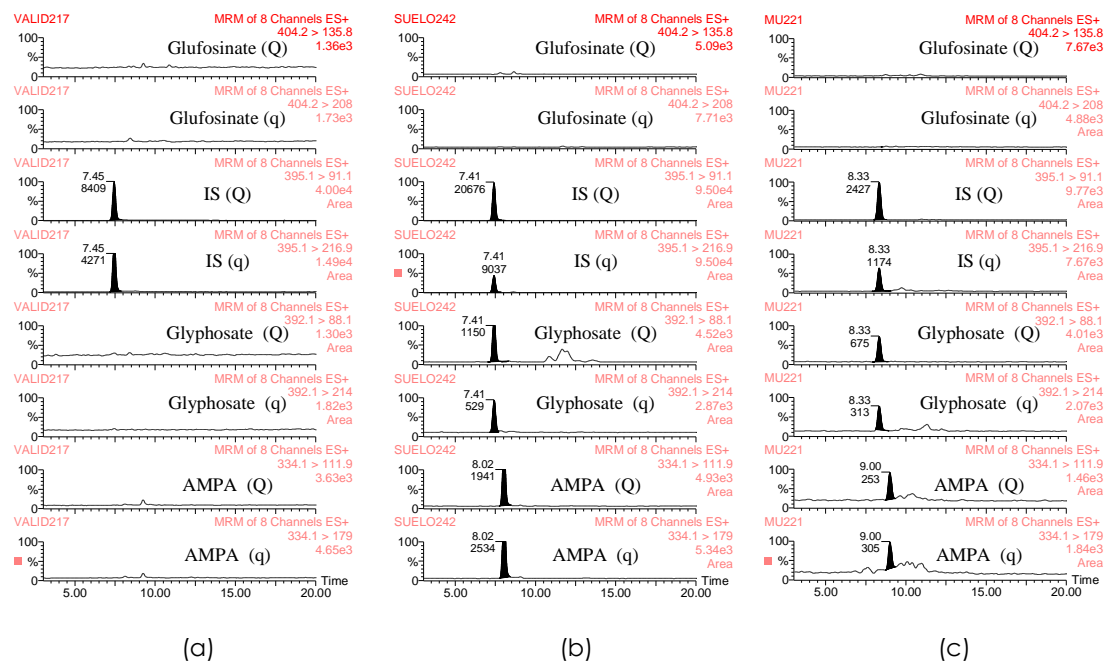


Figure 5. LC-MS/MS chromatograms corresponding to (a) blank soil sample, (b) soil sample, containing glyphosate and AMPA at concentration level of 0.28 and 0.38 mg/kg, respectively and (c) groundwater sample, containing glyphosate and AMPA at concentration level of 304 and 87 ng/L, respectively. (IS) Internal standard, (Q) quantitative transition, (q) qualitative transition.

CONCLUSION

The (SPE-)LC-ESI-MS/MS method developed in this work allows the rapid, accurate and selective determination of very low residue levels of glyphosate, AMPA and glufosinate in water and soil samples. All efforts to determine these compounds without any previous derivatization were not enough satisfactory, mainly due to problems in the preconcentration step. Therefore, a pre-derivatization with 9-fluorenmethylchloroformate in borate buffer was carried out to decrease the polarity of analytes and to improve their retention time in the RPLC system employed. Although these compounds had been traditionally recorded in negative ion mode, positive ionisation has been selected in this paper, as the specificity and sensitivity of the selected transitions improved under this ionisation mode. The developed method achieves excellent LODs for both water (5 ng/L) and soil (5 µg/kg), and allows the correct quantification and confirmation of positive samples at 50 ng/L for water and 0.05 mg/kg for soil. As the method does not require off-line preconcentration of the sample, 10 mL of water are sufficient to perform the derivatization step. Thus, 4.3 mL of the derivatized mixture are sufficient to be preconcentrated on-line in the SPE-LC system in order to reach the required sensitivity, allowing an easy automation and rendering a high analytical throughput. The higher complexity of the soil samples leads to considerable matrix effects, inhibiting the ionisation of the analytes. A 10-fold dilution of the soil extract previously to the derivatization step, together with the use of isotopically labelled glyphosate as internal standard, is a simple way to minimize and compensate for this undesirable effect and to obtain satisfactory quantification. The developed methodology has been applied to the determination of glyphosate, glufosinate and AMPA in real-world water and soil samples. All the detections were confirmed by the use of two MS/MS transitions.

ACKNOWLEDGEMENTS

The authors are very grateful to Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for using the triple quadrupole mass spectrometer (Quattro LC). This work has been developed under financial support of the Ministry of Science and Technology (Ref REN2002-01818), Spain.

M.I. is very grateful to Generalitat Valenciana for her pre-doctoral grant.

REFERENCES

- (1) Terry Roberts (Ed.). *Metabolic Pathways of Agrochemicals, Part 1, Herbicides and Plant Growth Regulators*, The Royal Society of Chemistry, 1998
- (2) N. Tsunoda, *J. Chromatogr. A* 637 (1993) 167
- (3) Z.H. Kudzin, D.K. Gralak, J. Drabowicz, J. Luczak, *J. Chromatogr. A* 947 (2002) 129
- (4) C. D. Stalikas, G. A. Pilidis, *J. Chromatogr. A*, 872 (2000) 215
- (5) A. Royer, S. Beguin, J.C. Tabet, S. Hulot, M.A. Reding, P.Y. Communal, *Anal. Chem.* 72 (2000) 3826
- (6) C. D. Stalikas, G.A. Pilidis, M.I. Karayannis, *Chromatographia* 51 (2000) 741
- (7) E. Borjesson, L. Torstensson, *J. Chromatogr. A* 886 (2000) 207
- (8) Z. H. Kudzin, D.K. Gralay, G. Andrijewski, J. Drabowicz, J. Luczak, *J. Chromatogr. A* 998 (2003) 183
- (9) E. A. Lee, L. R. Zimmerman, B. S. Bhullar, E. M. Thurman, *Anal. Chem.* 74 (2002) 4937
- (10) R. J. Vreeken, K. Speksnijder, I. Bobeldijk-Pastorova, Th. H. M. Noij, *J. Chromatogr. A* 794 (1998) 187
- (11) C. Hidalgo, C. Rios, M. Hidalgo, V. Salvadó, J.V. Sancho, F. Hernández, *J. Chromatogr. A* 1035 (2004) 153
- (12) E.A Hogendoorn, F.M. Ossendrijver, E. Dijkman, R.A. Baumann, *J. Chromatogr. A* 833 (1999) 67
- (13) J.V. Sancho, F. Hernández, F.J. López, E.A. Hogendoorn, E. Dijkman, P. van Zoonen, *J. Chromatogr. A* 737 (1996) 75
- (14) J.V. Sancho, F.J. López, F. Hernández, E.A. Hogendoorn, P. van Zoonen, *J. Chromatogr.* 678 (1994) 59
- (15) J.V. Sancho, C. Hidalgo, F. Hernández, F.J. López, E.A. Hogendoorn, E. Dijkman, *Int. J. Environ. Anal. Chem.* 62 (1996) 53
- (16) E. Mallat, D. Barcelo, *J. Chromatogr. A* 823 (1998) 129
- (17) J. Patsias, A. Papadopoulou, E. Papadopoulou-Mourkidou, *J. Chromatogr. A* 932 (2001) 83
- (18) G. Morovjan, J. Fekete, J. Repasi, *J. Liq. Chromatogr.* 18 (1995) 3219

- (19) Y. Zhu, F. Zhang, C. Tong, W. Liu, J. Chromatogr. A 850 (1999) 297
- (20) L. Goodwin, J. R. Startin, B. J. Keely, D. M. Goodall, J. Chromatogr. 1004 (2003) 107
- (21) S. Chang, C. Liao, J. Chromatogr. A 959 (2002) 309
- (22) F. Hernández, C. Hidalgo, J.V. Sancho, Int. J. AOAC 83 (2000) 728
- (23) K.H. Bauer, T. P. Knepper, A. Maes, V. Schatz, M. Voihsel, J. Chromatogr. A 837 (1999) 117
- (24) B.B.M. Sadi, A.P. Vonderheide, J.A. Caruso, J. Chromatogr. A 1050 (2004) 95
- (25) F. Hernández, M. Ibañez, J.V. Sancho, O.J. Pozo, Anal. Chem. 76 (2004) 4349
- (26) F. Hernandez, J.V.Sancho, O.J. Pozo, A.Lara, E.Pitarch, J. Chromatogr. A 939 (2001) 1
- (27) J.V.Sancho, O.J. Pozo, F. Hernandez, The Analyst 129 (2004) 38
- (28) J.V.Sancho, O.J. Pozo, F.J. López, F. Hernandez, Rapid Commun. Mass Spectrom. 16 (2002) 639
- (29) Official Journal of The European Communities L221, 8-36, Commission Decision (2002/657/EC) of 12 August 2002, Brussels, Belgium, 2002

b. Ejercicio de intercomparación: Optimización del método 2

El método descrito anteriormente en aguas fue aplicado a un ejercicio de intercomparación organizado por la compañía Monsanto Europa (Bruselas), en colaboración con el Instituto Fresenius (Alemania), y en el que participaron 30 laboratorios de 11 países europeos diferentes, siendo nuestro laboratorio el único participante español. Se analizaron 12 muestras de agua superficial y 12 de agua subterránea, conteniendo diferentes niveles de glifosato y AMPA, así como un patrón mezcla de ambos compuestos.

Las muestras llegaron congeladas en botellas de plástico de, aproximadamente, 250 mL, y se mantuvieron a -18°C hasta el momento de ser analizadas. Antes de su análisis, se procedió a su descongelación durante una noche en frigorífico a 4°C . Una vez descongeladas, se dejaron atemperar y se procedió al análisis.

En las siguientes gráficas pueden verse los resultados obtenidos por nuestro laboratorio y los datos que nos facilitó la compañía organizadora del ejercicio de intercomparación. Como puede verse en la Figura 3.18, los resultados obtenidos en las aguas superficiales fueron satisfactorias tanto en el caso del AMPA como en el del glifosato. En el caso de las aguas subterráneas (Figura 3.19), los resultados obtenidos para el AMPA fueron bastante satisfactorios, mientras que para el glifosato, reportamos unas concentraciones inferiores a las reales en algunas de las muestras analizadas. Por ejemplo, en la muestra de agua número 735, se reportó una concentración de $0.2\ \mu\text{g/L}$ en lugar de $0.9\ \mu\text{g/L}$. Algo semejante ocurrió con otras aguas, como por ejemplo las muestras 809 y 908

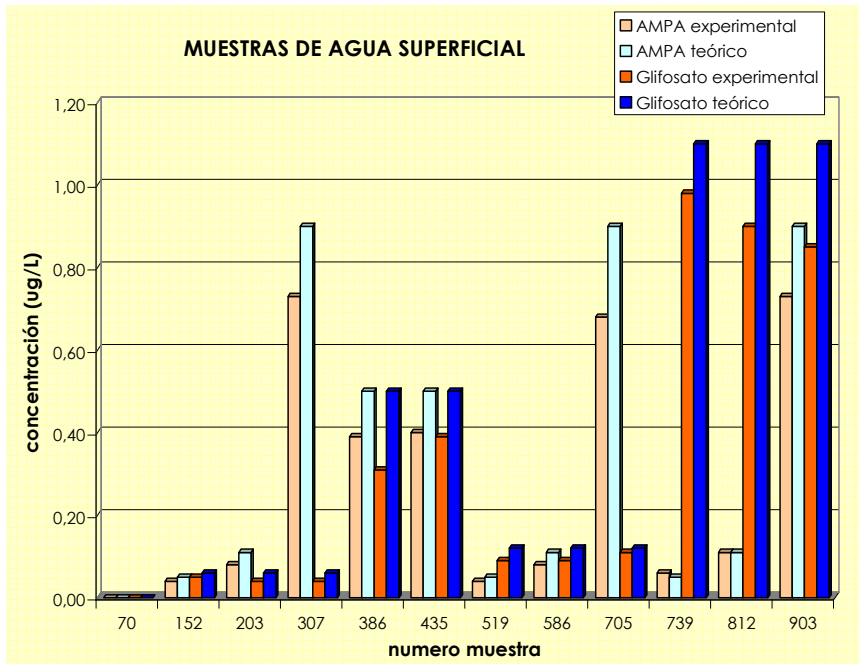


Figura 3.18 Resultados obtenidos por nuestro laboratorio en el análisis de aguas superficiales

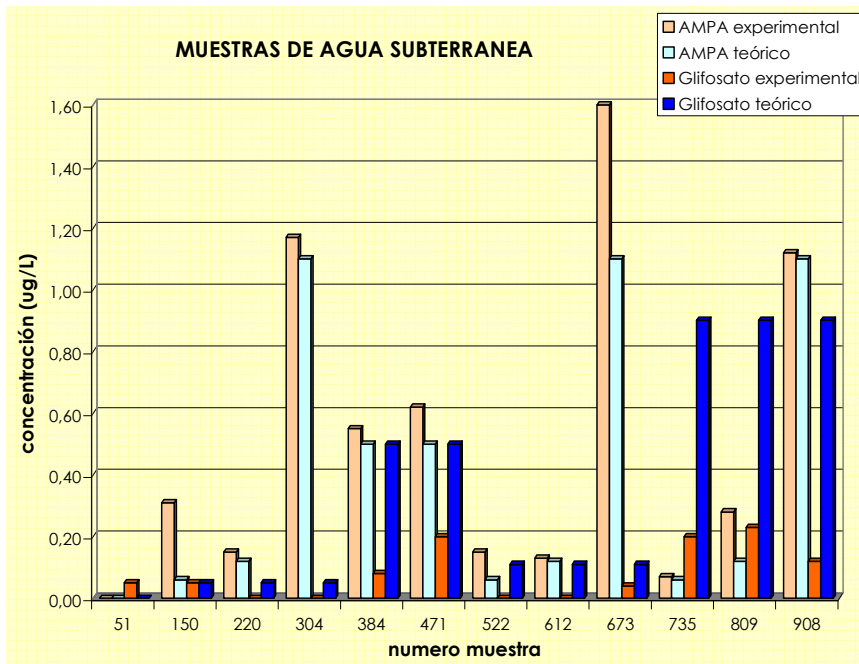


Figura 3.19 Resultados obtenidos por nuestro laboratorio en el análisis de aguas subterráneas

La empresa organizadora nos informó de que esto mismo había ocurrido en la mayoría de los laboratorios, y que sólo aquellos que utilizaron cromatografía de intercambio catiónico como etapa de preconcentración o de tratamiento de muestra, habían reportado resultados correctos para las muestras de aguas subterráneas. Estos resultados nos condujeron a realizar una serie de experiencias con el fin de mejorar el método. El desarrollo de las mismas, los resultados y las conclusiones obtenidas pueden verse con más detalle en el *artículo científico* 6.

Brevemente, dado que en nuestro caso utilizábamos patron interno de glifosato marcado, consideramos dos posibles explicaciones: 1) el tratamiento de muestra llevado a cabo con las columnas intercambiadoras era efectivo y capaz de eliminar los posibles interferentes que afectaban a la determinación del glifosato, 2) la acidificación previa llevada a cabo en este tipo de tratamiento facilitaba la correcta cuantificación del analito. En el primer caso, se habría observado una pérdida de sensibilidad en nuestro método, pero el IS habría sido capaz de corregir este efecto, obteniendo por lo tanto recuperaciones aceptables. En cuanto a la segunda posibilidad, la acidificación podría romper las posibles interacciones entre el glifosato y algún componente de la matriz. Aunque en principio un IS adecuado habría corregido este efecto, en caso de formarse un compuesto con una cinética lenta, al IS no le daría tiempo de formar dicho compuesto y se obtendrían, por tanto, bajas recuperaciones con lo que la corrección del IS no sería efectiva. Con el fin de averiguar las causas de esta menor recuperación, que por otro lado no experimentaban los QCs, procedimos a evaluar el efecto de la acidificación de las muestras con HCl (hasta pH = 1), con el fin de romper el hipotético complejo, procediendo a una posterior neutralización con KOH 6M hasta pH 6-7, previa a la derivatización con FMOC. Como se muestra en la Figura 2 del *artículo* 6, tras reanalizar de nuevo las muestras 735, 809 y 908 (fortificadas a 0.9 ppb y que nosotros habíamos reportado como 0.1-0.2 ppb), observamos que las recuperaciones obtenidas aumentaban sensiblemente dando lugar a las concentraciones esperadas (0.8-1.0 ppb). El método fue finalmente modificado incluyendo la acidificación previa de la muestra con HCl. El método propuesto puede verse en la Figura 1 del mismo artículo. Tras reanalizar 13 muestras de agua de la Comunidad Valenciana positivas en glifosato, (Tabla 2 del *artículo* 6), observamos que aproximadamente la mitad de ellas aumentaron su concentración 3 veces o más al aplicar el método modificado. En el caso de la muestra 12, aumentó hasta 14 veces. Sólo 3 muestras dieron el mismo resultado usando los dos métodos.

Tras una búsqueda bibliográfica sobre este tema se encontraron algunos artículos científicos sobre la formación de complejos del herbicida glifosato con metales como cobre, plomo, hierro, aluminio, zinc o cadmio, e incluso con materia orgánica. Con el fin de comprobar si se producía la formación de un complejo glifosato-metal, se realizó un análisis de metales de

todas las muestras, no encontrándose diferencias significativas entre ellas. Así pues la causa de este efecto no pudo ser identificada, siendo necesario realizar estudios más detallados con el fin de investigar la problemática existente.

Artículo científico 6

J. Chromatogr. A (2006) 1134: 51-55

RE-EVALUATION OF GLYPHOSATE DETERMINATION IN WATER BY LIQUID CHROMATOGRAPHY COUPLED TO ELECTROSPRAY TANDEM MASS SPECTROMETRY

María Ibáñez, Óscar J. Pozo, Juan V. Sancho, Francisco López, Félix Hernández
Research Institute for Pesticides and Water, University Jaume I, E-12071, Castellón, Spain

ABSTRACT

An analytical method based on on-line solid phase extraction liquid chromatography coupled to electrospray tandem mass spectrometry (SPE-LC-ESI-MS/MS) for the determination of glyphosate and aminomethylphosphonic acid (AMPA) residues has been applied to the analysis of water samples within a Round Robin Study. The method had been previously validated in a variety of water samples and it fulfilled all the parameters of precision, accuracy, sensitivity and unequivocal confirmation. The results within the study that we participated were highly satisfactory in all cases with the only exception of glyphosate in ground water samples, where surprisingly recoveries around 15% were obtained despite the use of isotope-labeled glyphosate as internal standard (IS). A slight modification has been introduced in the method, simply consisting in the acidification of sample with hydrochloric acid (HCl) to pH 1. Then, the sample is neutralized and immediately derivatized with 9-fluorenmethylchloroformate (FMOC) before LC-MS/MS determination. Round Robin Study samples were reanalyzed using this approach, and the recoveries increased up to 98%. A possible explanation might be the slow kinetic interaction between glyphosate and some components of the matrix. These components might act as chelating agents, making glyphosate unavailable for derivatization and therefore for analysis. Several water samples collected at the Mediterranean area of Spain, and previously analyzed and being found to contain glyphosate, were also reanalyzed using this approach, obtaining higher concentrations (between 2 and 14 times) in most of cases.

INTRODUCTION

Glyphosate [N-(phosphonomethyl)glycine] is a broad spectrum, nonselective, post-emergence herbicide extensively used worldwide in various applications for weed and vegetation control. The lack of adequate chemical groups in glyphosate molecule (e.g. chromophores or fluorophores) hampers its measurement by conventional detectors. Moreover, due to its ionic character, low volatility and low mass, its determination at the low concentrations required in water by the present Legislation (sub $\mu\text{g/L}$ levels) is very difficult. The use of mass spectrometry (MS) has been investigated due to the high sensitivity and unequivocal confirmation required in pesticide residue analysis. Thus, MS has been combined with ion chromatography (IC) [1], inductively coupled plasma (ICP) [2-3], capillary electrophoresis (CE) [4], gas chromatography (GC) [5-11] and liquid chromatography (LC) [12-14] for glyphosate residue determination. Most of these methods have been briefly commented in our previous paper [14], where we developed a rapid, sensitive and robust method for the determination of glyphosate, its principal degradation product, aminomethylphosphonic acid (AMPA), and glufosinate in water based on the use of solid phase extraction (SPE) coupled on-line with liquid chromatography with electrospray-tandem mass spectrometry detection (SPE-LC-ESI-MS/MS). The method developed included a derivatization step with FMOC, previously to LC-MS/MS determination, in order to improve the chromatographic behaviour of the analytes. The method fulfilled the requirements of excellent sensitivity and unequivocal confirmation of the residues detected by using two MS/MS transitions for each compound. Moreover, the use of isotope-labeled glyphosate as internal standard (IS) minimised derivatization variations and matrix effects, allowing in principle, a correct quantification. The method was validated in both ground and surface water samples, obtaining mean recoveries ranging from 89% to 106% for glyphosate (RSD=9%), from 97% to 116% for AMPA (RSD=10%) and from 72% to 88% for glufosinate (RSD=12%). Limits of quantification (LOQ) for all compounds were 50 ng/L with limits of detection as low as 5 ng/L. The procedure was applied to the analysis of ground and surface water (approximately 50 samples) from the Spanish Mediterranean region, an important agricultural area where glyphosate is widely used. Quality control (QC) samples prepared at the LOQ and 10 x LOQ levels were alternately inserted every three samples analyzed. Satisfactory QC recoveries were obtained (between 70-120%) demonstrating the robustness of the method along the period of time of the analysis. Therefore, the method was considered satisfactory as reported in our paper [14]. However, within a Round-robin study organized by Monsanto Europe at summer 2004, the method led to satisfactory results when applied to surface water but poor recoveries were obtained for groundwater. All data for AMPA were fully satisfactory.

The aim of this paper is to explore the reasons of the low concentrations reported for some samples, and to improve the previously developed method when applied to different types of water samples.

EXPERIMENTAL

Chemicals

Glyphosate reference standard (98%) and isotope-labeled glyphosate (1,2-¹³C, ¹⁵N), used as internal standard, were purchased from Dr Ehrenstorfer (Augsburg, Germany). Analytical reagent-grade disodium tetraborate decahydrate was obtained from Scharlab (Barcelona, Spain) and 9-fluorenmethylchloroformate (FMOC-Cl) was purchased from Sigma. Reagent-grade hydrochloric acid (HCl), formic acid, potassium hydroxide (KOH), acetic acid (HAc) and ammonium acetate (NH₄Ac) as well as LC-grade acetonitrile were purchased from Scharlab. LC-grade water was obtained by purifying demineralised water in a Nanopure II system (Barnstead Newton, MA, USA).

Glyphosate standard stock solution was prepared dissolving approximately 50 mg powder, accurately weighted, in 100 mL of water obtaining a final concentration of approximately 500 mg/L. Standard working solutions for the LC-MS/MS analysis and for fortification of samples were prepared by dilution of the 500-mg/L standard with water. All standard solutions were stored in nonsilanized glass.

The isotope-labeled glyphosate was purchased as 1.1 mL of 100- μ g/mL stock solution in water. A 11- μ g/mL standard solution was prepared by dissolving 1.1 mL of the stock solution in 10 mL of water. Standard working solutions were prepared by diluting the intermediate standard solution with water.

Solutions of 5% borate buffer (pH approximately 9) in HPLC-grade water and solutions containing 12000 mg/L of FMOC-Cl in acetonitrile were used for the derivatization step prior to the analysis.

Instrumentation

The instrument as well as operational conditions can be seen elsewhere [14]. Briefly, a Quattro LC (quadrupole-hexapole-quadrupole) mass spectrometer (Micromass, Manchester, UK) was interfaced to a LC system based on a 233XL autosampler with a loop of 4300 μL (Gilson, Villiers-le-Bel, France) and 2 pumps: an Agilent 1100 (Agilent, Waldbron, Germany) binary pump used to condition and wash the cartridge (P-1) and a Waters Alliance 2695 (Waters, Milford, MA, USA) quaternary pump used for the chromatographic separation (P-2). The SPE preconcentration was performed using an Oasis HLB cartridge, 20 x 2.1 mm i.d. (Waters). For the LC separation, a Discovery column C_{18} , 5 μm 50 x 2.0 mm i.d. (Supelco, Bellefonte, PA, USA). Mobile phase consisted of water pH 2.5 (adjusted with formic acid) in P-1, and mixtures of 5mM acetic acid/ammonium acetate (pH 4.8) water and acetonitrile in P-2.

The selected reaction monitoring (SRM) transitions chosen for the residue determination of glyphosate, as well as the optimised MS/MS parameters, are shown in [14].

Sample procedure

Sample treatment consisted on a derivatization with FMOC [14]. Previously, the water sample was acidified in order to glyphosate being available. The procedure is shown in Figure 1. 10-mL of water sample was introduced into a glass tube and acidified with 200 μL of 6M HCl to pH 1. After that, 100 μL of isotope-labeled glyphosate standard (110 $\mu\text{g}/\text{L}$) was added to the sample and neutralized with 200 μL of 6M KOH. Samples were then derivatized by adding 0.6 mL of 5% borate buffer (pH 9) followed by 0.6 mL of FMOC-Cl reagent (12000 mg/L), and allowing the reaction to take place overnight at room temperature. Derivatized samples were filtered through a 0.45 μm syringe filter and acidified with hydrochloric acid to pH 1.5. Finally, 4.3 mL of the acidified derivatized samples were directly injected into the SPE-LC-ESI-MS/MS system.

Re-evaluation of glyphosate determination

Precision (repeatability, expressed as relative standard deviation, in %) and recoveries were re-evaluated, by analysing a ground water and a surface water sample spiked at 100 ng/L (n=3 each) under the new conditions.

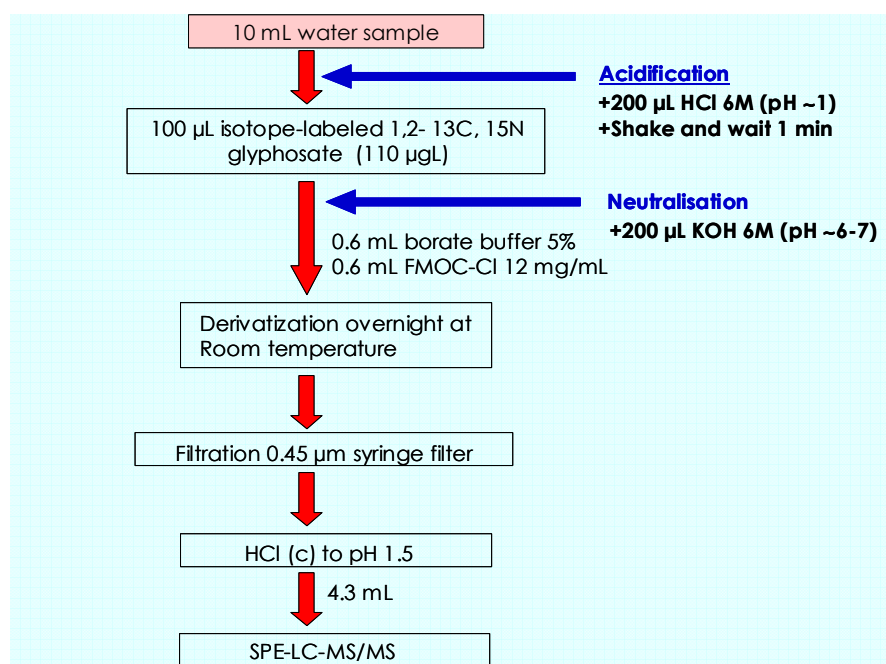


Figure 1. Sample pre-treatment in the determination of glyphosate residues in water. Bold letter (right side) modifications introduced in relation to the previously reported method [14].

RESULTS AND DISCUSSION

Round Robin Study Results

The SPE-LC-MS/MS method previously developed for glyphosate, glufosinate and AMPA at our laboratory [14] was validated, obtaining satisfactory recoveries and precision for all the compounds. Besides, the method was applied to the analysis of around 50 water samples. Quality control (QC) recoveries were also satisfactory (between 70-120%) for the three analytes, along the period of analysis (three months). Therefore, the method was considered satisfactory, assuming that quantification was correct. Later, the method was applied to the analysis of surface and ground water samples spiked at 12 different levels with glyphosate and AMPA, within a Round Robin Study organised by Monsanto Europe. Our results for AMPA were highly satisfactory in both groundwater and surface water. However, in the case of glyphosate, we reported good results in surface water but, surprisingly, recoveries around 15% were obtained in groundwater samples even using labeled glyphosate as surrogate internal standard. Although the use of surrogate/internal standards is widely accepted to correct for matrix effects and

possible losses during the sample pre-treatment allowing a correct quantification, in this case the IS did not lead to satisfactory results.

Method improvement

Low glyphosate recoveries in groundwater samples were reported by all laboratories participating in the study, except for only those that used cationic exchange chromatography as clean-up/preconcentration step (Chelex resin columns).

On the basis of these results, two possibilities were plausible:

- i) the clean up performed by Chelex resin columns was effective and able to remove the interferences that affected to the final measurement; or ,
- ii) the previous acidification required in this clean-up before loading the sample into the column facilitated the correct determination of the analyte.

In the first case, it is well known that the presence of an interferent in the sample matrix could alter analyte ionization (so called matrix effect), obtaining unacceptable recoveries when using LC-MS/MS. However, the use of an analogue IS should solve this problem as the interferent would affect both the analyte and the IS ionization in the same way. Then, a loss of sensitivity could be observed if ionization suppression takes place, but satisfactory recoveries would be reached.

Regarding the second possibility, the acidification step could break some interactions between analyte and matrix components. These interactions might hamper the determination, mainly affecting to the derivatization step applied in the method. An adequate labeled IS should correct this effect, but the effectiveness of this approach would depend on the kinetics of the process involved. Thus, the IS approach does not take into account the kinetic of the processes and it can lead to incorrect data if slow kinetic processes are involved, because the analyte is normally present in the sample during a time substantially longer than the freshly spiked with IS.

Therefore, acidification of the samples previously to the derivatization step was checked. A slight modification was introduced in the method consisting on an acidification and posterior neutralization of the samples previously to the derivatization step (see Figure 1). After reanalysing the samples where poor recoveries were obtained, these values increased up to 98%. This can be seen in Figure 2, where the response glyphosate/IS ratio increased from 0.09 to 0.63 after acidifying the sample. It is illustrative to notice that the area of the IS remained more or less constant in both standard (Figure 2a) and spiked samples (Figures 2b and 2c), while glyphosate

peak area in the non-acidified spiked sample decreased around 8 times. This problem was solved after acidification of the spiked sample, obtaining an adequate area similar to that of the reference standard.

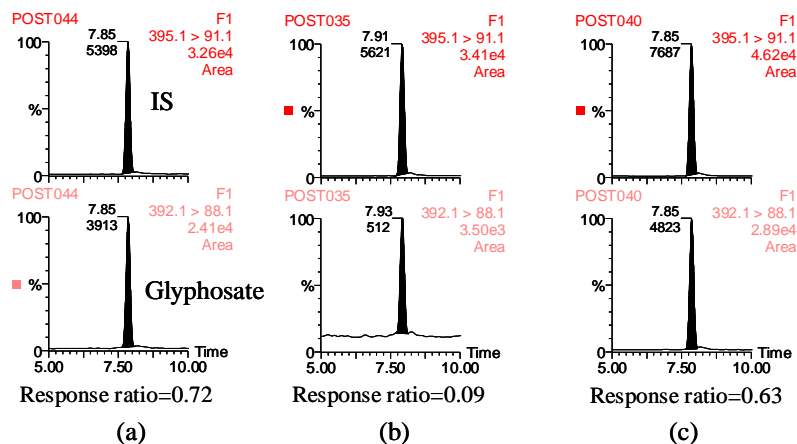


Figure 2. LC-MS/MS chromatograms for glyphosate and IS corresponding to (a) 1 µg/L standard (b) groundwater sample spiked at 0.9 µg/L without previous acidification (c) groundwater sample spiked at 0.9 µg/L acidified previously to the derivatization

The low concentrations reported for glyphosate in some of the samples might be due to the formation of a complex between compounds present in the sample and the glyphosate. This complex would involve the amino group of the glyphosate, making it unavailable for derivatization with FMOC and therefore for analysis. Moreover, this complex formation would follow a slow kinetic, justifying the different behavior observed for analyte and IS. This would explain that the amount of glyphosate available for derivatization is lower than that of IS, which would be unaffected by the matrix components in a freshly spiked sample. Therefore, the IS would not be able to correct this interference. After acidification, the complex would be broken making glyphosate available for derivatization. After that, the following neutralization step would affect in a lower extent because the IS is added immediately before neutralization. Then, all processes that might affect to glyphosate during neutralization (including the formation of the complex glyphosate-interferent) will also affect to the IS in a similar extent.

Formation of complexes of the herbicide glyphosate with some metals as copper, lead, iron, aluminium, zinc or cadmium [15-19], and with organic matter [20-23] has been previously reported. Although we have performed some experiments with different types of water samples and several analysis have been made on trace metals, the identification of the cause of this effect has not been established yet, and additional experiments would be required.

Method re-evaluation

The method was found to be precise and accurate, obtaining satisfactory results. Thus, mean recoveries of 100% (RSD=9%) and of 91% (RSD=2%) were obtained in groundwater and surface water samples, respectively. As it was expected, the use of the IS allowed a correct quantification, not being affected by the previous acidification and posterior neutralization of the matrix.

Application to real samples

The Round Robin Study involved a blank surface water and a blank groundwater spiked at different levels. As we obtained unacceptable glyphosate recoveries only in the groundwater but not in surface water samples, we also reanalyzed 13 real samples positive for glyphosate to check if this was either a general problem or a particular behaviour of this sample. The 13 samples selected had been previously analyzed according to our SPE-LC-MS/MS method [14] and they were stored at $<-18^{\circ}\text{C}$ until re-analysis. As can be seen in Table 1, the majority of the samples were affected by acidification, and only three of them gave the same result using both approaches. Around 50% of the water samples reanalyzed gave concentrations three-fold or even higher than the reported [14]. In one case (sample 12) the concentration increased up to fourteen times after being reanalyzed with the new approach.

Table 1. Effect of the acidification on the glyphosate concentration in real-world water samples

Sample	Water Source	Glyphosate Conc ^a (µg/L)	Glyphosate Conc ^b (µg/L) after reanalysis	Increasing factor
1	Surfacer	<0.05 (0.03)	0.21	7
2	Ground	0.29	0.59	2
3	Surface	0.20	0.31	2
4	Surface	<0.05 (0.03)	< 0.05 (0.03)	1
5	Groundr	0.13	0.21	2
6	Groundr	0.11	0.85	8
7	Surface	0.12	0.11	1
8	Ground	0.06	0.17	3
9	Ground	<0.05 (0.03)	0.17	6
10	Ground	0.11	0.44	4
11	Surfacer	0.06	0.10	2
12	Ground	0.06	0.84	14
13	Surfacer	0.10	0.11	1

^a Samples analyzed using our previous reported method [14]

^b Samples analyzed using the approach described in this paper

Thus, the results reported will have to be reconsidered as glyphosate concentrations were surely underestimated. After a bibliographic search, we have noticed that only two, among all papers dealing with glyphosate determination by MS detectors, acidified the samples before derivatization [8, 10]. The acidification was performed previously to a purification step using an ion exchange resin. Thus, it seems that acidification was carried out as a requirement for ion-exchange clean-up/preconcentration more than to have glyphosate available for subsequent analysis. However, in most of papers [2-7, 9, 11-14], sample pretreatment consisted only on a filtration and/or evaporation step. According to our data, it seems reasonable to question some of the results reported until now even after using fully validated methods which use QCs to assure the quality of the results obtained in real samples, because the problem described in the present paper remains unnoticed along method validation and analysis of real samples. The non availability of certified samples for non-persistent pesticides in the environmental field makes that spiked samples are normally used in validation processes and for QCs preparation. They are normally prepared by fortification of a blank usually equilibrated for short time periods. Therefore,

the added pesticide would not be affected by slow kinetic processes, which interfere in the analysis, in the same way than the analyte present in sample.

CONCLUSION

This paper shows the problems observed in glyphosate determination for some type of water samples. As the IS approach does not take into account the kinetic of some processes that may occur in the sample, its use may not be effective in those cases where the analyte can be interfered by a matrix component following slow kinetics. This situation seems to occur for glyphosate in some types of water samples, as the labeled IS is added just before the derivatization with FMOC, and it is not able to correct this interference. Moreover, this problem remains unnoticed along both the method validation and the analysis of real samples, because spiked samples and quality controls are usually prepared by fortification of blank samples. The problems associated to glyphosate were observed only after applying our LC-MS/MS method in a Round Robin Study. Although the results were satisfactory for surface water, the recoveries for groundwater were around 15%. The problem was solved by acidification to pH 1 and posterior neutralization of the samples prior to derivatization, obtaining in this way quantitative recoveries. When applying this simple approach to a number of samples previously analyzed without acidification, around 80% of them gave higher concentrations than the initially obtained.

Most of methods reported for the analysis of glyphosate, including our previous LC-MS/MS paper, did not perform acidification of sample before derivatization. Therefore, some data reported on glyphosate concentrations in water might be questionable in the light of the results obtained in the present paper. Possible causes of this effect are the presence of some metal ions or organic matter, which may act as chelating agents forming a complex with glyphosate and making it unavailable for the derivatization step. It is pointed out that the problems described in this paper are not dependent upon the analytical technique applied, as they come from the non-availability of glyphosate for analysis. Even applying powerful approaches as on line SPE-LC with MS/MS detection, using labeled glyphosate as internal standard, the application to some real-world samples failed. The problems observed seemed to depend on the matrix of the sample, as glyphosate was available for analysis in several of the samples analyzed while not in other. More studies would be necessary to elucidate the nature of the formed complex and to establish whether acidification of samples is a general approach to be applied to all water samples.

ACKNOWLEDGEMENTS

The authors are very grateful to Monsanto Europe (Brussels, Belgium) for inviting us to participate in the Round Robin Study, providing all the results from the involved laboratories and for their useful comments. The authors also thank to Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for using the triple quadrupole mass spectrometer (Quattro LC) and to Ramon Díaz for his collaboration.

This work has been developed under financial support of the Ministry of Science and Technology (Ref REN2002-01818), Spain.

M.I. is very grateful to Generalitat Valenciana for her pre-doctoral grant.

REFERENCES

- (1) K.H. Bauer, T. P. Knepper, A. Maes, V. Schatz, M. Voihsel, J. Chromatogr A 837 (1999) 117
- (2) B.B.M. Sadi, A.P. Vonderheide, J.A. Caruso, J. Chromatogr A 1050 (2004) 95
- (3) Z.X. Guo, Q.T. Cai, Z.G. Yang, J. Chromatogr A 1100 (2005) 160
- (4) E. A. Lee, L. R. Zimmerman, B. S. Bhullar, E. M. Thurman, Anal Chem 74 (2002) 4937
- (5) N. Tsunoda, J Chromatogr A 637 (1993) 167
- (6) Z.H. Kudzin, D.K. Gralak, J. Drabowicz, J. Luczak, J. Chromatogr A 947 (2002) 129
- (7) C. D. Stalikas, G. A. Pilidis, J. Chromatogr A, 872 (2000) 215
- (8) A. Royer, S. Beguin, J.C. Tabet, S. Hulot, M.A. Reding, P.Y. Communal, Anal Chem 72 (2000) 3826
- (9) C. D. Stalikas, G.A. Pilidis, M.I. Karayannis, Chromatographia 51 (2000) 741
- (10) E. Borjesson, L. Torstensson, J. Chromatogr A 886 (2000) 207
- (11) Z. H. Kudzin, D.K. Gralay, G. Andrijewski, J. Drabowicz, J. Luczak, J. Chromatogr A 998 (2003) 183
- (12) L. Goodwin, J. R. Startin, B. J. Keely, D. M. Goodall, J. Chromatogr 1004 (2003) 107
- (13) R. J. Vreeken, K. Speksnijder, I. Bobeldijk-Pastorova, Th. H. M. Noij, J. Chromatogr A 794 (1998) 187
- (14) M. Ibáñez, O.J. Pozo, J.V. Sancho, F.J. López, F. Hernández J Chromatogr A 1081 (2005) 145-155
- (15) T. Undabeytia, E. Morillo, C. Maqueda J. Agric. Food Chem. 50 (2002) 1918
- (16) J.Sheals, P. Persson, B. Hedman Inorg. Chem. 40 (2001) 4302
- (17) B.C. Barja, M.D. Afonso Environ. Sci. Technol. 32 (1998) 3331
- (18) T. Undabeytia, M.V. Cheshire, D. McPhail Chemosphere 32 (1996) 1245
- (19) P.G. Daniele, C. DeStefano, E. Prenesti, S. Sammartano Talanta 45 (1997) 425
- (20) Y. Yu, Q. Zhou Chemosphere 58 (2005) 811
- (21) O.K. Borggaard, B. Raben-Lange, A.L. Gimsing, B.W. Strobel Geoderma 127 (2005) 270
- (22) H.M. Dion, J.B.Harsh, H.H.Hill J. Radional. Nucl. Chem. 249 (2001) 385

- (23) A.L. Gimsing, O.K. Borggaard, M. Bang Eur. J. Soil Sci. 55 (2004) 183

3.4.3 Bibliografía

Kiehnuis PGM, Geerdink RB

Liquid chromatography-tandem mass spectrometric analysis of surface and waste water with atmospheric pressure chemical ionisation. II. Applications

Trends Anal. Chem. 19 (2000) 460-474

Lagerwerf FM, van Dongen WD, Steenvoorden RJJM, Honing M, Jonkman JHG

Exploring the boundaries of bioanalytical quantitative LC-MS/MS

Trends Anal. Chem. 19 (2000) 418-427

Sancho JV, López FJ, Hernández F, Hogendoorn EA, van Zoonen P

Rapid determination of glufosinate in environmental water samples using 9-fluorenylmethoxycarbonyl precolumn derivatization, large-volume injection and coupled-column liquid chromatography

J. Chromatogr. 678 (1994) 59-67

Sancho JV, Hidalgo C, Hernández F, López FJ, Hogendoorn E.A., Dijkman E

Rapid determination of glyphosate residues and its main metabolite AMPA in soil samples by liquid chromatography

Intern. J. Environ. Anal. Chem. 62 (1996) 53-63

Sancho JV, Pozo OJ, López FJ, Hernández F

Different quantitation approaches for xenobiotics in human urine samples by liquid chromatography/electrospray tandem mass spectrometry

Rapid Commun. Mass Spectrom. 16 (2002) 639-645

Capítulo 4.

QqQ versus QTOF en el proceso de confirmación de contaminantes orgánicos

4.1 Introducción

4.2 Experimental

4.2.1 Triple cuadrupolo

4.2.2 (Q) TOF

4.2.3 Comparación "233 XL" versus "ASPEC XL"

4.3 Resultados y discusión

4.3.1 Triple cuadrupolo

4.3.2 TOF

4.3.3 (Q) TOF

Artículo científico 7

Comparison of different mass spectrometric techniques combined with liquid chromatography for confirmation of pesticides in environmental water based on the use of identification points
Anal. Chem. (2004) 76: 4349-4357

Artículo científico 8

Confirmation of organic micropollutants detected in environmental samples by liquid chromatography tandem mass spectrometry: achievements and pitfalls
Trends Anal. Chem. (2006) 15: 1030-1042

4.4 Bibliografía

4.1 Introducción

Es evidente la necesidad de garantizar la calidad en las determinaciones analíticas con el fin de asegurar, por un lado, que los datos de concentración reportados son fiables y, por otro, evitar incorrectas identificaciones que conducirían a falsos positivos o falsos negativos. Por este motivo es importante conocer con total seguridad si uno o varios analitos se encuentran presentes en una muestra, es decir, es necesario confirmar de una manera inequívoca su presencia en la muestra. Dependiendo de la decisión tomada por el laboratorio, la presencia de un compuesto en una muestra puede acarrear acciones legales con importantes consecuencias sociales, económicas y/o personales. La confirmación de los compuestos detectados debe ser objetiva y fiable, especialmente cuando se encuentran a niveles de concentración superiores a los máximos permitidos. Para ello, se requieren métodos específicos y sensibles. En este tipo de análisis destaca el uso de la resonancia magnética nuclear (NMR) y sobretodo de LC-MS/MS, que se ha convertido en una herramienta cada vez más poderosa. Esta técnica ha empezado a utilizarse en diferentes campos, como son la toxicología forense, farmacología, análisis de alimentos o análisis medioambiental.

Como ya se ha comentado en la introducción, un aspecto fundamental para garantizar la calidad de un resultado es la especificidad de la determinación. Cuando se utiliza LC-MS/MS como técnica analítica podría parecer que el uso de un ion precursor y de un ion producto, junto con el tiempo de retención, proporciona la especificidad adecuada en la detección. Sin embargo, esta técnica puede dar lugar a falsos positivos/negativos, principalmente en el análisis de muestras complejas, ya que algunas interferencias pueden presentar las mismas propiedades espectrométricas que el analito.

Esto podría ocurrir más fácilmente cuando se seleccionan transiciones poco específicas, como por ejemplo pérdidas de agua. La utilización del *pattern* isotópico tampoco asegura que se vayan a evitar falsos positivos. Por ejemplo, para un compuesto con un átomo de Cl, se seleccionan las transiciones $M \rightarrow A$ (como transición Q) y $M+2 \rightarrow A+2$ (como transición q). Si el

ion A conserva el átomo de Cl en su estructura, cualquier interferente con un átomo de Cl que comparta la transición Q con el analito cumplirá el *ion ratio* Q/q, ya que la transición q proviene de la distribución isotópica natural del átomo de Cl. Otro caso sería el análisis de compuestos previamente derivatizados, donde su fragmentación puede generar iones productos poco selectivos originados a partir del propio grupo derivatizante.

A pesar de las limitaciones indicadas anteriormente, la mayoría de guías para la determinación de residuos de plaguicidas no contemplan esta problemática con suficiente detalle. La Unión Europea ha publicado dos guías donde se definen los criterios que deben cumplir los métodos analíticos desarrollados con fines de pre-registro (SANCO/3029/99 rev 4) y de post-registro (SANCO/825/00 rev 7) de plaguicidas. En nuestra opinión, esta normativa no contempla adecuadamente los problemas que, como hemos comentado, pueden aparecer a la hora de confirmar la presencia o no de un analito.

Así, en estudios pre-registro se indica que no es necesaria una confirmación adicional cuando el método resulta específico para la matriz de estudio, ya que la mayoría de estos estudios suelen desarrollarse en un ambiente controlado, es decir, teniendo una muestra blanco idéntica a la tratada y sabiéndose con total seguridad qué producto se ha aplicado. Sólo cuando el análisis se realiza por técnicas de inmunoensayo se aconsejan ensayos de confirmación. Sin embargo, en programas de control post-registro, donde no se dispone generalmente de blancos idénticos a la muestra ni se sabe con seguridad que plaguicida se ha utilizado, la normativa indica la necesidad de utilizar técnicas de confirmación como por ejemplo GC-MS o HPLC-DAD, si el espectro UV es característico. En el caso de utilizarse GC-MS como técnica de análisis se necesitan al menos tres iones relevantes para la confirmación; sin embargo, se considera que la especificidad de la técnica LC-MS/MS es suficientemente alta como para no ser necesario un método adicional de confirmación, lo cual es, como mínimo, debatible.

Recientemente se ha publicado una Decisión Europea (2002/657/EC) donde se establecen los criterios para la cuantificación y confirmación de contaminantes y residuos orgánicos en muestras de alimentos de origen animal, con el fin de garantizar un control seguro y eficaz. Debido posiblemente al mayor uso del LC-MS/MS en el campo farmacéutico y veterinario, la legislación sobre determinación de residuos en este tipo de muestras va por delante de la de muestras vegetales o medioambientales. En estos dos últimos campos no hemos encontrado ninguna normativa detallada, por lo que algunos autores han empezado a aplicar esta guía en el análisis toxicológico o ambiental, con la dificultad añadida de llevar a cabo la confirmación a niveles extremadamente bajos.

La mencionada Decisión Europea divide los contaminantes en 2 grupos. Se incluyen en el grupo A aquellas sustancias que están prohibidas por la ley y en el grupo B aquellos compuestos con un límite máximo de residuo permitido (MRL). Cabe señalar que todos los compuestos estudiados en la presente Tesis pertenecen al grupo B.

Esta Decisión hace uso de una nueva aproximación para unificar criterios en la confirmación espectrométrica de contaminantes y residuos orgánicos, basada en el uso de puntos de identificación (IPs), de tal forma que a cada técnica se le asigna un número de IPs en función de su especificidad inherente. La principal ventaja del uso de los IPs es que la verificación de la identidad puede realizarse de una manera bien descrita e internacionalmente aceptada. Así pues, un laboratorio puede emplear cualquier técnica espectrométrica molecular o combinación de las mismas, con el fin de obtener el mínimo número de IPs necesario para la confirmación de un compuesto. Para confirmar la identidad de compuestos del grupo A se requiere un mínimo de 4 IPs mientras que para sustancias del grupo B se requieren 3. Obviamente este es el número mínimo de IPs requerido, pudiendo ser aumentado en función de la técnica utilizada.

En la Tabla 4.1 se muestran los valores de IPs asociados a cada técnica:

Tabla 4.1 Relación entre las distintas técnicas MS y sus IPs asociados	
Técnica MS	IPs obtenidos por cada ion
MS de baja resolución (LR)	1.0
LR-MS ⁿ ion precursor	1.0
LR-MS ⁿ ion producto	1.5
MS de alta resolución (HR)	2.0
HR-MS ⁿ ion precursor	2.0
HR-MS ⁿ ion producto	2.5

Cabe indicar que la sola presencia de los iones seleccionados para un compuesto no es suficiente, ya que para alcanzar la confirmación satisfactoria de su identidad, se ha de medir al menos una relación de intensidad para cada uno de los iones seleccionados (*ion ratio*). Esta relación iónica tiene que coincidir con la obtenida para un patrón de concentración similar a la obtenida en las muestras supuestamente positivas, de acuerdo con unas tolerancias establecidas en función de su intensidad relativa, y que se detallan en la Tabla 4.2.

Tabla 4.2 Tolerancias máximas permitidas en la medida de relaciones iónicas para la confirmación de contaminantes orgánicos en muestras de origen animal

Intensidad relativa	GC-EI-MS	Otras técnicas
>50%	± 10%	± 20%
20-50%	± 15%	± 25%
10-20%	± 20%	± 30%
≤10%	± 50%	± 50%

Si atendemos a esta normativa, muchos de los métodos multiresiduo desarrollados hasta la fecha únicamente se podrían considerar como métodos de *screening* o como métodos cuantitativos, pero no permitirían la confirmación inequívoca de la identidad de los contaminantes detectados.

En este capítulo se estudia con detalle esta problemática y se compara el potencial de tres analizadores (QqQ, TOF, QTOF) acoplados a LC con fines de confirmación de residuos de plaguicidas.

4.2 Experimental

En nuestro laboratorio ya se había desarrollado un método multiresiduo basado en LC-MS/MS QqQ para la determinación rápida de plaguicidas seleccionados en aguas de la Comunidad Valenciana (Sancho et al, 2004). En este método únicamente se adquiría una transición MS/MS por compuesto, por lo cual había sido usado con fines de *screening* y cuantificación, requiriéndose experiencias adicionales para la confirmación inequívoca de la identidad del compuesto detectado. En la presente Tesis Doctoral se reanalizaron varias muestras positivas, tanto de agua superficial como subterránea, usando QqQ y QTOF, con el fin de estudiar detalladamente la problemática de la confirmación.

4.2.1 Triple Cuadrupolo

Con el fin de alcanzar la sensibilidad requerida, se utilizó un sistema de SPE (233XL, Gilson) acoplado en línea al LC, tal como se ha descrito en el apartado 1.3. Se disponía de una jeringa de 10 mL, por lo que éste era el volumen máximo que se podía inyectar. Finalmente, se utilizó un *loop* de 4.3 mL.

En cuanto a la inyección en LC se realizó mediante llenado total del *loop*. Se tomó un pequeño volumen de aire antes y después de la aspiración de la muestra (*air gap*). De esta manera, el aire desplaza al líquido presente en el *loop* haciendo que no haya mezcla entre éste y la muestra, permitiendo así su llenado completo sin necesidad de aspirar varias veces su volumen.

Para la confirmación de plaguicidas detectados en las muestras bastó con añadir una transición MS/MS más para cada compuesto en el método de *screening* cuantitativo ya desarrollado por LC-MS/MS.

4.2.2 (Q)TOF

Debido a la menor sensibilidad del QTOF respecto al triple cuadrupolo trabajando en SRM, se procedió a preconcentrar volúmenes más grandes de muestra (aproximadamente 20-30 mL). Para ello se modificó el equipo disponible, un procesador de muestra ASPEC XL medioambiental de Gilson (Villiers-le-Bel, Francia), con el fin de llevar a cabo un acoplamiento en línea SPE-LC de manera completamente automatizada.

Adaptación del equipo ASPEC XL

El procesador de muestra ASPEC XL (*Automatic Sample Processing System using Extraction Columns*) es un equipo automático desarrollado por Gilson para llevar a cabo de forma rápida y eficaz la purificación y/o preconcentración de muestra, utilizando cartuchos desechables de SPE con diferentes capacidades (1, 4, 6 y 8 mL). Aunque se trata de un equipo inicialmente diseñado para llevar a cabo SPE *off line* de manera automatizada, aprovechando la versatilidad del ASPEC se planteó su utilización para llevar a cabo la preconcentración en modo *on-line*. Para ello se requirieron importantes modificaciones en cuanto a *hardware* y *software*. En la Figura 4.1 se muestra un esquema del equipo finalmente utilizado en nuestro laboratorio.

En cuanto a las modificaciones de hardware introducidas, se agregó una válvula de 8 vías de baja presión (LP), modelo Gilson 817, que permitió seleccionar cada uno de los canales. También se incorporó una bomba de HPLC (P-1) para llevar a cabo el acondicionamiento de la precolumna y posterior carga de muestra. La primera válvula de alta presión (HV-1) permite purgar los diferentes canales seleccionados en su posición de *inject* y, acondicionar la columna o cargar la muestra, en su posición de *load*. Así pues, el puerto de inyección no es utilizado (IP). También se incorpora otra bomba de HPLC (P-2), que se utiliza para llevar a cabo la transferencia de los analitos desde la precolumna hasta la columna analítica C-2, donde tiene lugar la posterior separación de los mismos, pasando finalmente al sistema de detección, en este caso un QTOF MS.

Las modificaciones en cuanto a software consistieron en el desarrollo de un programa informático utilizando Turbo Pascal como lenguaje de programación, que tomara control directo de todos los módulos del ASPEC. Una vez escrito este programa, se compiló dando lugar a un archivo ejecutable desde el pequeño ordenador suministrado junto con el procesador de muestra, que no era modificable desde este último módulo. El programa desarrollado y utilizado en este trabajo se muestra en el Apéndice 1.

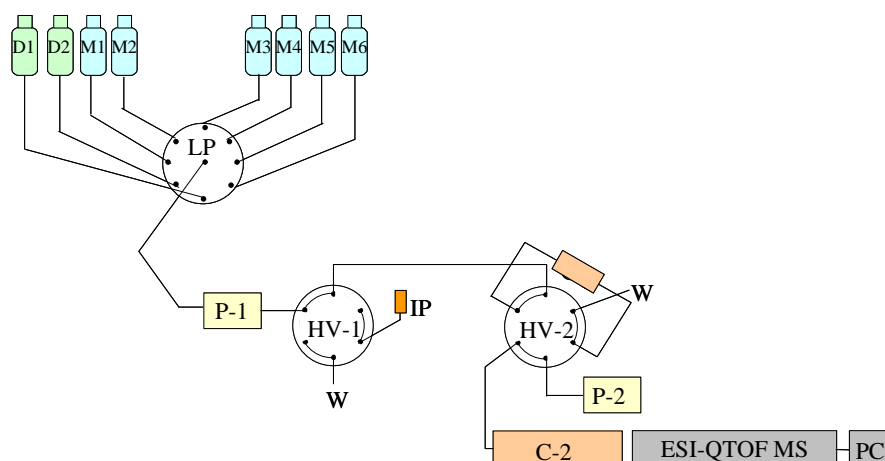


Figura 4.1 Representación esquemática del equipo instrumental utilizado en el acoplamiento SPE-LC-QTOF MS (ASPEC XL, Gilson, modificado): disolventes acondicionadores; M: muestras de agua; P-1: Bomba preconcentradora; C-1: Columna/cartucho preconcentrador; P-2: Bomba para la elución y separación cromatográfica; C-2: columna analítica; LP: válvula de baja presión; HV-1 y HV-2: válvulas de alta presión. IP: puerto de inyección para inyecciones mediante *loop*. W: Deshecho

4.2.3 Comparación “233 XL” vs “ASPEC XL”

Los dos sistemas de SPE utilizados en esta Tesis (SPE-LC con el equipo de QqQ y SPE-LC con el QTOF) presentan claras diferencias entre ellos. En cuanto al volumen de muestra, con el sistema SPE del QTOF se pueden inyectar volúmenes mucho mayores que con el usado con el QqQ. Así, en el caso del triple cuadrupolo se puede inyectar como máximo 10 mL, ya que este es el volumen de la jeringa disponible. En el caso del QTOF el máximo volumen a inyectar viene determinado más por el cartucho y la posibilidad de *breakthrough* que por una limitación propia del sistema. Sin embargo, un mayor volumen de muestra repercute en un mayor tiempo de análisis, pues más tiempo tardará el sistema en cargar/inyectar la muestra. Por otra parte, el número de muestras que se pueden procesar es mucho más limitado en el caso del QTOF, siendo el máximo 6. Con el sistema usado en QqQ, se pueden procesar muchas más muestras de manera automatizada, lo que permite dejar secuencias nocturnas con total comodidad. En base a estas observaciones, y teniendo en cuenta que la cromatografía no variaba sustancialmente de un sistema al otro en cuanto a tiempo, podríamos concluir que la capacidad en cuanto al número de muestras analizadas (*sample throughput*) es mucho mayor en el caso del triple cuadrupolo. Finalmente, la comodidad del software en el caso del triple es mucho mayor que en el QTOF, donde cada vez que se desea realizar un cambio en el programa hay que compilarlo.

4.3 Resultados y discusión

En este capítulo se incluyen dos artículos científicos que se consideran suficientemente ilustrativos sobre el tema objeto de estudio, como para no requerir una discusión adicional en estas líneas. En el *artículo 7* se evalúa el potencial de LC-MS con analizadores QqQ y QTOF en la confirmación de contaminantes orgánicos en muestras medioambientales, centrándonos en los logros y limitaciones de este proceso. En este trabajo se han evaluado alrededor de 1000 detecciones positivas en muestras de agua, observando ciertas limitaciones (falsos positivos/falsos negativos) al aplicar los criterios de confirmación establecidos. Este es un tema relevante, al que sin embargo, no se le ha prestado mucha atención hasta la fecha, especialmente cuando se usa LC-MS, con el consiguiente riesgo de generar datos de pobre calidad analítica en cuanto a la confirmación de la identidad del analito. En este artículo se propone una estrategia para mejorar la fiabilidad del proceso de confirmación, usando ambos instrumentos.

El *artículo 8* se centra exclusivamente en el estudio del (Q)TOF en cuanto a la confirmación de contaminantes orgánicos. En él se muestran, con ejemplos, las aplicaciones y limitaciones de esta técnica. Así, a pesar del cumplimiento de las guías vigentes algunas limitaciones pueden ser involuntariamente ignoradas, especialmente en la confirmación de analitos a bajos niveles de concentración, con lo que aún existe la posibilidad de reportar falsos positivos/ negativos.

A continuación, se muestran los resultados obtenidos al analizar una muestra de agua subterránea mediante los tres analizadores (QqQ, TOF y QTOF), con el fin de ilustrar con mayor detalle el proceso general seguido en este estudio comparado.

4.3.1 Triple cuadrupolo

La posibilidad de añadir transiciones MS/MS adicionales para algunos plaguicidas hizo posible la confirmación de su identidad utilizando el triple cuadrupolo. Así, se confirmaron diversos positivos en muestras de agua superficial y subterránea calculando la relación entre las áreas de los picos para las dos transiciones seleccionadas (Q/q). La muestra se consideraba confirmada si la diferencia entre el valor obtenido para la muestra y para un patrón de concentración similar no excedía las tolerancias permitidas (Tabla 4.2). Comentar que en el caso de la simazina, terbutrina y terbumetona se obtuvieron 4 IPs ya que se utilizó un mismo ion

precursor para las dos transiciones, mientras que en el caso de la terbutilazina se obtuvieron 5, debido al uso de dos precursores distintos.

En la Figura 4.2 se muestran los cromatogramas obtenidos para los 4 plaguicidas detectados en una muestra de agua subterránea procedente de Carcaixent, tomada el día 11 de julio de 2003, tras ser analizada con el triple cuadrupolo. Pueden observarse los cromatogramas para las dos transiciones correspondientes a terbutilazina (230.1>174.1 y 232.1>176.1), simazina (202.1>132.1 y 202.1>124.2), terbutrina (242.1 >186.2 y 242.1>71.1) y terbumetona (226.1>170.2 y 226.1>114.1), tanto para la muestra como para el patrón. Todos los compuestos fueron confirmados, pues presentaron una relación Q/q similar en muestras y patrones. Así, en el caso de la terbutilazina, la relación Q/q para el patrón es 3.26 mientras que en la muestra real se obtuvo una relación de 3.34. La desviación es muy pequeña (2.45%), lo que permite confirmar su presencia. De la misma manera se confirmó la presencia de los otros herbicidas mostrados en la Figura 4.2. Los resultados obtenidos para la relación teórica (patrones) y experimental (muestras), así como la desviación y la concentración obtenida para cada compuesto se muestran en la Tabla 4.3.

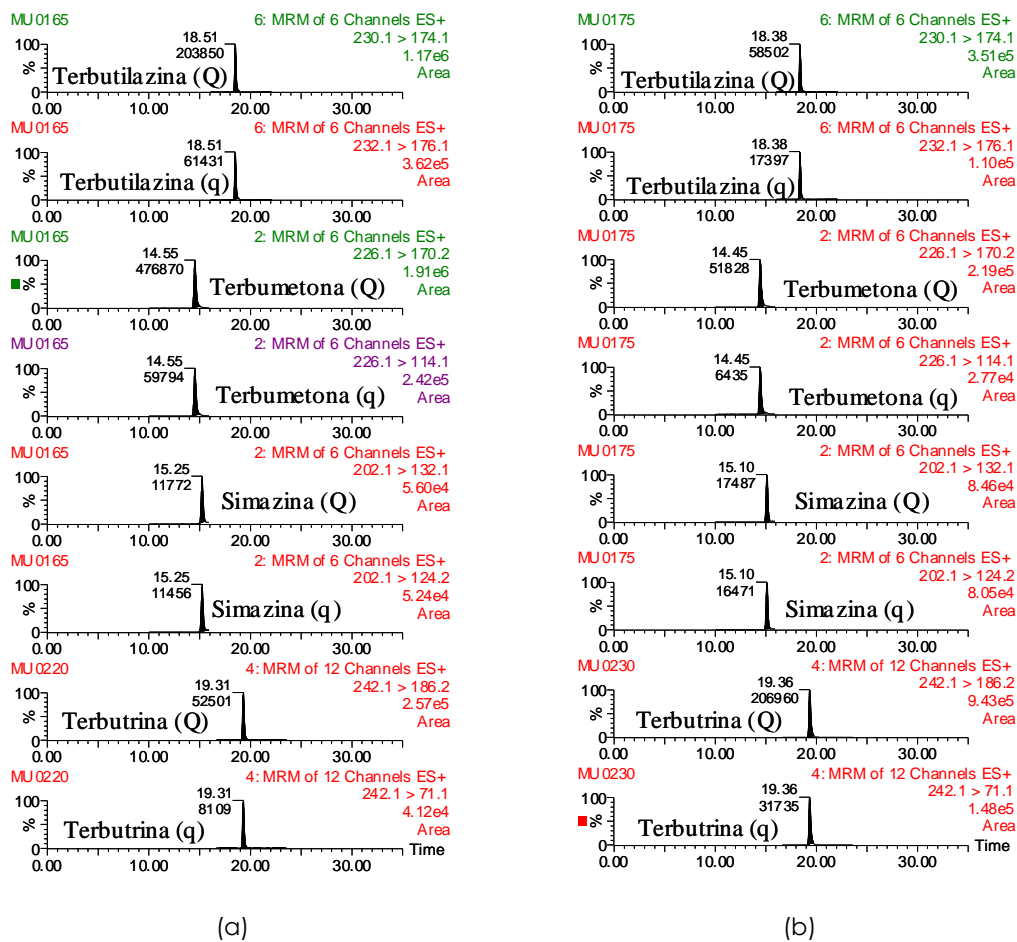


Figura 4.2 Análisis realizado por SPE-LC-MS/MS (QqQ). Confirmación de los plaguicidas detectados en una muestra de agua subterránea procedente de Carcaixent (11-07-03). (a) Muestra, (b) Patrón. (Q) transición de cuantificación y (q) transición de confirmación.

Tabla 4.3 Desviaciones obtenidas en el ion ratio en la confirmación por triple cuadrupolo de plaguicidas detectados en el agua subterránea de Carcaixent (11-07-03)

Compuesto	(Q/q) en patrón	(Q/q) en muestra	Desviación (%)	Concentración muestra (µg/L)
Terbutilazina	3.26	3.34	2.45	0.389
Simazina	1.05	1.02	-2.85	0.073
Terbumetona	7.94	7.9	-0.5	0.886
Terbutrina	6.58	6.3	-4.2	0.019

Como puede observarse en la tabla anterior, todas las desviaciones obtenidas para los compuestos cuantificados fueron inferiores al 5%, un valor mucho menor al permitido en la Decisión 2002/657/EC (ver Tabla 2.5). Este hecho es notorio, sobretodo en el caso de la terbutetona donde la decisión permite una desviación del $\pm 30\%$ ya que el ion producto presenta una abundancia del 10-20% (es decir, un valor de Q/q entre 5 y 10).

4.3.2 Tiempo de vuelo

Otra manera de confirmar muestras positivas es la utilización de instrumentos de alta resolución con capacidad de medida de masa exacta. En el caso del QTOF, en primer lugar se realizaron experiencias en *full scan* (modo TOF) para confirmar la presencia de los plaguicidas por medidas de masa exacta. Para ello, se procesaron 20 mL de muestra con el dispositivo ASPEC-XL anteriormente descrito. En este caso será necesaria la adquisición de dos iones junto con la medida de la relación de abundancia entre ellos para, en principio, obtener una confirmación adecuada de la identidad del analito.

La extracción a la masa de la terbutilazina con una ventana de 0.2 Da generó un único pico a 39.46 minutos. Como se puede apreciar en la Figura 4.3, el espectro correspondiente a este pico cromatográfico presentó una señal intensa a m/z 230.1189, cuya masa exacta difería 1.7 mDa de la masa exacta de la molécula protonada de la terbutilazina. En este espectro también puede observarse un ión a m/z 232.1132 con una abundancia relativa aproximada del 30% del pico base. Este ión indica claramente la presencia de un átomo de cloro en la molécula de la terbutilazina, proporcionando una confirmación adicional de la misma. Otra manera de obtener los dos iones necesarios para confirmar la presencia de este compuesto sería el uso de un fragmento producido en la fuente.

De modo análogo, se pudo confirmar la presencia del resto de plaguicidas y también algunos de sus productos de transformación. Se pudo incluso confirmar la presencia de terbutrina a una concentración tan baja como 0.019 $\mu\text{g/L}$.

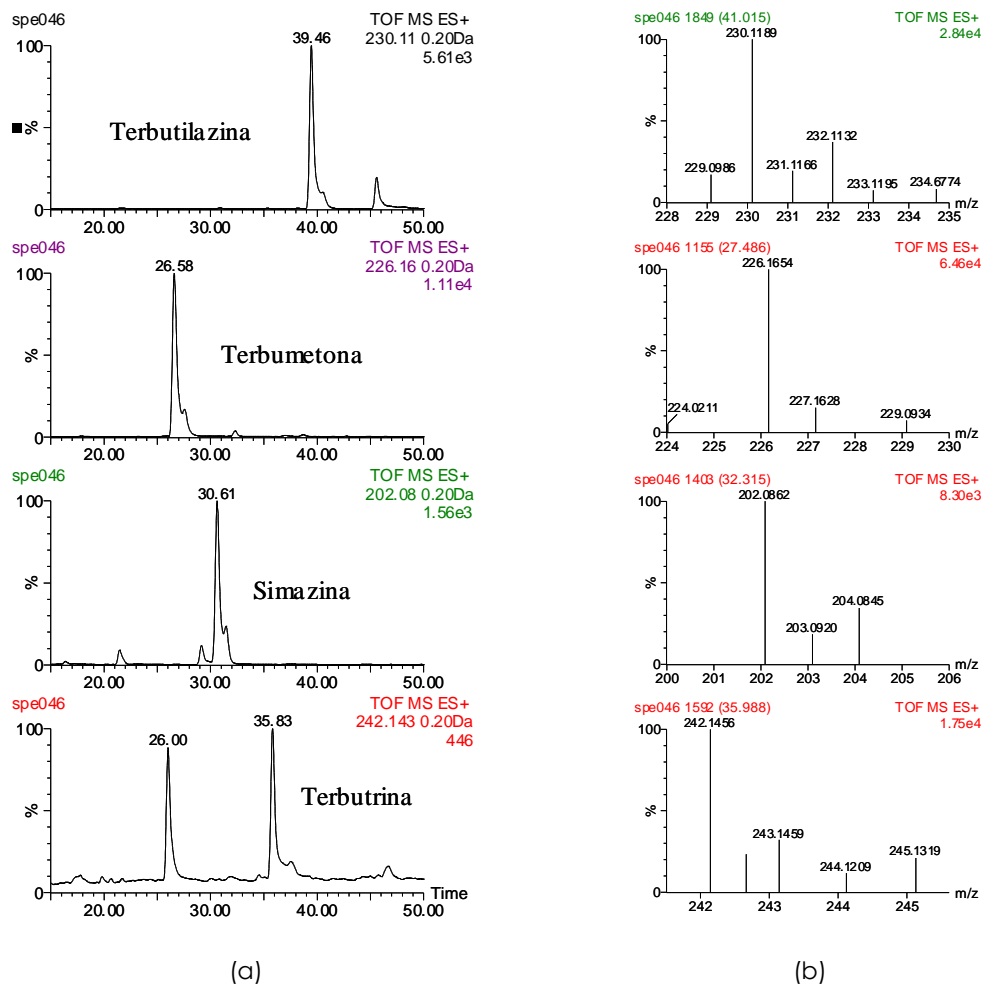


Figura 4.3 Confirmación mediante SPE-LC-TOF MS de la presencia de terbutilazina, simazina, terbumetona y terbutrina en un muestra de agua subterránea de Carcaixent. (a) XIC's a las correspondientes m/z (b) Espectro combinado del pico obtenido

En la Tabla 4.4 se muestra la masa teórica esperada para los iones encontrados en esta muestra, así como su masa experimental y el error en mDa. Se puede observar el bajo error obtenido, lo que permite confirmar la presencia de los plaguicidas indicados por TOF MS en modo *full scan*.

Tabla 4.4 Resultados obtenidos en la confirmación por TOF de plaguicidas detectados en el agua subterránea de Carcaixent (11-07-03)

Compuesto	Fórmula	m/z teórica [M+H] ⁺	m/z exper. [M+H] ⁺	Conc. en muestra (µg/L)	Error (mDa)
<i>Terbutilazina</i>	C ₉ H ₁₆ N ₅ Cl	230.1172	230.1189	0.389	1.7
<i>Terbumetona</i>	C ₉ H ₁₀ N ₅ O	226.1668	226.1654	0.886	1.4
<i>Simazina</i>	C ₇ H ₁₂ N ₅ Cl	202.0859	202.0862	0.073	0.3
<i>Terbutrina</i>	C ₁₀ H ₁₉ N ₅ S	242.1459	242.1456	0.019	0.3

4.3.3 Cuadrupolo-Tiempo de vuelo

Finalmente, dada la disponibilidad de un equipo híbrido QTOF, se pudo realizar una confirmación adicional determinando la masa exacta de los iones producto. Para ello se realizaron experiencias MS/MS con el equipo QTOF, utilizando el resto de ion precursor, todavía presente, como *lock mass*. A modo de ejemplo, se muestran los cromatogramas MS/MS obtenidos para las 4 triazinas anteriormente comentadas (Figura 4.4). La obtención de numerosos fragmentos con QTOF en todos los casos (ver Tabla 4.5), hizo que se obtuvieran un número elevado de IPs. Así, en el caso de la terbutilazina la obtención de 6 fragmentos con QTOF (Tabla 4.5) hizo que se consiguieran 13 IPs. Este número de IPs se podría incluso haber duplicado debido a la presencia de un átomo de Cl en la molécula, realizando la fragmentación del isótopo correspondiente al ³⁷Cl. De esta manera se comprueba la gran utilidad de estos instrumentos en la confirmación de muestras positivas.

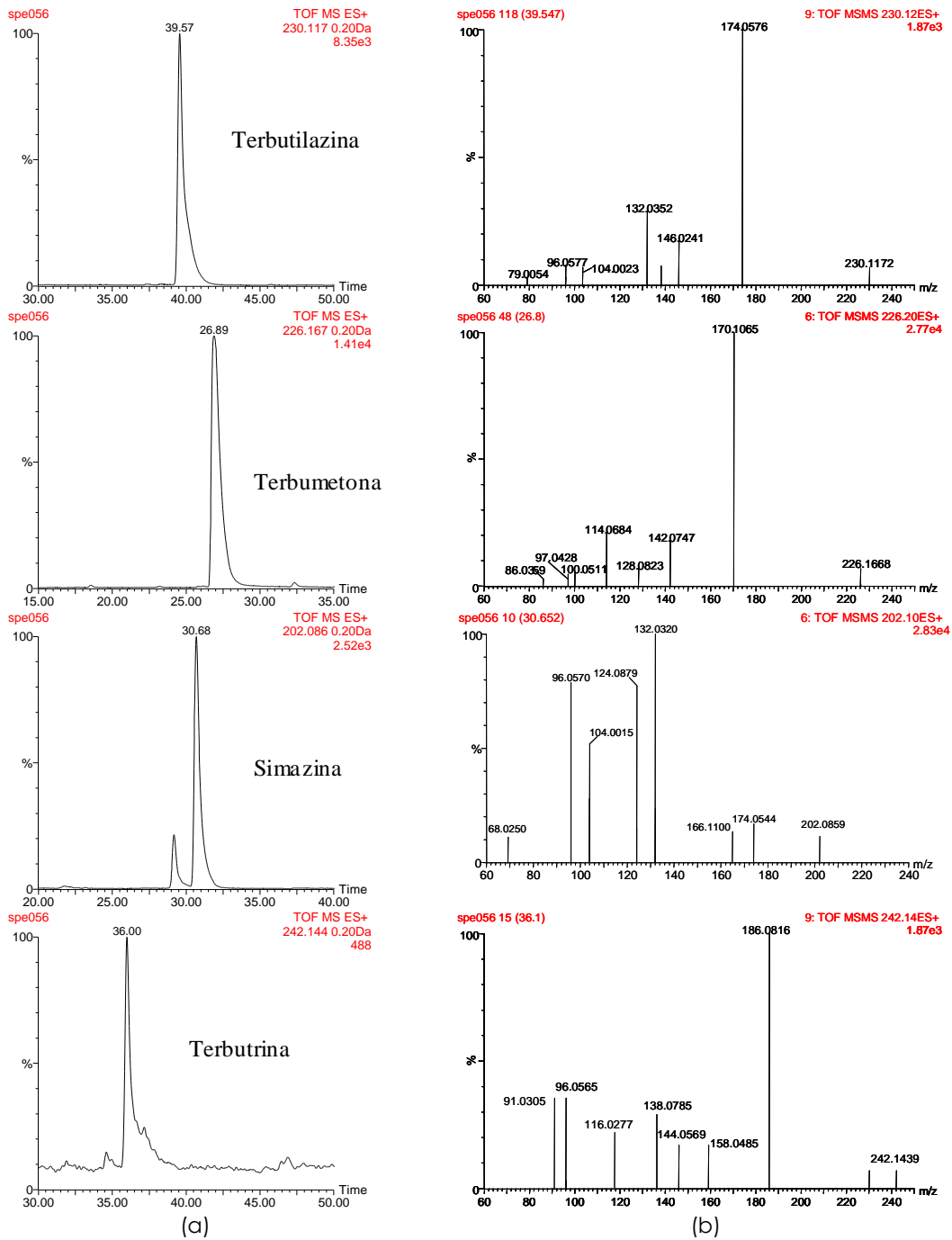


Figura 4.4 Confirmación mediante SPE-LC QTOF MS de la presencia de terbutilazina y terbumetona en una muestra de agua subterránea de Carcaixent. (a) Cromatogramas en modo MS/MS; (b) Espectros combinados de los picos obtenidos

Tabla 4.5 Resultados obtenidos en la confirmación por QTOF de los herbicidas terbutilazina, terbumetona, simazina y terbutrina detectados en el agua subterránea de Carcaixent (11-07-03)

<i>Terbutilazina</i>				
Pérdida	m/z teórica	m/z experimental	Error (mDa)	Error (ppm)
-(CH ₃) ₂ C=CH ₂	174.0546	174.0576	3	-17.2
-(CH ₃) ₂ C=CH ₂	146.0233	146.0241	0.8	5.4
-CH ₂ =CH ₂				
-(CH ₃) ₂ C=CH ₂	132.0329	132.0352	2.3	17.4
-NH ₂ -CN				
-(CH ₃) ₂ C=CH ₂	104.0015	104.0023	0.8	7.7
-NH ₂ -CN				
-CH ₂ =CH ₂				
-(CH ₃) ₂ C=CH ₂	96.0562	96.0577	-1.5	15.6
-NH ₂ -CN				
-HCl				
-(CH ₃) ₂ C=CH ₂	79.0063	79.0054	-0.9	-11.39
-NH ₂ -CN (x2)				
-CH ₂ =CH ₂				
<i>Terbumetona</i>				
Pérdida	m/z teórica	m/z experimental	Error (mDa)	Error (ppm)
-(CH ₃) ₂ C=CH ₂	170.1042	170.1065	2.3	13.5
-(CH ₃) ₂ C=CH ₂	142.0729	142.0747	1.8	12.7
-CH ₂ =CH ₂				
-(CH ₃) ₂ C=CH ₂	128.0824	128.0823	-0.1	0.78
-NH ₂ -CN				
-(CH ₃) ₂ C=CH ₂	114.0667	114.0684	1.7	14.9
-NH ₂ -CN				
-CH ₃				
-(CH ₃) ₂ C=CH ₂	100.0511	100.0511	0	0
-NH ₂ -CN				
-CH ₂ =CH ₂				
-(CH ₃) ₂ C=CH ₂	86.0354	86.0360	0.6	6.9
-NH ₂ -CN				
-CH ₂ =CH ₂				

Tabla 4.5 (cont) Resultados obtenidos en la confirmación por QTOF de los herbicidas terbutilazina, simazina, terbutrina y terbumetona detectados en el agua subterránea de Carcaixent (11-07-03)

<i>Simazina</i>				
Pérdida	m/z teórica	m/z experimental	Error (mDa)	Error (ppm)
-CH ₂ =CH ₂	174.0546	174.0544	-0.2	1.14
-HCl	166.1093	166.1100	0.7	4.2
-CH ₂ =CH ₂	132.0329	132.0320	-0.9	6.8
-NH ₂ -CN				
-HCl	124.0875	124.0879	-0.4	3.2
-NH ₂ -CN				
-CH ₂ =CH ₂ (x2)	104.0015	104.0015	0	0
-NH ₂ -CN				
-HCl	96.0562	96.0570	0.8	8.3
-NH ₂ -CN				
-CH ₂ =CH ₂				
-HCl	68.0241	68.0250	0.9	13.2
-NH ₂ -CN				
-CH ₂ =CH ₂ (x2)				
<i>Terbutrina</i>				
Pérdida	m/z teórica	m/z experimental	Error (mDa)	Error (ppm)
-(CH ₃) ₂ C=CH ₂	186.0813	186.0816	0.3	1.6
-(CH ₃) ₂ C=CH ₂	158.0500	158.0485	-1.5	9.4
-CH ₂ =CH ₂				
-(CH ₃) ₂ C=CH ₂	144.0595	144.0569	-2.6	18.0
-NH ₂ -CN				
-(CH ₃) ₂ C=CH ₂	138.0780	138.0785	0.5	3.6
-CH ₃ -S·				
-(CH ₃) ₂ C=CH ₂	116.0282	116.0277	0.5	4.3
-NH ₂ -CN				
-CH ₂ =CH ₂				
-(CH ₃) ₂ C=CH ₂	96.0562	96.0565	0.3	3.1
-NH ₂ -CN				
-CH ₃ -S·				
-(CH ₃) ₂ C=CH ₂	91.0330	91.0305	-2.5	27.5
-NH ₂ -CN (x2)				
-CH ₂ =CH ₂				

En la Tabla 4.6 se muestran las desviaciones observadas en el *ion ratio* de los cuatro compuestos a confirmar en relación con un patrón de concentración similar.

Tabla 4.6 Desviaciones obtenidas en el *ion ratio* en la confirmación de los herbicidas terbutilazina, terbumetona, simazina y tebutrina detectados en el agua procedente de Carcaixent (11-07-03)

Ion	m/z	Abundancia		
		Patrón	Muestra	Desviación(%)
<i>Terbutilazina</i>				
C ₅ H ₉ N ₅ Cl	174.0546	100	100	-
C ₃ H ₅ N ₅ Cl	146.0233	24.1	25.8	7.0
C ₄ H ₇ N ₃ Cl	132.0329	35.7	32.1	10.1
C ₂ H ₃ N ₃ Cl	104.0015	11.6	9.1	21.5
C ₄ H ₆ N ₃	96.0562	15.6	13.4	14.1
CH ₄ N ₂ Cl	79.0063	6.2	5.4	12.9
<i>Terbumetona</i>				
C ₆ H ₁₂ N ₅ O	170.1042	100	100	-
C ₄ H ₈ N ₅ O	142.0729	18.6	18.7	0.5
C ₅ H ₁₀ N ₃ O	128.0824	7.75	6.3	18.8
C ₄ H ₈ N ₃ O	114.0667	24.4	22	9.8
C ₃ H ₆ N ₃ O	100.0511	5.03	6	19.2
C ₂ H ₄ N ₃ O	86.0354	2.3	3.1	34.8
<i>Simazina</i>				
C ₅ H ₉ N ₅ Cl	174.0546	15.6	13.2	15.4
C ₇ H ₁₂ N ₅	166.1093	12.5	10.0	20
C ₄ H ₇ N ₃ Cl	132.0329	100	100	-
C ₆ H ₁₀ N ₃	124.0875	78.1	77.1	12.8
C ₂ H ₃ N ₃ Cl	104.0015	53.1	52.1	1.9
C ₄ H ₆ N ₃	96.0562	78.1	77.1	12.8
C ₂ H ₂ N ₃	68.0241	10	8	20
<i>Tebutrina</i>				
C ₆ H ₁₂ N ₅ S	186.0813	100	100	-
C ₄ H ₈ N ₅ S	158.0500	17.1	16.1	5.8
C ₅ H ₁₀ N ₃ S	144.0595	17.1	16.1	5.8
C ₅ H ₈ N ₅	138.0780	31.5	27.2	13.6
C ₃ H ₆ N ₃ S	116.0282	22.8	24.6	7.9
C ₄ H ₆ N ₃	96.0562	37.1	33.0	11.0
C ₂ H ₇ N ₂ S	91.0330	37.1	33.0	11.0

Como puede verse, las desviaciones obtenidas para los compuestos estudiados fueron en todos los casos inferiores a los permitidos por la Decisión 2002/657/EC, confirmando por lo tanto su presencia.

Siguiendo la misma metodología, se investigaron 20 muestras de agua con el fin de demostrar el potencial del QqQ y del QTOF en la confirmación de plaguicidas en aguas.

A modo de conclusión general, cabe indicar que con instrumentos de triple cuadrupolo, la manera más sencilla de confirmar un positivo es la adquisición de, al menos, dos transiciones selectivas para cada analito. De esta forma, se obtendrían 1.5 IPs por cada ion producto y 1 adicional debido a la medida del ion precursor (total 4 IPs, suficientes para una confirmación satisfactoria). Así, si se dispone de un método de *screening* ya desarrollado, normalmente adquiriendo una transición por analito, bastaría con añadir una transición más para cada uno. Sin embargo, la adquisición del doble de transiciones puede generar, como ya hemos comentado con anterioridad, algunos problemas debido a la disminución en el tiempo de adquisición de cada transición o en el número de puntos obtenidos por pico. Así, en métodos multiresiduo se debe alcanzar un compromiso entre sensibilidad y forma de pico. Una aproximación adecuada para la confirmación inequívoca es la de realizar una segunda inyección, añadiendo el máximo número de transiciones para cada compuesto que haya dado positivo en el primer análisis, en cuyo caso, sería necesario realizar al menos dos inyecciones por muestra.

En el caso de que se incluyan compuestos con propiedades analíticas tan diferentes que imposibiliten la optimización cromatográfica simultánea (por ejemplo, compuestos aniónicos, catiónicos y neutros), existe la posibilidad de dividir los analitos en varios grupos con el fin de determinar cada compuesto bajo sus condiciones óptimas. Esto permitiría seleccionar dos transiciones para cada compuesto, y utilizar la más sensible para la cuantificación y la menos sensible para la confirmación. Con esta aproximación, las muestras se analizarían por varios métodos complementarios, permitiendo cada uno de ellos la simultánea detección, cuantificación y confirmación de los analitos incluidos en los mismos con excelente sensibilidad y fiabilidad.

Otra manera de confirmar la presencia de plaguicidas es la utilización de instrumentos de alta resolución. En el caso del QTOF, debido a la posibilidad de obtener espectros tanto de MS como de MS/MS con masa exacta, es posible la obtención de 4 IPs con la medida de sólo dos iones en modo MS. Sin embargo, parece más conveniente la adquisición de todos los iones producto (modo MS/MS) con la mayor exactitud de masa. Con ello, se obtendría un mayor número de IPs debido al elevado número de productos que pueden estar presentes, número que podría ser duplicado en el caso de compuestos con *pattern* isotópico característico.

Sin embargo, la Decisión Europea en algunos casos resulta ambigua sobre si los instrumentos TOF pueden ser considerados como instrumentos de alta resolución, ya que normalmente no alcanzan la resolución requerida para esta clasificación. Así, aunque algunos autores consideran los TOF como instrumentos de alta resolución y por tanto asignan 2.5 IPs a cada ion producto, la asignación de un número intermedio de IPs sería una opción interesante, tal como discutiremos en el *artículo 7*. De todos modos, el uso de QTOF para la confirmación conduce al máximo número de IPs, permitiendo la confirmación última de muestras positivas. La principal limitación del QTOF se encuentra en su menor sensibilidad en comparación con equipos de QqQ en modo SRM, lo cual dificulta la confirmación a bajos niveles de concentración (normalmente inferiores a 0.05 µg/L)

Artículo científico 7

Anal. Chem. (2004) 76: 4349-4357

COMPARISON OF DIFFERENT MASS SPECTROMETRIC TECHNIQUES COMBINED WITH LIQUID CHROMATOGRAPHY FOR CONFIRMATION OF PESTICIDES IN ENVIRONMENTAL WATER BASED ON THE USE OF IDENTIFICATION POINTS

Félix Hernández, María Ibáñez, Juan V. Sancho, Óscar J. Pozo

Analytical Chemistry, Experimental Sciences Department, University Jaume I, E- 12071, Castellon, Spain

ABSTRACT

Three mass spectrometric techniques have been used and compared for the confirmation of the presence of several pesticides that had been detected in environmental water samples by a previously reported SPE-LC-MS/MS screening method. The 2002/657/EC European Commission Decision establishes the need to obtain at least 3 identification points (IPs) in order to confirm organic residues and contaminants in live animals and animal products. In this paper, a similar approach has been applied for confirmation of pesticides in water samples, using triple quadrupole mass spectrometry (QqQ), time-of-flight mass spectrometry (TOF) and hybrid quadrupole time-of-flight mass spectrometry (QTOF) to achieve the required IPs. The number of IPs collected, the sensitivity and practical advantages and disadvantages of these techniques have been discussed. In summary, the QqQ instrument allowed the confirmation of detected pesticides even at very low concentrations (ng/L) achieving between 4 and 5 IPs when adding confirmatory transitions. The direct confirmation with TOF instrument was only feasible for those compounds showing sufficient sensitivity, isotopic pattern or easy in-source fragmentation. In other cases, the required IPs could be reached by adding IPs earned with this technique to those obtained from the MS/MS screening method. Finally, the use of a QTOF instrument allowed obtaining up to 20 IPs in a single run at relatively high concentrations (sub- $\mu\text{g/L}^{-1}$) as no "ion shopping" was required. Additionally, the application of TOF and QTOF techniques made it possible to detect some non-target organic contaminants, which were not included in the screening method.

INTRODUCTION

Several LC-MS(/MS) methods, based on quadrupole instruments, have been developed in the last few years in order to determine pesticides in environmental waters¹⁻⁴. Although these methods do not permit the determination of some important compounds, such as organochlorine pesticides and PCBs, they are chosen ahead of GC-MS based methods as they present obvious advantages, for example reducing sample pre-treatment and capability to determine very polar compounds and/or transformation products (TPs). In the last years, applications in this field using tandem mass spectrometers have increased due to these instruments becoming more commercially affordable⁵. These methods are usually based on the monitoring of one MS/MS transition for each analyte.

Although MS based methods could be considered as highly selective methods, the occurrence of false positives is possible mainly in the analysis of relatively dirty samples, as some interferences can share the same MS properties as the analyte. This can also occur in water sample analysis and has been reported in some papers, producing some very constructive discussions on the subject⁶⁻⁸.

A new European Commission Guideline for identification and quantification of organic residues and contaminants has been proposed to guarantee effective and reliable control of residues in animals and fresh meat. The EC criteria are based on the use of identification points (IPs), a new approach to set up quality criteria for the spectrometric identification and confirmation of organic residues and contaminants. A laboratory is able to use any molecular spectrometric technique or combination of techniques in order to obtain the minimum number of IPs necessary for the identification of a compound. The number of IPs "earned" by the detection of a precursor/product ion depends on the technique used. If mass fragments are measured, the minimum number of points that must be obtained for group A (banned compounds) is set at four. For compounds with an established maximum residue level (MRL) (group B), a minimum of three IPs is required for satisfactory confirmation of the compound's identity. In order to qualify for the IPs, at least one ion ratio must be measured, all measured ion ratios must agree within specified tolerances (in LC-MS, maximum permitted tolerances range from $\pm 20\%$, for a relative ion intensity of $>50\%$, to $\pm 50\%$, for a relative ion intensity of $<10\%$)⁹⁻¹⁰ and a maximum of three separate techniques can be combined to achieve the minimum number of IPs. Table 1 shows the number of IPs that each of the basic mass spectrometric techniques can earn. The main advantage of using IPs is that the verification of identity can be done in a well-described and internationally accepted way.

Therefore, in the determination of pollutants in animal samples two MS/MS transitions or three different MS ions are necessary in order to have a confident positive. In this way, most of the LC-MS/MS published methods should be considered as screening methods as only one transition is monitored. It can even be found in recent literature^{11,12} examples where single ion analysis is used for identification purposes. In the environmental field, legislation still allows determination with only one MS/MS transition in spite of the possibility of generating false positives¹³. In general, there is a lack of discussion on confirmation requirements in environmental chemistry, especially when using LC-MS based methods, and where the concept of IPs could be useful. Thus, some authors have started to carry out confirmation in the trace analysis of drugs and contaminants in environmental matrices by LC-MS/MS, according to the new EC Guidelines^{14,15}. The specificity of the transitions selected will have to be also considered in the near future, as it plays an important role in the confirmation process.

The use of (Q)TOF in the environmental field is still unexploited mainly due to its high cost, although some authors have started to use the LC/TOF/MS for the analysis of emerging contaminants in the aquatic environment^{16,17}. The first application in this field using LC-QTOF dealt with the screening and identification of 6 selected compounds in spiked water extracts. Moreover, structures of three real unknown compounds were also elucidated; however, the structure of a fourth unknown detected remained unresolved¹⁸. Very recently, QTOF has been applied for the quantification and/or confirmation of organic micro-constituents in a variety of environmental and biological samples^{15,19}. In a previous work²⁰ we demonstrated the potential of the QTOF in this field to elucidate pesticide transformation products (TPs) in surface water samples. In this paper, we explore the capabilities of three mass spectrometric techniques (triple quadrupole mass spectrometry (QqQ), time-of-flight mass spectrometry (TOF) and hybrid quadrupole time-of-flight mass spectrometry (QTOF)) for the identification and confirmation of pesticide residues in water based on the use of IPs.

Additionally, the QTOF instrument is used for the detection of other non-target compounds also present in the water samples. The acquisition of the product ion spectra, together with the exact mass of the fragments for these potential unknowns, will favour their correct identification.

EXPERIMENTAL

Liquid chromatography

The selected mass spectrometer was interfaced to a LC system (Figure 1) based on a 233XL autosampler with a loop of 2000 μL (Gilson, Villiers-le-Bel, France) and 2 pumps: an Agilent 1100 (Agilent, Waldbron, Germany) binary pump used to condition and wash the cartridge (P-1) and a Waters Alliance 2695 (Waters, Milford, MA, USA) quaternary pump used for the chromatographic separation (P-2). The SPE pre-concentration was performed using a PRP-1 polymeric cartridge, 10 x 2 mm i.d. (Teknokroma), as C-1. For the LC separation, a Kromasil column C_{18} , 5 μm 125 x 2.0 mm i.d. (Scharlab), was used as C-2. Mobile phases consisted of water and acetonitrile in P-1 and mixtures of 0.01% HCOOH in acetonitrile – 0.01% HCOOH in water in P-2.

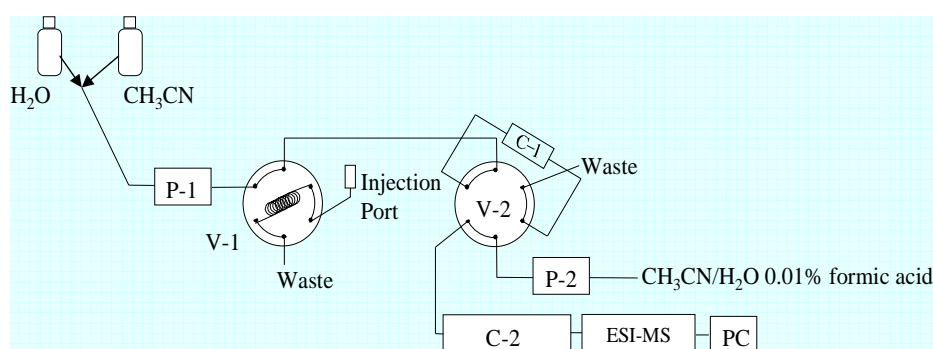


Figure 1. SPE-LC set up; HV = six-port high-pressure valve; P-1 = binary LC pump; P-2 = quaternary LC pump; C-1 = SPE cartridge; C-2 = separation column; MS = mass spectrometer detector; PC = data system; W = waste.

Mass spectrometry

Two mass spectrometers were used: a triple quadrupole mass spectrometer and a hybrid quadrupole time-of-flight (QTOF) mass spectrometer. With these instruments, three different mass spectrometric modes were tested.

The first consisted of a Quattro LC (quadrupole-hexapole-quadrupole) mass spectrometer (Micromass, Manchester, UK) with an orthogonal Z-spray-electrospray interface. Drying gas as well as nebulising gas was nitrogen, generated from pressurized air in a NG-7 nitrogen generator (Aquila, Etten-Leur, NL). The nebuliser gas flow was set to approximately 80 L/h. and the desolvation gas flow to 800-900 L/h. Datastation operating software was MassLynx v4.0.

For operation in MS/MS mode, collision gas was Argon 99.995% (Carbueros Metalicos, Valencia, Spain) with a pressure of approximately $1 \cdot 10^{-3}$ mbar in the collision cell. Capillary voltages of -3 kV and 3.5 kV were used in negative and positive ionization modes respectively. The interface temperature was set to 350°C and the source temperature to 120°C. Dwell times of 0.1 s/scan were chosen.

The hybrid quadrupole time-of-flight (QTOF) mass spectrometer provided with an orthogonal Z-spray-electrospray interface was from Micromass (Manchester, UK). Full scan TOF experiments were carried out with this instrument using the first quadrupole only as a RF-guide; in the case of QTOF experiments, a full product ion spectrum for each detected compound was acquired.

Drying gas as well as nebulising gas was nitrogen, generated from pressurized air in a nitrogen generator from Peak Scientific (Inchinnan, Scotland). The nebuliser gas flow was set to approximately 15 L/h. and the desolvation gas flow to 700-800 L/h. TOF-MS resolution was approximately 5000 (FWHM). MS data were acquired over a m/z range of 60 to 600. The MCP detector potential was set to 2700 V in positive ionization mode and to 2800 V in negative ionization mode.

For operation in MS/MS mode, collision gas was Argon 99.995% (Carbueros Metalicos, Valencia, Spain) with a pressure of approximately $2 \cdot 10^{-5}$ mbar in the collision cell. A cone voltage of 25 V and a capillary voltage of 3.5 kV were used in both positive and negative ionization modes. The interface temperature was set to 350°C and the source temperature to 120°C. Dwell times of 1 s/scan were chosen. A suitable MS profile was used. Data station operating software was MassLynx v4.0.

Calibration experiments were performed daily using a Model 11 single syringe pump (Harvard instruments, Holliston, MA, USA), directly connected to the interface. Calibration in both positive and negative electrospray modes was conducted from 60 to 600 m/z with a mixture of NaOH 0.1M:HCOOH 10% (50:50) diluted (1:25) with acetonitrile:water (20:80), at a flow rate of 20 $\mu\text{L}/\text{min}$.

3,5-diiodo-L-tyrosine, used as lock mass, was introduced post-column using a model 2150 isocratic HPLC pump from LKB (Bromma, Sweden) at a flow rate of 100 $\mu\text{L}/\text{min}$ by means of a PEEK T-union²⁰.

SPE-LC procedure

The conditioning of the PRP-1 cartridge was performed with acetonitrile at a flow-rate of 1 mL/min for 3 min, followed by 4 min more with LC-grade water. An aliquot of 2 mL of water sample was preconcentrated (1 mL/min) into the cartridge and washed with 4 mL of LC-grade water. After washing, the sample was transferred in backflush mode to the C-2 column and a gradient in P-2 started. To perform the chromatographic separation in the three MS instruments assayed, the gradient was water 0.01% HCOOH/acetonitrile 0.01% HCOOH, where the percentage of organic modifier was changed linearly as follows: 0 min., 5%; 2 min., 5%; 55 min., 90%; 58 min., 90 %; 59 min., 5%. The chromatographic separations were completed within 60 minutes.

The determination was carried out by mass spectrometry under optimized conditions, using the three techniques mentioned along this paper.

Sample procedure

Water samples were acidified with formic acid at 1%. 2 mL of the acidified samples were loaded in the loop and directly injected into the SPE-LC-ESI-MS system. Ground and surface water samples had been previously collected in selected sites from the Spanish Mediterranean region.

RESULTS AND DISCUSSION

Screening

A triple quadrupole based screening method for 47 preselected compounds (pesticides and TPs) was developed in our laboratory and applied to approximately 100 ground and surface water samples, collected in selected sites from the Spanish Mediterranean coastal region, where there is a notable agricultural activity. In this MS/MS method, only a single transition for each target compound was used for its determination²¹. Some of the monitored samples contained several pesticides at relatively high concentrations (ppb levels) while other pesticides were detected at the sub- $\mu\text{g/L}$ level. With this method only 2.5 IPs could be obtained (1.5 IPs for the transition + 1 IP for the parent ion in low resolution MS, according to Table 1) and no ion ratio was measured. Thus, it would be convenient that positive samples, especially those containing pesticide levels higher than $0.1 \mu\text{g/L}$, were reanalyzed for the confirmation of the analytes, as this screening method did not achieve the minimum 3 IPs required.

Although with a gradient of about 20-30 minutes the chromatographic separation would be satisfactory for both QqQ and QTOF instruments, the run time was increased up to 60 min to obtain an adequate separation between possible isobaric compounds when TOF analyzer in scan mode was used.

For the subsequent confirmation of the analytes detected in water, three mass spectrometric techniques were applied and their confirmatory capabilities compared: triple quadrupole mass spectrometry (QqQ), time-of-flight mass spectrometry (TOF) and hybrid quadrupole time-of-flight mass spectrometry (QTOF).

Ten selected positive samples where several herbicides, particularly triazines, had been detected were reanalyzed.

Table 1. Relationship between nature of MS information and IPs earned

MS Technique	Ips obtained for each ion ^a
Low resolution (LR) MS	1.0
LR-MS precursor ion	1.0
LR-MS product ion	1.5
High Resolution (HR) MS	2.0
HR-MS precursor ion	2.0
HR-MS product ion	2.5

^a Criterium proposed by the Commision Decision

Table 1 (cont). Relationship between mass accuracy and IPs earned

Mass accuracy	Ips obtained for each ion ^b
<i>Error higher than 10 mDa:</i>	
-Single ion	1.0
-Precursor ion	1.0
-Product ion	1.5
<i>Error between 2-10 mDa:</i>	
-Single ion	1.5
-Precursor ion	1.5
-Product ion	2.0
<i>Error below 2 mDa:</i>	
-Single ion	2.0
-Precursor ion	2.0
-Product ion	2.5

^b Criterium proposed in this paper

Triple quadrupole mass spectrometer

For the confirmation of the identity of the detected pesticides by using triple quadrupole instruments, additional transitions had to be added in order to reach the required IPs. These confirmatory transitions were selected by infusing 5 µg/mL standard solutions and choosing only the two most sensitive. Two transitions were selected as a compromise between sensitivity and correct chromatographic peak shape. Although other available transitions occurred in most of cases, the recording of more transitions usually required a decrease in the dwell time in order to maintain satisfactory chromatography, affecting the sensitivity and difficulting the confirmation at low concentration levels. According to Table 1, the detection of two transition product ions in low resolution MS yields 3 IPs (1.5 IP each) and one additional IP for the parent ion, resulting in 4 points. However, when the product ion spectra showed several abundant ions, it is possible that the most selective transitions were not monitored as we only chose two of them ("ion shopping"). This "ion shopping" could generate false positives, although with very low probability, if the

selected transitions are not specific enough, i.e., loss of water, CO₂, etc. In this sense, the number and concept of earned IPs would have to be revised considering the inherent specificity of the product ion selected. On the contrary, it is sometimes difficult for some compounds to obtain more than one transition that can be used for confirmation, due to the low collision energy applied in the collision cell.

Previously detected pesticides in the samples re-analyzed were confirmed with this technique, even if they were found at very low concentrations (below 0.1 µg/L) as a result of the high sensitivity of this instrument in selected reaction monitoring (SRM) mode. As an example, Figure 2a shows the confirmation of diuron in a sample, at 0.01 µg/L, by the use of an additional transition (q). The confirmation at this low concentration level was feasible due to its favorable product ion spectrum, which allows the use of several transitions with similar sensitivity rendering an ion ratio of nearly 2 (see table 2).

Table 2. Ion ratios obtained in the confirmation of several pesticides found in environmental water samples using triple quadrupole mass spectrometer.

Compound	Ion ratio (Q/q) standard	Ion ratio (Q/q) sample	Deviation (%)
<i>terbutylazine</i>	2.31	3.14	-0.6
<i>simazine</i>	1.00	1.05	5.0
<i>terbumeton</i>	7.37	7.32	-0.7
<i>desisopropylatrazine (DIA)</i>	8.20	8.49	3.5
<i>dimethoate</i>	1.17	1.18	1.0
<i>bromacil</i>	1.46	1.49	2.2
<i>terbacil</i>	3.84	3.86	0.5
<i>diuron</i>	2.30	2.39	3.9

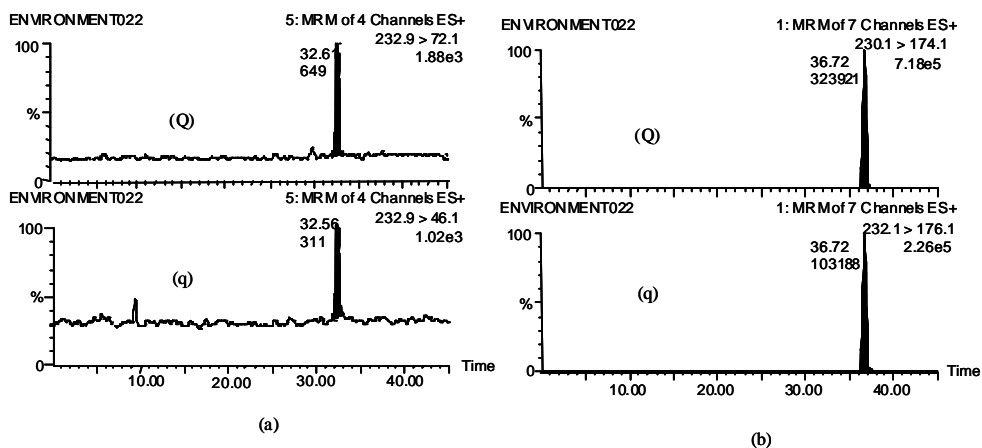


Figure 2. LC-MS/MS (SRM) chromatograms of an environmental water sample containing (a) diuron at $0.01 \mu\text{g L}^{-1}$ and (b) terbutylazine at $0.6 \mu\text{g } \mu\text{g L}^{-1}$; (Q) Quantification transition and (q) confirmation transition.

However, it is common in triple quadrupole instruments to obtain a predominant ion product with higher sensitivity than for the rest. This is the case of desisopropylatrazine (DIA), a triazine transformation product, where the transition $174.1 \rightarrow 104.0$ is much more sensitive than the other one selected ($174.1 \rightarrow 96.1$). For this compound, an ion ratio of 8.20 was obtained, meaning that this TP could only be confirmed at a concentration around 8 times higher than its limit of detection.

This problem could easily be solved in the case of chlorinated or brominated molecules. The presence of an advantageous isotopic pattern in these compounds offers the possibility of choosing the most abundant transitions by selecting different precursor ions, leading to two transitions with the same order of sensitivity. As an example, terbutylazine presented an abundant fragment due to the loss of the terbutyl group ($230.1 \rightarrow 174.1$), with the next fragment ($230.1 \rightarrow 132$) giving a sensitivity almost ten times lower. However, the presence of a chlorine atom in this molecule, allowed us to include the transition $232.1 \rightarrow 176.1$ (corresponding to the ^{37}Cl ion) for confirmation, obtaining an ion ratio of three (see peak areas in Figure 2b). Besides, the selection of two different precursor ions increased the IPs earned to 5.

The ion ratios measured in the water samples for the most frequently detected compounds were compared with those obtained in reference standards (Table 2). The confirmation was considered successful if the ion ratio deviation was lower than 20 %, according to Decision 2002/657/EC.

Time of flight mass spectrometer

To confirm the previously detected pesticides using TOF, an extracted ion chromatogram with a 0.2 Da mass window at the exact m/z corresponding to the pesticide was carried out. If a peak was present at the appropriate retention time, its corresponding spectrum was centered by using lock mass correction in order to measure the exact mass. This mass was then compared with the theoretical value.

The use of exact mass as a confirmatory tool is not explicitly mentioned in the legislation⁹, where the IPs are assigned based on the resolution of the mass analyzer used. The European Guidelines assumes that an ion measured with a high resolution MS would give twice as many IPs as a low resolution MS. This assignment of 2 IPs versus 1 IP, is possibly based on the exact mass capabilities of traditional high resolution mass spectrometers (magnetic sector field, FT-ICR...) rather than on their resolution power. The difficulties possibly appear when one try to classify mass analyzers as low or high resolution mass spectrometers.

TOF analyzers can nowadays reach resolutions between 5000-20000 FWHM, comparable to the traditional sector instruments¹⁶. However, the procedure to measure exact mass differs slightly from that of magnetic sector field mass spectrometers, usually obtaining worse mean mass measurement accuracy than sectors using peak matching mode, but similar values when using magnetic scanning modes²². Moreover, even using quadrupole mass analyzers one can reach high mass accuracy following a sound calibration protocol and working with a sample free of unresolved isobaric interferences²².

In this paper, we propose an alternative criterium for IPs assignment based on mass measurement accuracy rather than on resolution power. Mass errors below 2 mDa are generally accepted as accurate mass measurements¹⁵. However, mass errors slightly higher than 2 mDa still provides better confidence in contrast to low resolution mass analyzers. Therefore, an intermediate position for ions measured with any mass analyzer that renders mass errors between 2 and 10 mDa has been introduced (see Table 1). Thus, using TOF mass analyzers rendering errors below 2 mDa, 2 IPs would be assigned per ion. Obtaining mass errors between 2 and 10 mDa, would lead to only 1.5 IPs per ion. Thus, in order to achieve the minimum IPs required, at least two ions would need to be monitored.

For some compounds, for example terbutryn, increasing the cone voltage of the electrospray source results in an increase of the in-source fragmentation process, and an additional fragment ion could be monitored. In this molecule, the easy loss of the terbuthyl group allowed us to obtain an additional ion at m/z 186.0810, by increasing the cone voltage to 25V.

The mass deviation observed for the two ions measured was lower than 2 mDa and therefore 4 IPs were obtained (Table 3, Figure 3a). However, this is not a common situation as many compounds do not present in-source fragmentation even after increasing the cone voltage. In these cases, only one peak is obtained achieving 1.5 or 2 IPs, insufficient for the correct confirmation by only one analysis, but enough if the earned IPs are added to the 2.5 IPs obtained in the screening method. Nevertheless, this solution implies the sometimes-unaffordable situation of using both triple quadrupole and TOF instruments. The most favorable situation would occur when the analyte presents characteristic isotopic pattern, as occurs for chlorinated compounds. Then, the required IPs could be achieved by determining the exact mass of both ^{35}Cl and ^{37}Cl isotopes as well as their ion ratio. A typical example of this situation is simazine, where its chlorine atom allows the exact mass measurement of the ions 202.0859 and 204.0831 with mass errors of -0.5 and 1.3 mDa respectively, as can be seen in Figure 3b and Table 3. With these two exact mass measurements 4 IPs were earned.

An optimum case is terbuthylazine, as this compound presents both requirements in order to get satisfactory confirmation: a chlorine atom and easy in-source fragmentation. The measurement of four exact masses (Table 3, Figure 3c) allowed earning 7 IPs for this compound, because two of the ions presented mass errors slightly above 2 mDa.

Exact mass measurements and mass errors for some herbicides found in real water samples are shown in Table 3.

A limitation of the TOF instrument is its lower sensitivity relative to the triple quadrupole in SRM mode. For example, the presence of diuron in a water sample was confirmed by QqQ instruments as low as $0.01\mu\text{g}\cdot\text{L}^{-1}$ (see Figure 2a), but no peak was detected in TOF when an extracted ion chromatogram at m/z 233.02 was carried out (Figure 3d). Despite the lack of sensitivity, the confirmation of pesticides at concentrations higher than $0.05\mu\text{g}\cdot\text{L}^{-1}$ was always successful.

However, the main difference of TOF instruments in relation to QqQ instruments is the achievement of the full spectrum acquired at high resolution and mass accuracy, therefore favoring the detection of additional (non target) compounds present in the water samples that were not detected during the screening method. As an example, Figure 3b shows the extract ion chromatogram for the determination of simazine (m/z 202.08, t_r 25.6 min). An additional peak appeared in the chromatogram evidencing the presence of an isobaric compound at 27.3 min, which was confirmed by measuring its exact mass. This unknown isobaric compound also contained a chlorine atom as occurs in simazine, with its typical isotopic pattern.

Table 3. Exact mass measurements. Mass errors for several pesticides found in environmental water samples using time-of-flight mass spectrometer.

Compound	theoretical mass	measured mass	deviation (mDa)	Assigned IPs
<i>terbutryn</i>	242.1439	242.1440	0.1	2
	186.0813	186.0810	-0.3	2
	<i>Total IPs</i>			4
<i>simazine</i>	202.0859	202.0854	-0.5	2
	204.0831	204.0844	1.3	2
	<i>Total IPs</i>			4
<i>terbutylazine</i>	230.1172	230.1177	0.5	2
	232.1145	232.1166	2.1	1.5
	174.0546	174.0530	-1.6	2
	176.0517	176.0539	2.2	1.5
<i>Total IPs</i>			7	
<i>unknown at 27.3 min</i>	202.0859	202.0865	0.6	2
	204.0831	202.0825	-0.6	2
	<i>Total IPs</i>			4
<i>dimethoate</i>	230.0074	230.0131	5.7	1.5
	<i>Total IPs</i>			1.5
<i>bromacil</i>	259.0082	259.0124	4.2	1.5
	261.0062	261.0096	3.4	1.5
	<i>Total IPs</i>			3
<i>terbacil</i>	215.0588	215.0571	-1.7	2
	217.0560	217.0554	-0.6	2
	<i>Total IPs</i>			4
<i>diuron</i>	233.0248	-	-	-

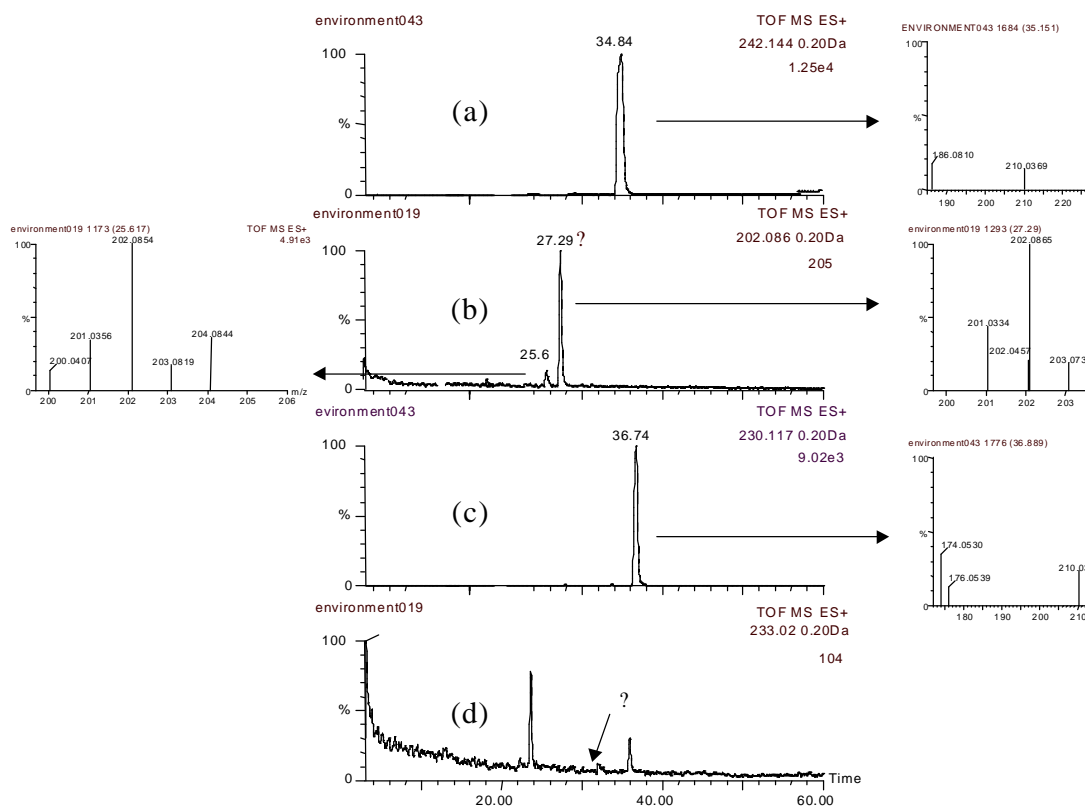


Figure 3. LC-TOF chromatograms and full scan mass spectra of selected environmental water samples containing (a) terbutryn, (b) simazine and unknown, (c) terbuthylazine and (d) diuron (not detected).

Hybrid quadrupole time-of-flight mass spectrometer

The confirmation of pesticides using a hybrid quadrupole time-of-flight analyzer was carried out by selecting the precursor ion of the target analyte in the quadrupole filter and measuring its product ion spectrum, after breakdown in the collision cell by scanning the TOF analyzer (see Figure 4). The exact masses obtained for all product ions were compared with the theoretical ones, and the ion ratios were measured. In order to obtain the exact mass of product ions, the theoretical mass of the precursor ion was used as lock mass. The number of IPs obtained with this technique was 1 for the parent ions and 1.5-2.5 for each product ion measured, depending on their mass accuracy. As the precursor ion was used as lock mass, accuracy mass capabilities of TOF analyzer were lost for this ion. Thus, the IPs assigned were only 1 due to its previous selection using a quadrupole filter. However, although not with our equipment, when using specialized sources that allow the separate introduction of the lock mass calibrant, the remaining precursor ion will receive between 1-2 IPs depending on its mass accuracy, as it can

now be measured adequately. Therefore, the minimum number of required IPs (3) could usually be achieved, even using post-column addition of lock mass and obtaining only one MS/MS transition (1 IP for the parent and 2 IPs for the product ion).

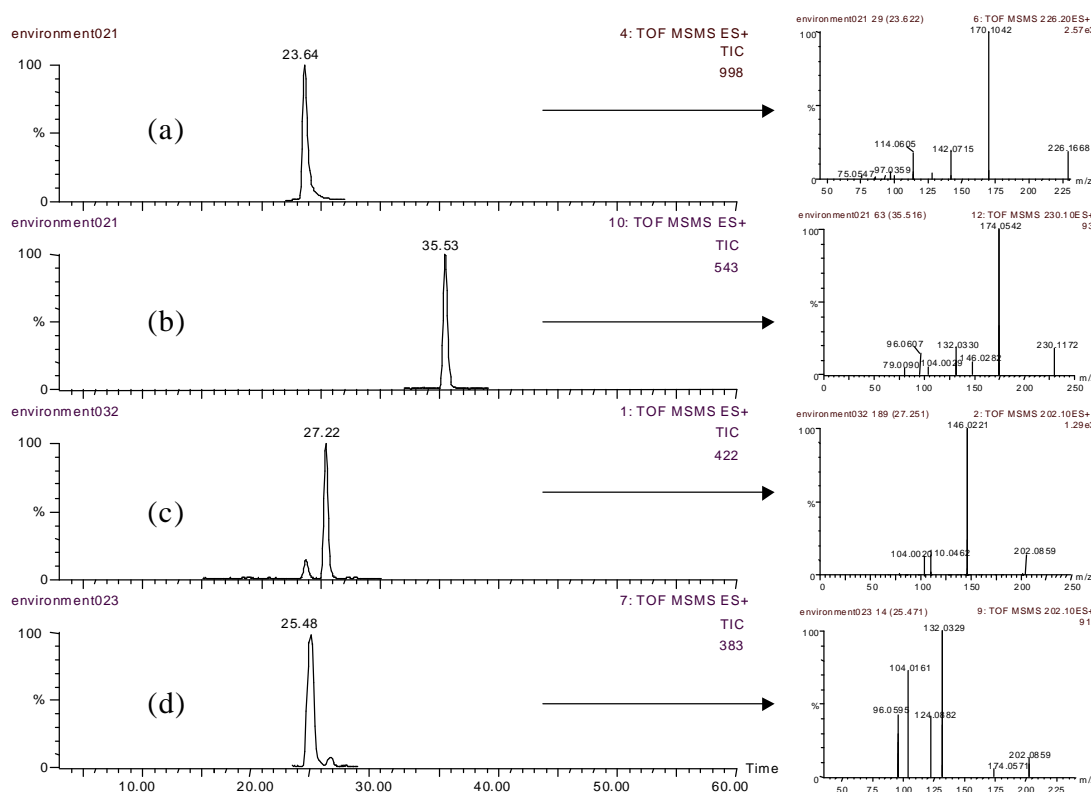


Figure 4. LC-QTOF chromatograms and product ion spectra of selected environmental water samples containing (a) terbumeton, (b) terbuthylazine and (c) simazine and desethylterbuthylazine.

This is one of the most important advantages of the QTOF as confirmatory tool, since some compounds only present one important product ion. In these cases, when this transition is monitored by QqQ instruments, only 2.5 IPs are earned and no ion ratio can be measured; in contrast, using QTOF, the number of IPs obtained is the minimum of 3 required and the ion ratio precursor/product can also be measured.

Moreover, the acquisition of the full product ion spectra in the QTOF allows the potential use of all fragments in order to get the correct confirmation without the need of specifically pre-selecting some of them, i.e. "ion shopping" is not compulsory in QTOF instruments. This characteristic made it possible to increase the IPs up to 19 when the product ion spectra contained 8 fragments, as for example, terbumeton (Table 4, Figure 4a.). For the terbuthylazine confirmation (Figure 4b), up to 29 IPs could be achieved by measuring the exact mass of six major fragments present in the product ion spectra of each precursor ion. In this case, two IPs were earned due to the use of quadrupole filters for the selection of the two parents ions (230 and 232, corresponding to the ^{35}Cl ion and ^{37}Cl ion, respectively). The product ion spectrum for each precursor showed 6 fragment ions with abundance higher than 10%, which added 13.5 IPs per ion (see Table 4), reaching 14.5 IPs for each precursor ion, giving a total number of 29 IPs for this molecule.

Thus, the main advantage of QTOF compared with QqQ instruments comes from the fact that TOF always works in scan mode without loss of sensitivity, generating the full data of the product ion spectra and avoiding "ion shopping". It can be concluded that, because of these features, QTOF instruments allowed an ultimate confirmation for pesticides detected in water.

The main limitation of QTOF is its lower sensitivity in relation to the triple quadrupole instrument¹⁵ (about one order of magnitude), which hampered the confirmation of some pesticides that were detected in the screening method. However, all analytes were confirmed at significant environmental levels (0.1 $\mu\text{g/L}$) in the 10 water samples that were re-analyzed.

Table 4 shows the exact mass measurements and mass errors for the fragment ions selected for several pesticides found in the environmental water samples analyzed.

Additionally, QTOF offered the possibility of obtaining the product ion spectra for unknown (non target) compounds present in the water samples that were detected by a previous TOF scan, helping in their final identification. For example, the product ion spectrum of the compound at 27.3 min with m/z 202.0865 and isobaric with simazine (Figures 4c and 4d), showed fragments at m/z 146.0221, 110.0462 and 104.0020. Combining this information with our knowledge about the fragmentation pathway of triazines and their different transformation products²⁰, we were able to identify this abundant compound as desethylterbuthylazine due to the losses of $(\text{CH}_3)_2\text{C}=\text{CH}_2$, $(\text{CH}_3)_2\text{C}=\text{CH}_2 + \text{HCl}$ and $(\text{CH}_3)_2\text{C}=\text{CH}_2 + \text{NH}_2\text{-CN}$. Moreover, the relatively high concentration of parent terbuthylazine (600 ng/L) in the water sample supported this finding.

Table 5 shows a summary comparing the identification points earned by each mass analyzer for several pesticides selected from those most frequently detected in environmental water using the screening SPE-LC-MS/MS method. Regarding QqQ instruments, the minimum IPs were obtained for all compounds as we always achieved two abundant transitions for each compound. Thus, the confirmation achieved with this technique is in accordance with the European Union Decision, even in the case of banned compounds. The number of IPs increased up to 5 when the analyte presented a halogen atom, due to its characteristic isotopic pattern. As Table 5 shows, IPs obtained with TOF instrument are normally 3 or 4, depending on the exact mass achieved by the instrument and the number of ions that can be measured. In the case where the instrument can not measure the exact mass with less than 2 mDa of error and assuming that either an abundant isotopic pattern or in-source fragmentation is possible, the number of IPs obtained would be 3. In this case, the number of IPs earned by TOF instruments would be insufficient to confirm the identity of banned pollutants. This situation can also occur in other high-resolution mass spectrometers, such as magnetic sectors, where exact mass measurement errors higher than 2 mDa can be also reported. However, the use of QTOF for confirmatory purposes generates the highest number of IPs, normally more than 15, even when errors higher than 2 mDa are obtained. In this way, the QTOF is shown to be the most powerful approach for identification and confirmation of analytes present in water at sub-ppb level.

Our current research involves the use of Metabolynx Application Manager for enhanced detection of unknowns in environmental water samples followed by product ion spectra acquisition in order to elucidate their structures.

Table 4. Exact mass measurements. Fragment mass errors for several pesticides found in environmental water samples using quadrupole time-of-flight mass spectrometer.

Compound	Precursor ion ^a	Theoretical mass fragments	measured mass fragments	Deviation (mDa)	Assigned IPs
<i>terbuthylazine</i>	230	174.0546	174.0542	-0.4	2.5
		146.0233	146.0282	4.9	2
		132.0329	132.0330	0.1	2.5
		104.0015	104.0029	1.4	2.5
		96.0562	96.0607	4.5	2
		79.0063	79.0090	2.7	2
		Parent measured with LR-MS			
<i>Total IPs (³⁵Cl)</i>					14.5
<i>simazine</i>	202	174.0546	174.0571	2.5	2
		132.0329	132.0329	0	2.5
		124.0875	124.0882	0.7	2.5
		104.0015	104.0161	14.6	1.5
		96.0562	96.0595	3.3	2
		Parent measured with LR-MS			
<i>Total IPs</i>					11.5
<i>desethyl terbuthylazine</i>	202	146.0233	146.0221	-1.2	2.5
		110.0467	110.0462	-0.5	2.5
		104.0015	104.0020	0.5	2.5
<i>Total IPs</i>					8.5
<i>terbumeton</i>	226	170.1042	170.1042	0	2.5
		142.0729	142.0715	-1.4	2.5
		114.0667	114.0605	-6.2	2
		100.0511	100.0566	5.5	2
		97.0276	97.0359	8.3	2
		86.0354	86.0360	0.6	2.5
		96.0562	96.0539	-2.3	2
		75.0558	75.0547	-1.1	2.5
		Parent measured with LR-MS			
<i>Total IPs</i>					19

^a Precursor ion measured with LR-MS**Table 5.** IPs earned by each mass analyzer applied in this paper for the confirmation of pesticides found in environmental water samples

Compound	concentration in the water sample (µg/L)	isotopic pattern	in-source fragmentation	QqQ	TOF	QTOF
<i>terbuthylazine</i>	0.60	YES	YES	4-5	6-8	29
<i>simazine</i>	1.36	YES	NO	4-5	3-4	23
<i>terbumeton</i>	0.95	NO	YES	4	3-4	19
<i>diuron</i>	0.01	YES	YES	4-5	-	-

CONCLUSION

The identification of pesticide residues in environmental samples is a problem that remains unresolved. Analytical Guidelines legislation still allow for their determination using only one MS/MS transition in spite of the possibility of generating "false positives".

In a recent European Commission Decision the use of identification points (IPs) is proposed to confirm organic residues and contaminants in live animals and animal products. This approach can also be very useful in environmental matrices to avoid "false positives". A problem arises with the European Commission Decision definition of "high resolution mass spectrometry instruments" independent of the mass accuracy obtained by them. We feel this definition does not take into account sufficiently a relevant parameter for the confirmation of a pollutant, that of exact mass, and therefore disadvantages instruments with high accuracy mass capabilities that do not meet the Decisions "high resolution" specifications. Our work and data demonstrate the importance and advantages of using exact mass and we propose a different approach for the IPs earned depending on the exact mass obtained.

Using triple quadrupole instruments, confirmation of detected pesticides is easily performed by monitoring a secondary confirmatory transition, obtaining the minimum 3 identification points required. Maximum sensitivity is achieved allowing the confirmation of some analytes at concentrations as low as 0.01 µg/L although "ion shopping" of two product ions is often required to confirm several compounds in one run, at the same time avoiding the selection of the less selective transitions, such as loss of water, CO₂, etc.

The use of a time-of-flight mass analyzer allows the confirmation of analytes using its exact mass capability, leading to mass errors normally lower than 3 mDa. However, the number of IPs required can only be reached for compounds with a favorable isotopic pattern or with easy in-source fragmentation, as two ions should always be monitored. Another important limitation in the environmental field is the lower sensitivity of these instruments, which impedes analyte confirmation at concentrations lower than 0.05 µg/L. As an advantage, the TOF analyzer made it possible to detect non-target compounds present in the water samples that were not found by the screening method, giving the exact mass for these unknown compounds.

The use of a hybrid quadrupole time-of-flight analyzer allows an unequivocal confirmation of the pesticides detected, as complete, full scan product ion spectra in exact mass are obtained and, therefore, no "ion shopping" is carried out. With these instruments, a number of IPs higher than 10 can easily be achieved when the product ion spectrum contains several

fragment ions. Even in cases of low fragmentation, enough IPs are reached. However, the lower sensitivity of QTOF instruments compared with the triple quadrupole analyzers made the confirmation of analytes at very low concentration levels (i.e., below 0.1 µg/L) difficult. On the other hand, the QTOF allows obtaining the product ion spectra for unknowns favoring their final identification.

ACKNOWLEDGEMENTS

The authors are very grateful to Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for using the triple quadrupole mass spectrometer (Quattro LC) and the Q-TOF hybrid quadrupole time-of-flight mass spectrometer. This work forms a part of the research project "Aplicación del acoplamiento cromatografía líquida-espectrometría de masas (LC-MS/MS y LC-QTOF MS) a la identificación de metabolitos de plaguicidas en muestras de interés ambiental y toxicológico" (Ref: BQU2003-02685) developed under financial support of the Ministry of Science and Technology, Spain.

REFERENCES

- (1) Yu, K.; Krol, J.; Balogh, M. *Anal. Chem.* **2003**, 75, 4103-4112
- (2) Wang, N.; Budde, W.L.; *Anal. Chem.* **2001**, 73, 997-1006
- (3) Hernández, F.; Sancho, J.V.; Pozo, O.; Lara, A.; Pitarch, E. *J Chromatogr. A.* **2001**, 939, 1-11
- (4) Hogenboom, A.C.; Hofman, M.P.; Kok, S.J.; Niessen W.M.A., Brinkman, U.A.T. *J Chromatogr. A* **2000**, 829, 379-390
- (5) Reemtsma, T. *Trends Anal. Chem.* **2001**, 20, 500-517
- (6) Kolpin, D.W.; Furlong, E.T.; Meyer, M.T.; Thurman, E.M.; Zaugg, S.D.; Barber, L.B.; Buxton, H.T. **2002** *Environ. Sci. Technol*, 36, 1202-1211
- (7) Ericson, J.F.; Laenge, R.; Sullivan, D.E. **2002** *Environ. Sci. Technol*, 36, 4005-4006
- (8) Kolpin, D.W.; Furlong, E.T.; Meyer, M.T.; Thurman, E.M.; Zaugg, S.D.; Barber, L.B.; Buxton, H.T. **2002** *Environ. Sci. Technol*, 36, 4007-4008
- (9) Commission Decision 2002/657/CE of 12 August 2002
- (10) André, F.; De Wasch, K.K.G.; De Brabander, H.F.; Impens, S.R.; Stolker, L.A.A.; van Ginkel, L.; Stephany, R.W.; Shilt, R.; Courtheyn, D.; Nonnaire, Y.; Fürst, P.; Gowik, P.; Kennedy, G.; Kuhn, T.; Moretain, J.P.; Sauer, M. *Trends Anal Chem* **2001**, 20, 435-445
- (11) Yu, K.; Krol, J.; Balogh; Monks, I. M.; *Anal Chem* **2003**, 75, 4103-4112
- (12) Barco, M.; Planas, O.; Palacios, F.; Ventura, F.; Rivera, J.; Caixach, J. *Anal Chem* **2003**, 75, 5129-5136
- (13) Commission Decision SANCO/825/00 rev.6 of 20 June 2000
- (14) Stolker, A.A.M.; Dijkman, E.; Niesing W.; Hogendoorn, E.A. *Liquid chromatography Mass Spectrometry, MS/MS and Time-of-Flight MS: Analysis of Emerging Contaminants.* **2003**, American Chemical Society Symposium Series 850, New York
- (15) Stolker, A.A.M.; Niesing W.; Fuchs, R.; Vreeken, R. J.; Niessen, W. M. A.; Brinkman, U. A. Th. *Anal. Bioanal. Chem.* **2004**, 378, 1754-1761
- (16) Ferrer, I.; Thurman, E.M. *Trends Anal. Chem.* **2003**, 22, 750-756
- (17) Ferrer, I.; Thurman, E.M. *Liquid chromatography Mass Spectrometry, MS/MS and Time-of-Flight MS: Analysis of Emerging Contaminants.* **2003**, American Chemical Society Symposium Series 850, New York

- (18) Bobeldijk, I.; Vissers, J.P.C.; Kearney, G.; Major, H.; van Leerdam, J.A. *J. Chromatogr. A* **2001**, 929, 63-74
- (19) Thurman, E. M.; Ferrer, I.; Benotti, M.; Heine, C. H. *Anal Chem* 2004, 76, 1228-1235
- (20) Ibañez, M.; Sancho, J.V.; Pozo, O.J.; Hernández, F. *Anal. Chem.* 2004, 76, 1328-1335
- (21) Sancho, J.V.; Pozo, O.J.; Hernández, F. *The Analyst.* 2004, 129, 38-44
- (22) Bristow, A.W.T.; Wee, K.S. *J. Am. Soc. Mass Spectrom.* 2003, 14, 1086-1098

Artículo Científico 8

Trends Anal. Chem. (2006) 15: 1030-1042

CONFIRMATION OF ORGANIC MICROPOLLUTANTS DETECTED IN ENVIRONMENTAL SAMPLES BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY: ACHIEVEMENTS AND PITFALLS.Oscar J. Pozo¹, Juan V. Sancho¹, María Ibáñez¹, Félix Hernández^{1*}, W. M. A. Niessen²¹Research Institute for Pesticides and Water, University Jaume I, E-12071, Castellón, Spain²hyphen MassSpec Consultancy, De Wetstraat 8, 2332 XT Leiden, Netherlands**ABSTRACT**

Different approaches to the confirmation of pesticides and antibiotics detected in environmental waters have been evaluated using liquid chromatography (LC) hyphenated to mass spectrometry (MS) -both triple quadrupole (QqQ) and hybrid quadrupole-time of flight (QTOF) analyzers. After analyzing more than 100 groundwater and surface-water samples, we discuss in this article the main achievements and pitfalls of LC-MS in confirmation of positive findings. The acquisition of, at least, two specific Selected Reaction Monitoring (SRM) transitions for each analyte when using LC-tandem MS (LC-MS²) QqQ was found to be an efficient and simple way of achieving safe confirmation, while the use of only one transition would have resulted in some false positives in the samples analyzed.

The use of non-specific transitions, such as those involving the loss of water, might sporadically result in false positive findings even acquiring two transitions, as the probability of finding interferences sharing the same transition increases. Moreover, some false negatives might be reported when acquiring two transitions in when one of them is shared by an interferent, as it would affect the experimental ion-abundance ratio. This possibility is more likely to occur at sub-ppb analyte concentrations than at higher concentration levels. For this reason, we found that the acquisition of the highest number of available transitions was an efficient way to improve reliability in the confirmation process.

The use of QTOF MS can provide an ultimate, unequivocal confirmation of positive findings, due to its inherent advantages of accurate mass and acquisition of complete product-ion spectra. However, false negatives could be reported if a co-eluting isobaric interference was present. In that case, we found that a more efficient chromatographic separation was the best way to improve the confirmation process. The lower sensitivity of QTOF in comparison to QqQ can

be one drawback of this approach, which can hamper the confirmation at very low analyte concentration levels.

INTRODUCTION

Confirmation of positive findings in the environmental field has become a matter of concern due to the undesirable effects associated with erroneous confirmations (i.e. false positives or false negatives). Analytical chemists have to develop reliable methods that allow not only accurate quantification of targeted analytes but also, and even more important, their unequivocal confirmation. Ideally, confirmation should be objective and safe, not depending on the subjective interpretation of the analyst, so predefined and efficient confirmation rules would be required.

Confirmation by gas chromatography coupled to mass spectrometry (GC-MS) has been widely applied in the last years and it is highly supported by the abundant, valuable information contained in electron ionization (EI)-MS spectra and by automated searching of standardized libraries. This confirmation is normally accepted as an ultimate confirmation, due to the large amount and the good quality of the information obtained. When the acquisition is performed in Selected Ion Monitoring mode (SIM), the measurement of three or four ions is normally considered sufficient for a correct confirmation of analytes at low concentration levels [1-3].

In the last decade, increased use of liquid chromatography (LC) coupled to MS (LC-MS) or tandem mass spectrometry (LC-MS²) with atmospheric pressure interfaces (API) has forced reconsideration of the confirmation requirements in order to include these techniques. For this purpose, it is necessary to take into account that the structural information obtained from API-MS spectra is more limited than that from EI-MS spectra [4]. However, the use of MS² provides valuable information from API-MS spectra with the additional benefit of knowledge of the origin of the selected product ions.

There are some guidelines for confirmation that consider different types of ionization and/or scan modes, such as those elaborated by WADA [1], FDA [2] or the European Union [3,5], and a comparison between the different criteria employed was made recently [6-7]. In general, confirmation is based on the acquisition of a limited amount of information. Normally, the acquisition of several ions in single MS, or SRM transitions in MS², together with retention time data and measurement of the ion-intensity ratios, gives sufficient information for a safe confirmation. One of the most detailed criteria regarding comparison of techniques was proposed by the European Union for confirmation of contaminants in samples of animal origin [3] (2002/657/EC),

where confirmation is based on the accumulation of identification points (IPs). This criterion is a valuable tool for a rapid, easy comparison of two MS techniques from the point of view of confirmation. Thus, the number of IPs reached depends on the approach used, differentiating between MS, MSⁿ, and low resolution and high resolution instruments. However, guidelines make normally reference to the quantity of information (number of ions or IPs required) rather than to the quality of this information, as the selection of these ions/transitions may be quite arbitrary and lead to poor-quality information that is insufficient for a safe confirmation. Despite the increased interest in obtaining reliable data by applying confirmation criteria, to our knowledge, there is not specific regulation about confirmation in the environmental field.

Environmental analysis, particularly water analysis, presents several particular differences from other fields which make confirmation of organic pollutants more troublesome. Among these are the extremely high sensitivity needed in some cases and the lack of analytical information on the samples analyzed (e.g., pesticides have to be detected at concentrations around and lower than 0.1 µg/L in drinking waters [8], and some pharmaceuticals, such as antibiotics, are found in environmental water typically at low ng/L levels [9, 10]).

Papers published on the determination of organic contaminants in the environment by LC-MS or LC-MS² rarely deal with the confirmation of analytes detected in samples. This situation may lead one to question the validity of the data reported using these methods [11, 12], unless additional confirmation of the compounds detected is carried out.

Recently, some authors have started to apply confirmation rules to environment samples, normally by extrapolation from previously existing guidelines in other fields. The most usual approach has been acquisition of two transitions in LC-MS/MS [13-15] or several ions in LC-MS methods [16, 17] which conforms to most of existing guidelines. Instruments with high mass accuracy capabilities, such as time-of-flight (TOF)- MS and hybrid quadrupole (Q)-TOF MS have recently been used for confirmation in this field, by acquiring the full-scan spectrum or the product-ion spectrum, respectively [18-20]. The great potential of QTOF analyzers for qualitative analysis makes them valuable, even for elucidating non-target analytes [21-22]. A detailed review of TOF and QTOF applications in environmental analysis was published recently [23].

The goal of this article is to study in depth confirmation of the identity of positive findings in groundwater and surface water samples by LC-MS² (QqQ and QTOF). We report on several examples of false positives for pesticides and antibiotics in water, revealed by applying existing guidelines, and we describe some limitations that would result in false negatives being reported. We point out the importance of selecting specific SRM transitions in QqQ instruments and the need to apply efficient chromatographic separations in some complex situations.

EXPERIMENTAL

SPE-LC-MS/MS

Different LC-MS/MS methods were previously developed in our laboratory for the targeted determination of around 50 polar pesticides and transformation products (TPs) [24,25] and 18 antibiotics [10], the experimental conditions being described elsewhere [10, 25]. In summary, these methods are based on on-line pre-concentration of a small volume of water sample into a solid phase extraction (SPE) cartridge using a Gilson 233XL autosampler. Once pre-concentrated, the analytes are transferred by on-line back-flush to the analytical column where the chromatographic separation is carried out by an Alliance 2695 (Waters, Milford, MA, USA).

Detection and quantification of these target analytes were performed by a Quattro LC QqQ instrument (Micromass, Manchester, UK), using an electrospray interface and time-scheduled acquisition. Confirmation of positive findings revealed by both target methods was explored by two approaches: QqQ working in SRM mode and hybrid QTOF (Micromass) instrument working in product ion full spectrum mode [10, 26].

Samples analyzed

The analytical methodology developed for pesticides was applied to groundwater and surface water samples from Valencia, a Mediterranean area of Spain, while the method for antibiotics was applied to surface water samples collected from The Netherlands. In summary, around 100 samples (including groundwater and surface water) were analyzed for pesticides and 50 surface water samples were analyzed for antibiotics.

QqQ confirmation of positives samples

Two different strategies were applied in order to confirm positive findings by QqQ instruments. In the determination of polar pesticides and TP, confirmation was performed at the same time as detection and quantification using a unique multiresidue method based on the acquisition of two MS/MS transitions for each analyte. The ion concentration ratio was calculated by quantifying the concentrations of the analytes based on the use of two separate transitions. Theoretically, both concentrations should be equal, so the expected ratio must be close to 1. A finding was accepted as positive if the ion concentration ratio was in the range 0.8-1.2. This approach was selected as the software used in our LC-MS instrument (QuanLynx) made it easier to calculate concentration ratios rather than ion intensity ratios. Using this approach, slight variations in ratios with time were minimized as the full calibration curve was used for quantification. Retention times for the reference standard and sample were also compared and

accepted when a deviation lower than 2.5% was obtained [3]. Around 100 samples were analyzed for pesticides and nearly 1000 findings were investigated using the strategy described above.

For the determination of antibiotics, a rapid screening method was developed by acquiring only one SRM transition for each analyte. Positive samples were re-injected for a second analysis, where a confirmatory method was individually applied for each antibiotic and included the highest possible number of available transitions. The confirmation batch included a standard, a blank and the positive sample to be confirmed. In this case, intensity ratios between different transitions were calculated and compared to those of the reference standard. Positives were confirmed if the ion ratio and retention time deviations were between the limits established by the European Union [3]. Around 50 samples were analyzed for antibiotics and 18 findings were investigated using the above mentioned strategy.

QTOF confirmation of positives samples

Confirmation by QTOF was performed by the acquisition of the full spectrum product ion spectrum selecting the m/z of the suspected positive as precursor ion. In the confirmation batch the suspected positive was injected together with a LC-water blank and a standard. The abundance of product ions was compared with that obtained for the reference standard and the experimental accurate masses were compared with the theoretical ones. The use of accurate mass as a confirmation criterion was also used despite its no-inclusion in most guidelines, because in our opinion it seems a more adequate criterium than the resolution power of the instrument [18]. Results were assumed to be confirmed when mass accuracies were better than 2 mDa and the ion ratio deviations of, at least, two ions were between limits established by the European Union [3]. Around 200 positive pesticide findings revealed by QqQ, out of near 1000 total findings, were subsequently investigated by QTOF.

RESULTS AND DISCUSSION

Achievements.

The application of LC-MS/MS with QqQ or QTOF to a considerable number of samples allowed us to evaluate the suitability of confirmation criteria in the environmental field. Thus, around 1000 positive findings were evaluated, resulting in more than 90% of positives (revealed by the acquisition of one SRM transition) being confirmed when applying confirmation criteria using both QqQ and QTOF analysers.

- *Triple quadrupole*

The main drawback when acquiring only one SRM transition is the need for ion selection (i.e. the analyst has to select just one of the available transitions in order to determine the analyte). Ideally, ion selection would be performed by selecting the most specific transition, in this way maximizing the selectivity of the method. However, it is impossible to assure the absence of interferences that might share the selected transition. Generally, the most sensitive transition is selected to increase the method sensitivity, but in that case not much attention is paid to the selectivity, so a negative sample containing an interferent that shares the transition selected for a target analyte would be reported as a false positive if only this transition is monitored.

The selection of two SRM transitions greatly minimizes the problems relating to ion selection, as the potential interferent would have to share not only the retention time and one transition but also a second specific transition. For this reason, most guidelines advise the use of two SRM transitions. In the case of the EU 2002/657/EC [3], the need to have a minimum of 3 IPs forces the analyst to acquire at least two transitions (2.5 IPs are reached by the acquisition of one transition meanwhile the acquisition of two leads to 4 or 5 IPs). We proved the convenience of this approach in our work, as a few findings reported using only one transition were revealed as false positives when two transitions were acquired. Figure 1 shows some examples of false positives for pesticides (1b) and antibiotics (1a) that were revealed by the application of this confirmation criterion.

Investigating the reasons of false positives when using only one SRM transition led us to establish two possible causes:

- i) the low specificity of the transition selected; and/or,
- ii) a significant amount of interferences in the water sample, mainly when searching for analytes at sub-ppb levels.

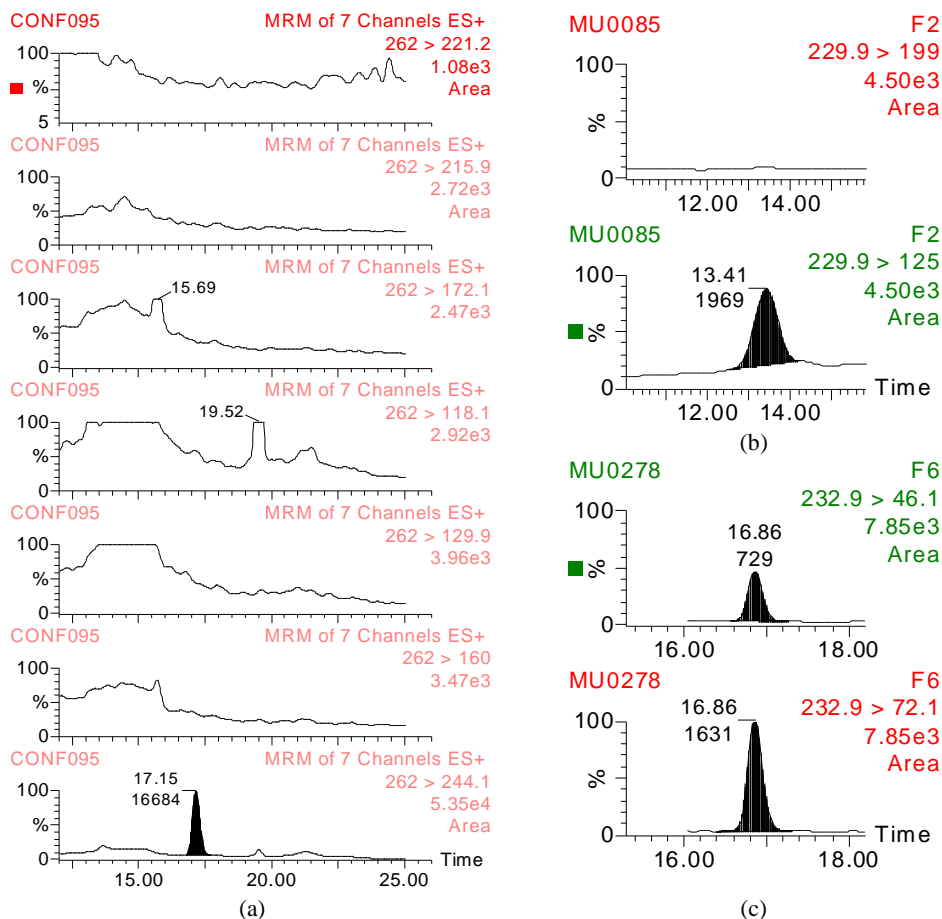


Figure 1. SRM chromatograms for: (a) sample suspected to be positive for oxolinic acid revealed as negative when acquiring the highest number of available transitions, (b) sample that would be reported as positive for dimethoathe if only one transition was acquired and (c) sample confirmed to be positive for diuron at a concentration of 18 ng/L

In our experience, the selection of one low specific transition notably increased the possibility of finding interferences. This was the case of the antibiotic oxolinic acid (Figure 1a) where the most sensitive SRM transition, the loss of water, was selected for quantification (m/z 262 \rightarrow m/z 244). The low specificity of this loss led to finding several positives, which were revealed as false positives when all the available transitions were acquired. As can be seen in the Figure 1a, although the acquisition of the highest number of available transitions clearly demonstrated that the sample was negative, this fact would also be revealed if only one additional SRM transition was acquired.

Although the selection of low specific transitions seems to be one of the main reasons for false positives occurring, the selection of only one more specific transition did not assure a safe confirmation. This was the case for the insecticide dimethoate (Figure 1b) where the transition m/z 230 \rightarrow m/z 125 was selected for quantification. The product ion at m/z 125 seemed to be highly specific as it corresponds to the neutral loss of $\text{CH}_3\text{NHCOCH}_2\text{SH}$. However, one interferent in several water samples analyzed also shared this transition with only 0.9% deviation in the retention time of dimethoate reference standard. The acquisition of two SRM transitions (m/z 230 \rightarrow m/z 125 for quantification and m/z 230 \rightarrow m/z 199 for confirmation) allowed us to reveal this false positive finding.

This example illustrated potential problems observed in confirmation at very low levels. The presence of analytes at sub-ppb levels increases the possibility of finding interferents as a large number of compounds can be present in environmental samples at those levels. In addition, the great variability in the composition of water samples, depending on their origin, means that analytical chemists have to solve the problems resulting from a great variety of interferents, which, although present at low concentrations, may share some SRM transitions with the analyte, and therefore hamper the confirmation. The great variability in water matrices also makes it difficult to predict the occurrence of these interferents by analysing blank samples.

One advantage of using QqQ instruments for confirmation of positive findings was the high sensitivity obtained in the SRM mode, so the simultaneous acquisition of two or more transitions for each compound allowed confirmation of positive samples even at concentrations close to the limit of detection, although this possibility depended on the relative abundance of the selected transitions. In our work, due to the usual presence of at least one additional product ion with similar relative abundance in both methods applied, for pesticides and antibiotics, the confirmation of positive findings was always feasible at the limit of quantification (LOQ), i.e. the lowest concentration that was fully validated applying the overall analytical procedure (10-25 ng/L, depending on the method applied). As an example, Figure 1c shows the confirmation of the herbicide diuron in a water sample at a concentration as low as 18 ng/L.

- QTOF

Although the accurate mass obtained by TOF mass analyzers is as a valuable tool for confirmation of positive samples [27], its potential is limited when using API sources, due to the difficulty of achieving more than one significant ion. In this sense, the use of QTOF for confirmation presents inherent advantages due to the acquisition of the full scan product ion spectrum with accurate mass. This characteristic makes of QTOF an interesting alternative to QqQ for the purposes of confirmation. Data acquisition in QTOF is always performed in full scan mode, so, it is not necessary to select any product ion (i.e. ion selection is not required), and all available product ions from a pre-selected precursor ion are acquired. This fact leads to a notable increase in the number of IPs obtained, it being usual to reach more than 10 IPs [18].

In addition, the high resolution and mass accuracy obtained by QTOF allows the measurement of all product ions with accurate mass, increasing the confirmation capabilities and reliability due to the great amount and quality of information gathered in comparison with QqQ instruments. Confirmation performed by QTOF can therefore be considered ultimate, unequivocal confirmation, as the risk of reporting false positive findings is extremely low. As an example, Table 1 shows confirmation of terbumeton by QTOF in a water sample, presenting the accurate mass and the relative intensities of the main ions in the sample and compared with the theoretical ones. The mass accuracy obtained for all six product ions presented errors lower than 3 mDa, and typically lower than 2mDa. Relative abundance deviations were normally lower than 20%, except for the less intense ion (m/z 86.0354) which presented a deviation of a 68%.

Table 1. Confirmation of terbumeton (0.056 µg/L) in a water sample by QTOF

Ion	Mass accuracy			Abundance		
	Theoretical	Sample	Deviation(mDa)	Standard	Sample	Deviation(%)
C ₆ H ₁₂ N ₅ O	170.1042	170.1047	0.5	100	100	-
C ₄ H ₈ N ₅ O	142.0729	142.0729	0.0	18.6	20.5	10
C ₅ H ₁₀ N ₃ O	128.0824	128.0835	1.1	7.7	8.5	10
C ₄ H ₈ N ₃ O	114.0667	114.0697	3.0	24.4	22.9	-6
C ₃ H ₆ N ₃ O	100.0511	100.0516	0.4	5.0	5.8	15
C ₂ H ₄ N ₃ O	86.0354	86.0365	1.1	2.3	3.9	68

Apart from their high cost, the main drawback of QTOF instruments in the environmental field is their lower sensitivity compared with QqQ in SRM mode. In principle, this limitation could be minimized by preconcentrating higher sample volumes (normally in off-line SPE procedures). Despite this possibility, several positives for pesticides and TPs could not be confirmed by QTOF

when they were present at concentrations below 50 ng/L. However, confirmation of all positives investigated was feasible when they were found at concentrations above regulatory limits (i.e. > 0.1 µg/L). In the case of antibiotics, their low concentrations in water (normally below 50 ng/L) impeded their confirmation by QTOF [10].

Pitfalls

The acquisition of two SRM transitions in QqQ instruments together with the measurement of the ion ratio was found to be a valuable approach for confirmation. However, the application of this criterion did not completely eradicate findings of false positive and its strict application might even lead to the possibility of reporting false negatives. This result was rather unusual, but a few cases were certainly observed. Some illustrative examples are reported in the next section

False positive findings

Reporting false positives can cause economical losses, social alarm or unnecessary concern, so analytical methods for organic priority pollutants should provide safe, reliable confirmation of the identity of detected analytes. The use of two transitions (together with the measurement of the ion ratio) as confirmation criteria drastically reduced the number of false positives in the water samples investigated, although there was still some possibility finding them when using QqQ instruments. The use of QTOF instruments actually eliminated this risk, provided that analyte concentrations were high enough to be detected by QTOF MS.

- *Triple quadrupole*

The occurrence of a false positive with QqQ implies the presence of an interferent sharing the analyte-retention time and two transitions with similar relative abundance to those of the analyte. It seems quite difficult for this situation to occur, so one would expect that reporting a false positive under these conditions would not be feasible. However, one false positive was found in the determination of antibiotics in surface water. Figure 2 shows the SRM chromatograms for a sample suspected of containing the quinolone flumequine. As can be seen, the two most sensitive transitions for flumequine were present in the sample (Figure 2b) with a retention time deviation of 0.75% with respect to the reference standard (Figure 2a). In addition, the ion intensity ratio calculated in this sample presented a deviation of only 10% when compared to the reference standard (1.50 for standard, 1.65 for sample). This detection would therefore have been reported as positive if only the two most sensitive transitions were acquired. Fortunately, in

this case, confirmation analysis applied for antibiotics included acquisition of the highest number of available transitions for each analyte [10]. As can be seen in Figure 2, the acquisition of four additional transitions revealed that the compound detected did not correspond to flumequine, because none of the additional transitions available were observed in the sample.

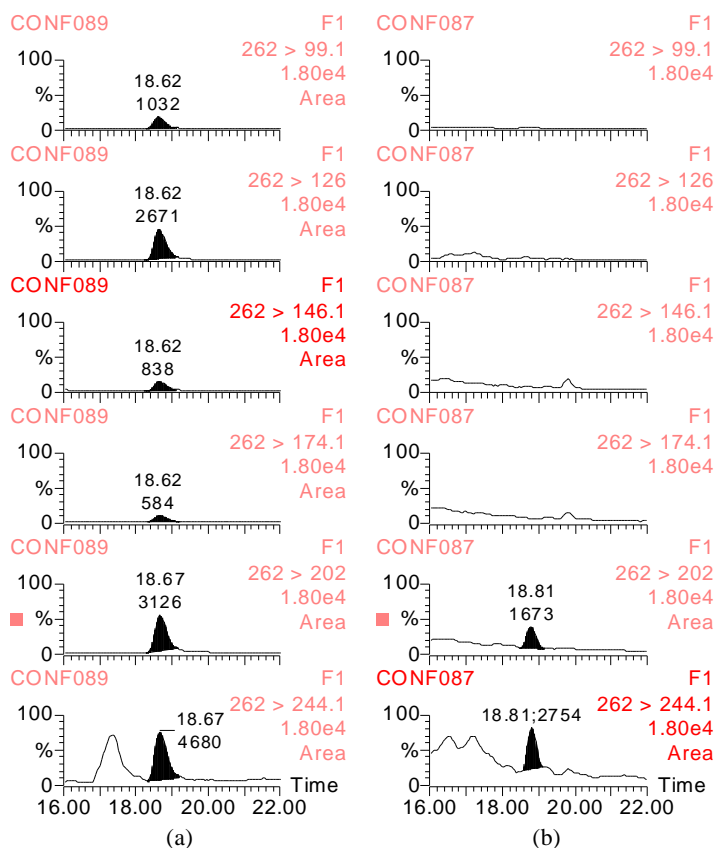


Figure 2. SRM chromatograms for: (a) 25 ng/L standard of flumequine (ion ratio between m/z 262 \rightarrow m/z 244 and m/z 262 \rightarrow m/z 202: 1.50) and (b) sample revealed as negative when the highest number of available transitions was acquired (ion ratio between m/z 262 \rightarrow m/z 244 and m/z 262 \rightarrow m/z 202: 1.65)

The occurrence of this false positive was undoubtedly related to the ion selection required when working with QqQ in SRM and could be justified by the low specificity of the transitions selected. The most abundant product ion was therefore due to the neutral loss of water (m/z 262 \rightarrow m/z 244), which meant that this transition should not be recommendable for the purposes of confirmation. Regarding the second transition (m/z 262 \rightarrow m/z 202), the $[M-60]^+$

fragment may also be shared by other compounds (e.g., that present a neutral loss of acetic acid).

It is remarkable that this false positive of flumequine was the only one found among all samples investigated for antibiotics. Thus, only one positive out of 18 investigated was found to be a false positive when acquiring the two most sensitive transitions.

With respect to the determination of pesticides and TPs, two transitions were acquired in the confirmatory method (the most sensitive for quantification (Q), and the additional one for confirmation (q)), and positive findings were not further investigated. In this case, the absence of transitions with low specificity in the confirmation of pesticides and TPs allows one to expect no false positives in the reported data. This assumption was supported by the fact that all positive samples with concentrations higher than 0.1 µg/L were subsequently re-confirmed by QTOF.

- *QTOF*

The occurrence of false positives when using QTOF for confirmation was assumed to be eradicated due to the absence of ion selection in this approach and to the high quality of the information provided by this instrument, where all product ions present at a defined collision energy were measured with accurate mass. As stated above, the main problem of this approach was the lower sensitivity achieved, and that allowed us to confirm findings at concentrations at or higher than 0.1 µg/L, including the flumequine false positive showed in Figure 2. It might be assumed that, by using higher pre-concentration factors (normally by employing larger sample volumes in off-line SPE procedures), the QTOF instrument would allow show this false positive.

False negative findings

Even more problematic than reporting false positives is the possibility of reporting false negatives because the presence of relevant target pollutants, although investigated, would be ignored.

In most criteria used for confirmation by MS/MS, the measurement of at least one ion ratio and its comparison to that of the reference standard is required in order to ensure a safe confirmation. The maximum tolerance allowed depends on the relative abundance of the selected ions (normally 10%-50%). However, available guidelines do not define any protocol to be followed when this tolerance is out of the established range. Thus, a finding would be reported as negative when this ion ratio is out of the tolerance window. It is interesting to go in detail into this

subject, as the strict application of this criterion might lead to positive findings being ignored in some cases by both QqQ and QTOF instruments.

- *Triple quadrupole*

When two transitions are acquired in a positive sample, a false negative might be reported if one of these transitions is shared by an interferent. The ion ratio would be affected and the experimental value obtained might be out of the tolerance window.

As a general approach, when an interferent co-elutes and shares a transition with the analyte, different situations might occur, depending on the number of transitions acquired:

(i) if only one transition is monitored:

(a) a false positive would be reported if the sample does not contain the analyte; and,

(b) an overestimation of the concentration would be reported if the sample is positive actually; or

(ii) if two transitions are monitored, a false negative would be reported if only one transition is interfered.

An illustrative example can be found in a groundwater sample that contained 2-hydroxy-terbutylazine. As can be seen in Figure 3, a chromatographic peak was observed when acquiring the two SRM transitions selected (m/z 212 \rightarrow m/z 156 for quantification, and m/z 212 \rightarrow m/z 86 for confirmation). However, when the ion ratios for standard and sample were calculated and compared, a deviation of 82% was obtained, so the sample could not be confirmed to be positive. However, given the existence of the two transitions in the sample, it was further investigated to ensure whether this compound was the targeted analyte or not. Two possibilities were considered:

(i) the sample is positive but one of the selected transitions is interfered; or,

(ii) the sample is negative actually.

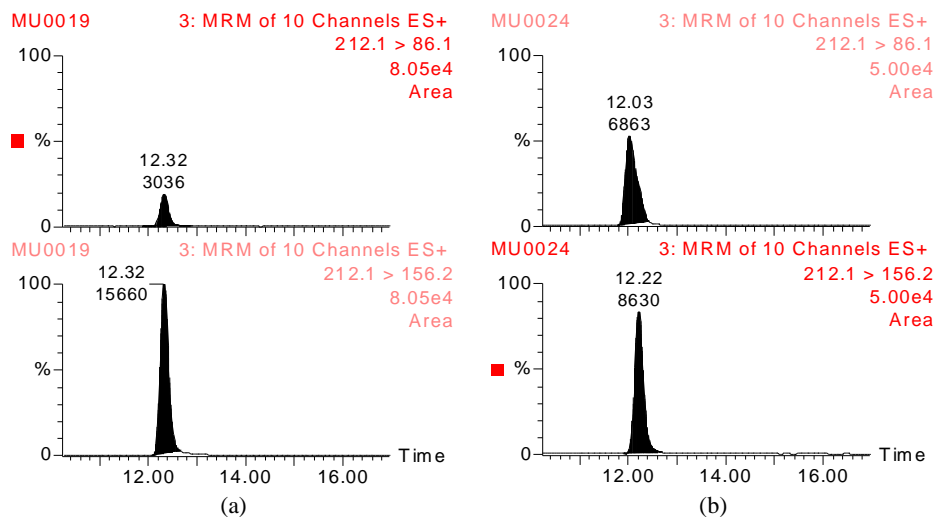


Figure 3: SRM chromatograms for (a) 25 ng/L standard of 2-hydroxy-terbutylazine (ion ratio 5.2) and (b) sample suspected to be positive for 2-hydroxy-terbutylazine (ion ratio 1.3)

In order to solve this dilemma, the highest number of available transitions were acquired in a second analysis (Figure 4). The monitoring of up to eight SRM transitions for this compound allowed us to confirm the presence of 2-hydroxy-terbutylazine in the sample. However, three of these transitions seemed to be shared by an interferent present in the sample leading to ion-ratio deviations as high as 99%. Four of the ion ratios calculated were within the tolerance window (deviation always lower than 7%), confirming the presence of this triazine TPs at a concentration level of 0.02 $\mu\text{g/L}$ (Table 2). In addition, the slightly lower retention time obtained at the interfered transitions supported the hypothesis of an interferent sharing up to three transitions with the analyte. This example illustrates the importance of selecting several transitions for confirmation, as we found in our research more examples of interferents sharing transitions with analytes.

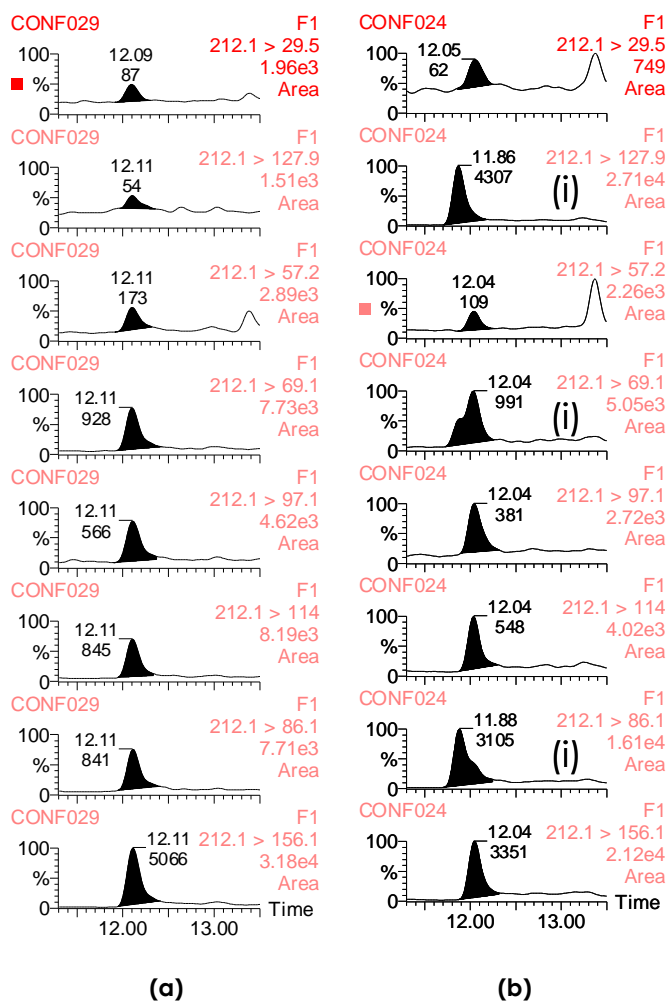


Figure 4. SRM chromatograms for (a) 25 ng/L standard of 2-hydroxy-terbutylazine when the highest number of available transitions was acquired and (b) sample suspected to be positive by 2-hydroxy-terbutylazine when the highest number of available transitions was acquired

(i) interfered transition.

Table 2. Comparison of confirmation by QqQ and QTOF of a water sample containing 2-hydroxy-terbutylazine (0.02 µg/L) and two co-eluting isobaric interferents

Triple quadrupole						
Transition	Retention time			Ion Ratio		
	Standard	Sample	Deviation(%)	Standard	Sample	Deviation(%)
212>156	12.1	12.0	0.8	100	100	-
212>86	12.1	11.9	1.6	6.0	1.1	-82
212>114	12.1	12.0	0.8	6.0	6.1	2
212>97	12.1	12.0	0.8	8.9	8.8	-1
212>69	12.1	12.0	0.8	5.5	3.4	-38
212>57	12.1	12.0	0.8	29.3	30.7	5
212>128	12.1	11.9	1.6	93.8	0.8	-99
212>29	12.1	12.0	0.8	58.2	54.0	-7
QTOF						
Ion	Mass accuracy			Abundance		
	Theoretical	Sample	Deviation(mDa)	Standard	Sample	Deviation(%)
C ₅ H ₁₀ N ₅ O	156.0885	156.0868	-1.7	100	100	-
C ₃ H ₆ N ₅ O	128.0572	128.0653	8.1	42.0	3.8	-91
C ₄ H ₈ N ₃ O	114.0667	114.0679	1.2	2.8	2.6	-7
C ₄ H ₅ N ₂ O	97.0402	97.0359	-4.3	10.5	8.4	-20
C ₅ H ₄ N ₃ O	86.0354	86.0359	0.5	7.0	3.1	-56

The small difference in the retention times suggested another feasible way of confirming the presence of this triazine TP: an improvement in the chromatography. Chromatographic separation is normally considered to be less relevant in LC-MS/MS methods when compared to conventional LC or LC-MS, however in this case helped us to confirm the presence of 2-hydroxy-terbutylazine. Thus, the interference was correctly resolved after performing a 30 minutes gradient on the basis that all seven measured ion ratios were within the tolerance window.

- QTOF

In our opinion, a positive sample confirmed by QTOF should be considered as unequivocally confirmed; consequently, many IPs are obtained by this technique. However, the presence of co-eluting isobaric interferences might yield difficulties in confirmation when using QTOF because a product ion spectrum with low spectral purity would contain a number of peaks not related to the analyte. This situation may hamper the comparison between the product ion spectra of the reference standard and the sample if a library search is performed.

This was the case when applying QTOF for confirmation in the example showed before: a positive finding of 2-hydroxy-terbutylazine. As can be seen in Figure 5, when the product ion spectrum of the *m/z* 212 precursor ion was acquired, only a peak was observed in the

chromatogram. The significant differences between the spectrum obtained for this peak (Figure 5b) and for a 2-hydroxy-terbutylazine reference standard (Figure 5c) might lead to report the sample as negative. However, as in the case of QqQ, two different possibilities were evaluated:

- (i) the sample was positive but the product ion spectrum was interfered by an isobaric compound; or,
- (ii) the sample was negative

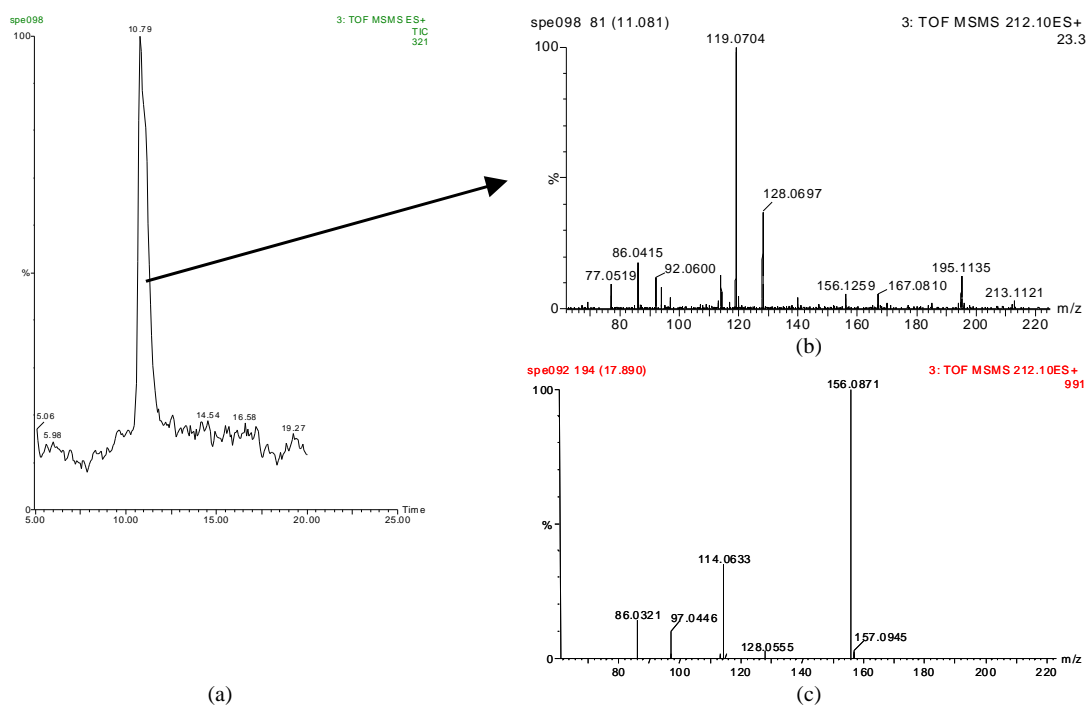


Figure 5. Confirmation by QTOF of a sample suspected to be positive for 2-hydroxy-terbutylazine: (a) Total product ion chromatogram from precursor ion at m/z 212, (b) product ion spectrum obtained from the sample chromatographic peak and (c) product ion spectrum obtained from a 2-hydroxy-terbutylazine standard.

In the case of QTOF, the only way to distinguish between these two possibilities was found to be to improve the chromatography. Using a 30 minutes gradient allowed the analytes and interferents to be separated (Figure 6). Unexpectedly, this separation showed that up to three isobaric compounds were separated using this gradient, so that there were three unknowns compounds sharing the same m/z for the precursor ion and co-eluting in the first gradient applied.

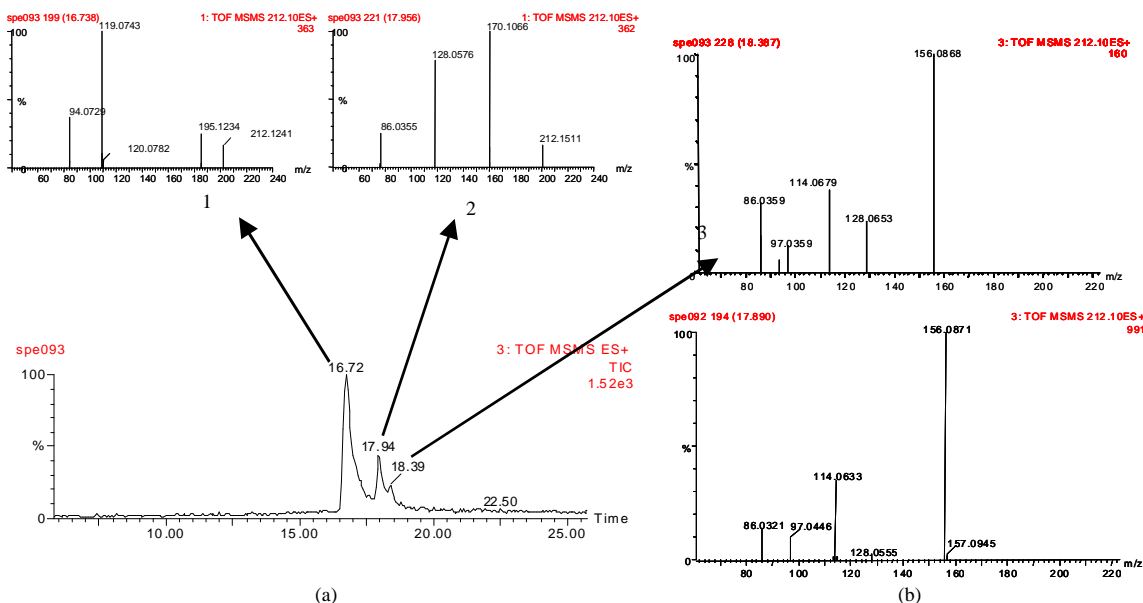


Figure 6. Effect of the chromatographic separation in the confirmation by QTOF of a sample suspected to be positive for 2-hydroxy-terbutylazine: (a) Total product ion chromatogram from precursor ion at m/z 212 and product ion spectra obtained for each chromatographic peak: 1: isobaric interference, 2: isobaric interference and 3: analyte, (b) product ion spectrum spectrum obtained from a 2-hydroxy-terbutylazine standard

This situation shows that a remarkable number of possible interferents may occur in an environmental sample when the analytes are present at very low concentration levels. Besides, these interferents were present in a few samples only, while being undetected in others despite several of the samples being collected from the same area. This example illustrates the variability in the composition of matrices of environmental samples. As a result, it is very difficult to predict the occurrence of these interferences by analyzing blank samples.

As can be seen in Figure 6 and Table 2, the chromatographic separation between the analyte and interferences allowed final confirmation of this positive, although the spectrum still showed two product ions (m/z 128.0572 and m/z 86.0354) that interfered due to the co-elution of the analyte (numbered 3, retention time: 18.39 min) and the peak tail of one interferent (numbered 2, retention time 17.94 min). This interferent made confirmation by QTOF difficult as these two ions presented ion abundance deviations out of established range (deviations higher than 50%). However, the availability of three non-interfered ions together with their accurate mass measurement allowed us to confirm the presence of this analyte in the water sample. In this case, both product ions of the interferent 2 presented an accurate mass similar to that of the analyte ions and therefore, so the use of their accurate mass was not helpful to discriminate which ions came from the interferent 2 and which from 2-hydroxy-terbutylazine.

This similarity between 2-hydroxy-terbutylazine and interferent 2 made us believe that they could be structurally related, as the product ion at m/z 86.0354 is common to several triazine compounds. As can be seen in Figure 6a, this isobaric compound shows two neutral losses of propene (mass accuracies better than 2.4 mDa). This data, along with our previous knowledge of fragmentation of triazines [28], allowed us to suggest that interferent 2 was 2-hydroxy-propazine.

CONCLUSIONS

Confirmation of the identity of organic micropollutants in environmental samples based on the acquisition of only one MS/MS transition in QqQ instruments is not sufficient for a safe confirmation. This approach might be used for a rapid screening and quantification of organic pollutants in water, but the application of more confident confirmation criteria is necessary, normally by means of a second independent analysis.

Figure 7 shows the analytical strategy that we proposed for reliable confirmation based on using LC-MS/MS methods, so a sample suspected to be positive (e.g. after application of a screening method) can be confirmed by using either QqQ or QTOF instruments. Two transitions in QqQ instruments (together with the measurement of the retention time and the ion intensity ratio) is the minimum required for a safe confirmation, as we have proved in this article. Confirmation can be considered definitive if both selected transitions are highly specific and the Q/q ratio is in agreement with the reference standard. Moreover, when one of the SRM transitions does not show any signal, the sample can be considered as negative without the need of obtaining more additional information.

However, false positives may be reported if the selected transitions are not specific, and also false negatives when an interferent shares one of the transitions with the analyte, resulting in changing the expected ion ratio.

In both cases, the acquisition of all available transitions has been found to be an easy, efficient way to achieve a reliable confirmation. In our opinion, it is advisable to use this approach as a default strategy for confirmation, whenever possible. This allows one to obtain much more information that is useful for identification purposes. In the unexpected situation where the confirmation still remains unclear after acquiring all available transitions, chromatographic improvement undoubtedly helps confirmation by resolving interferent and analyte peaks.

In confirmation by QTOF, the possibility of reporting false positive findings is extremely low, so a positive confirmed by this technique should be considered to be unequivocal. However, there are some risks of reporting false negatives when a co-eluting isobaric interference is present in the sample matrix. The presence of this type of compounds can lead to additional peaks in the sample spectrum, making it difficult to compare spectra from the sample and the standard. The most reliable way of reducing this limitation is to improve chromatographic resolution.

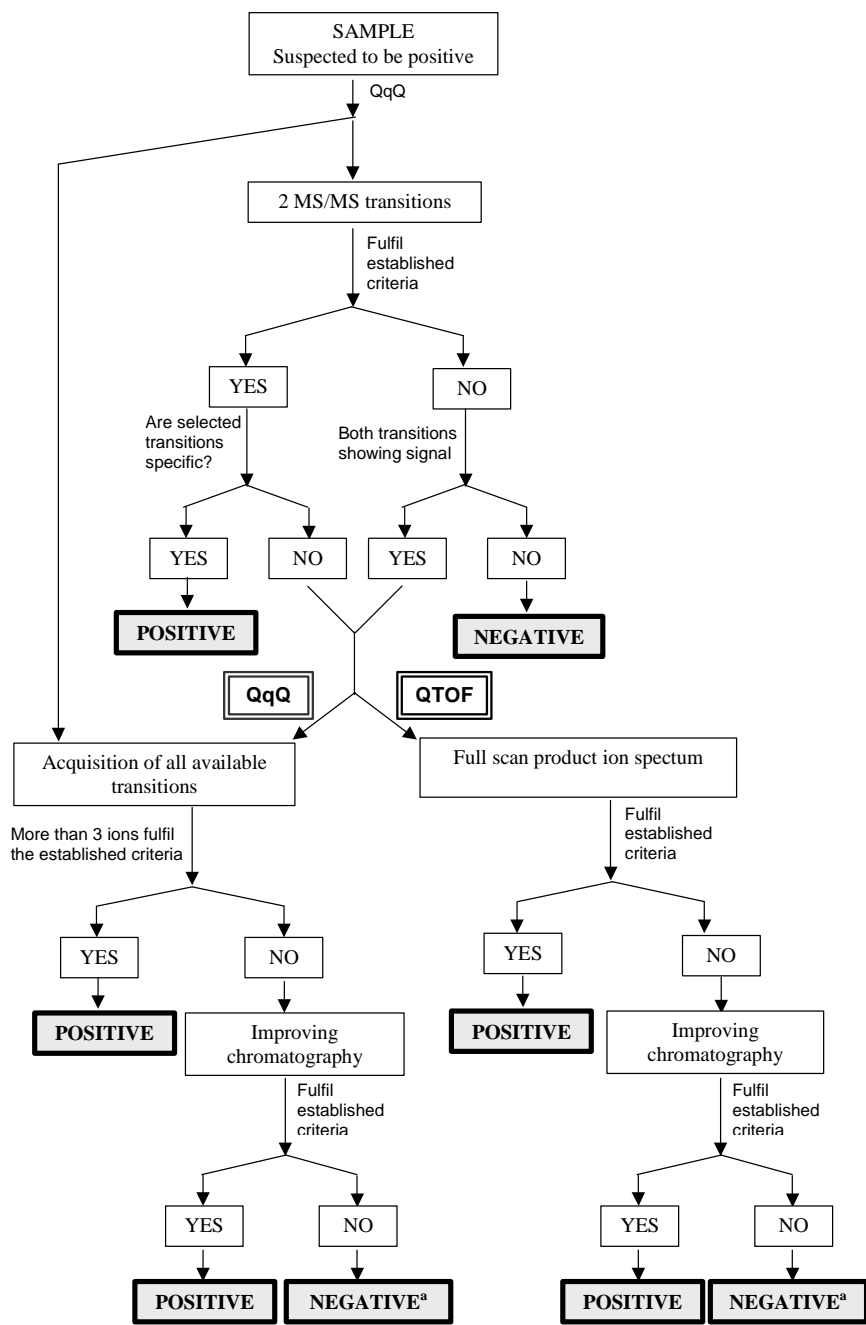


Figure 7. Strategy proposed for confirmation of positive findings.

^a More research might be required to ensure that the sample is negative actually.

After investigating around 1000 findings of pesticides and antibiotics in environmental samples, we found that the acquisition of two SRM transitions together with the measurement of Q/q ratio was not sufficient in only 1% of findings (only one false positive because of the low specificity of both selected transitions, and a few false negatives due to the non-compliance of Q/q ratio as a consequence of co-eluting isobaric interferences). Obviously, a method based on monitoring only one transition would have resulted in a larger number of false positives, as we proved in our work, which makes questionable some of the data reported in the scientific literature where only one transition was selected.

When only two transitions are acquired, they should be selected to be as specific as possible, avoiding the use of common losses (e.g. H₂O, CO₂ or HCl) in order to prevent false positives. In addition, a suitable chromatographic separation is advisable in order to reduce the possibility of finding isobaric co-eluting interferences. Chromatographic separation and the specificity of the transitions and not just the sensitivity attainable should therefore be considered carefully when developing analytical methods in the environmental field. However, it seems quite difficult to define or establish general rules for the adequate selection of chromatographic conditions and for the MS/MS transitions to be acquired. The expertise and MS background of the analytical chemists is of outstanding importance in this process.

This article presents a strategy to achieve reliable confirmation of organic pollutants in water. However, other sources of false positive or false negative findings have also to be taken into account, such as carryover or the reporting of analyte concentrations above or below a defined threshold, mainly in those samples that present levels slightly higher or slightly lower than that value [29].

ACKNOWLEDGEMENTS

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for using the Quattro LC triple quadrupole and QTOF I mass spectrometers. M. Ibáñez is very grateful to Generalitat Valenciana for her pre-doctoral grant. This work forms a part of the research project "Aplicación del acoplamiento cromatografía líquida-espectrometría de masas (LC-MS/MS y LC-QTOF MS) a la identificación de metabolitos de plaguicidas en muestras de interés ambiental y toxicológico" developed under financial support of the Ministry of Science and Technology ((Ref: BQU2003-02685), Spain.

REFERENCES

- (1) Identification criteria for qualitative analysis, WADA technical document TD2003IDCR 1.2, January 1, 2004
- (2) Guidance for industry: Mass spectrometry for confirmation of the identity of animal drugs residues. Draft Guidance 118, Food And Drug Administration June 6, 2001
- (3) European Union Decision 2002/657/EC 17 August 2002. Off. J. Eur. Commun. 221 (2002) 8
- (4) R.B. Geerdink, W.M.A.Niessen, U.A.Th. Brinkman, J. Chromatogr. A. 70 (2002) 65
- (5) Quality control procedures for pesticide residue analysis (doc. SANCO/10476/2003) February 5, 2004
- (6) L. Rivier, Anal. Chim. Acta 492 (2003) 69
- (7) P. Van Eenoo, F.T. Delbeke, Chromatographia 59 (2004) S39
- (8) EC Council Directive 98/83/EC, Off J. European Communities, No. L 330, December 5, 1998 p32
- (9) R. Hirsch, T.A.Ternes, K. Haberer, A. Mehlich, F. Ballwanz, K.L. Kratz, J. Chromatogr. A, 815 (1998) 213.
- (10) O.J. Pozo, C. Guerrero, J.V. Sancho, M. Ibáñez, E. Pitarch, E. Hogendoorn, F. Hernández, J. Chromatogr. A, 1103 (2006) 83
- (11) T.Reemtsma, J. Chromatogr. A 1000, (2003), 477
- (12) A.E. Till, Sci. Total Environ. 350 (2005) 273
- (13) M. Ibáñez, O.J. Pozo, J.V. Sancho, F.J. López, F. Hernández, J. Chromatogr. A 1081 (2005) 145
- (14) M.D. Hernando, E. Heath, M. Petrovic, D. Barceló, Anal. Bioanal. Chem. DOI: 10.1007/s00216-006-0394-5
- (15) J.B. Quintana, R. Rodil, T. Reemtsma, Anal. Chem., 78 (2006) 1644
- (16) A.B. Vega, A.G. Frenich, J.L.M Vidal, Anal. Chim. Acta 538 (2005) 117
- (17) S. Seccia, P. Fidente, D.A. Barbini, P. Morrica, Anal. Chim. Acta 553 (2005) 21
- (18) F. Hernández, M. Ibáñez, J.V. Sancho, O.J. Pozo, Anal Chem 15 (2004) 4349
- (19) W.T. Ma, K. Steinbach, Z.W. Cai, Anal. Bioanal. Chem. 378 (2004) 1828

- (20) A.A.M. Stolker, W. Niesing, E.A. Hogendoorn, J.F.M Versteegh, R. Fuchs, U.A.Th Brinkman, *Anal. Bioanal. Chem.* 378 (2004) 955
- (21) M. Ibáñez, J.V. Sancho, O.J. Pozo, W.M.A Niessen, F. Hernández, *Rapid Commun. Mass Spectrom.* 19 (2005) 169
- (22) M. Ibáñez., J.V. Sancho, O.J. Pozo, F. Hernández, *Anal. Bioanal. Chem.* 384 (2006) 448
- (23) J.V. Sancho, O.J. Pozo, M. Ibáñez, F. Hernández, *Anal. Bioanal. Chem.* In press, DOI: 10.1016/j.trac.2006.06.012
- (24) F Hernández, J.V. Sancho, O. Pozo, A. Lara, E. Pitarch, *J. Chromatogr.A.* 939 (2001) 1
- (25) J.V. Sancho, O.J. Pozo, F. Hernández *Analyst* 129 (2004) 38
- (26) F. Hernández, O.J. Pozo, J.V. Sancho, F.J. López, J.M. Marín, M. Ibáñez, *Trends Anal Chem.* 24 (2005) 569
- (27) I. Ferrer, E.M. Thurman, A.R. Fernández-Alba, *Anal. Chem.* 77 (2005) 281
- (28) M. Ibáñez, J.V. Sancho, O.J. Pozo, F. Hernández., *Anal. Chem.* 76 (2004) 1328
- (29) S. Cárdenas, M. Valcárcel, *Trends Anal Chem.* 24 (2005) 477

4.4 Bibliografía

2002/657/CE Decisión de la Comisión de 12 de Agosto de 2002, por la que se aplica la Directiva 96/23/CE del Consejo en cuanto al funcionamiento de los métodos analíticos y la interpretación de los resultados.

Sancho JV, Pozo OJ, Hernández F

Liquid chromatography and tandem mass spectrometry: a powerful approach for the sensitive and rapid multiclass determination of pesticides and transformation products in water.

Analyst 129 (2004) 38-44

SANCO/3029/99 rev 4 11/07/00

Residues: Guidance for genotyping and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, section 4) and Annex III (part A, section 5) of directive 91/141

European Commission, Directorate General Health and Consumer Protection

SANCO/825/00 rev 6 20/06/00

Guidance document on residue analytical methods

European Commission, Directorate General Health and Consumer Protection

Capítulo 5.

Estrategias para la determinación de plaguicidas en aguas por LC-MS(/MS)

5.1 Introducción

5.2 Resultados y discusión

Artículo científico 9

Strategies for quantification and confirmation of multi-class polar pesticides and transformation products in water by LC-MS/MS using triple quadrupole and hybrid quadrupole time-of-flight analyzers
Trends Anal. Chem. (2005) 24: 596-612

Artículo científico 10

Potential of liquid chromatography/time of flight mass spectrometry for the determination of pesticides and transformation products in water.
Anal. Bioanal. Chem.(2006) 386: 987-997

5.3 Bibliografía

5.1 Introducción

Uno de los principales campos de aplicación de la Química Analítica moderna es la evaluación de la contaminación derivada de la actividad humana. El elevado número de potenciales contaminantes orgánicos, sus diferentes características físico-químicas y las bajas concentraciones normalmente presentes en las muestras analizadas, hacen que el control de estos compuestos en el medioambiente sea especialmente problemático. En la actualidad, la comunidad científica está haciendo grandes esfuerzos con el fin de desarrollar métodos para el control de contaminantes orgánicos, que sean fiables y con un amplio ámbito de aplicación. Para este fin, se pueden aplicar diferentes estrategias dependiendo del objetivo perseguido. Los métodos analíticos pueden clasificarse en diferentes categorías: (i) métodos de cribado (*screening*), que permiten detectar (rápidamente) la presencia de uno o más compuestos, basándose en una o más características comunes de una clase de analitos, a un determinado nivel de concentración, (ii) métodos cuantitativos, que deben proporcionar información precisa sobre la cantidad de analito que está presente en las muestras, (iii) métodos confirmatorios, que permiten confirmar inequívocamente la identidad del compuesto detectado y (iv) métodos de elucidación, que deben descubrir la identidad de un compuesto sospechoso o desconocido.

Hasta ahora, la mayoría de los esfuerzos se han dirigido a los dos primeros aspectos, principalmente debido a que los analizadores de masas normalmente usados en combinación con las técnicas cromatográficas, instrumentos de cuadrupolo (Q), triple cuadrupolo (QqQ) o trampa de iones (IT), son adecuados para el cribado y cuantificación de manera simultánea de un número preseleccionado de analitos. Así pues, la mayoría de los métodos publicados hasta ahora se centran en la determinación de un limitado número de compuestos preseleccionados (*target analysis*), con especial énfasis en la cuantificación. Sin embargo, existe un limitado número de métodos publicados que aborden la problemática de la confirmación inequívoca de los compuestos detectados o la identificación de posibles contaminantes no incluidos en los métodos aplicados.

Los **métodos de cribado** son muy útiles ya que permiten discriminar muestras con residuos no detectables (muestras negativas) de aquellas en las que se detectan posibles contaminantes que, en una etapa posterior, será necesario confirmar. Idealmente deberían hacerlo de una forma rápida, fiable y con poca manipulación de muestra. Esto permitiría centrar los esfuerzos analíticos en la correcta cuantificación e inequívoca confirmación de las posibles muestras positivas. Para fines de cribado, tradicionalmente se han aplicado diferentes aproximaciones basadas en técnicas de inmunoensayo, biosensores o bien en técnicas cromatográficas usando detectores tales como FPD, NPD, FD o UV. El uso de MS mejora ampliamente la aplicabilidad de los métodos de cribado pues permite incluir un elevado número de compuestos orgánicos de diferentes familias químicas. Así pues, aunque el cribado de contaminantes orgánicos usando acoplamiento cromatografía/MS es un tema ampliamente tratado, la mayoría de los métodos publicados hacen referencia a un limitado número de analitos, normalmente pertenecientes a unas pocas familias químicas. En este sentido, la elevada sensibilidad alcanzada por el triple cuadrupolo en el modo SRM hace que sea ideal para este tipo de aplicaciones. Sin embargo, el número de compuestos orgánicos que potencialmente pueden contaminar el medio ambiente es extremadamente alto, presentando además propiedades físico-químicas muy diversas. Esto hace que, con toda seguridad, algunos contaminantes no sean reportados en los análisis al no estar incluidos en los métodos de cribado. Esta situación es aún más notoria en el análisis de aguas debido a la falta de conocimiento sobre la composición de las muestras ambientales. Por ello, se requiere el desarrollo de métodos de cribado poderosos y fiables, con técnicas analíticas avanzadas, que incluyan el mayor número posible de contaminantes orgánicos y que, además, sean capaces de detectar posibles compuestos no incluidos en los análisis.

La aparición de analizadores TOF abre un nuevo escenario analítico en el desarrollo de métodos de cribado. Las ventajas de la tecnología TOF en este campo derivan de la posibilidad de llevar a cabo adquisiciones en modo de barrido completo con datos de masa exacta y con mayor resolución y sensibilidad que los analizadores MS más convencionales. Por ello, no es necesario predefinir la masa específica del contaminante antes del análisis, con lo que los analitos pueden ser seleccionados después de la adquisición de los datos. Esta aproximación, que consistiría en un "cribado a posteriori de compuestos diana", permite la detección, en principio, de un número ilimitado de potenciales contaminantes, sin necesidad de reanalizar las muestras. De este modo, el número de analitos incluidos en el método de cribado vendría limitado solamente por las propias limitaciones del instrumento, es decir, sólo aquellos compuestos sin el adecuado comportamiento cromatográfico o sin la adecuada ionización en MS, serían excluidos del método.

El alto nivel de multiresidualidad requerido en este tipo de análisis no puede ser fácilmente alcanzado mediante analizadores de cuadrupolo (tanto Q como QqQ, trabajando en modos SIM o SRM, respectivamente) debido a la necesidad de predefinir las masas que han de ser adquiridas y a la dificultad de reducir el tiempo de monitorización (*dwell time*) de cada transición por debajo de un determinado valor sin perder sensibilidad. Por otro lado, la elevada resolución de los analizadores TOF permite reducir la ventana de masa, pudiendo realizar la extracción de una masa específica a partir de los datos *full scan*. Una ventana más estrecha conduciría a una reducción sustancial del ruido químico, facilitando la detección de los compuestos a través del cromatograma generado a partir de la extracción de un ión (XIC). Sin embargo, esta aproximación presenta una limitación importante, como es la menor sensibilidad del TOF respecto al triple cuadrupolo trabajando en modo SRM, lo que dificulta la detección e identificación de analitos a niveles de sub $\mu\text{g/L}$.

Además de un cribado adecuado para discriminar entre muestras positivas y negativas, la **confirmación** de la identidad de contaminantes orgánicos es motivo de preocupación en el campo medioambiental, a consecuencia de los efectos indeseables asociados a confirmaciones erróneas, es decir, el hecho de reportar falsos positivos o falsos negativos. Por ello, son necesarios métodos fiables que permitan no sólo la correcta cuantificación de compuestos diana, sino también, e incluso más importante, su inequívoca confirmación. Idealmente, la confirmación debe ser objetiva y segura, no ha de depender de la interpretación subjetiva del analista, requiriéndose por lo tanto, normas de confirmación predefinidas, rigurosas y eficientes. La medida de tres iones se ha considerado tradicionalmente como una buena opción para la confirmación de contaminantes orgánicos por GC-MS usando EI como fuente de ionización. Sin embargo, el aumento del empleo de las técnicas LC-MS y LC-MS/MS con interfases a presión atmosférica (API) obliga a reconsiderar los requisitos de confirmación con el fin de incluir estas técnicas debido a sus características diferenciales con GC-MS. Existen algunas guías sobre confirmación que consideran los diferentes tipos de ionización y/o modos de barrido, tales como las desarrolladas por la WADA, FDA, o la Unión Europea. En general, la confirmación se basa en la adquisición de una cantidad de información limitada. Como ya se ha comentado en el capítulo 4, uno de los criterios más detallados es el propuesto por la Unión Europea para la confirmación de aditivos y contaminantes en muestras de alimentos de origen animal (Decisión 2002/657/EC), donde la confirmación se basa en la acumulación de puntos de identificación (IP), y el número de IPs alcanzado depende de la resolución del instrumento y la estrategia de MS utilizada. Para la confirmación de los compuestos estudiados en la presente Tesis Doctoral son necesarios 3 IPs.

La mayoría de las publicaciones sobre determinación de contaminantes orgánicos en el medioambiente por LC-MS(/MS) apenas tratan la problemática de la confirmación de los analitos detectados en las muestras. Esta situación puede llevar a cuestionar la validez de los resultados obtenidos mediante la aplicación de estos métodos (Reemtsma, 2003), debido a la posibilidad de reportar falsos positivos usando métodos LC-MS(/MS) basados en la adquisición de sólo un ión o una transición. En el caso de instrumentos de triple cuadrupolo, la manera más sencilla de confirmar un positivo es la adquisición de dos transiciones selectivas para cada analito. Así, si se dispone de un método de cribado ya desarrollado, bastaría con añadir más transiciones para cada analito. Sin embargo, como ya hemos comentado anteriormente, la adquisición del doble de transiciones puede generar algunos problemas debido a la disminución del tiempo de adquisición de cada transición o en el número de puntos obtenidos por pico, por lo que es necesario alcanzar un compromiso entre sensibilidad y forma de pico. Otra solución consistiría en realizar dos inyecciones: en la primera se descartarían las muestras negativas sobre la base de la adquisición de una sola transición, mientras que en la segunda se confirmaría la presencia o no del analito, añadiendo transiciones adicionales. En todos estos casos es necesario preseleccionar las transiciones que se van a monitorizar (*ion shopping*).

Otra manera de reducir notablemente el riesgo de falsos positivos es mediante el uso de los analizadores TOF, gracias a su elevada resolución, exactitud de masa y sensibilidad en modo *full scan*. En estos instrumentos, la adquisición de dos iones junto con la medida de la relación de abundancia entre ellos es, en principio, suficiente para una confirmación adecuada de la identidad del analito. Aunque este número de iones puede ser fácilmente obtenido mediante instrumentos GC-TOF, su obtención por LC-TOF es, en ocasiones, más complicada debida a la suave ionización producida en las interfases empleadas.

En términos de análisis confirmatorio el analizador híbrido QTOF es todavía más poderoso, pues permite la preselección de un ión precursor en el cuadrupolo que actúa como filtro de iones y la posterior adquisición del espectro completo de sus iones producto con elevada exactitud de masa, siendo actualmente una de las técnicas más valiosas para el análisis cualitativo (Stolker et al, 2004). En el proceso de confirmación por QTOF, tanto la masa exacta como la intensidad relativa de todos los iones producto disponibles de una muestra pueden ser comparados con los correspondientes a patrones de referencia. El número de IPs obtenido en QTOF suele ser mucho más elevado que el mínimo requerido, y la confirmación de la identidad del analito puede considerarse como inequívoca y definitiva (Hernández et al, 2004). En este sentido, aunque algunos autores han usado LC-TOF para la confirmación de la identidad del analito basándose en la medida de la masa exacta de un único ión (Benotti et al, 2003), esta confirmación podría ser insuficiente, siendo más adecuada la confirmación por QTOF

(Hernández et al, 2004; Stolker et al, 2004). Además, la elección de un ión precursor en el primer cuadrupolo aumenta la seguridad sobre el origen del ión producto y disminuye el número de interferentes y el ruido químico. Este bajo ruido químico y/o la eficiente fragmentación producida en la celda de colisión aumenta el número e intensidad relativa de los iones seleccionados cuando se utiliza QTOF, permitiendo no sólo mejorar la calidad de la confirmación, sino también confirmar muestras positivas a bajos niveles de concentración.

Además de las aplicaciones con fines de confirmación, las especiales características de los analizadores TOF pueden ser de gran ayuda en la **elucidación** de compuestos desconocidos. En este sentido, el uso de la masa exacta junto con las distribuciones isotópicas observadas en los compuestos investigados han sido utilizados por diferentes autores para la elucidación de algunos metabolitos de plaguicidas (García-Reyes, 2005). En la elucidación de desconocidos, la fórmula molecular obtenida puede usarse para realizar búsquedas en las bases de datos, obteniendo así un número reducido de posibles estructuras. Sin embargo, esta aproximación presenta algunas desventajas que limitan su aplicación en el proceso de elucidación, como por ejemplo la imposibilidad de distinguir entre isómeros. Cuando se hace uso del QTOF, la información estructural obtenida en el espectro de iones producto con masa exacta es de gran ayuda pues permite en numerosas ocasiones distinguir entre compuestos isoméricos. En algunos casos, cuando el experimento se lleva a cabo bajo condiciones controladas, la elucidación obtenida con el QTOF puede ser considerada como definitiva. Así, el QTOF se ha utilizado para la elucidación de metabolitos de plaguicidas en muestras biológicas (Ibáñez et al, 2006) o en estudios de fotodegradación (Detomaso et al, 2005; Ibáñez et al, 2004; Kouloumbos et al, 2005). Esta elucidación puede llevarse a cabo por comparación de una muestra fortificada con el plaguicida y una muestra blanco sometida al mismo procedimiento experimental. La aparición de un pico en el cromatograma TOF MS de la muestra y su ausencia en la muestra control revelaría la existencia de un metabolito/producto de transformación, el cual puede ser identificado posteriormente mediante los iones producto obtenidos en modo TOF MS/MS.

Aunque la aplicación de esta aproximación resulta de gran ayuda en la elucidación, puede dar lugar en ocasiones a intentos fallidos. La desventaja más frecuente es la ausencia de una posible fórmula molecular en las bases de datos disponibles, ya que muchos compuestos, incluidos la mayoría de los metabolitos/productos de transformación de diversos contaminantes conocidos, no están incluidos en las bases de datos comerciales. Así, aunque el análisis por QTOF puede proporcionar la fórmula molecular y algunos fragmentos específicos, la asignación de una estructura concreta es casi imposible en muchos casos. Esto hace que la elucidación de contaminantes orgánicos en muestras medioambientales sea especialmente problemática (Ibáñez et al, 2005; Bobeldijk et al, 2001), y todavía más cuando no hay ninguna fragmentación

específica en la molécula del compuesto investigado. Esto se ilustra en el trabajo de These et al (2004) donde la elucidación de ácidos húmicos y fúlvicos fue complicada debido a que las pérdidas observadas correspondían a agua y dióxido de carbono, las cuales no son específicas y, de hecho, son comunes para todos los compuestos de esta familia. En el caso de triple cuadrupolo, su menor resolución (se suele trabajar con resolución unidad) junto con su menor sensibilidad en modo *scan*, los hacen inadecuados para la elucidación estructural.

Finalmente, en términos de **análisis cuantitativo**, las características de los métodos LC-MS/MS con analizadores de triple cuadrupolo son excelentes en términos de sensibilidad, selectividad y rango lineal, con límites de cuantificación típicos del orden de 0.05-0.20 µg/L, lo que los hace muy adecuados para la cuantificación de residuos. La aplicación del (Q)TOF en la cuantificación de residuos de plaguicidas sigue siendo limitada, debido a su bajo rango lineal de respuesta, normalmente menor de 2 órdenes de magnitud.

Así pues, el desarrollo de metodología fiable para el cribado, cuantificación, confirmación y elucidación de contaminantes orgánicos presenta dificultades específicas cuando se trata con muestras medioambientales. Particularmente en el análisis de aguas, destaca la elevada sensibilidad requerida y la falta de información analítica sobre las muestras analizadas (muestras desconocidas). Los plaguicidas deberían ser detectados (y obviamente confirmados) a concentraciones alrededor de 0.025 µg/L en aguas potables (ECC Council Directive 98/83/EC). La presencia de analitos en muestras a niveles de ppb o ppt aumenta la posibilidad de encontrar interferencias ya que un gran número de compuestos pueden ser encontrados en estas muestras a dichos niveles. Además, la variabilidad en la composición de las muestras de agua dependiendo de su origen obliga a enfrentarse a una alta variedad de interferentes que podrían presentar la misma masa que el analito, dificultando la confirmación.

5.2 Resultados y discusión

En los dos artículos científicos que se muestran a continuación (*artículos 9 y 10*), se explican, de manera detallada y con ejemplos ilustrativos, diferentes estrategias a seguir cuando se utiliza LC-MS/MS en el análisis de residuos de plaguicidas, en función del objetivo perseguido. En el *artículo 9* se exploran las aplicaciones de los analizadores QqQ y QTOF, mientras que el *artículo 10* se centra exclusivamente en este último analizador.

En resumen, se puede concluir que los filtros cuadrupolares son ideales para el análisis cuantitativo de moléculas objetivo debido a su elevado rango lineal así como a la buena reproducibilidad en las medidas y elevada sensibilidad en modo SRM, características que los hacen adecuados para métodos multiresiduo. Finalmente, y de forma general, este instrumento puede utilizarse de manera eficiente para la confirmación de muestras positivas cuando existen varios iones producto, lo que permite seleccionar varias transiciones MS/MS. El uso de 2 transiciones SRM es una alternativa válida, siempre que se realice un *ion shopping* acertado, para cuantificar/confirmar de modo simultáneo.

Por otra parte, las características de los analizadores de masa TOF en cuanto a su alta resolución, capacidad para realizar medidas con masa exacta y su elevada sensibilidad en modo *scan*, hacen que sean muy útiles en el desarrollo de metodología analítica para la elucidación de microcontaminantes orgánicos. Por lo que se refiere al cribado y la confirmación de positivos, el QTOF en su modo de barrido de iones producto presenta ventajas importantes como son la no necesidad de realizar a priori *ion shopping* y que los interferentes quasi-isobáricos no afectan siempre que existan iones producto específicos del analito a confirmar. Sin embargo, este modo de operar también presenta una limitación importante como es su menor sensibilidad, por lo que en ocasiones impide utilizarlo para confirmar ciertos positivos obtenidos previamente mediante QqQ a bajos niveles de concentración. Esta menor sensibilidad podría ser compensada mediante una preconcentración previa del extracto, aunque ello supone una mayor laboriosidad del análisis. Por otro lado, las aplicaciones de los analizadores (Q)TOF en el campo cuantitativo siguen siendo escasas a consecuencia del bajo rango dinámico de linealidad que presentan estos instrumentos.

Artículo Científico 9

Trends Anal. Chem. (2005) 24: 596-612

STRATEGIES FOR QUANTIFICATION AND CONFIRMATION OF MULTI-CLASS POLAR PESTICIDES AND TRANSFORMATION PRODUCTS IN WATER BY LC-MS/MS USING TRIPLE QUADRUPOLE AND HYBRID QUADRUPOLE TIME-OF-FLIGHT ANALYZERS

Félix Hernández, Óscar J. Pozo, Juan V. Sancho, Francisco J. López, José M. Marín, María Ibáñez
Research Institute for Pesticides and Water, University Jaume I, P.O. Box 224 E-12080 Castellón,
Spain

ABSTRACT

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an excellent technique for the determination of polar pesticides and transformation products in water. On-line trace enrichment (SPE-LC) in combination with MS/MS satisfies most analytical demands of modern laboratories for pesticide-residue analysis, with respect to sensitivity (limits of detection and limits of quantification at sub- $\mu\text{g/L}$ levels), selectivity, reliability and rapidity, and all with very little sample manipulation. However, several obstacles have to be overcome when developing SPE-LC-MS/MS multi-residue and confirmative methods. The different physicochemical characteristics and wide polarity range of pesticides and their transformation products make their simultaneous preconcentration, chromatographic separation and determination difficult. Also, for the correct confirmation of the identity of compounds, it is necessary to increase the number of monitored MS transitions, which normally leads to a loss in sensitivity. This may hamper confirmation at low concentrations in a single chromatographic run (i.e. when a second analysis is not performed).

In this paper, we discuss different strategies for developing sensitive multi-residue methods for pesticides in water, able to correctly quantify and confirm compounds detected in samples. We discuss the use of different analyzers, triple quadrupole and hybrid quadrupole time-of-flight, and compare their performances for screening purposes, and for the quantification and confirmation of positive samples. We present several practical cases of water samples, including some positive findings that would result in false positives if no criteria were applied for unequivocal confirmation of the identity of detected compounds.

INTRODUCTION

Liquid chromatography-mass spectrometry (LC-MS) using atmospheric pressure ionization interfaces (API) has dramatically changed the analytical methods used to determine polar organic pollutants in water, as evidenced by the growing number of papers concerning the determination of polar pesticides in environmental water by LC-API-MS published in the scientific literature in the last few years. Interesting, detailed discussions on the use of LC-MS for the determination of organic pollutants, including pesticides, in water can be found in recent articles [1-4]. The great potential of LC-MS for pesticide residue analysis (PRA), focused on polar compounds, and the advantages of this technique in water analysis are widely recognised. However, several difficulties have to be overcome in qualitative and quantitative determination of organic compounds. The main obstacles are matrix effects that affect the ionisation of the analytes and obviously the quantification, the limited resolution of quadrupole and ion-trap MS, and the limited fragmentation encountered in collision-induced dissociation (CID), this latter restricting its application to qualitative analysis [5]. Additionally, high sensitivity is required in PRA of water, and one faces a challenging variety of pesticides of quite different polarities and chemical forms (anionic, cationic and neutral forms).

Some of the problems associated with LC-MS methods may be reduced by improved instrumentation [e.g. tandem MS (MS/MS) or time-of-flight (TOF)-MS] or improved chromatographic separation. Besides, an efficient sample clean-up is a common approach to remove interfering matrix components prior to the ionization process. For example, solid-phase extraction (SPE) with various sorbents has frequently been employed in water analysis, as a simple way of performing sample clean-up, and preconcentration. Thus, most published papers dealing with PRA in water by LC-MS use preconcentration by SPE, either off-line or on-line, before injection into the LC-API-MS system, in order to reach the sensitivity required. The sample volume processed normally depends on the instrument used, the limit of detection (LOD) required and the SPE mode applied (off-line or on-line). High sample volumes (500-1000 mL) are typically used when performing the SPE step off-line and using single MS instruments [6,7], but they can be lower (50-100 mL) when on-line SPE is used [8,9]. Moreover, if an MS/MS instrument is used, the volumes processed on-line can be decreased down to 10-25 mL [10,11] or even below 5 mL [12,13] and still achieve the required LODs. The excellent sensitivity reached by LC-MS/MS with triple quadrupole (QqQ) instruments may facilitate the detection of some polar pesticides in water, without performing any preconcentration step [14,15]. On-line SPE offers the possibility of reducing the sample volume in comparison to off-line procedures without loss in sensitivity, also minimizing sample manipulation and the use of organic solvents, with the advantage of being easily automated, facilitating its application in monitoring programs.

Efficient, practical monitoring of pesticides in water would require the use of multi-residue methods focused on those compounds most widely applied in the area under investigation, although other parameters, such as toxicity, persistence in the aquatic environment and mobility (leachability), would also have to be taken into account [16,17]. The wide range of polarities of pesticides that can potentially reach the water is one of the main obstacles in the development of multi-residue methods. This problem is increased by the current interest in monitoring the transformation products (TPs) of polar pesticides in water, considered as emerging contaminants by some authors [18]. Eventually, the list of selected analytes will surely include pesticides of different polarities and a number of relevant metabolites or TP [19]. The wide polarity range of analytes and the existence of different chemical forms in water will demand some kind of compromise to develop efficient multi-residue methods able to determine simultaneously highly polar TPs and medium polar pesticides, sacrificing sensitivity in some cases.

Different strategies could be applied for monitoring pesticides in water depending on the objectives pursued. The method requirements will differ, depending on whether it is intended simply to detect, to quantify or to confirm the presence of a possible residue. Analytical methods might be therefore classified into three categories:

- (i) screening methods, which may (quickly) detect the presence of one or more compounds based on one or more common characteristic of a class of pesticide in a qualitative or semi-quantitative manner at a specified concentration limit;
- (ii) quantitative (or determinative) methods, which should provide precise information concerning the amount of an analyte that may be present, but may provide only indirect information about the identity of the analyte; and,
- (iii) confirmatory methods, which should confirm the identity of the analyte but may or may not have a quantitative or semi-quantitative component [20].

Screening methods are useful because they provide greater analytical efficiency and allow samples with no detectable residue to be discriminated from those with pesticide residues at any appropriate level of interest. This would allow the analytical effort to focus on quantitation and confirmation of presumptive positive samples.

From this point of view, multi-residue SPE-LC-MS/MS methods used for screening and quantification satisfy the analytical demands of sensitivity, selectivity and accuracy required nowadays in PRA, but their screening capabilities are reduced due to the need to pre-select the MS/MS transitions before acquisition (target analysis), and they cannot reveal the presence of any other pesticide residues. It is very important that MS/MS methods can also unequivocally

confirm the identity of the compounds detected in water. Although MS/MS detection can be considered a very selective technique, this selectivity may be overestimated due to the high complexity of environmental samples. This might result in false positive findings, especially when low-resolution MS detection is used. In order to minimize this risk a recent European Commission Decision [22] established interesting criteria for the confirmation of positive findings based on the collection of identification points (IPs). Thus, adequate confirmation is made in low-resolution instruments [e.g., QqQ] acquiring two MS/MS transitions and measuring the ratio between them [21-23]. Although this decision was originally defined for the determination of organic contaminants in food samples, it has recently been used for confirming positive findings in other matrices, such as environmental samples [3,24].

The sensitive full-scan, high-mass resolution, and mass accuracy provided by TOF-MS are especially suited to screening and confirmation. For example, it has been shown that the higher mass resolution of TOF-MS allows the detection of some pesticides in river water, even when they are accompanied by isobaric compounds [25]. The potential of TOF-MS for unequivocally detecting emerging contaminants has also been recently pointed out [18]. This identification relies on the mass accuracy obtained, with mass errors below 2 mDa or 5 ppm normally being accepted for a positive identification [18,24]. Even more useful in terms of confirmatory analysis is a quadrupole-TOF combination (Q-TOF), as it allows MS/MS experiments to be performed to provide more structural information after the selection of a precursor ion, and that improves selectivity [3]. The use of hybrid Q-TOF instruments has allowed us to identify several unknowns in water [26], and its potential for the confirmation of pesticide TPs has also been proved in our laboratory [24,27].

Stolker et al [28,29] recently compared the potential of LC-QqQ-MS versus LC-Q-TOF-MS for the determination (screening and confirmation) of microconstituents in a variety of samples. It is remarkable that, although the later analyzers, QqQ and TOF, are gradually increasing their use and applicability in PRA, other analysers, as quadrupole ion traps (ITs) are also being applied in this field for similar purposes. The high sensitivity in scan mode along with the capability of performing MS^n experiments make this analyser adequate mainly for identification and confirmation.

In this paper, we discuss different strategies based on the use of SPE-LC-(ESI)-MS/MS for the determination of pesticides and TPs in water, with special emphasis on polar compounds present in water as ionic (anionic or cationic) or neutral forms. We consider several approaches using QqQ and TOF instruments for both quantification and confirmation of selected compounds widely used in the Mediterranean area. The potential of these techniques for screening,

quantitative and confirmatory analysis is also discussed, and some practical cases of water samples are presented for illustration.

LC-MS/MS DETERMINATION

The procedure used for determination by QqQ can be found elsewhere in more detail [12,13]. It is based on automated injection of a 2-mL aliquot of centrifuged sample into an SPE cartridge, where the analytes are preconcentrated. After washing the cartridge, analytes are transferred by back-flushing to an analytical column, where the separation of the analytes is performed.

Regarding Q-TOF analysis, the chromatographic system was similar to the previously described for QqQ. In this case, on-line preconcentration of 20-mL water is carried out in a longer analytical column in order to have better separation and spectrum purity. Experimental conditions are shown elsewhere [24].

In a previous paper [12], we developed a multi-residue SPE-LC-ESI-MS/MS method for the determination of 35 pesticides in groundwater and surface water, including 29 parent pesticides and 6 TPs that were previously selected from the most frequently applied pesticides in the Comunidad Valenciana Region, Spain. Later, the list of analytes was increased to 47 compounds, adding some acidic herbicides and several more TPs. The preconcentration cartridge was then changed to polymeric material, instead of the C18 used before, in order to increase the retention of the most polar compounds [13].

After applying the developed methodology to water samples collected from the Spanish Mediterranean area, we detected relevant concentrations of some pesticides (e.g., above 0.1 µg/L), mainly triazine herbicides; meanwhile, other (normally the less polar analytes and the majority of insecticides investigated) were not detected. As reported by Kolpin et al [30,31], in some situations, it is more appropriate to look for metabolites than for unchanged pesticides as the metabolites are more commonly detected and can be as toxic as unchanged pesticides. The use of LC-MS in environmental analysis is moving away from monitoring priority pollutants towards intensive identification and detection of TPs [3]. This trend was also pointed out by Richardson [4] in her detailed review on environmental MS, with the recognition that degradation products (often hydrolysis products) can be present at greater levels in the environment than the parent pesticide itself. Keeping in mind the need to update our previous methods, including the main metabolites of the most frequently detected compounds, we added several triazine metabolites to the list of selected compounds, removing pesticides that had not been detected.

Table 1 shows all the selected compounds as well as the optimal MS conditions of this updated method, based on the use of QqQ [32].

As a consequence of the high variation in the physico-chemical properties of the compounds selected, different approaches might be considered for their correct chromatographic separation and ionization, which depend mainly on the analyte charge. In some cases, a compromise will have to be reached between chromatographic and MS requirements in order to determine all selected analytes simultaneously (anionic, cationic and neutral).

Anionic compounds

Anionic compounds usually present abundant response in negative ionization, as they are inherently deprotonated. Normally, the main problem for LC-MS determination is to achieve correct chromatography, as the anionic compounds are eluted as non-retained compounds in reversed-phase liquid chromatography (RPLC).

Typically, the anionic forms come from weakly acidic compounds and, as a consequence, their dissociation depends on the working pH, so the acid-base equilibrium can be modified by changes in the pH of the mobile phase. This is the case with carboxylic acids, which, at neutral pH, are in the form of carboxylate anions, while, after acidifying the mobile phase, they become neutral carboxylic acids, in this way improving their chromatographic retention.

For very acidic compounds such as sulphates, which are deprotonated in water, other options are feasible to increase their chromatographic retention, such as using ionic chromatography [33] or forming ion-pairs in RPLC. In the latter case, the ion-pairing reagent must be sufficiently volatile to avoid deposition in the interface [34,35]. As an example, tetrabutyl ammonium has been used as ion-pairing reagent for the LC-MS determination of acidic compounds in environmental samples [36].

In our case, all the anionic compounds were carboxylic acids and therefore correct chromatographic behaviour could be obtained after acidifying the mobile phase. Although the presence of acid as modifier was necessary to obtain an efficient chromatography, the continuous introduction of its corresponding anion into the interface might inhibit the ionization of the anionic analytes, thus decreasing the sensitivity of the method. In order to minimize this undesirable effect, volatile acids have to be added at the lowest concentration that still generates sufficient chromatographic retention. Formic and acetic acids can be added into the

mobile phase for this purpose. We obtained satisfactory results using 0.01% formic acid in both aqueous and organic mobile phases.

Cationic compounds

The LC-MS determination of cationic compounds is usually performed in positive-ionization mode due to their inherent positive charge. Similarly to anionic analytes, one of the main problems for their residue determination is obtaining sufficient chromatographic retention.

The presence of the cationic form can also be a function of the working pH, mainly for amine compounds. An initial approach could be to add volatile bases in the mobile phase in order to deprotonate basic compounds. However, it is difficult to obtain a correct chromatography in this way due to the pH working range for common reversed phase stationary columns, normally between pH 2 and pH 7.

A more suitable approach in RPLC is to make use of ion-pairing chromatography, where a long-chain carboxylic acid is added to form an ion pair, thus improving retention in the column. Two of the most characteristic long-chain carboxylic acids used are heptafluorobutyric acid (HFBA) and tridecafluoroheptanoic acid (TDFHA). This approach has been applied to the determination of cationic pesticides, such as quaternary ammonium herbicides, in water with a satisfactory chromatographic behaviour [37].

The increase in the retention of the analyte achieved in this way is directly related to the size of the ion-pairing chain (i.e., the longer the chain the higher the retention). One also has to consider that the acidic character of these ion-pairing reagents would favour the protonation of analytes (cationic or neutral) measured in positive-ionization mode, increasing the sensitivity in positive mode. However, the ion-pairing reagent can also inhibit the ionisation, possibly because it can act as surfactant filling the droplet surface, making the desolvation of the analyte into the gas phase difficult [38]. From this point of view, the longer the chain, the higher the inhibition [39]. Another possible explanation for this loss of sensitivity might be the formation of persistent neutral ion-pairs, which are not introduced in the mass spectrometer and therefore not detected by MS.

Table 1. MS/MS conditions, exact mass and charge status for all the selected compounds

Compound	Polarity		Precursor ion		Product ions		Exact mass precursor ion	Status ^c
			(m/z)	Cone (V)	(m/z)	Collision (eV)		
desisopropyl-2-hydroxy-atrazine ^b	ESI+	Q ^c	156.1	30	86.1	15	156.0885	C
		q ^c			69.0	25		
2-hydroxy-simazine ^b	ESI+	Q	184.2	35	114.1	20	184.1198	N
		q			69.0	30		
DIA ^b	ESI+	Q	174.1	35	96.1	10	174.0546	C
		q			68.0	15		
2-hydroxy-terbutylazine ^b	ESI+	Q	212.1	30	156.2	15	212.1511	N
		q			86.1	30		
desethyl-2-hydroxy-terbutylazine ^b	ESI+	Q	184.1	30	128.0	15	184.1198	C
		q			86.1	25		
desethyl-terbutylazine ^b	ESI+	Q	202.1	25	146.1	15	202.0859	C
		q	204.1	25	148.1	15		
desethyl-terbumeton ^b	ESI+	Q	198.2	30	142.1	15	198.1355	C
		q			86.1	25		
Terbutryne	ESI+	Q	242.1	30	186.2	20	242.1439	N
		q			71.1	35		
Carbendazim	ESI+	Q	192.1	25	160.0	15	192.0773	N
		q			132.0	30		
2-aminobenzimidazol ^b	ESI+	Q	134.1	45	92.1	25	134.0718	C
		q			65.1	35		
3,4-dichloroaniline ^b	ESI+	Q	161.8	40	127.0	20	161.9877	C
		q			109.0	30		

(continued on next page)

Table 1 (cont). MS/MS conditions, exact mass and charge status for all the selected compounds

Compound	Polarity		Precursor ion		Product ions		Exact mass precursor ion	Status ^a
			(m/z)	Cone (V)	(m/z)	Collision (eV)		
propanyl	ESI+	Q ^c	218.0	35	162.0	15	218.0139	N
		q			127.0	25		
imazalyl	ESI+	Q	296.9	35	158.9	25	297.0561	N
		q			41.1	25		
Methidathion	ESI+	Q	302.9	20	145.1	10	302.9697	N
		q			85.1	25		
thiobencarb	ESI+	Q	258.0	25	125.1	15	258.0719	N
		q			100.2	10		
Simazine	ESI+	Q	202.1	30	132.1	20	202.0859	N
		q			124.2	20		
terbutylazine	ESI+	Q	230.1	30	174.1	15	230.1172	N
		Q	232.1	30	176.1	15		
Terbumeton	ESI+	Q	226.1	35	170.2	20	226.1668	N
		q			114.1	30		
Diuron	ESI+	Q	232.9	30	72.1	15	233.0248	N
		q			46.1	15		
oxamyl	ESI+	Q	242.0	25	72.0	15	242.0575	N
		q			121.0	15		
dimethoathe	ESI+	Q	229.9	20	125.0	20	230.0074	N
		q			199.0	10		

(continued on next page)

Table 1 (cont). MS/MS conditions, exact mass and charge status for all the selected compounds

Compound	Polarity		Precursor ion		Product ions		Exact mass precursor ion	Status ^c
			(m/z)	Cone (V)	(m/z)	Collision (eV)		
molinate	ESI+	Q ^c	188.1	25	126.2	15	188.1109	N
		q			55.1	25		
Terbacil	ESI+	Q	215.1	25	159.1	15	215.0588	N
		q	217.1	25	161.1	15		
MCPA	ESI+	Q	198.9	25	141.0	10	199.0162	A
		q	200.9	25	143.0	10		
Bantazone	ESI+	Q	238.9	40	132.0	30	239.0491	A
		q			197.0	20		
fluoxypyr	ESI+	Q	253.0	20	195.0	10	252.9583	A
		q	255.0	20	197.0	10		
bromacil	ESI+	Q	259.0	40	203.0	20	259.0082	N
		Q	261.0		205.0	20		

^a C, Cationic analyte; A, Anionic analyte; N, Neutral (nor cationic nor anionic) analyte;

^b Transformation product;

^c Q, Quantification transition; q, Confirmation transition

Some of the selected analytes in our work presented a primary amine group and would therefore be found as cationic compounds in an acidic-neutral medium. The absence of hydrophobic groups in some of them hampered their correct retention in RPLC, making it necessary to add long-chain acids, acting as ion-pairing reagents. This effect was observed for some transformation products of triazine, such as desethyl-2-hydroxy-terbutylazine, as can be seen in Figure 1(a). The two ion-pairing reagents tested, HFBA and TDFHA, led to satisfactory chromatography, and, as expected, retention time was higher when using TDFHA. However, the addition of TDFHA increased the ion suppression decreasing the sensitivity, mainly for compounds which did not require ion-pairing chromatography. This is the case with terbumeton (Figure 1.(c))

where the sensitivity was around 20-fold lower using TDFHA, so HFBA was selected as the ion-pairing reagent, as a compromise between sensitivity and retention (Figure 1.(b))

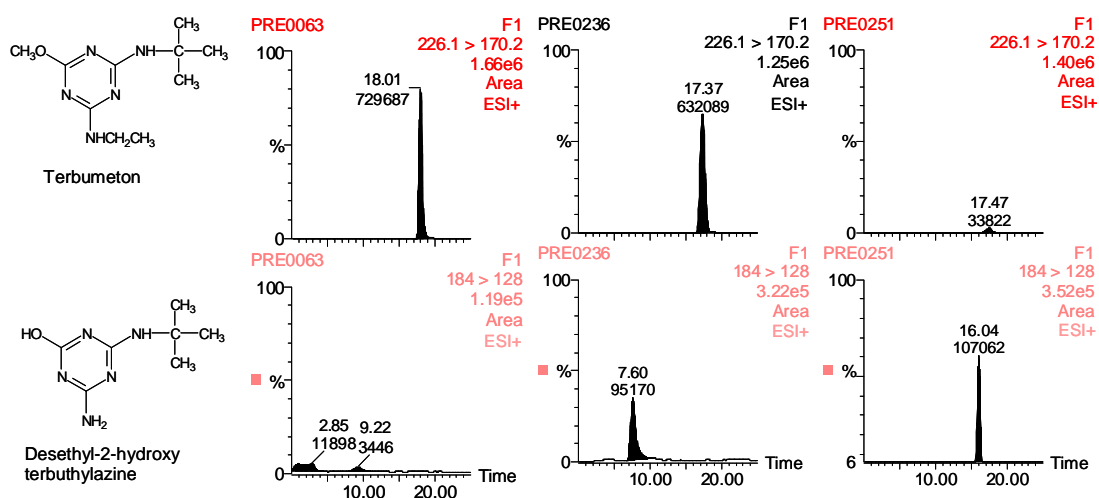


Figure 1. Effect of the ion-pairing reagent in the chromatography and the sensitivity in LC-MS/MS for terbutometone (top) and desethyl-2-hydroxy-terbutylazine (bottom): (a) without ion-pairing reagent, (b) using HFBA, and (c) using TDHFA

Neutral compounds

The least problematic situation regarding the behaviour of chromatography occurs with neutral analytes (without acid or basic groups), as they normally show satisfactory chromatographic retention without the need for adding modifiers into the mobile phase. However, in LC-API-MS determinations, adequate modifiers could be added to favor the ionization of neutral compounds. Thus, a small amount of acid would decrease the sensitivity for analytes measured in negative-ionization mode, while it would favor the positive-ionization mode.

As an example, Figure 2 shows the chromatograms for three compounds (anionic, cationic and neutral) obtained by adding HCOOH or HFBA to the mobile phase. While HFBA produced strong ion suppression for anionic compounds, such as fluoroxypir (Figure 2(a)), its presence was required for cationic compounds, such as 2-aminobenzimidazol, which was not detected in the absence of this ion-pairing reagent (Figure 2(b)). The neutral compound, carbendazim, was not significantly affected by these modifiers. This situation illustrates the difficulty of developing a method for the simultaneous residue determination of multi-class pesticides including cationic and anionic compounds.

In the following sections we will discuss the feasibility and the interest in determining all selected compounds in the same method depending on both the LC-MS instrument and the aim of the method (i.e., screening, or quantitative or confirmatory analysis)

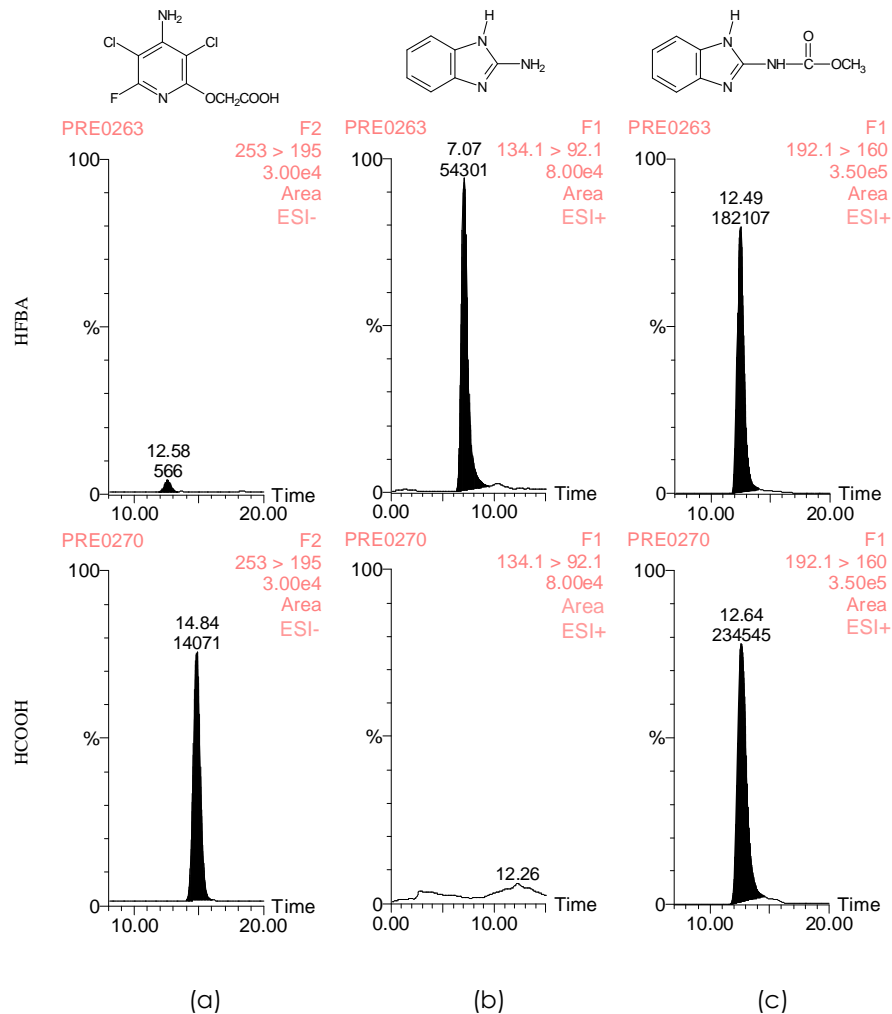


Figure 2. SPE-LC-MS/MS chromatograms obtained for 0.5 µg/L standard under different conditions (top: P-1 1mM aqueous HFBA:acetonitrile, P-2 water:acetonitrile, sample 25mM HFBA; bottom: P-1 1mM HCOOH: acetonitrile, P-2: 0.01% aqueous HCOOH: acetonitrile, sample: 0.5% HCOOH) for three analytes: (A) fluoroxypir (anionic), (B) 2-aminobenzimidazol (cationic) and (C) carbendazim (neutral).

SCREENING AND QUANTITATIVE METHODS

Screening methods are normally developed to determine the presence of contaminants in a sample rapidly. In some cases, they can also give a semi-quantitative estimation of the concentration level, but, in general, a second analysis is required to quantify correctly the analytes detected and confirm their identity.

In the case of PRA in water, screening methods should be able to detect a large number of compounds at sub- $\mu\text{g/L}$ level. Three alternatives might be considered for LC-MS screening methods in this field:

- (i) the development of pre-target screening methods, where the analytes are pre-selected and therefore other positives cannot be revealed;
- (ii) the development of post-target screening methods, where all the compounds eluted from the chromatographic column are measured by MS and the m/z of post-selected analytes are extracted afterwards from the total ion current chromatogram; and
- (iii) the development of non-target screening, sometimes named "general unknowns screening" (GUS), where all compounds eluted from the analytical column can be detected and identified without any kind of selection (with the obvious limitations derived from the chromatographic and ionization processes in the LC-MS interfaces used nowadays)

Selecting one of these alternatives depends on the objective of the screening and, especially on the instrument available.

The low sensitivity in full-scan mode of QqQ instruments makes it inadvisable to use them in post-target and non-target screening of pesticide residues. However, the sensitivity achieved in selective reaction monitoring (SRM) mode makes this instrument ideal for the pre-target screening, due to its excellent characteristics of sensitivity, selectivity, little sample manipulation and rapid analysis. But (Q)-TOF instruments are well suited to screening purposes, as their high resolving power may be sufficient to provide molecular formulae and to confirm or deny a suggested structure. The ability of TOF-MS to provide full scan spectra with high sensitivity makes this equipment an interesting choice for post-target and non-target screening.

- **Triple quadrupole instruments**

The main application of QqQ instruments is to develop target-screening methods. Considering the list of 27 selected analytes, including 18 polar and ionic pesticides and 9 TPs, two possible strategies could be followed: a single multi-residue method including all the analytes; or, multiple methods focussed on different chemical families.

Bearing in mind the quick detection of the maximum number of analytes, we initially tried to develop a single LC-MS/MS method for screening these compounds. As stated before, the main problem was the presence of different chemical forms (anionic, cationic and neutral) and, consequently, the conditions required for adequate simultaneous retention (SPE) and chromatographic separation (LC) as well as optimum MS response. Therefore, a compromise must be reached in order to determine all selected analytes simultaneously.

As stated in the previous section, HFBA was chosen as ion-pairing reagent as a compromise between sensitivity and chromatographic retention for cationic analytes. Additionally, its acidic character favored the chromatographic retention of anionic compounds. Thus, the use of HFBA was tested for the simultaneous determination of all selected compounds. The high ion suppression produced by the heptafluorobutirate ion hampered the determination of several analytes, mainly those measured in negative-ionization mode, making unfeasible the determination of several analytes at 0.1- $\mu\text{g/L}$ level. In order to minimize this problem, HFBA was removed from the mobile phase avoiding its continuous introduction in the interface. This approach has been applied successfully in our previous work dealing with the determination of fosetyl aluminium where the ion-pairing reagent (TBA) was added into the sample vial instead of the mobile phase [40].

To try to achieve the same effect, HFBA was removed from the P-2 mobile phase and its concentration was increased in P-1, used for loading the SPE cartridge. Thus, the retention in the SPE cartridge increased, minimizing breakthrough. Additionally, the ion-pair preformed in the SPE cartridge was transferred into the analytical column, facilitating the achievement of sufficient chromatographic retention and increasing the sensitivity of the method, as HFBA was not present in the mobile phase.

By using HFBA in both the sample vial and P-1, most compounds could be detected below the 0.1- $\mu\text{g/L}$ level, including some analytes with negative ionization (see Figure 2(a)). However, for several compounds (anionic), there was insufficient sensitivity, considering the present regulations for pesticide residues in water. Besides, using only one transition for every

analyte, quantification would be possible, but confirmation would not be achieved (see the next section).

In this way, only screening and quantification of a certain number of pesticides and TPs (around 22 out of 27) was found to be satisfactory with the sensitivity required by this LC-MS/MS method using QqQ (LODs around 0.01-0.02 µg/L for neutral and cationic analytes), and a second analysis would be required for confirmation.

We would like to emphasize that the scenario chosen in this example possibly corresponds to the worst case, as we are faced with anionic, cationic and neutral compounds. Certainly, the LC-MS/MS approach using QqQ can be an excellent way for the screening of multi-class pesticides, including quantification, if, for example, cationic compounds are not included in the pre-selection of target analytes. In such a case, no ion-pairing reagent would be necessary for these compounds, but obviously cationic compounds would not be retained in the cartridge (i.e., some triazines TPs would not be detected). This is illustrated in the method previously developed by our research group for 35 pesticides [12], subsequently increased to 47 compounds, including some acidic herbicides [13], and in some papers found in the literature [41-45]. Normally, the analytical characteristics of the LC-MS/MS methods are excellent in terms of sensitivity and selectivity [limits of quantification (LOQs) around 0.01-0.025 µg/L are typical], although matrix effects are a drawback of this technology, and they have to be carefully considered for correct quantification.

However, some general limitations of QqQ for target screening purposes should be pointed out;

- (i) the limited number of transitions that can be monitored in a chromatographic run limits the number of compounds to be included in the method (less problematic in the new generation of instruments);
- (ii) the required selection of the analytes prior to the development of the method (always target analysis); and,
- (iii) the need for re-analysis where other compounds are suspected to be present in the sample. These limitations are normally present in most of previously reported methods, where only one transition for every pre-selected compound has been normally acquired.

- **TOF instruments**

The use of LC-API-MS instruments could be of great interest in non-target screening methods for water analysis. Non-target screening is more common in clinical and forensic analysis, and some authors use the term of GUS in this field. For example, non-target procedures allow the unambiguous identification of xenobiotics in intoxication cases when there are no hints on the compound(s) involved. LC-MS/MS shows strong potential for GUS, especially when using information-dependent acquisition (IDA), where, at each time unit, the m/z ions of high intensity in scan mode are automatically selected for fragmentation in product-ion scan mode (automated MS/MS). In this way, MS/MS information is obtained for only those compounds abundant enough to give a MS response above a threshold value, allowing their unambiguous identification after searching in a home-made library or comparing with the MS or MS/MS spectra of reference standards, if available [46-48]. Q-TOF instruments are well suited for this purpose due to their improved performance in terms of sensitivity and resolving power in comparison with other mass analyzers such as QqQ or IT. Q-Trap, a hybrid mass analyzer that has recently appeared, might also be an adequate alternative for performing non-target analysis due to its sensitivity in both full-scan and product-ion scan modes, although with lower resolution than TOF analyzers.

In spite of the potential of MS for non-target screening, the GUS approach presents several limitations in the field of residue analysis, the most important being the low sensitivity of TOF instruments, compared with QqQ in SRM mode, which hampers the detection and identification of the analytes at the sub- $\mu\text{g/L}$ level. This limitation is even more important for IDA procedures, as the compound, present at the residue level, needs to give enough response to be identified by the system as an abundant compound in complex matrices and this can be difficult. Another problem in this field is that a preconcentration step is normally required, and there is a need to achieve a compromise in the experimental conditions in order to include the maximum number of analytes. But, during the analytical process, it is almost impossible to avoid the "loss" of some compounds that may be present and that are relevant from an environmental and toxicological point of view.

A more realistic approach using TOF would be the post-target screening of pesticide residues acquiring the full-scan spectra at high resolution and performing subsequent ion-extract chromatograms (XIC) at post-selected m/z . In addition to the high sensitivity of TOF in full-scan mode, another advantage of these instruments is the possibility of obtaining XIC with a narrow mass window (0.05 Da), which decreases the chemical noise, improving the sensitivity and the reliability of the data [25].

In a post-target screening, only selected analytes can be detected, but the number of selected compounds might be unlimited. Thus, TOF instruments offer the possibility in screening or investigating afterwards (once the analysis have been performed and data acquired) other compounds that could be present in the samples, without performing additional analysis. This is a strength of this technology in comparison with QqQ, where the pre-selection of analytes before analysis is compulsory in SRM methods. Moreover, the presence of either an abundant isotopic pattern in the analyte molecule or an in-source fragment with accurate mass measurement helps to confirm the presence of the post-selected analyte (see the next section).

The main problems of this approach, compared with QqQ in SRM mode, are the lower sensitivity achieved, which hampers the identification of analytes at low concentrations, and limited quantification. We performed the analysis of several water samples by LC-TOF-MS in order to investigate the presence of pesticides and TPs by means of the full-scan spectra obtained at high resolving power. Our data showed that all the analytes present at relatively high concentrations (higher than 0.05 µg/L) could be detected by both QqQ and TOF-MS methods. However, when the concentration in water was lower, the sensitivity with TOF-MS was insufficient to detect these compounds. As an example, Figure 3 compares the determinations of 2-hydroxy-simazine (0.03 µg/L) and terbutylazine (0.08 µg/L) by QqQ and TOF. The higher sensitivity of the QqQ allowed 2-hydroxy-simazine to be detected in this sample while it was not detected by TOF.

The use of TOF for the simultaneous screening and quantification of pesticide residues is still problematic because of the low linear range of the MS response in this type of instrument. Some attempts have been described, but the dynamic linear range was around or lower than two orders of magnitude [49,50]. Although it is predictable that the development of new TOF instruments will gradually solve this drawback and facilitate its applicability to quantitative analysis, the main application of TOF remains in the qualitative field.

When a QTOF instrument is available, pre-selection of a precursor ion in the quadrupole filter and the recording of the full scan product ion spectra with high mass accuracy are also possible, so it is the most valuable tool for confirmatory analysis, as discussed in the next section.

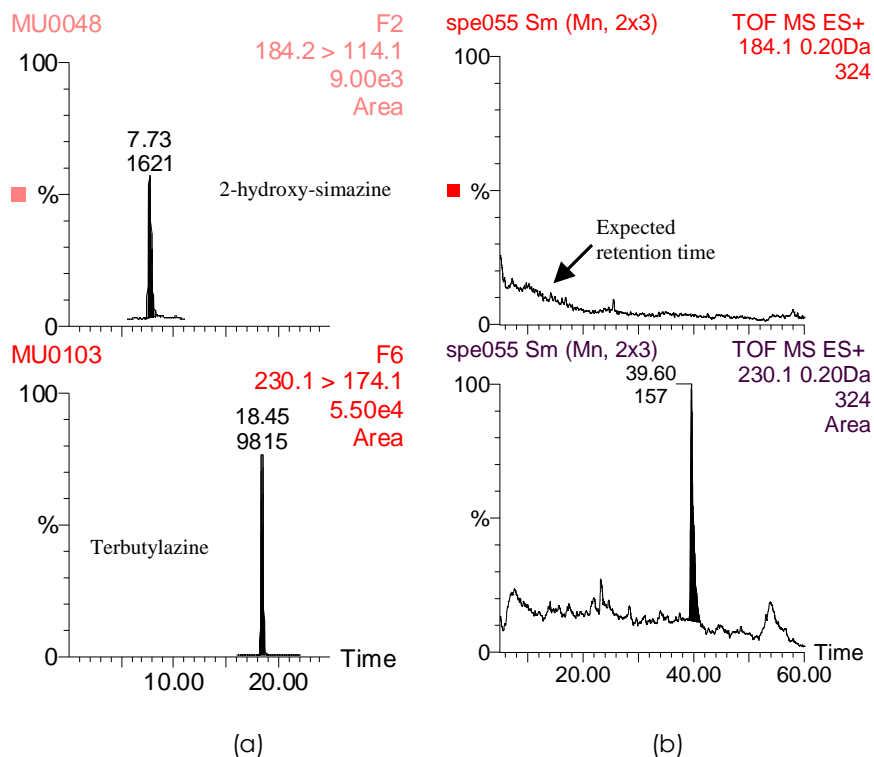


Figure 3. Chromatograms obtained in the measurement of terbutylazine (0.08 $\mu\text{g/L}$) and 2-hydroxy-simazine (0.03 $\mu\text{g/L}$) in a positive sample by: (a) QqQ acquiring one transition and (b) TOF showing a XIC with a 0.2 Da mass window.

CONFIRMATORY METHODS

Increasing concern about the confirmation of positive data, favored the development of different criteria to assure data quality and to avoid the reporting of false positives. This subject was treated in detail by Reemstma [3] in his excellent review on LC-MS analysis of polar organic pollutants.

The criteria established in a Commission Decision of the EU are becoming the most important in Europe [22]. In summary, this Decision proposes a system of Identification Points (IPs), where at least three IPs are required (four in the case of banned compounds) to confirm a positive finding. In addition, the deviation of the relative intensity of the recorded ions must not exceed a certain percentage of the reference standard, and the retention time must not deviate more than 2.5%. This means that it is necessary to acquire three ions in single-MS

instruments (3 IPs), or the selection of one precursor ion and the recording of two product ions at low resolution in MS/MS instruments, resulting in 4 IPs. However, many published LC-MS or LC-MS/MS methods acquired only one ion or transition for every analyte (1 and 2.5 IPs, respectively), so they should be considered as screening methods or determinative methods (normally quantification is possible), but some of the positive findings reported might become questionable [3].

Different strategies could be used in order to reach the number of IPs required for the confirmation of positive findings, depending basically on the instrument available. Two approaches are considered in this paper. Both are suitable regarding sensitivity and the number of IPs obtained:

- (i) the acquisition of two (or more transitions) in QqQ instruments; and,
- (ii) the acquisition of the total product ion spectra with QTOF instruments.

- **Triple quadrupole instruments**

The simplest way to confirm a positive finding when using QqQ instruments is to acquire two selective transitions for each analyte. Therefore, upgrading a target-screening and quantitative LC-MS/MS method to a confirmative method could easily be performed modifying the method by adding one transition more for every selected compound. However, the two-fold increase in acquired transitions could generate some problems in the MS determination, as it usually involves a decrease in either the time of acquiring the transition or the number of points obtained per peak. When developing multi-residue methods, a compromise between sensitivity and peak shape has to be reached. This problem might be minimised by the use of a new design of instruments where the acquisition time can be decreased without loss of sensitivity. These instruments allow increasing the number of MS/MS transitions without any loss in either sensitivity or peak shape, but their cost is still too high for environmental laboratories, so, although their use in routine environmental analysis is expected in the near future, at present analytical chemists are forced to develop other strategies in order to perform confirmative analysis.

The most logical approach for the confirmation of positive findings would be to perform a second injection, adding the maximum number of selective transitions for every detected compound. This strategy was suggested in our previous work for the confirmation of positive findings [13] and it has been applied by Stolker et al [28] for the confirmation of pharmaceuticals in surface-water and wastewater effluents. However, this approach presents several

disadvantages such as the need to perform two injections or the need to use different confirmative methods depending on the detected analyte.

As stated in previous sections, the development of a target-screening method for a variety of anionic, cationic and neutral compounds presents several obstacles. The incompatibility in the chromatographic optimization between anionic and cationic analytes made difficult the simultaneous screening of all selected analytes at the regulatory or relevant concentration levels. Thus, the determination of anionic compounds with enough sensitivity was only feasible in the absence of ion-pairing reagents. However the addition of HFBA was required to achieve a satisfactory retention for cationic compounds. Consequently, we have developed two different, complementary multi-residue methods for the simultaneous determination and confirmation of selected polar pesticides and TPs [32]. Analytes were divided into two groups:

- (i) one formed by anionic compounds that were preconcentrated and chromatographically separated using formic acid: and,
- (ii) one formed by cationic compounds, where HFBA was added into the vial sample and to P-1 (used for loading and washing the SPE cartridge).

Neutral compounds were added into one or other group depending on their retention time in order to obtain an adequate number of transitions in each method. Two transitions were selected for each analyte and included in the development and optimization of each method. The ion ratios were monitored and the ion-abundance ratios had to be within a tolerance of 20% relative to that of reference standards to confirm a finding as positive. Normally, the most sensitive transition was used for quantification and the other for confirmation. In this way, all the water samples were routinely analyzed by both methods, which allowed simultaneous screening, quantification and confirmation of all pre-selected analytes with excellent sensitivity.

This strategy presented some advantages. Firstly, the determination of each analyte could be performed under its optimum conditions, due to the separation into groups and to the lower number of compounds to be determined in each chromatographic run. This led to a considerable increase in sensitivity in some cases and allowed us to determine all selected compounds at the sub- $\mu\text{g/L}$ level, the method being validated at $0.025 \mu\text{g/L}$, reaching LODs lower than $0.01 \mu\text{g/L}$ for all the analytes [32]. Moreover, the simultaneous acquisition of two transitions per analyte allowed us to obtain directly 4 or 5 IPs (the same or different precursor ion selected in each transition), so confirmation of the analyte was performed at the same time.

Confirmation without a second analysis can be seen as an advantage, but it also has a drawback. Re-injecting the sample implies a repetition of the analysis (normally on a different day), which minimises the risk of sample contamination as it is manipulated twice independently. This is important when there are potential non-compliant residues that should be quantitatively confirmed by analysis of at least one additional test portion [51]. Performing quantification and confirmation simultaneously, might lead to the conclusion that a second analysis is superfluous. Certainly, in on-line SPE-LC-MS/MS methods, sample manipulation is significantly reduced and the risk of contamination during the analytical procedure is minimized, as the sample is directly injected immediately after adding either formic acid or HFBA. However, one should not ignore the possibility of contamination and, consequently, a second injection might be necessary, or at least recommendable, in the case of positive results at non-compliant levels.

- **Q-TOF instruments**

The most interesting aspects of Q-TOF are its high sensitivity in the scan mode and the possibility of obtaining product ion spectra with high mass accuracy. These advantages facilitate the confirmation of analytes suspected to be positive, making the ultimate confirmation by the acquisition of the totality of product ions with high mass accuracy possible. With this approach, the number of IPs is considerably increased due to the high number of product ions that could be present and the mass accuracy obtained. However, the European Decision is, in some ways, ambiguous whether TOF instruments can be considered high-resolution instruments, as they normally do not achieve the required resolution for this classification [18]. Despite this, some authors consider TOF instruments as high-resolution detectors [52], so 2.5 IPs would usually be associated with each product ion, although the assignment of an intermediate number of IPs would be an interesting option [25]. Regardless, using Q-TOF for confirmation leads to the maximum number of IPs, allowing the ultimate and unequivocal confirmation of positive findings.

Despite their great potential for confirmation purposes compared with QqQ, there are a limited number of QTOF instruments being used in the field of residues, mainly due to the lower sensitivity and high price of Q-TOF instruments. In our experience, the confirmation of residues by QqQ was feasible for most of analytes at concentrations around the LOD (normally close to 0.01 µg/L). However, the confirmation by Q-TOF of some positive findings detected previously in pre-target QqQ screening was not feasible. In order to minimize this limitation, a higher (10-fold) sample volume was preconcentrated by SPE and reanalysed by Q-TOF MS, improving the sensitivity of the method in this way. In spite of this, some compounds detected and confirmed by QqQ could still not be confirmed by Q-TOF at low sub µg/L levels.

Figure 4 shows a representative water sample, where confirmation of positive findings for terbumeton and terbuthylazine was made by both QqQ and QTOF MS. As can be seen in Figure 4, Q-TOF could confirm the presence of terbumeton at 0.89 µg/L and terbuthylazine at 0.39 µg/L level. The use of Q-TOF allowed more than 10 IPs to be obtained for both compounds, while confirmation by QqQ only reached 4 and 5 IPs for terbumeton and terbuthylazine, respectively. However, the presence of DIA at 0.02 µg/L could be confirmed using only QqQ due to the higher sensitivity of this technique. In summary, it can be concluded that both instruments are useful and, in some way, complementary regarding confirmation, as QTOF allows the maximum number of IPs to be obtained and QqQ allows confirmation at lower concentration levels. Table 2 presents data on positive findings in water by both instruments.

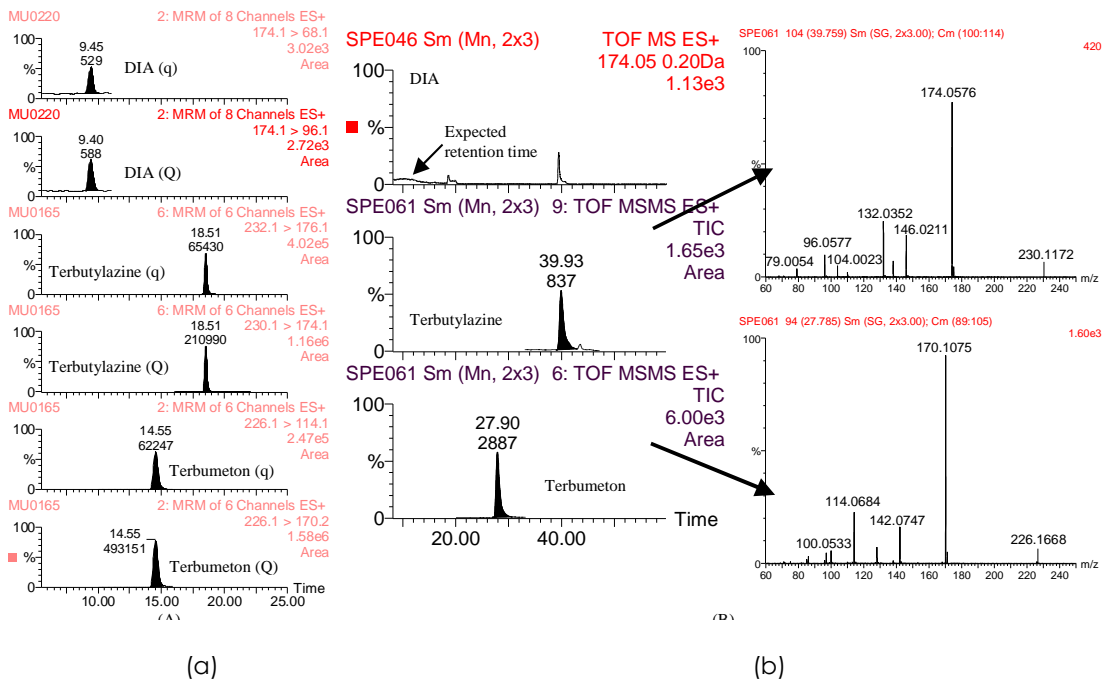


Figure 4. Chromatograms obtained in the confirmation of terbumeton (0.89 µg/L), terbuthylazine (0.39 µg/L) and DIA (0.02 µg/L) in a positive sample by (a) QqQ acquiring two transitions and (b) QTOF showing the product ion spectra with accurate mass obtained for each detected analyte. Q: quantification transition, q: confirmation transition.

Table 2. Confirmation of some positive findings. Comparison between QqQ and QTOF capabilities

Compound	Quantification	Confirmation QqQ				Confirmation Q-TOF				
	Conc ^a (µg/L)	Conc ^b (µg/L)	Conc ^c (µg/L)	relative error (%)	IPs ^d	Ion	Exact mass (m/z)	Measured mass (m/z)	Deviation (mDa)	IPs ^e
Desethyl- terbumeton C ₈ H ₁₅ N ₅ O	1.37	1.371	1.309	4	4	[M+H] ⁺	198.13	198.1339	1.6	8
						[M-C ₄ H ₈] ⁺	142.07	142.0729	1.2	
						[M-C ₅ H ₁₀ N ₂] ⁺	100.05	100.0545	3.4	
						[M-C ₆ H ₁₂ N ₂] ⁺	86.035	86.0361	0.7	
Imazalil C ₁₄ H ₁₄ N ₂ OCl ₂	0.17	0.169	0.192	14	4	[M+H] ⁺	297.05	297.0582	2.1	10.
						[M-C ₃ H ₆] ⁺	255.00	255.0110	1.8	
						[M-C ₅ H ₈ N ₂] ⁺	200.98	200.9863	1.1	
						[M-C ₇ H ₁₀ N ₂ O] ⁺	158.97	158.9762	0.6	
						[C ₃ H ₅ N ₂] ⁺	69.045	69.0491	3.8	
Carbendazi C ₉ H ₁₀ N ₃ O ₂	0.37	0.366	0.360	2	4	[M+H] ⁺	192.07	192.0744	2.9	6
						[M-CH ₄ O] ⁺	160.05	160.0522	0.8	
						[M-C ₂ H ₄ O ₂] ⁺	132.05	132.0562	1.7	
Desethyl- terbutylazin C ₈ H ₁₅ N ₅ O	0.02	0.018	0.017	3	5	[M+H] ⁺	2020.0	-	-	-

^a Reported concentration^b Concentration calculated from the quantification transition^c Concentration calculated from the confirmation transition^d IPs calculated as in European Decision [22]^e IPs calculated as shown in [24]

APPLICATION TO SURFACE AND GROUNDWATER

The strategy presented in this paper was applied to around 100 surface water and groundwater samples collected from the Valencian Region, a Spanish Mediterranean area with a long history in agriculture and with a predominance of citric crops. The most useful approach for application in actual samples was found to be the use of QqQ-MS-based methodology, which allowed the rapid screening, quantification and confirmation of residues detected using the two complementary methods developed. This strategy allowed a compromise between sensitive determination and confirmation, which proved successful for all selected analytes at concentration levels as low as 0.01-0.02 µg/L. The excellent characteristics of this approach can be deduced from Figure 4, where low levels of pesticides/TPs were detected and confirmed (0.02 µg/L in the case of DIA).

Results obtained in our analyses are summarised in Table 3. Most TPs included in the screening method were amply detected, mainly triazine TPs, showing the interest in including these compounds in the analysis. More than 10% of the samples contained concentrations higher than 0.1µg/L for several of the triazines and their TPs. These results agreed with our previous monitoring programme, where triazines were the most frequently detected pesticides in both groundwater and surface water [13]. In addition to triazines and their TPs (normally cationic analytes), other compounds frequently detected were bromacil (21% of samples with concentration > 0.1µg/L), MCPA and bentazone (6% of samples with concentration > 0.1µg/L), which presented negative ionization. The most commonly detected analytes were therefore either cationic or anionic compounds, proving the usefulness of applying two complementary methods as their simultaneous determination in a general, unique screening method is difficult, as we have illustrated in this article.

Table 3. Summary of positive findings for pesticides and TPs in groundwater and surface-water of the Mediterranean Valencian region (n = aprox 100).

Compound	% Positive samples	% Samples > 0.1 µg/L	Maximum level (µg/L)
Simazine	61	3	0.45
2-hydroxy-simazine	3	0	0.07
desisopropyl-2-hydroxy-atrazine	3	0	0.03
DIA	38	6	0.25
Terbutylazine	45	4	0.40
2-hydroxy-terbutylazine	58	13	0.16
desethyl-2-hydroxy-terbutilazine	72	14	0.21
desethyl-terbutilazine	54	4	0.63
terbumeton	28	4	0.96
desethyl-terbumeton	68	11	1.62
Terbutrin	18	0	0.07
carbendazim	39	1	0.37
Diuron	30	4	0.37
3,4-Dichloroaniline	4	4	9.92
Propanil	1	0	0.03
Imazalil	11	4	0.22
methidathion	1	0	0.03
dimethoate	4	0	0.03
molinate	1	0	0.01
terbacil	7	0	0.04
MCPA	8	6	0.65
bentazon	10	6	0.42
bromacil	38	21	0.57

Regarding confirmation of positives, the established criteria was achieved in 95% of cases, which means that no false positives would have been reported if only one transition was acquired in 95% of cases. Thus, in only a few cases, the monitoring of one transition did not assure the presence of the analyte, as the second transition did not fit the established requirements, and consequently a positive was not reported. As an example, Figure 5 shows comparative chromatograms for a reference standard of the insecticide dimethoate and for a water sample that could have been reported as positive for this compound (concentration estimated around 0.025 µg/L). The signal obtained when acquiring the quantification transition (230>125) together

with the similar retention time would lead to reporting this sample as positive for dimethoate. However, no signal was obtained for the confirmation transition (230>199) and therefore the ion ratio abundance did not fit the criteria established. Consequently, we concluded that the compound detected was not dimethoate but a co-eluting compound, which shared the quantification transition. This situation was repeated in a few more water samples, there being false positives for dimethoate. Some other cases were found for 2-hydroxy-terbutylazine and carbendazim, where expected ion-abundance ratios were not obtained. As a result, although it is unlikely, there is a potential risk of reporting false positives when acquiring only one transition using LC-MS/MS (QqQ), and this supports the value of the European Union Decision [22].

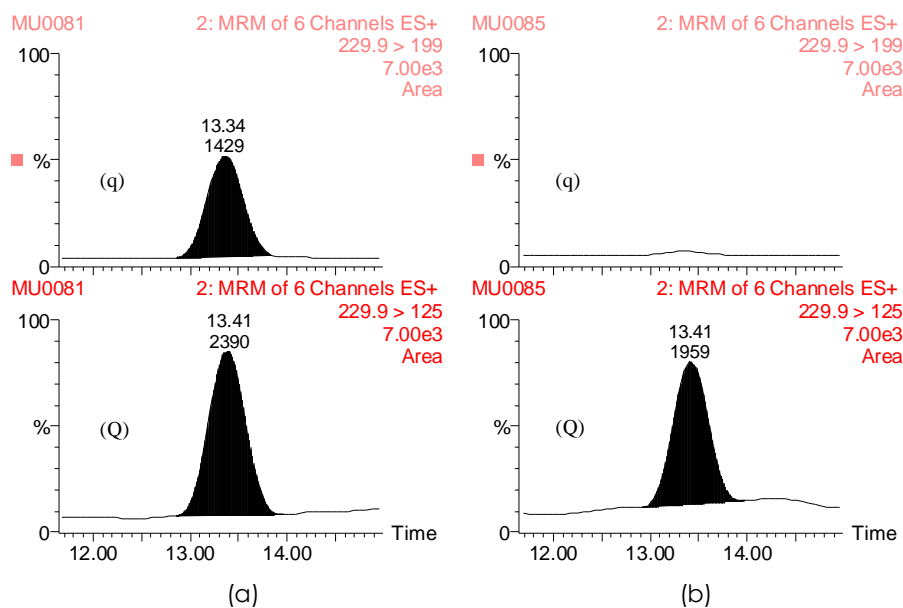


Figure 5. Chromatograms of (a) dimethoate standard, 0.025 µg/L and (b) water sample suspected to be positive for dimethoate. Q, quantification transition; q, confirmation transition.

Although in SPE-LC-MS/MS procedures there is very little sample manipulation, one should not ignore the possibility of contamination during the analytical procedure. For this reason, and also for comparison and re-confirmation of positives, several of the positive samples (normally groundwater with non-compliant concentrations, i.e. higher than 0.1 µg/L) were reanalyzed by injecting 20 mL into the SPE system coupled on-line with an LC-Q-TOF instrument. This additional confirmation was carried out by recording the product ion spectra of the selected parent pesticide at different collision energies, in this way recording the total number of product ions.

Our results showed that all positives detected and confirmed by QqQ- MS were reconfirmed with Q-TOF normally obtaining more than 7 IPs (see Figure 4 (b)). The ultimate confirmation by Q-TOF allowed us to be sure about the presence of the analytes in the sample, verifying that the use of two transitions in QqQ was a satisfactory approach.

CONCLUSIONS

We have discussed different strategies based on the use of SPE-LC-API-MS for the screening, quantification and confirmation of polar pesticides and TPs. The most common and single approach for the multi-residue screening and quantification is to use QqQ instruments to record just one transition for each pre-selected analyte. However, this approach presents some drawbacks:

- the differences in polarities and chemical forms of the analytes (anionic, cationic and neutral) make difficult the simultaneous preconcentration, chromatographic separation and MS determination of all compounds;
- additional analysis is required for confirmation of compounds detected.; and,
- pre-selection of the analytes (target analysis) is required, and that hampers the detection of other relevant compounds that might be present in the samples.

TOF instruments have the advantage of allowing the non-target screening, although it is difficult to search for relevant peaks and to process all information acquired in order to establish the identity of the "unknowns" present in samples. Besides, the lower sensitivity and limited quantification capabilities of this technique, compared with QqQ working in SRM mode, make it inadvisable for PRA. A more suitable strategy using TOF instruments could be to develop post-target screening methods where analytes are selected after acquiring the MS data in full -scan mode, allowing the searching of (theoretically) an unlimited number of compounds, but again with limitations in sensitivity and quantification.

In this article, which deals with the residue determination of anionic, cationic and neutral compounds, we found that the development of two multi-residue methods by QqQ was the most powerful approach. In one method, formic acid was added in the mobile phase, giving satisfactory chromatography for anionic and neutral analytes. In the other method, HFBA was added into the vial and into the cartridge-conditioning solvent, obtaining satisfactory chromatographic and MS behaviour for cationic and neutral compounds. Thus, analytes were included in one or other method depending on their ionic character, allowing their determination in optimal conditions as regards sensitivity. The reduction in the number of monitored compounds as a consequence of the distribution in two methods allowed us to include two transitions for each analyte, favoring the confirmation of positive findings by collecting sufficient number of IPs. The application of these two methods allowed the rapid direct detection, quantification and confirmation of all pre-selected compounds at the 0.025- $\mu\text{g/L}$ level. If an ultimate confirmation of analytes is needed, the use of Q-TOF instruments allows the

maximum number of IPs to be reached by acquiring the product ion spectrum with mass accurate. However, the lower sensitivity of this technique made difficult the confirmation of positive samples at low concentration levels. New hybrid tandem mass spectrometers, such as quadrupole-linear IT (Qq-LIT) or LIT-ion cyclotron resonance (LIT-ICR), might also be useful for achieving this ultimate confirmation due to their inherent characteristics, and their potential in this field will surely be widely investigated in the near future.

Although the confirmation criterion based on collection of sufficient number of IPs is useful, the quality of the transitions in tandem MS-based methods has to be carefully considered, as non-selective transitions (e.g., loss of water, carbon dioxide, and hydrochloric acid) are more prone to be hampered and might lead to the reporting of false positives or negatives in the samples.

The usefulness of the methods developed was tested by analyzing around 100 groundwater and surface-water samples. We proved that it is necessary to acquire two transitions in QqQ-MS in order to confirm the identity of the compounds detected and to avoid reporting false positives. For positive real samples at non-compliant levels (i.e., >0.1 µg/L), additional and ultimate confirmation was achieved by Q-TOF.

ACKNOWLEDGEMENTS

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for using the Quattro LC triple quadrupole mass spectrometer and the Q-TOF. This work has been developed with the financial support of the Ministry of Science and Technology (Ref REN2002-01818 and Ref BQU2003-02685), Spain.

M. Ibáñez is very grateful to Generalitat Valenciana for her pre-doctoral grant.

REFERENCES

- (1) A.C. Hogenboom, W.M.A. Niessen, U.A.Th. Brinkman, *J. Sep. Sci.* 24 (2001) 331
- (2) R.B. Geerdink, W.M.A. Niessen, U.A.Th. Brinkman, *J. Chromatogr. A* 970 (2002) 65
- (3) T. Reemstma, *J. Chromatogr. A* 1000 (2003) 477
- (4) S.D. Richardson, *Anal. Chem.* 76 (2004) 3337
- (5) T. Reemstma, *Trends Anal. Chem.* 20 (2001) 533
- (6) K. Martínez, I. Ferrer, D. Barceló, *J. Chromatogr. A* 879 (2000) 27
- (7) S. Rodríguez-Mozaz, M.J. López de Alda, D. Barceló, *J. Chromatogr. A* 1045 (2004) 85
- (8) I. Ferrer, D. Barceló, *J. Chromatogr. A* 854 (1999) 197
- (9) T.C.R. Santos, J.C. Rocha, D. Barceló, *J. Chromatogr. A* 879 (2000) 3
- (10) A.C. Hogenboom, M.P. Hofman, D.A. Jolly, W.M.A. Niessen, U.A.Th. Brinkman, *J. Chromatogr. A* 885 (2000) 377
- (11) A.C. Hogenboom, W.M.A. Niessen, U.A.Th. Brinkman, *Rapid Commun. Mass Spectrom.* 14 (2000) 1914
- (12) F. Hernández, J.V. Sancho, O. Pozo, A. Lara, E. Pitarch, *J. Chromatogr. A* 939 (2001) 1
- (13) J.V. Sancho, O.J. Pozo, F. Hernández, *Analyst (Cambridge, UK)* 129 (2004) 38
- (14) O. Pozo, E. Pitarch, J.V. Sancho, F. Hernández, *J. Chromatogr. A* 923 (2001) 75
- (15) B.A. Ingelse, R.C.J. van Dam, R.J. Vreeken, H.G.J. Mol, O.M. Steijger, *J. Chromatogr. A* 918 (2001) 67
- (16) D. Barceló, *J. Chromatogr.* 643 (1993) 117
- (17) D. Barceló, M. C. Hennion, *Trace Determination of Pesticides and Their Degradation Products*, Elsevier, Amsterdam, The Netherlands, 1997, pp 541.
- (18) I. Ferrer, E.M. Thurman, *Trends Anal. Chem.* 22 (2003) 750
- (19) European Commission, SANCO 221/2000, rev. 10 Final, Guidance document on assessment of the relevance of metabolites in groundwater of substances regulated under Council Directive 91/414/EEC, European Commission, Brussels, Belgium, February 2003 (http://europa.eu.int/comm/food/fs/sc/scp/out139_ppp_en.pdf)

- (20) World Health Organisation, Codex Alimentarius Commission CX/RVDF 04/15/7, General Consideration on Analytical Methods for Residue Control, FAO/WHO, Geneva, Switzerland, October 2004.
- (21) F. André, K.K.G. de Wasch, H.F. de Brabander, S.R. Impens, L.A.M. Stolker, L. Van Ginkel, R.W. Stephany, R. Schilt, D. Courtheyn, Y. Bonnaire, P. Furst, P. Gowik, G. Kennedy, T. Kuhn, J.P. Moretain, M. Sauer, Trends Anal. Chem. 20 (2001) 436
- (22) European Commission Decision 2002/657/EC, Off. J. Eur. Commun., L221 (2002) 8
- (23) L.A.M. Stolker, W. Niessing, E.A. Hogendoorn, A.B. Rambali, W. Vleeming, J. Chromatogr. A 1020 (2003) 35
- (24) F. Hernández, M. Ibáñez, J.V. Sancho, O.J. Pozo, Anal. Chem. 76 (2004) 4349
- (25) A.C. Hogenboom, W.M.A. Niessen, D. Little, U.A.Th. Brinkman, Rapid Commun. Mass Spectrom. 13 (1999) 125
- (26) M. Ibáñez, J.V. Sancho, O.J. Pozo, W.M.A. Niessen, F. Hernández, Rapid Commun. Mass Spectrom. 19 (2005) 169
- (27) M. Ibáñez, J.V. Sancho, O.J. Pozo, F. Hernández, Anal. Chem. 76 (2004) 1328
- (28) L.A.M. Stolker, W. Niessing, E.A. Hogendoorn, J.F.M. Versteegh, R. Fuchs, U.A.Th. Brinkman, Anal. Bioanal. Chem. 378 (2004) 955
- (29) L.A.M. Stolker, W. Niessing, R. Fuchs, R.J. Vreeken, W.M.A. Niessen, U.A.Th. Brinkman, Anal. Bioanal. Chem. 378 (2004) 1754
- (30) D.W. Kolpin, E.M. Thurman, S.M. Linhart, Sci. Total Environ. 248 (2000) 115
- (31) D.W. Kolpin, E.M. Thurman, S.M. Linhart, Environ. Sci. Technol. 35 (2001) 1217
- (32) F. Hernández, O. J. Pozo, J. V. Sancho, F. J. López, J. M. Marín, in preparation
- (33) K.H. Bauer, T.P. Knepper, A. Maes, V. Schatz, M. Voihsel, J. Chromatogr. A 837 (1999) 117
- (34) T.C. Schmidt, U. Buetehorn, K. Steinbach, Anal Bioanal Chem 378 (2004) 926
- (35) M. Holpacek, K. Volna, P. Jandera, L. Kolarova, K. Lemr, M. Exner, A. Cirkva, J. Mass Spectrom. 39 (2004) 43
- (36) M.J.F. Suter, S. Riediker, W. Giger, Anal. Chem. 71 (1999) 897
- (37) R. Castro, E. Moyano, M.T. Galcerán, J. Chromatogr. A 914 (2001) 111
- (38) N.B. Cech, C.G. Enke, Mass Spectrom. Rev. 20 (2001) 362

- (39) K. Petritis, H. Dessans, C. Elfakir, M. Dreux, LC-GC 15 (2002) 98
- (40) F. Hernández, J.V. Sancho, O.J. Pozo, C. Villaplana, M. Ibáñez, S. Grimalt, J. AOAC Int. 86 (2003) 832
- (41) R. Jeannot, H. Sabik, E. Sauvard, E. Genin, J. Chromatogr. A 879 (2000) 51
- (42) R. Bossi, K.V. Vejrup, B.B. Mogensen, W.A.H. Asman, J. Chromatogr. A 957 (2002) 27
- (43) A. Laganà, A. Bacaloni, I. De Leva, A. Faberi, G. Fago, A. Marino, Anal. Chim. Acta 462 (2002) 187
- (44) L. Gomides-Freitas, C.W. Götz, M. Ruff, H.P. Singer, S.R. Müller, J. Chromatogr. A 1028 (2004) 277
- (45) A.C. Borba da Cunha, M.J. López de Alda, D. Barceló, T.M. Pizzolato, J.H.Z. dos Santos, Anal. Bioanal. Chem. 378 (2004) 940
- (46) P. Marquet, F. Saint-Marcoux, T.N. Gamble, J.C.Y. Leblanc, J. Chromatogr. B 789 (2003) 9
- (47) T.N. Decaestecker, S.R. Vande Castele, P.E. Wallemacq, C.H. Van Peteghem, D.L. Defore, J.F. Van Bocxlaer, Anal. Chem. 76 (2004) 6365
- (48) B. Van den Driessche, W. Van Dongen, F. Lemièrre, E.L. Esmans, Rapid Commun. Mass Spectrom. 18 (2004) 2001
- (49) F. Guan, C.E. Uboh, L. R. Soma, Y. Luo, R. Li, E.K. Birks, D. Teleis, J.A. Rudy, D.S. Tsang, Rapid Commun. Mass Spectrom. 16 (2002) 1642
- (50) S. Marchese, A. Gentili, D. Perret, G. D'Ascenzo, F. Pastori, Rapid Commun. Mass Spectrom. 17 (2003) 879
- (51) European Commission, SANCO 10476/2003, Quality Control Procedures for Pesticide Residue Analysis, European Commission, Brussels, Belgium, February 2004 (http://europa.eu.int/comm/foog/plant/protection/resources/qualcontrol_en.pdf)
- (52) B. Maralikova, W. Weinmann, J. Chromatogr. B 811 (2004) 21

Artículo científico 10

Anal. Bioanal. Chem. (2006) 386: 987-997

POTENTIAL OF LIQUID CHROMATOGRAPHY/TIME OF FLIGHT MASS SPECTROMETRY FOR THE DETERMINATION OF PESTICIDES AND TRANSFORMATION PRODUCTS IN WATER.

Juan V. Sancho, Óscar J. Pozo, María Ibáñez and Félix Hernández

Research Institute for Pesticides and Water, University Jaume I, E-12071, Castellón, Spain

ABSTRACT

Until now, time of flight (TOF) mass analysers have only been very rarely used until in pesticide residue analysis (PRA) of water samples. However, the inherent characteristics of TOF MS make these analysers well-suited to this field, mainly for qualitative purposes. Thus, the high sensitivity in full scan acquisition in comparison to other MS analysers and the high resolution of TOF MS suggest its suitability for screening purposes; it also increases the multiresidue capabilities of methods based on it and decreases the chance of reporting false positives. Although these characteristics can also be helpful for quantification, confirmation and elucidation, some limitations on the use of TOF for these purposes have been observed. These limitations are more noticeable when dealing with samples containing very low analyte concentrations, which is the normal situation for PRA in water. The use of hybrid quadrupole-time of flight instruments (QTOF) minimises the limitations of TOF, facilitating the simultaneous detection and unequivocal confirmation of pesticides found in the sample. Additionally, the acquisition of accurate product ion full-scan mass spectra can help to elucidate the structure of unknown compounds. In this paper, the potential of TOF and QTOF hyphenated to liquid chromatography for PRA in water is explored, emphasizing both the advantages and limitations of this approach for screening, quantification, confirmation and elucidation purposes. Emphasis is placed in the determination of polar pesticides and transformation products-the analytes that fit well with LC-API-(Q)TOF MS technology.

INTRODUCTION

In recent years, liquid chromatography-mass spectrometry (LC-MS) using an atmospheric pressure ionization interface (API) has become an increasingly popular analytical method used for determining polar organic pollutants in water. This is proved by, for example, the growing number of papers published in the last five years concerning the determination of polar pesticides in water by LC-API-MS. Interesting and detailed discussions on the use of LC-MS for the determination of organic pollutants, including pesticides, in water can be found in recent articles [1-7]. One of the main obstacles to the development of multi-residue methods for determining pesticides comes from the wide range of polarities of these compounds -including both nonionic and ionic compounds-, that can potentially reach the water. This problem is increased by the current interest in monitoring transformation products (TPs), considered to be emerging contaminants by some authors [8], which are normally more polar than the parent pesticide.

Different strategies can be applied when monitoring pesticides and TPs in water, depending on the objectives pursued. The method requirements will differ depending on whether it is intended to simply detect, to quantify, to confirm the presence of a detected target analyte, or to elucidate a possible residue corresponding to a non-target analyte (5). Thus, analytical methods can be classified into different categories: (i) screening methods, able to (quickly) detect the presence of one or more compounds based on one or more common characteristics of a class of pesticides in a qualitative or semi-quantitative manner at a specified concentration limit; (ii) quantitative (determinative) methods, which should provide precise information concerning the amount of an analyte that may be present, but may only provide indirect information about the identity of the analyte, (iii) confirmatory methods, which should confirm the identity of the suspected analyte, but may or may not have a quantitative or semiquantitative component, and (iv) elucidation methods, which should discover the identity of a suspected or unknown analyte that was previously detected by a screening method but not confirmed afterwards.

Screening methods are very useful because they allow samples with no detectable residues (negative samples) to be distinguished from those with evident pesticide residues, ideally in a rapid manner, and with little sample manipulation. This would allow us to focus analytical efforts on the accurate quantitation and reliable confirmation of samples presumed to be positive.

When determining pesticide residues in water by LC-API-MS, most efforts so far have focused on the first two type of methods, mainly due to the mass analyzers used, single (Q) and triple (QqQ) quadrupole instruments [9-11], which are adequate for the simultaneous screening and quantification of a number of pre-selected pesticides, especially when tandem MS with QqQ instruments are used. However, one must also ensure that the detected and quantified signal truly belongs to the suspected target analyte. Therefore, reliable confirmatory methods should be applied in order to avoid false-positive findings. Potentially QqQ is well-suited to use for confirmatory purposes, although it has not been investigated much in this regards up to now in real-world samples. However, special care must be taken when selecting the number and specificity of the selected reaction monitored (SRM) transitions chosen [5, 12].

The risk of false-positive findings is significantly reduced with TOF analysers due to their increased mass resolution, mass accuracy and sensitivity in full-scan mode. Also, this analyzer is particularly suited to both non-target and post-target screening [5], as no pre-selection is required before data acquisition.

Even more useful in terms of confirmatory analysis is the hybrid analyser quadrupole-TOF (QTOF), as it permits the pre-selection of a precursor ion in the quadrupole filter and the recording of the full scan product ion spectra with high mass accuracy, which is one of the most valuable tools for confirmatory analysis nowadays. These capabilities have led to QTOF being investigated as a technique for elucidating of unknowns in environmental waters [13]. The accurate masses of both the precursor and product ions obtained using this hybrid analyser has facilitated the elucidation of pesticides metabolites and TPs in degradation studies under controlled laboratory conditions (14-19).

All of these characteristics make TOF and, particularly, QTOF analyzers very attractive for organic pollutants analysis in water. Their increasing popularity is demonstrated by the growing number of papers that have appeared in the literature since the pioneering work of Hogenboom et al (20) in 1999, and by the trends observed in scientific meetings and specialized workshops within this field.

In spite of its enormous analytical potential, not much has been published as yet until now on pesticide residue analysis (PRA) in water by LC-(Q)TOFMS. Only five papers were found in our search and a few more on the degradation of pesticides under controlled laboratory conditions (Table 1). Thus, in this paper we will give an overview of the scarce existing scientific literature that exists in this field, focusing on the potential, advantages and drawbacks of TOF MS technology in LC-based methods for the screening, quantification, confirmation and elucidation

of pesticide residues in water. Due to the scarcity of the literature, some examples from other fields, such as pharmaceuticals in water or pesticides in food, have been used to illustrate the potential of TOF for residue analysis, as they can easily be extrapolated to PRA in water.

Table 1. Selected bibliography dealing with the screening, quantification, confirmation or elucidation of pesticides in water samples using TOF or QTOF analysers

Analytes	Matrix ^a	Instrument	Use	Year	Ref
10 Pesticides	SW	TOF	screening and quantification	1999	20
Alachlor	SW	TOF	elucidation (photodegradation)	2000	14
Acetolachlor, alachlor 2 TPs	GW	TOF	Screening	2002	39
Diuron	STD	TOF	elucidation (photodegradation)	2003	15
Diazinon	STD	QTOF	elucidation (photodegradation)	2003	16
Rotenone	SW	TOF	Quantification	2003	23
47 Pesticides and TPs	SW/GW	QTOF	confirmation	2004	27
1 Insect repellent	WW	TOF	elucidation	2004	40
Triazines	SW	QTOF	elucidation (photodegradation)	2004	17
Quaternary ammonium herbicides	DW	TOF	quantification	2004	25
18 Pesticides/9 TPs	GW/SW	QTOF	screening, quantification and confirmation	2005	5
Carbofuran	STD	QTOF	elucidation (photodegradation)	2005	18
Diazinon	SW	QTOF	elucidation (photodegradation)	2006	19

^a SW: Surface water, GW: groundwater, STD: standards, WW: waste water, DW: drinking water.

• TOF

Recent advances in mass spectrometry have meant that a new analyzer has become available, the orthogonal-accelerated time of flight (oa-TOF) mass spectrometer. Due to inherent advantages associated with its ion separation and detection principles compared to other mass analysers (quadrupoles, ion traps), this type of instrument is often used to identify either small or big molecules. Its high mass resolving power (> 5000 FWHM) provides better confirmatory ability and signal-to-noise ratios than single quadrupole analysers, especially when dealing with complex matrix samples. A unique feature of accurate mass determinations (< 5 ppm) performed

using TOF is the useful information obtained about elemental compositions, which can confirm or rule out potential molecular formulae. Additionally, the inherent high sensitivity of the TOF analysers when used in full-scan mode is useful for detecting organic pollutants at relevant environmental levels.

The characteristics of TOF mass analyzers make them useful when developing analytical methodologies for screening, quantifying, confirming and elucidating pesticide residues and their TPs in water. LC-TOFMS methods are well-suited for polar (and ionic), non-volatile, thermolabile compounds -a wide range of non-GC amenable analytes- which are often found in water, mainly in groundwater as a consequence of their higher leachability from soil environments.

Screening

Screening methods should detect the presence of target compounds in a qualitative or semiquantitative manner at a specified concentration limit. In MS-based methods, the characteristic mass of an analyte is screened for when monitoring its presence in water.

The benefits of using a TOF analyzer comes from its measuring principle, which allows it to perform full-scan acquisitions with superior sensitivity and high mass accuracy. Therefore, the monitoring of a specific mass of an analyte is not predefined before data acquisition, and post-target screening can also be performed if desired. This fact allows us to detect the presence of an unlimited number of potential contaminants without re-analysis, provided that all of these compounds share both ionization and separation modes, even in cases where no sample is left to be reanalysed. An illustrative example has been reported by our research group [13] when analyzing an urban wastewater sample in the Castellon province, an area with a predominance of citrus crops. The presence of the post-harvest fungicide imazalil led us to suspect that other post-harvest fungicides also used in citrus crops may be present too. The previously acquired dataset was re-evaluated extracting chromatograms at the specific masses of different fungicides. In this way, the presence of thiabendazole ($[M+H]^+$ m/z 202.0439) was detected in a post-target style.

The high multiresiduality of this approach is not easily achieved by quadrupole mass analyzers (both Q and QqQ, working in SIM or SRM mode, respectively) due to the need to predefine the masses to be monitored because it is difficult to reduce the dwell time below a threshold value while maintaining a suitable sensitivity.

On the other hand, the elevated mass resolution of TOF analyzers allows us to reduce the mass window when extracting a specific mass from the full-scan dataset. A smaller mass window

leads to a substantial reduction in the chemical noise, facilitating the detection of the screened compound in the eXtracted Ion Chromatogram (XIC, also named EIC or RIC depending on the manufacturer). Some authors call these chromatograms microwindow XICs (mwXICs) and the benefits of reducing the mass window from 1 Da scale (similar to that for quadrupole or ion trap analyzers) down to 10-20 mDa were reported some time ago [20] in the environmental field. However, a drawback of using narrower mass windows, apart from the reliability of the mass accuracy attainable by the TOF analyzer used, is the significant mass errors produced by coeluting isobaric interferents that cannot be resolved by the analyser. The increase in mass errors can be so high that the compound being screened for may fall out of the monitored mass window, leading to a false negative being reported. This situation was observed by Benotti et al. [21] when screening some pharmaceuticals in wastewater effluents. Mass errors as high as 20 mDa were observed in their work for caffeine due to the presence of the ^{13}C isotope peak from a coeluting compound with a mass 1 Da lower than the analyte. Therefore, if the mwXICs would have been reconstructed with a small mass window, typically $\pm 5\text{-}10$ mDa, the presence of caffeine would have been masked and a false negative sample would have been reported.

Thus, when performing screening in real samples, one should be cautious and avoid using unreasonably narrow mass windows. As a compromise between improving baseline noise and signal-to-noise ratio and preventing reporting false negatives, a 50 mDa mass window is recommended, when reconstructing mwXICs. However, this mass window will presumably be narrowed following the expected increase in resolving power achievable by TOF analysers.

Quantitation

When dealing with pesticide residues in water, the sensitivity attainable is possibly the key issue. In this sense, TOF offers high sensitivity under full-scan conditions compared to other analyzers, but triple quadrupole instruments working in SRM mode show their superiority in target pesticide quantitation. Thus, in the determination of pesticides and transformation products in water, TOF was found to be around one order of magnitude less sensitive than a triple quadrupole instrument used in SRM mode [5]. This lower sensitivity hampered the detection of some pesticides by TOF, which were easily detected by triple quadrupole instruments. This gap of around one order of magnitude was also found in the determination of other pesticides (carbamates, organophosphorus and triazines) in water [20] and in other fields such as the determination of pharmaceuticals [21] and cyanobacteria toxins [22] in water. However, in all

cases, the sensitivity achieved by TOF was sufficient regarding the required detection limit of each application.

In addition to sensitivity, the feasible linear dynamic range of the TOF response is of paramount importance when applied for quantitative purposes. These instruments usually suffer from narrow dynamic ranges. Thus, linear ranges of a maximum of two orders of magnitude are typically used for quantitative purposes, as in the case of the multiresidual determination of pesticides in water [20, 23] or in vegetables [24]. Although this range can be sufficient for quantitative purposes, there are some applications, such as the quantification of cyanobacteria toxins in water [22], which have been performed by using a single point calibration.

As an illustrative example of the potential of TOF instruments for quantification purposes, the determination of quaternary herbicides in water by on-line SPE was performed (25). Using this approach, suitable LODs (lower than 0.1 µg/L) were achieved for mineral water samples loaded with 30 mL of sample, although higher values (around 0.5 µg/L) were obtained in tap and groundwater samples. The use of triple quadrupole instruments in SRM mode decreased the LODs, which were lower than 0.06 µg/L. In terms of the linear dynamic range, a calibration plot covering around 3 orders of magnitude was feasible in the determination by triple quadrupole, while the calibration range was reduced down to 1.5 orders of magnitude when working with TOF instruments.

Confirmation

Confirmatory methods should verify the identity of the suspected analytes detected during the screening process in order to confirm the finding and avoiding the reporting of false positives.

The confirmation process is typically achieved by acquiring more mass information regarding the suspected compound and applying various criteria in order to ensure data quality. The criterium established in a Commission Decision of the EU is one of the most useful and widely applied [26]. This Decision proposes the use of the so-called identification points (IPs), with at least three IPs being required to confirm a positive finding. IPs are earned by detecting mass ions, both precursor or product ions, and the number of IPs earned depends on the technique used. The EU Decision assumes that an ion measured with a high-resolution MS instrument (resolving power of 10,000 at the m/z being measured, based on 5% valley or 20,000 resolving power for FWHM) would give twice the number of IPs given by a low-resolution MS. This assignment of 2 IPs versus 1

IP is based on resolution power, rather than on mass accuracy, although the accurate mass measurements provided by these instruments seem to be more relevant within this subject [27]. Thus, although TOF analysers do not normally reach resolving powers of up to 20,000, they have been considered to be high-resolving instruments in the confirmation of pesticides in vegetables, and 2 IPs per ion measured were assigned in this case [28]. Thus, the potential of TOF analysers for confirmation is evidenced by the higher number of IPs assigned in comparison to low resolution instruments.

To qualify for IPs, at least one ion ratio must be also measured, and it must be within specified tolerances. This means that a minimum of two ions must be measured with TOF instruments, earning 4 IPs and allowing us to confirm both regulated and even banned compounds. However, the measurement of two ions using API interfaces may be troublesome if the suspected analyte does not show a rich characteristic isotopic pattern or an abundant in-source fragmentation. The presence of elements with abundant heavier isotopes, can help us to achieve the required number of IPs, but no additional structural information is obtained. In this sense, the measurement of in-source fragment ions seems to be more valuable. Thus, Ferrer et al. [28] proposed a confirmatory approach based on the in-source fragmentation of three chloronicotinyl pesticides in vegetable samples. In this methodology, four different ions were obtained for imidacloprid and two ions for acetamiprid and thiacloprid at two different fragmentor voltages. The halogen isotopic pattern must be taken into account in order to obtain a correct confirmation in this case.

However, some drawbacks may be encountered when using the in-source fragmentation approach in the environmental field, where confirmation at low analyte concentrations might be troublesome due to the lower abundance of fragment ions compared to the precursor ones. Besides, the low m/z value usually obtained for fragment ions is more prone to interference, and the origin of the fragment ions may not be unequivocal, which can complicate the confirmation process, particularly for complex matrix samples.

Despite the growing understanding in the scientific community about the need to provide reliable confirmations of positive findings, there are still some papers where confirmations have been made by using only the mass error obtained on one ion. Thus, some pharmaceuticals [21] and nitrotoluensulfonic acids [29] in water have been confirmed with this approach. The number of compounds sharing the same empirical formula and therefore exact mass can be surprisingly high, which makes information on fragments necessary.

Elucidation

The high resolution and accurate mass measurements obtained by TOF analysers, can be a great advantage in the elucidation of unknown compounds. Thus, the combination of the accurate mass together with a detailed study of the isotopic pattern of the unknown allows the number of potential molecular formulae for the compound detected to be reduced. This strategy has been used by different authors in the elucidation of some pesticide metabolites. Thus, Garcia-Reyes et al (30) successfully elucidated different chlorinated pesticide metabolites using the isotopic pattern as a filter and the measured accurate mass for discriminate between all possible formulae.

In the elucidation of unknowns, the molecular formula obtained can be fed into databases, leading to a reduced number of possible structures for the unknown compound. However, this approach presents several drawbacks which limit its application in the elucidation process. The most important one is the inability to distinguish between isomeric compounds, as compounds with the same molecular formula can not be distinguished using TOF instruments. Other limitations arise from the absence of a characteristic isotopic pattern for the investigated compound or the lack of unequivocal information about possible candidates with the exact mass obtained, which can also reduce the applicability of TOF for this purpose.

The complementary use of other techniques is normally required to obtain the molecular formula [14-17]. As an example, Hogenboom et al. complement the data obtained by TOF with those obtained with triple quadrupole and GC-MS in order to elucidate alachlor metabolites [14]. In the same way, GC-MS and TOF analysis were used in the elucidation of some diazinon metabolites [16], and diuron metabolites have been elucidated with complementary information obtained from ion trap and TOF analysis [15]. In the elucidation of triazine herbicides metabolites, this complementary information was obtained by QTOF [17], which had the additional advantage that both TOF and QTOF analysis could be performed with the same instrument. The potential of QTOF for elucidation will be discussed in the next section.

As an example of the potential and limitations of TOF for elucidation, Figure 1 shows a XIC chromatogram obtained when investigating a compound that seemed to be the herbicide diuron. Although the retention time and the nominal mass were similar to those for this compound, both the accurate mass (m/z 233.1121) and the isotopic pattern differed significantly from the expected ones (m/z 233.0248); therefore, the water sample was reported as negative for this herbicide. In an attempt to gain a wider knowledge about the sample composition, TOF was used for the elucidation of this isobaric compound. A search for the molecular formula was attempted without any restriction, neither in the number of atoms (C 0-100, H 0-200, N 0-20, S 0-3,

P 0-3, F 0-20, O 0-20) nor in the double bond equivalent (DBE -0.5-50). Under these conditions, 66 possible formulae were obtained for this compound. The restriction imposed by the isotopic pattern reduced the number of possible formulae down to 9. The plausible molecular formulae, together with isotopic abundance pattern, are shown in Table 2. Despite the great potential of TOF to reduce the number of possible molecular formula, the absence of a characteristic isotopic pattern (presence of chlorine or bromine atoms) hampered the elucidation. Additionally, Table 2 also shows the results obtained when searching for plausible formulae in different databases. Thus, only 3 molecular formulae presented entries in databases, with a total of 14 possible structures. The use of TOF could not be used to distinguish between these 14 structures and additional experiments with other instruments, such as GC-MS, IT or QTOF, would be required in order to unequivocally elucidate this unknown.

Table 2. Feasible molecular formulae for an unknown (m/z 233.1112) found in a wastewater sample

Unknown compound	Absolute abundance			Relative abundance (%)		
	M	M+1	M+2	M+1	M+2	
Intensity in combined spectrum	532	76	42	14.2	7.9	
+ Acceptable error ^a				17.0	11.8	
- Acceptable error ^a				11.4	3.9	

Feasible molecule formulae	Absolute abundance			Relative abundance (%)		NIST database entries
	M	M+1	M+2	%M+1	%M+2	
C ₁₀ H ₁₇ N ₂ OFS	8402	1088	454	12.9	5.4	-
C ₁₁ H ₂₁ OPS	8366	1120	456	13.4	5.5	-
C ₁₃ H ₁₆ N ₂ S	8148	1323	461	16.2	5.7	9
C ₁₀ H ₂₀ N ₂ S ₂	7999	1099	779	13.7	9.7	-
C ₈ H ₁₆ N ₄ O ₂ S	8508	977	462	11.5	5.4	-
C ₁₂ H ₁₈ F ₂ S	8297	1196	447	14.4	5.4	-
C ₇ H ₁₆ N ₆ OS	8560	947	444	11.1	5.2	-
C ₁₁ H ₂₀ OS ₂	7950	1126	794	14.2	10.0	4
C ₁₁ H ₂₀ O ₃ S	8327	1120	488	13.5	5.9	1

^a Tolerance range extracted from ref [11]: $\pm 20\%$ for M+1 and $\pm 50\%$ for M+2

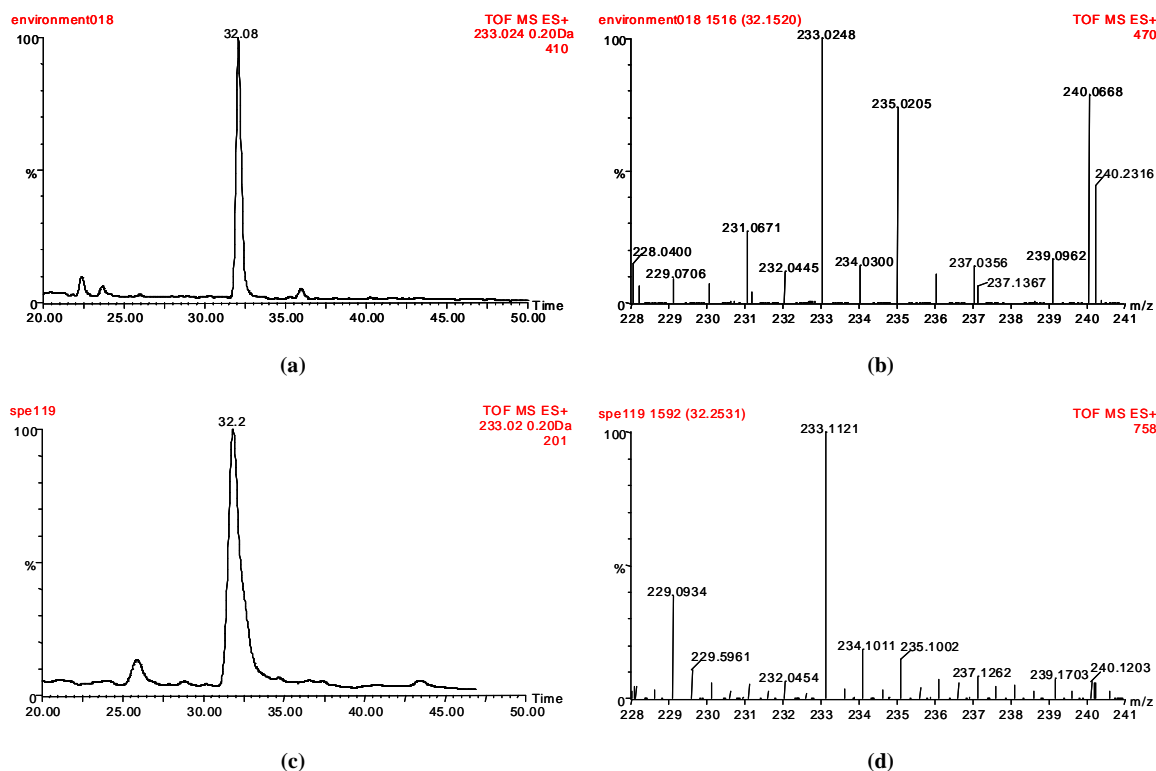


Figure 1. Use of TOF for diuron screening. (a) Chromatogram for a diuron standard (cone 25V), (b) TOF spectrum of the standard at the diuron retention time, (c) Chromatogram for a water sample suspected to be positive (cone 25V) and (d) TOF spectrum of the water sample at the diuron retention time.

Application to water samples

Although TOF instruments are a valuable tool in environmental analysis, their potential can be substantially limited in the determination of organic micropollutants, like pesticides and transformation products, in water samples due to the high sensitivity normally required. This fact can complicate the detection of some analytes at regulatory low levels (e.g., 0.1 µg/L in drinking water). The application of higher preconcentration factors during sample treatment is an obvious and efficient alternative that can be used to improve the sensitivity of the method, but it may also lead to undesirable effects such as analyte breakthrough in SPE procedures or the preconcentration of matrix interferences, which can produce ionization suppression or enhancement. Controlling these adverse effects is crucial in quantitative methods, although it

seems less important for qualitative purposes (i.e. screening, confirmation and elucidation). As an example, Nuñez et al. (25) preconcentrated 30 mL of water sample as a compromise between sensitivity and breakthrough. When using this sample volume, although some analytes such as difenzoquat present satisfactory recoveries (90%) that achieve the required detection limits, for other compounds such as chlormequat significant breakthrough starts to occur, resulting in recoveries of around 50 %. In any case, this volume was not enough to obtain LODs below the required levels (0.1 µg/L) in the matrices tested.

The low concentrations normally present in the environment may make some of the TOF limitations previously stated more noticeable. Thus, the analyte mass deviation produced by the presence of an isobaric co-eluting interference increases with the relative abundance of the interferent compound. Therefore, the lower the analyte concentration, the higher the mass deviation observed when this type of interference is present. Even some of the mass ions produced by the mobile phase might interfere in the analyte mass measurement when present at residue concentration levels.

Other aspect should also be emphasized when confirming positive findings at low concentrations. In-source fragments are required for a safe confirmation in many cases. The ions obtained from this type of fragmentation are frequently less abundant than $(M+H)^+$ or $(M-H)^-$ ions, and this fact can hamper confirmation at low concentration levels. Besides, in complex matrices it can be difficult to obtain information about the origin of the ion because some interferences may share the same mass as the in-source fragment. Some nonisobaric interferences may even produce isobaric fragments that would interfere with the confirmation. This situation is more problematic when low m/z fragments are selected, as this region of the spectrum tends to be noisier when the cone voltage is increased. The use of QTOF drastically minimizes these limitations, increasing confidence about the origin of the ion and also reducing the isobaric interferences, as discussed in the next section.

- **QTOF**

The development of hybrid quadrupole-time-of-flight (QTOF) instruments has presented the analyst with an attractive new tool for the determination of pesticides in the environment. Although its use is still very limited in this field, mainly due to its high cost, QTOF has undoubted potential due to its inherent characteristics. QTOF presents all of the advantages indicated above for TOF, as it can be used in TOF mode with the first quadrupole acting as an ion guide. However, the biggest advantage of QTOF is not obtained when it is used in TOF mode.

The main advantage of QTOF is its ability to perform accurate product ion mass scan. Thus, while the accurate mass obtained from TOF allows us to establish the elemental composition of a compound, QTOF allows us to establish the elemental composition of all of the product ions obtained, which is very helpful when attempting to elucidate unknowns. The accurate mass, when combined with the acquisition of the full-scan spectra for the product ions, also provides a powerful tool for the unequivocal confirmation of positives (target analytes).

The emergence of QTOF opens up new possibilities for the determination of pesticides in the environment regarding screening, quantification, confirmation and elucidation, although some limitations should be taken into account in order to properly evaluate the potential of this powerful analysis technique in this field.

Screening

The use of QTOF in MS/MS mode implies the preselection of the analytes, which makes it necessary to know the analyte mass in order to filter it in the quadrupole. This is a limitation on its use for screening purposes as it would impede the performance of a post-target screening. Thus, the great potential of QTOF for screening purposes is actually found in pre-target applications. Obviously, to avoid pre-selection of the analytes, the QTOF instrument could be used in TOF mode, but the main advantages of this hybrid analyser are not apparent when working in MS mode.

The screening by QTOF also presents a drawback related to the limited number of compounds that can be monitored simultaneously. The sequential mode acquisition performed by the quadrupole and the relatively long time needed by the TOF to get an adequate response for the measured product ion spectrum (around a second, with the instruments used up to now) significantly reduces the number of analytes that can be selected in a short time period. Under these conditions, efficient chromatographic separation is essential for reliable screening.

For these reasons, the use of QTOF for screening purposes has normally been limited to a few pre-target compounds. Thus, QTOF was investigated for the multiresidue screening of around 30 pesticides and transformation products, but due to the limitations stated above, QTOF was considered to be more valuable for confirmative analysis [5]. Another example of the use of QTOF for screening purposes can be found in the determination of pharmaceuticals in water [31]. In this case, a multiresidue method that included 13 pharmaceuticals was developed for the simultaneous screening and confirmation at the low $\mu\text{g/L}$ level.

The number of analytes that can be included in the method could be increased by using automated MS to MS/MS switching, as demonstrated by Bobeldijk et al. [32]. This approach is based on the possibility of automatically changing from MS to MS/MS mode when the compound of interest is eluting from the analytical column. The instrument is initially set-up as TOF acquiring in full-scan mode; when a specific mass exceeds a predefined number of counts, the instrument automatically changes to MS/MS mode recording the product ion spectrum of this mass, and returning to TOF mode when the spectrum is acquired. This approach was tested for 6 pesticides used as model compounds, which showed its suitability for screening and identifying them. Subsequently, the developed methodology was extended to 4 unknowns, which demonstrated one of the most important advantages of this approach: it avoids the need to pre-select the analytes before screening. The most noticeable limitation is the need to predefine a threshold value above which a compound is considered relevant.

Despite some limitations, the use of QTOF for screening purposes presents important advantages derived from the inherent characteristics of this analyser: the acquisition of the complete and accurate product ion mass spectrum allows the simultaneous screening and confirmation of the selected analyte. Thus, when using QTOF in screening applications, an additional injection for confirmation is not necessary. Recent QTOF instruments, that have acquire spectra faster, will surely allow us to increase the number of compounds screened in the near future.

Quantitation

The application of QTOF to the quantification of organic pollutants in environmental samples has been quite limited so far [31, 33-35], and to our knowledge no study quantifying pesticides in environmental samples by QTOF has been performed. The main reasons are almost certainly its lower sensitivity and linear dynamic range compared to triple quadrupole in SRM mode, as well as its high price. The sensitivity achieved by a QTOF is of the same order as that achieved by TOF, and normally around tenfold lower than QqQ in SRM mode (5,33). The linear range achieved by QTOF is also limited, tending to be around one order of magnitude (33, 35) or even lower (31, 34).

However, the use of QTOF for quantification may have some advantages over TOF. For example, mw-XIC is not normally necessary for a correct quantification as most of the interferences are filtered in the quadrupole. Only the isobaric interferences isolated in the quadrupole that produces isobaric product ions would affect the quantification of the analyte, which only happens very rarely in real samples. Therefore, correct quantification, without noticeable interferences and with an extremely low background, can be obtained without the need to achieve an extremely accurate mass. Thus, some deviations in accurate mass measurements, such as those produced by high concentrations of analyte or by the presence of quasi-isobaric compounds (21) which can produce poor quantification when using TOF with mw-XIC, are less important when using QTOF for quantification purposes.

In our opinion, the most important advantage of QTOF is its inherent ability to confirm analyte identity at the same time as performing quantification, without the need for an additional confirmatory analysis.

Confirmation

As stated before, one of the main advantages of QTOF instruments is the inherent confirmation provided by the acquisition of the accurate full product ion mass spectrum. When confirming positive findings by QTOF, both the exact masses and the relative intensities of all of the available product ions of a sample can be compared with those of the reference standard. The number of IPs reached when using QTOF much higher than the minimum required, and the confirmation achieved by QTOF can be considered to be the ultimate confirmation of analyte identity [5]. In this way, although some authors propose LC-TOF for confirmation of the analyte identity based on the measurement of the accurate mass of one ion [21], this confirmation could be still not sufficient, being more adequate the confirmation by QTOF [5, 27, 31, 33].

Another important advantage of QTOF in confirmatory applications is its ability to obtain abundant fragmentation without any significant interference. The use of QTOF minimises the limitations of TOF instruments when working with in-source fragment ions, as the selection of a precursor ion in the first quadrupole increases confidence about the origin of the product ion and decreases the chemical noise. Additionally, the low chemical noise and/or the efficient fragmentation produced in the collision cell increase the number and relative intensities of product ions when using QTOF, improving the quality of the confirmation and also enabling us to confirm positive samples at concentration levels close to the limit of detection.

This approach has been successfully applied by Stolker et al. [33] to the confirmation of different drugs in several matrices, including environmental waters, showing the suitability of QTOF compared to other analysers such as triple quadrupole. Going back to pesticide examples, Figure 2 and Table 3 show the ultimate confirmation achieved in a groundwater sample suspected to be positive for terbumeton. The deviations obtained in the measured masses of all product ions were lower than 2 mDa. Additionally, when comparing the relative abundances observed in the sample suspected to be positive with the relative abundances obtained for a reference standard, all of the deviations were within the limits proposed by the European Decision 2002/657/EC [26] except for the less sensitive product ion (relative abundance 2.3%). Therefore, this sample was confirmed by QTOF to be positive for terbumeton, obtaining 13.5 IPs.

Table 3. Results obtained in the confirmation of the herbicide terbumeton ($C_{10}H_{19}N_5O$) detected in a groundwater sample from the Spanish Mediterranean area ($0.056 \mu\text{g/L}$). See also Fig. 2

Product ion	Accurate mass			Relative Abundante (%)		
	theoretical	experimental	Deviation (mDa)	theoretical	experimental	Deviation (mDa)
$C_6H_{12}N_5O$	170.1042	170.1047	0.5	100	100	-
$C_4H_8N_5O$	142.0729	142.0729	0	18.6	20.5	10.2
$C_5H_{10}N_3O$	128.0824	128.0835	1.1	7.8	8.5	9.7
$C_4H_8N_3O$	114.0667	114.0697	3	24.4	22.9	6.3
$C_3H_6N_3O$	100.0511	100.0516	0.4	5.0	5.8	15.3
$C_2H_4N_3O$	86.0354	86.0365	1.1	2.3	3.9	68.2

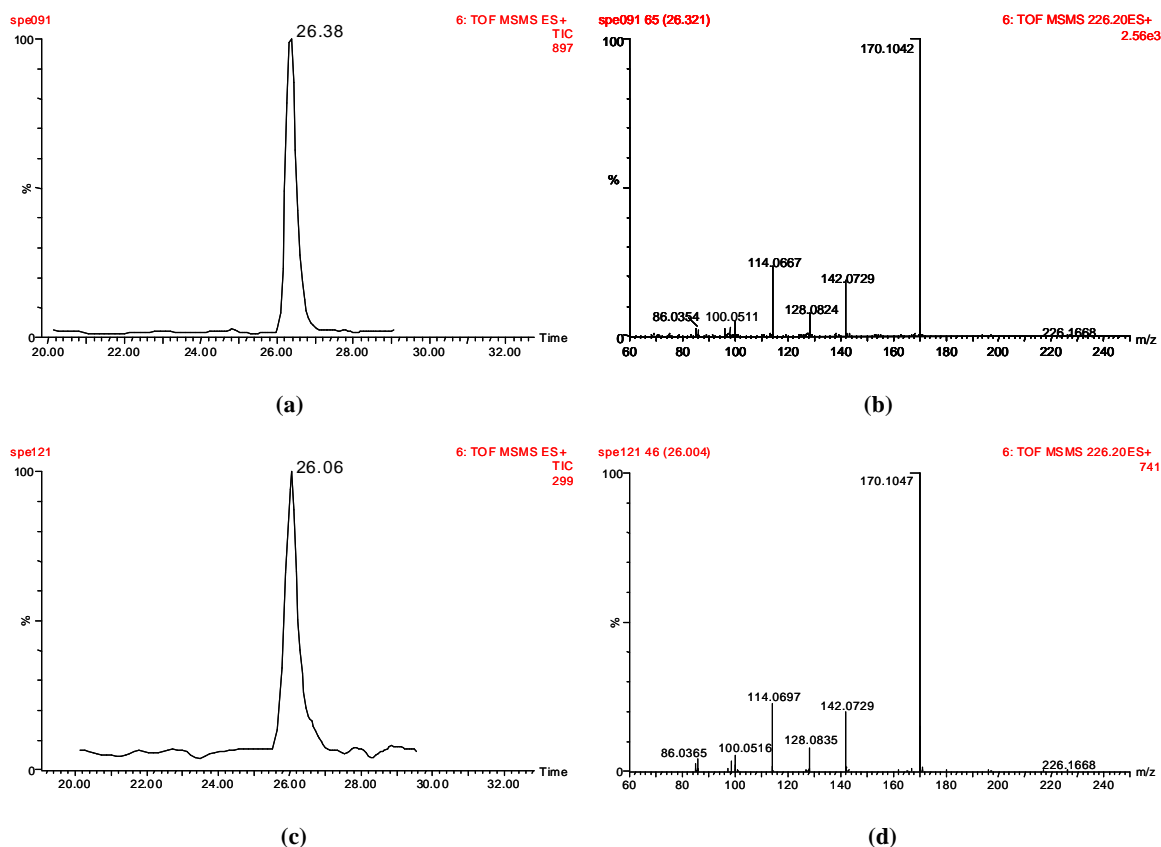


Figure 2. Confirmation of a positive water sample by QTOF. (a) QTOF chromatogram and (b) product ion spectrum of m/z 226 (cone 25 V, collision energy 25 eV) from a terbumeton standard, (c) QTOF chromatogram and (d) product ion spectrum of m/z 226 (cone 25 V, collision energy 25 eV) from a water sample suspected of being positive for terbumeton (0.056 $\mu\text{g/L}$). See Table 3

Elucidation

Apart from providing the ultimate confirmation of positive findings, the valuable information yielded by the accurate product ion mass spectra aids to elucidate the structures of unknown compounds. In some cases, when the experiment is carried out under controlled conditions, the elucidation achieved via QTOF can be considered to be definitive, even without the use of any additional technique.

Thus, QTOF has been used for the elucidation of pesticide metabolites in human fluids [19] and in photodegradation studies [16-18]. The elucidation of metabolites under controlled conditions can be performed by comparing a sample spiked with the pesticide to a blank

sample subjected to the same experimental process. The occurrence of a peak in the sample chromatogram which is not present in the control sample reveals the existence of a metabolite/photodegradation product, which can be identified by means of the product ions obtained. As an example, triazine photodegradation products have been elucidated in detail using LC-QTOF (17). In a first step, the fragmentation pathways of the parent herbicides were proposed based on the accurate masses of the product ions in order to obtain detailed knowledge of the MS/MS behaviour of this family of analytes. Then, the molecular formulae of the observed photodegradation products were obtained from their accurate masses. In order to elucidate the final structure of each photodegradation product, its MS/MS spectrum was compared with the parent compound assuming a similar fragmentation pathway. Using this approach, most of photodegradation products of triazines were unequivocally elucidated.

The potential of QTOF to elucidate unknowns is more limited when dealing with samples where no previous knowledge is available about the possible structures of the investigated compounds. In these cases, the most common approach is to obtain the molecular formula and to then search in a database. The accurate product ion mass spectrum provides additional structural information, which is useful for discriminating between possible isomeric structures, making elucidation feasible in some cases. Comparison of the retention time and the MS/MS spectrum with a reference standard, if available, tends to be the final way to unequivocally identify the unknown compound [13].

In the previous example related to an isobaric compound of diuron, which could not be solved by TOF, the use of QTOF permitted discriminate ion between most of the 14 chemical structures found in NIST database. As Figure 3 shows, after acquiring the product ion spectrum of the parent ion m/z 233, only one intense product ion was observed at m/z 151.0325, and this was obtained after the loss of cyclohexene (C_6H_{10} , theoretical m/z 82.0783, experimental error 0.4 mDa). Only two out of the 14 potential compounds presented a cyclohexane moiety, and therefore the number of plausible structures was reduced down to these two candidates. In order to get the ultimate confirmation of the unknown, a comparison between the product ion spectra and the retention times for both the sample and the standard, if available, would have been necessary.

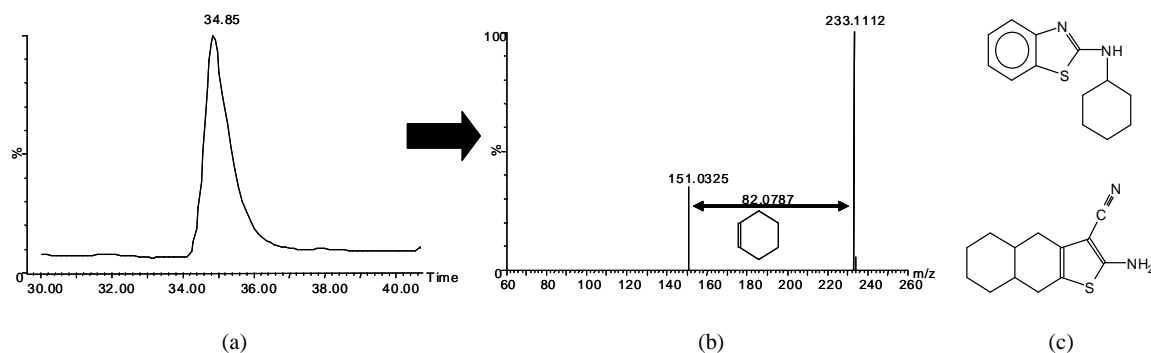


Figure 3. Elucidation of unknowns by LC-QTOF. (a) Chromatogram and (b) product ion spectrum of m/z 233 from a sample containing a diuron isobaric compound. (c) Feasible structures for this unknown.

The application of this approach is helpful in the elucidation of unknowns elucidation, but unsuccessful attempts frequently occur. The most common drawback is the absence of the predicted molecular formula in the database as many compounds, including most pesticide metabolites/transformation products, are not included in commercial databases. Thus, although analysis by QTOF can provide the molecular formula and some specific fragments, the assignment of a concrete structure is almost impossible in many cases. This means that the elucidation of organic pollutants in environmental samples is troublesome [13, 36]. The elucidation by QTOF is more problematic when there is no specific fragmentation in the analyte molecule. This is illustrated in the work of These et al. [37], where the elucidation of fulvic acids was hampered because the only losses observed corresponded to water and carbon dioxide, which are not specific and are in fact common to all compounds of this family.

Application to water samples

To our knowledge, there are almost no works on the application of LC-QTOF to pesticide residue analysis in the environment, despite its strong potential in this field. This hybrid analyser circumvents most of the limitations of TOF regarding quantification and confirmation, just as tandem MS is far more powerful than single MS in terms of its analytical characteristics. The most significant limitation when applied in environmental field is probably its relatively low sensitivity, mainly when compared to triple quadrupole instruments, which can hamper the detection of some analytes at sub-ppb levels. As an example, the detection of several antibiotics in water by QTOF was not feasible at the concentrations found in water (normally between 10 and 30 ng/L,

determined by QqQ) even after increasing the preconcentration factor in off-line SPE procedures 6 fold [38].

The unequivocal confirmation of the analyte identity is automatically achieved by QTOF when used in both screening and quantitative methods. Theoretically, this ultimate confirmation would only be hampered by the unusual presence of an analyte coeluting with isobaric interference that also presents several common product ions. Due to the low probability of encountering this scenario, one can conclude that QTOF is the ideal tool for confirmation purposes. However, one should be aware that coeluting isobaric interferences may hamper the confirmation, even in the case that they do not share any isobaric product ion, a situation that is more likely to occur in complex matrix samples. As the confirmation of the analyte is usually performed by comparing of the product ion spectra for both the standard and the sample, the presence of coeluting isobaric interferences may lead to a complex composite spectrum containing product ions from the analyte and the interferent, which would make a comparison of both spectra troublesome. This situation is favoured at low analyte concentrations, such as those that occur in environmental samples. Thus, the lower the required concentration level, the higher the number of potential compounds that can interfere with the analysis [12]. This effect is more problematic when elucidating unknowns, as a composite spectrum can result in misinterpretation of the observed fragmentation, causing either an impossible or a false elucidation.

The easiest way to reduce this limitation is to improve the chromatographic separation between the analyte and the interference. This solution is relatively easily applied when confirming of positive findings, but its application to the elucidation of unknowns is more limited because the analyst does not have any evidence of the occurrence of a co-eluting interferent.

More than 100 water samples from the Valencian region, a Spanish Mediterranean area with a long agricultural tradition and a predominance of citric crops, were investigated, and the most relevant positive findings were confirmed by LC-QTOF (5). Most of the confirmed findings (reaching typically a minimum of 7 IPs) were herbicides, mainly triazines and their TPs, normally present at concentrations between 0.1 and 0.5 $\mu\text{g/L}$. Some samples with lower analyte concentrations could also be confirmed with a high number of IPs. As an example, Figure 2 and Table 3 shows the confirmation of terbumeton at 0.056 $\mu\text{g/L}$.

CONCLUSIONS

The inherent characteristics of TOF MS (mass accuracy and high resolution) make this analyser very attractive in the pesticide residue analysis of water samples. Its potential is more evident when used for screening purposes because the acquisition of full-scan spectra with high sensitivity increases the multiresidue capability of the method and facilitates the application of LC-TOF methods to the screening of, in principle, an unlimited number of compounds. Additionally, the use of mw-XIC also allows the number of interferents to be reduced, making the screening more efficient. On the other hand, the use of hybrid QTOF for screening limits the number of analytes that can be included in the method, although an unequivocal confirmation of the analyte identity is achieved at the same time.

In terms of quantification, both TOF and QTOF present a common limitation, which derives from the low linear dynamic range of TOF analysers. This limitation is expected to be minimised in the upcoming generation of instruments equipped with ADC digitizers, which will surely be able to increase the linear range, improving the applicability of these instruments to quantification.

The advantages of using TOF for screening, quantification, confirmation and elucidation purposes can be decreased when a (quasi)isobaric interferent co-elutes with the analyte. The presence of this interferent can affect the accurate mass obtained by TOF, generating an erroneous quantification, even after using mw-XICs, and it also makes the assignment of the correct molecular formula more difficult. In such a case, the confirmation of the analyte identity and, obviously, the elucidation of unknown (non target) compounds is hampered. Under these circumstances, the applicability of TOF instruments is quite limited, making it necessary to perform an efficient chromatographic separation in order to minimise the risk of obtaining co-eluting interferents.

This problem is minimised when using QTOF because the chances of finding a coeluting isobaric interferent which also present an isobaric fragment are much less. Thus, the quantification can be correctly performed by selecting a product ion that does not encounter interference in the MS/MS mode. However, the presence of a co-eluting isobaric interferent might affect the confirmation and elucidation processes, even if no isobaric fragments are shared with the analyte. In this situation, a composite product ion spectrum would be obtained, causing difficulties when attempting confirmation by comparison with a reference standard and making it particularly difficult when elucidating an unknown structure based on the fragments obtained.

In summary, when a finding is confirmed by QTOF, this confirmation can be taken as being unequivocal. However, a finding not confirmed by QTOF may be in some cases actually be a positive hampered by co-eluting isobaric interferences. The possibility of reporting false negatives is certainly quite low, but it increases when the analyte is present at very low concentrations, such as sub-ppb levels. More research would be necessary for samples not confirmed by QTOF, including a detailed study of the product ion mass spectra and the chromatographic separation, in order to obtain reliable results.

The potential of QTOF for elucidating unknowns comes from the accurate mass measurements it can take in the product ions spectrum. Although the utility of this hybrid analyser has been demonstrated in recent scientific articles, the elucidation of non target compounds in environmental samples is still a challenge for analytical chemists due to understandable difficulties associated with this subject. A considerable increase in the number of publications dealing with TOF-MS applications in pesticide residue analysis is expected in the very near future, as expected from the great potential of this technique.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of the Ministerio de Educación y Ciencia (Project BQU 2003-02685) in their research on LC-TOFMS applications in the environmental and toxicological fields.

M.I. is very grateful to Generalitat Valenciana for her predoctoral grant.

REFERENCES

- (1) Hogenboom A.C., Niessen W.M.A., Brinkman U.A.Th. (2001) *J. Sep. Sci.* 24: 331-354
- (2) Geerdink R.B., Niessen W.M.A., Brinkman U.A.Th. (2002) *J. Chromatogr. A* 970: 65-93
- (3) Reemtsma T. (2003) *J. Chromatogr. A* 1000:477-501
- (4) Richardson S.D. (2004) *Anal. Chem.* 76: 3337-3364
- (5) Hernández F., Pozo O.J., Sancho J.V., López F.J., Marín J.M., Ibáñez M. (2005) *Trends Anal. Chem.* 24:596-612
- (6) Zwiener C., Frimmel F.H. (2003) *Anal Bioanal Chem* 378: 851-861
- (7) Zwiener C., Frimmel F.H. (2003) *Anal Bioanal Chem* 378: 862-874
- (8) Ferrer I., Thurman E.M. (2003) *Trends Anal. Chem.* 22: 750-756
- (9) Vega A.B., Frenich A.G., Vidal J.L.M. (2005) *Anal. Chim. Acta.* 538: 117-127
- (10) Sancho J.V., Pozo O.J., Hernandez F. (2004) *Analyst* 129: 38-44
- (11) Kampioti A.A., Borba Da Cunha A.C., De Alda M.L., Barcelo D. (2005) *Anal. Bioanal. Chem.* 382: 1815-1825
- (12) Hernández F., Sancho J.V., Pozo O.J. (2005) *Anal. Bioanal. Chem.* 382: 934-946
- (13) Ibáñez M., Sancho J.V., Pozo O.J., Niessen W.M.A., Hernández F. (2005) *Rapid Commun. Mass Spectrom.* 19: 169-178
- (14) Hogenboom A.C., Niessen W.M.A., Brinkman U.A.Th. (2000) *Rapid Commun. Mass Spectrom.* 14: 1914-1924
- (15) Ferrer I. and Thurman E.M. (2003) (eds) Malato S., Albanis T., Piedra L., Aguera A., Hernando D., Fernandez-Alba A. *Chromatography/Mass Spectrometry, MS/MS and Time-of-Flight MS Analysis of Emerging Contaminants.* ACS Symposium series 850: 66-95
- (16) Kouloumbos V.N., Tsiipi D.F., Hiskia A.E., Nikolic D., van Breemen R.B (2003) *J. Am. Soc. Mass Spectrom.* 14: 803-817
- (17) Ibáñez M., Sancho J.V., Pozo O.J., Hernández F. (2004) *Anal. Chem.* 76: 1328-1335
- (18) Detomaso A., Mascolo G., Lopez A. (2005) *Rapid Commun. Mass Spectrom.* 19: 2193-2201
- (19) Ibáñez M., Sancho J. V., Pozo O. J., Hernández F. (2006) *Anal. Bioanal. Chem.* 384: 448-457

- (20) Hogenboom A.C., Niessen W.M.A., Little D., Brinkman U.A.Th. (1999) *Rapid Commun. Mass Spectrom.* 13: 125-133
- (21) Ferrer I. and Thurman E.M. (2003) (eds) Benotti M.J., Lee Ferguson P., Rieger R.A., Iden C.R., Heine C.E, Brownawell B.J. in *Liquid Chromatography/Mass Spectrometry, MS/MS and Time-of-Flight MS Analysis of Emerging Contaminants*, ACS Symposium series 850: 109-127
- (22) Maizels M., Budde W.L (2004) *Anal. Chem.* 76: 1342-1351
- (23) Holm A., Molander P., Lundanes E., Greibrokk T. (2003) *J. Chromatogr. A.* 983: 43- 50
- (24) Ferrer I., García-Reyes J.F., Fernández-Alba A. (2005) *Trends Anal. Chem.* 24: 671-682
- (25) Núñez O., Moyano E., Galcerán M.T. (2004) *Anal. Chim. Acta* 525: 183-190
- (26) European Commission Decision 2002/657/EC, *Off. J. Eur. Commun.* L221 (2002) 8
- (27) Hernández F., Ibáñez M., Sancho J.V., Pozo O.J. (2004), *Anal. Chem.* 76: 4349-4357
- (28) Ferrer I., Thurman E.M., Fernández-Alba A.R. (2005) *Anal. Chem.* 77: 2818-2825
- (29) Ma W. T., Steinbach K., Cai Z.W. (2004) *Anal. Bioanal. Chem.* 378: 1828-1835
- (30) Garcia-Reyes J.F., Ferrer I., Thurman E.M., Molina-Diaz A., Fernandez-Alba (2005) *Rapid Commun. Mass Spectrom.* 19: 2780-2788
- (31) Stolker A.A.M., Niesing W., Hogendoorn E. A., Versteegh J. F. M, Fuchs R. Brinkman U.A.Th.(2004) *Anal. Bioanal. Chem.* 378: 955-963
- (32) Bobeldijk I., Vissers J.P.C., Kearney G., Major H., van Leerdam J. A. (2001) *J. Chromatogr. A.* 929: 63-74
- (33) Stolker A.A.M., Niesing W., Fuchs R., Vreeken R.J., Niessen W.M.A., Brinkman U.A.Th (2004) *Anal. Bioanal. Chem.* 378: 1754-1761
- (34) Marchese S., Gentili A., Perret D., D'Ascenzo G., Pastori F. (2003) *Rapid Commun. Mass Spectrom.* 17: 879-886
- (35) Marchese S., Gentili A., Perret D., Sergi M., Notari S. (2004) *Chromatographia* 59: 411-417
- (36) Bobeldijk I., Stoks P. G. M., Vissers J.P.C, Emke E., van Leerdam J. A., Muilwijk B., Berbee R., Noij T. H. M.(2002) *J. Chromatogr. A.* 970:167-181
- (37) These A., Winkler M., Thomas C., Reemtsma T. (2004) *Rapid Commun. Mass Spectrom.* 18: 1777-1786

- (38) Pozo O. J., Guerrero C., Sancho J. V., Ibáñez M., Pitarch E., Hogendoorn E., Hernández F. (2006) *J. Chromatogr. A.* 1103: 83-93
- (39) Thurman E.M., Ferrer I., Parry R. (2002) *J. Chromatogr. A* 957: 3-9
- (40) Knepper T.P. (2004) *J. Chromatogr. A.* 1046: 159-166

5.3 Bibliografía

2002/657/CE Decisión de la Comisión de 12 de Agosto de 2002, por la que se aplica la Directiva 96/23/CE del Consejo en cuanto al funcionamiento de los métodos analíticos y la interpretación de los resultados. Official Journal of the European Communities L221 (2002) pp 8-36

98/83/CE del Consejo de 5 de Diciembre de 1998, relativa a la calidad de las aguas destinadas al consumo humano. Diario Oficial L330 (1998) pp 32-54

Benotti MJ, Lee Ferguson P, Rieger RA, Iden CR, Heine CE, Brownawell BJ

HPLC/TOF-MS: An alternative to LC/MS/MS for sensitive and selective determination of polar organic contaminants in the aquatic environment in Liquid Chromatography/Mass Spectrometry, MS/MS and Time-of-Flight MS Analysis of Emerging Contaminants, I. Ferrer and E.M. Thurman eds., ACS Symposium series 850 (2003) pp 109-127

Bobeldijk I, Vissers JPC, Kearney G, Major H, van Leerdam JA

Screening and identification of unknown contaminants in water with liquid chromatography and quadrupole-orthogonal acceleration-time-of-flight tandem mass spectrometry. J. Chromatogr. A (2001) 929: 63-74.

Commission Decision SANCO/10476/2003 5 February 2004 on quality control procedures for pesticide residues analysis

Detomaso A, Mascolo G., López A

Characterization of carbofuran photodegradation by-products by liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry Rapid Commun. Mass Spectrom. (2005) 19: 2193-2202.

Hernández F, Ibáñez M, Sancho JV, Pozo OJ

Comparison of different mass spectrometric techniques combined with liquid chromatography for confirmation of pesticides in environmental water based on the use of identification points Anal. Chem. (2004) 76: 4349-4357

Ibáñez M, Sancho JV, Pozo OJ, Hernández F

Use of quadrupole time-of-flight mass spectrometry in environmental analysis: elucidation of transformation products of triazine herbicides in water after UV exposure Anal. Chem. (2004) 76: 1328-1335

Ibáñez M, Sancho JV, Pozo OJ, Niessen WMA, Hernández F

Use of quadrupole time-of-flight mass spectrometry in the elucidation of unknown compounds present in environmental water

Rapid Commun. Mass Spectrom. (2005) 19: 1-10

Ibáñez M, Sancho JV, Pozo OJ, Hernández F

Use of liquid chromatography quadrupole time-of-flight mass spectrometry in the elucidation of transformation products and metabolites of pesticides. Diazinon as a case study

Anal. Bioanal. Chem. (2006) 384: 448-457

García-Reyes JF, Ferrer I, Thurman EM, Molina-Diaz A, Fernandez-Alba AR

Searching for non-target chlorinated pesticides in food by liquid chromatography/time-of-flight mass spectrometry

Rapid Commun. Mass Spectrom. 19 (2005) 2780-2788

Guidance for industry: Mass spectrometry for confirmation of the identity of animal drugs residues.

Draft Guidance 118, Food And Drug Administration June 6, 2001

Kouloumbos VN, Tsiipi DF, Hiskia AE, Nikolic D, van Breemen RB

Identification of photocatalytic degradation products of diazinon in TiO₂ aqueous suspensions using GC/MS/MS and LC/MS with quadrupole time-of-flight mass spectrometry

J. Am. Soc. Mass Spectrom. (2003) 14: 803-817

Reemtsma T

Liquid chromatography-mass spectrometry and strategies for trace-level analysis of polar organic pollutants.

J. Chromatogr. A 1000 (2003) 477-501

Stolker AAM, Niesing W, Hogendoorn EA, Versteegh JFM, Fuchs R, Brinkman UATH

Liquid chromatography with triple-quadrupole or quadrupole-time of flight mass spectrometry for screening and confirmation of residues of pharmaceuticals in water.

Anal. Bioanal. Chem. 378 (2004) 955

These A, Winkler M, Thomas C, Reemtsma T

Determination of molecular formulas and structural regularities of low molecular weight fulvic acids by size-exclusion chromatography with electrospray ionization quadrupole time-of-flight mass spectrometry

Rapid Commun. Mass Spectrom. 18 (2004) 1777-1786

WADA Technical Document TD2003IDCR version number 1.2, de 1 de enero de 2004 Identification

criteria for qualitative assays. Incorporating chromatography and mass spectrometry

Capítulo 6. Conclusiones



De la presente Tesis Doctoral se pueden extraer las siguientes **conclusiones**:

I- El acoplamiento LC-QTOF MS se ha mostrado como una poderosa herramienta analítica en la identificación de productos de transformación y metabolitos de plaguicidas en muestras medioambientales y biológicas, respectivamente.

La elevada resolución de este analizador, junto con la posibilidad de llevar a cabo experiencias MS y MS/MS con elevada exactitud de masa, ha permitido elucidar la estructura de los diversos productos de transformación/metabolitos detectados en las experiencias de laboratorio en un ambiente controlado.

La metodología seguida en esta Tesis, basada en irradiación de muestras de agua fortificadas y posterior investigación de los compuestos formados por LC-QTOF MS ha sido aplicada con éxito a diversas familias de plaguicidas como triazinas, organofosforados o uracilos. La utilidad de estos experimentos de fotodegradación ha quedado demostrada al analizar muestras reales de agua, ya que han sido detectados muchos de los productos de transformación elucidados en estas experiencias.

Por otra parte, se ha mostrado la utilidad de LC-QTOF en los estudios de metabolismo, tanto *in vitro*, con microsomas, como *in vivo*, con ratas, tomando como ejemplo de estudio el insecticida diazinon. Las experiencias de laboratorio realizadas ha permitido identificar un buen número de metabolitos polares, gracias al gran potencial de la técnica LC-QTOF, Algunos de los compuestos detectados son coincidentes con los productos de transformación identificados en experiencias de fotodegradación en aguas.

El acoplamiento LC-QTOF también ha sido aplicado con éxito a la elucidación de compuestos desconocidos (*non target*), en distintos tipos de aguas (subterráneas, superficiales, potables, residuales urbanas y residuales industriales).

II- El acoplamiento LC-MS/MS con analizadores de triple cuadrupolo se ha mostrado como una herramienta analítica muy valiosa para la determinación rápida y selectiva de una gran diversidad de plaguicidas y productos de transformación en diferentes matrices de interés ambiental, con poco tratamiento de muestra, minimizando así los errores analíticos asociados a esta etapa y acortando el tiempo de análisis.

El amplio rango de aplicabilidad de esta técnica la hace idónea para la determinación simultánea de moléculas pertenecientes a diversas familias químicas mediante métodos multiresiduo. La principal limitación en este aspecto es que el número de transiciones utilizadas simultáneamente debe permitir obtener una buena definición del pico cromatográfico. En este sentido, la optimización cromatográfica es de gran importancia para minimizar el número de compuestos que coeluyen en un determinado tiempo.

La eficiente ionización en ESI de compuestos de diferentes familias químicas, el mínimo tratamiento de muestra que conlleva la SPE en línea, y su compatibilidad con la técnica LC-MS/MS, hacen del acoplamiento SPE-LC-ESI-MS/MS una herramienta analítica de gran utilidad para la determinación multiresiduo de plaguicidas en aguas a niveles de concentración de $\mu\text{g/L}$ y sub- $\mu\text{g/L}$.

Se han desarrollado dos métodos multiresiduo para el análisis de aguas basados en SPE-LC-MS/MS con el fin de incluir los plaguicidas más utilizados en el área mediterránea así como sus productos de transformación, obteniendo excelentes características analíticas al nivel de $0.05 \mu\text{g/L}$. La estrategia seguida se basa en realizar dos análisis independientes de cada muestra: una alícuota se trata con HCOOH (para el análisis de compuestos ácidos) y la otra con HFBA (para el análisis de compuestos básicos).

La metodología de trabajo aplicada unida a la elevada sensibilidad de SPE-LC-MS/MS ha permitido determinar los niveles de concentración objetivo con la inyección de tan sólo 2 mL de muestra en cada análisis. La purificación producida por el acoplamiento SPE-LC junto con la mínima cantidad de matriz introducida en el sistema MS/MS ha facilitado la correcta cuantificación de los compuestos sin necesidad de utilizar patrón interno. Así mismo, ha sido posible la cuantificación y confirmación simultánea de analitos ácidos, básicos y neutros en las muestras, gracias a la adquisición de al menos, dos transiciones MS/MS para cada compuesto.

Se ha desarrollado, así mismo, un método para la determinación de compuestos altamente polares como el glifosato, AMPA y glufosinato, en muestras de agua y de suelo, a niveles de 50 ng/L y 0.05 mg/Kg, respectivamente.

Se ha observado un importante efecto matriz producido por las aguas y los suelos en la determinación del glifosato y su producto de transformación AMPA, por lo que ha resultado necesario el uso de glifosato marcado isotópicamente como patrón interno. Adicionalmente, en el caso de suelos, es necesaria una dilución del extracto de la muestra para disminuir el efecto matriz.

La participación en un ejercicio interlaboratorio ha dejado en evidencia que la aproximación del patrón interno puede no ser efectiva en aquellos casos en los que el analito sea interferido por un componente de la matriz mediante algún mecanismo de reacción que siga una cinética lenta. Así, se han obtenido excelentes resultados en todos los casos excepto en el análisis de glifosato en aguas subterráneas, donde se reportaron recuperaciones del 15% a pesar del uso del compuesto marcado como patrón interno. Tras una reevaluación del método consistente en una acidificación con ácido clorhídrico hasta pH aproximadamente 1 previa a la derivatización con FMOC, las recuperaciones aumentaron hasta el 98%. Parece ser que la acidificación previa de las muestras conduce a la liberación del glifosato enlazado a algún componente de la matriz, quedando ya libre para su posterior análisis. Es importante remarcar que este problema pasa desapercibido tanto en la validación del método como en el análisis de muestras reales, ya que tanto las muestras fortificadas como las muestras control de calidad se preparan normalmente a partir de muestras blanco recientemente fortificadas.

Los métodos desarrollados en esta Tesis han sido validados siguiendo las guías SANCO de la Unión Europea y bajo el espíritu de las GLPs. Así, el menor nivel de concentración para el cual ha sido validado el método se ha tomado como límite de cuantificación, siempre que la recuperación fuera aceptable (70-110%) y la precisión adecuada (<20%).

Los métodos desarrollados en esta Tesis se han aplicado al análisis de aguas subterráneas y superficiales del área Mediterránea. Los resultados obtenidos muestran la presencia mayoritaria de herbicidas y productos de transformación, apareciendo sólo ocasionalmente algunos insecticidas. Entre los plaguicidas detectados destacan herbicidas de la familia de las triazinas así como el glifosato y el bromacilo. En cuanto a las

concentraciones obtenidas, se ha superado el valor de 0.1 µg/L por compuesto individual en varias muestras, llegando a superar 1 µg/L en algunos puntos de muestreo conflictivos. Cabe destacar la mayor frecuencia de detección de los productos de transformación en relación con los plaguicidas intactos, superando en muchos casos los niveles de concentración de los mismos. La mayoría de estos productos de transformación fueron elucidados en las experiencias de fotodegradación, demostrando la gran utilidad de este tipo de experimentos.

III- Se ha estudiado con detalle el potencial de LC-MS con equipos de triple cuadrupolo y QTOF con fines cualitativos. Con este fin se ha confirmado la identidad de los plaguicidas detectados en algunas muestras positivas seleccionadas mediante la utilización de ambos instrumentos.

La confirmación última mediante triple cuadrupolo se puede conseguir adquiriendo todas las transiciones MS/MS disponibles, lo que permite cumplir sobradamente con los requisitos de la Decisión Europea 2002/657/CE en cuanto a número de puntos de identificación requeridos (IPs) y cálculo de relaciones iónicas.

En general, la adquisición de dos transiciones SRM junto con la medida de un *ion ratio* suele ser suficiente para garantizar la confirmación del analito con equipos de triple cuadrupolo. La confirmación se considera como definitiva si ambas transiciones son suficientemente específicas y la relación iónica está en concordancia con la del patrón.

Sin embargo, se podrían reportar falsos positivos si las transiciones no son suficientemente específicas, y también falsos negativos, si una interferencia comparte una de las transiciones con el analito, alterando el valor de la relación Q/q. En estos casos, la mejor manera de asegurar una correcta confirmación es adquirir todas las transiciones disponibles.

En cuanto a la confirmación por QTOF se ha realizado un barrido de iones producto de los plaguicidas detectados midiendo la masa exacta de los fragmentos y calculando posteriormente las relaciones iónicas. La confirmación de un positivo por QTOF puede considerarse como inequívoca, dadas sus excelentes prestaciones en el campo cualitativo.

Sin embargo, existen algunos riesgos de reportar falsos negativos con QTOF cuando un interferente isobárico coeluya con el analito, ya que la presencia de otros picos adicionales en el espectro de iones producto puede dificultar la comparación con el espectro del patrón. Ante este problema la mejor solución sería mejorar la separación cromatográfica.

La utilización del triple cuadrupolo permite, en general, la confirmación a niveles de concentración más bajos mientras que mediante QTOF se puede alcanzar un mayor número de IPs debido a las medidas de masa exacta, aunque con la limitación de su menor sensibilidad.

Chapter 6.
Conclusions

LC-QTOF MS coupling has been shown to be a powerful analytical tool for the identification of pesticide transformation products and metabolites in environmental and biological samples, respectively.

The high resolution of this mass analyser, together with the possibility of performing both MS and MS/MS experiments with high mass accuracy, has allowed the elucidation of the structure of several transformation products/metabolites detected in laboratory experiments under controlled conditions.

The methodology followed in this Thesis, based on irradiation of spiked water samples and posterior elucidation of the compounds formed by LC-QTOF MS, has been successfully applied to different pesticide families such as triazines, organophosphorus compounds or uracils. The usefulness of photodegradation experiments has been shown as a lot of the transformation products detected in these experiments were detected analysing real water samples.

On the other hand, LC-QTOF usefulness has been shown in metabolism studies, both *in vitro*, with microsomes, and *in vivo*, with rats, using the insecticide diazinon as an example. The experiments carried out in the laboratory have allowed the identification of a large number of polar metabolites due to the high potential of the LC-QTOF technique. Some of the detected compounds coincide with the transformation products already identified in the photodegradation experiments.

LC-QTOF coupling has also been successfully applied to the elucidation of unknown compounds (*non target*), in different kind of water samples (groundwater, surface water, drinking water, wastewater and sewage water).

II- The LC-MS/MS coupling with triple quadrupole mass analysers has been shown to be a powerful analytical tool for the rapid and selective determination of a large number and variety of pesticides and transformation products in different environmental matrices. Additionally, the minimal sample pre-treatment required lowers the analytical errors associated with this step, and leads to shorter analysis times.

The broad scope application of this technique makes it ideal for the simultaneous determination of molecules relating to very different chemical families by means of multi-residual methods. The main limitation of the technique, apart from the compulsory analytes ionisation using the soft ionisation techniques employed nowadays, is related to the number of transitions monitored simultaneously in a concrete time window along the chromatographic run. This number has to be low enough to obtain satisfactory definition in the chromatographic peak. The chromatographic separation being of great importance to minimise the number of compounds co-eluting at the same time.

The efficient ionisation of different chemical families' compounds by ESI, the minimum sample pre-treatment in on-line SPE methods and its compatibility with LC-MS/MS technique make SPE-LC-ESI-MS/MS coupling a useful analytical tool for the multi-residual determination of pesticides in water samples at different $\mu\text{g/L}$ and sub- $\mu\text{g/L}$ concentrations.

Two multi-residue methods for water analysis based on SPE-LC-MS/MS were developed to include the pesticides most commonly used in the Mediterranean area as well as their transformation products. Excellent analytical characteristics at the $0.05 \mu\text{g/L}$ level were obtained. The strategy is based on the performance of two independent analysis of each sample: one treated with HCOOH (for acidic compounds analysis) and the other with HFBA (for the analysis of basic compounds).

The developed analytical methodology together with the high sensitivity of SPE-LC-MS/MS allowed achievement of the objective concentration levels after injection of only 2 mL of sample. The purification offered by the SPE-LC coupling and the minimum amount of matrix components introduced in the MS/MS system facilitated the correct quantification for selected compounds without using internal standard. The simultaneous quantification and confirmation of acidic, basic and neutral analytes has been possible by acquiring at least two transitions for each compound.

In the same way, a multi-residual method for the determination of highly polar compounds such as glyphosate, AMPA and glufosinate in water and soil samples, at 50 ng/L and 0.05 mg/Kg, respectively, has been developed.

An important matrix effect in the determination of glyphosate and its transformation product AMPA in water and soil samples has been observed, making the use of isotope labelled glyphosate as internal standard necessary. Additionally, in the case of soils, an extract dilution to decrease this matrix effect has been necessary.

This work has shown that the internal standard approach may not be effective in those cases where the analyte can be interfered by a matrix component following slow kinetics. Thus, the results of an inter-laboratory comparison study that we participated in, were highly satisfactory in all cases with the only exception of glyphosate in ground water samples, where surprisingly low recoveries, around 15%, were obtained despite the use of isotope-labelled glyphosate as internal standard. A slight modification was introduced in the method, simply consisting of the acidification of sample with hydrochloric acid (HCl) to pH 1 before derivatisation with FMOC. Samples were reanalyzed using this approach, and the recoveries increased up to 98%. A possible explanation might be the slow kinetic interaction between glyphosate and some components of the matrix, which might act as chelating agents. The acidification step could break these interactions, making glyphosate available for derivatization and therefore for analysis. It is important to comment that this problem remains undetected in both the method validation and the analysis of real samples, because spiked samples and quality controls are usually prepared by freshly fortification of blank samples.

Methods developed in this research have been validated following the European Union SANCO Guidelines, adhering to the principles of GLP. Thus, the lowest concentration level assayed in the validation process is taken as limit of quantification, when acceptable recovery (70-110%) and adequate precision (<20%) are obtained.

The methods developed in this Thesis have been applied to monitoring programmes for ground and surface water in the Mediterranean region. The results obtained show the presence of mainly herbicides and transformation products, with insecticides only occasionally detected. The pesticides triazines and bromacil were the most frequently found. Some compounds were present at concentrations higher than 0.1 µg/L in several samples,

reaching in some cases concentrations over 1 µg/L. It is important to note the higher number of transformation product findings in relation with the unchanged pesticides, and the higher concentration levels of these transformation products versus the parent pesticides. Finally, most of these transformation products had been previously elucidated in the photodegradation experiments, showing their usefulness.

III- LC-MS potential with triple quadrupole and QTOF analysers has been studied in detail for qualitative aspects. Thus, the identity of some pesticides detected in selected positive samples by means of the two instruments, has been confirmed.

The ultimate confirmation of positive samples using triple quadrupole can be achieved by acquiring all available MS/MS transitions, satisfying the requirements of the European Commission 2002/657/EC regarding the minimum number of identification points (IPs) and ion ratio calculation.

In general, two transitions in QqQ instruments (together with the measurement of the retention time and the ion intensity ratio) is the minimum required for a safe confirmation. Confirmation can be considered definitive if both selected transitions are highly specific and the Q/q ratio is in agreement with the reference standard.

However, false positives may be reported if the selected transitions are not specific, and also false negatives when an interferent shares one of the transitions with the analyte, resulting in changes to the expected ion ratio. In both cases, the acquisition of all available transitions has been found to be an easy and efficient way to achieve a reliable confirmation

Regarding confirmation by QTOF, product ion spectrum of the pesticides has been acquired with accurate mass measurement of the fragments and subsequent ion ratio calculation. A positive confirmed by this technique may be considered to be unequivocal due to its excellent features in the qualitative field.

However, there are some risks of reporting false negatives with QTOF when a co-eluting isobaric interference is present in the sample matrix. The presence of this type of compounds can lead to additional peaks in the MS/MS spectrum of the sample, making it difficult to compare spectra from the sample and the standard. The most reliable way of reducing this interference is to improve chromatographic resolution.

The use of triple quadrupole allows, in general, confirmation at lower levels, while in the case of QTOF, the number of IPs reached is higher due to the accurate mass measurements although with the limitation of its lower sensitivity.

Sugerencias para trabajos futuros

Con la presente Tesis Doctoral se ha puesto de manifiesto el gran potencial del acoplamiento LC-MS/MS con analizadores QqQ y QTOF para la determinación de residuos de plaguicidas y TPs/metabolitos principalmente en matrices ambientales. A partir de los resultados y conclusiones obtenidos en esta memoria, se pueden perfilar distintas líneas para la ampliación de los trabajos realizados. A continuación se muestran algunas sugerencias para posibles trabajos futuros:

- Analizar muestras de orina de personas expuestas a plaguicidas con el fin de elucidar sus principales metabolitos mediante LC-QTOF MS.
- Desarrollar métodos multiresiduo para la determinación de plaguicidas y sus metabolitos en orina basados en LC-MS/MS con analizadores QqQ.
- Utilizar los métodos desarrollados para la determinación de metabolitos de orina en programas de control de población potencialmente expuesta.
- Ampliar estos métodos a otras matrices biológicas que pueden presentar un interés particular, como placenta o meconium.
- Desarrollar métodos analíticos rápidos, sensibles, selectivos y con capacidad de confirmación para los antibióticos y hormonas más relevantes
- Aplicar los métodos desarrollados y validados a muestras de aguas residuales y medioambientales de la Comunidad Valenciana para determinar los niveles de antibióticos y hormonas que se están emitiendo al medioambiente a través de las aguas depuradas, así como los niveles que finalmente se alcanzan en las aguas superficiales y subterráneas.
- Desarrollar métodos rápidos para el análisis de nuevos contaminantes como perfluorados o toxinas marinas.
- Analizar mediante QTOF MS la posible existencia de impurezas en los formulados de pesticidas
- Aplicar el QTOF MS en el campo de la metabolómica, es decir, analizar las moléculas que son producto del metabolismo, con el fin de utilizar esta información en el diagnóstico de enfermedades con mayor antelación y precisión.

Artículos científicos presentados

Artículo científico 1

The efficient use of quadrupole-time of flight mass spectrometry in environmental analysis: elucidation of transformation products of triazine herbicides in water after UV exposure

M. Ibáñez, J. V. Sancho, O. J. Pozo, F. Hernández

Anal. Chem. (2004) 76: 1328-1335

Artículo científico 2

Use of liquid chromatography quadrupole time-of-flight mass spectrometry in the elucidation of transformation products and metabolites of pesticides. Diazinon as a case of study

M. Ibáñez, J.V. Sancho, O.J. Pozo, F. Hernández

Anal. Bioanal. Chem. (2006) 384: 448-457

Artículo científico 3

Use of quadrupole-time of flight mass spectrometry in the elucidation of unknown compounds present in environmental water

M. Ibáñez, J. V. Sancho, O. J. Pozo, F. Hernández, W. Niessen

Rapid Commun. Mass Spectrom. (2004) 19: 169-178

Trabajo incluido entre el 1% de los trabajos de mayor influencia en su campo, de acuerdo con Essential Science Indicators, THOMSON. Incluido entre los cuatro artículos clasificados como "core papers" en "Quadrupole TOF MS; LC-MS/MS; LC-TOF MS análisis; LC-TOF-MS; environmental water (información, Enero 2006)

Artículo científico 4

Liquid chromatography-mass spectrometry role in the elucidation and determination of transformation products of pesticides in water

M. Ibáñez, J. V. Sancho, O. J. Pozo, F. Hernández

(En preparación)

Artículo científico 5

Residue determination of glyphosate, glufosinate and AMPA in water and soil samples by liquid chromatography coupled to electrospray tandem mass spectrometry

M. Ibáñez, O.J. Pozo, J.V. Sancho, F.J. López, F. Hernández

J. Chromatogr. A (2005) 1081: 145-155

Artículo científico 6

Re-evaluation of glyphosate determination in water by liquid chromatography coupled to electrospray tandem mass spectrometry

M. Ibáñez, O.J. Pozo, J.V. Sancho, F.J. López, F. Hernández

J. Chromatogr. A (2006) 1134: 51-55

Artículo científico 7

Comparison of different mass spectrometric techniques combined with liquid chromatography for confirmation of pesticides in environmental waters

F. Hernández, M. Ibáñez, J.V. Sancho, O.J. Pozo

Anal. Chem. (2004) 76: 4349-4357

Artículo científico 8

Confirmation of organic micropollutants detected in environmental samples by liquid chromatography tandem mass spectrometry: achievements and pitfalls

O. J. Pozo, J. V. Sancho, M. Ibáñez, F. Hernández, W. Niessen

Trends Anal. Chem. (2006) 15:1030-1042

Artículo científico 9

Strategies for quantification and confirmation of multi-class polar pesticides and transformation products in water by LC-MS/MS using triple quadrupole and hybrid quadrupole-time of flight analysers

F. Hernández, O.J. Pozo, J.V. Sancho, F. J. López, J.M. Marín, M. Ibáñez

Trends Anal. Chem. (2005) 24: 596-612

Trabajo incluido entre el 1% de los trabajos de mayor influencia en su campo, de acuerdo con Essential Science Indicators, THOMSON. Incluido entre los cinco artículos clasificados como "research front" en: "multiresidue method", "easy method", "pesticide residue analysis", "LC-MS² using triple quadrupole" (información, Noviembre 2006).

Artículo científico 10

Potential of liquid chromatography/time of flight mass spectrometry for the determination of pesticides and transformation products in water.

J.V. Sancho, O.J. Pozo, M. Ibáñez, F. Hernández

Anal. Bioanal. Chem. (2006) 386:987-997

Nota de aplicación 1

UPLC-aa TOF MS for rapid screening of multiple pesticide residues

D. McMillan, M. Ibáñez

Waters application note (2006)

Otros artículos científicos relacionados

A critical overview on antibiotic residues determination in environmental waters by LC-MS

F. Hernández, J. V. Sancho, M. Ibáñez, C. Guerrero

Trends Anal. Chem. (Enviado)

Efficient approach in the determination of antibiotics in water using on line solid phase extraction liquid chromatography tandem mass spectrometry

O. J. Pozo, J.V. Sancho, C. Guerrero, E. Pitarch, M. Ibáñez, E. Hogendoorn, F. Hernández

J. Chromatogr. A (2006) 1103: 83-93

Residue determination of cyromazine and its metabolite melamine in chard samples by ion-pair liquid chromatography coupled to electrospray tandem mass spectrometry

J. V. Sancho, M. Ibáñez, S. Grimalt, Ó. J. Pozo, F. Hernández*

Anal. Chim. Acta (2005) 530: 237-243

Rapid determination of fosetyl aluminium residues in lettuce by liquid chromatography/ electrospray tandem mass spectrometry

F. Hernández, J. V. Sancho, O. J. Pozo, C. Villaplana, M. Ibáñez, S. Grimalt

J. AOAC Int. (2003) 86: 832-838

Apéndice 1

Programa utilizado en Turbo Pascal

A continuación se presenta el programa escrito en lenguaje de programación Turbo Pascal que permite llevar a cabo la preconcentración y posterior análisis de diferentes analitos mediante SPE-LC, utilizando el ASPEC XL y el programa de software 719 v2.01 suministrado por Gilson. Este programa permite preconcentrar diferentes volúmenes de muestras situados en diferentes canales, un determinado número de veces.

PROGRAM preconcent;

```
{ $X+ }  
USES Samplib, Sampglob, Crt;  
VAR facond : longint;  
    vmuestra: array [1..5] of longint;  
    nveces, ttransf, tespera, tana: integer;  
    q, p, nmuestra, tubo, i: integer;  
    canal:string;  
    l: char;  
{ Main Program }
```

PROCEDURE acondicionar;

```
Begin  
ClrEol;  
  
BufCmd(20, 'pa');  
SwitchValve(1, RIGHT, INJECT);  
SwitchValve(1, LEFT, LOAD);  
Writeln('purga del canal (a) con ACN ');  
DispenseToInjectionPort(1, 1, RIGHT, 5000 ,5.5);  
SwitchValve(1, RIGHT, LOAD);  
Writeln('acondicionamiento del cartucho con ACN ');  
DispenseToInjectionPort(1, 1, RIGHT, 10000, facond, facond);  
BufCmd(20, 'pb');
```

```
SwitchValve(1, RIGHT, INJECT);
Writeln('purga del canal (b) con H2O ');
DispenseToInjectionPort(1, 1, RIGHT, 5000, 5.5);
SwitchValve(1, RIGHT, LOAD);
Writeln('acondicionamiento del cartucho con agua ');
DispenseToInjectionPort(1,1, RIGHT, 5000, facond, facond);
End;
```

```
BEGIN
```

```
DefineSampler(1, 10, 183, 40, M233);
SetValveDefault(1, RIGHT, INJECT);
SetValveDefault(1, LEFT, INJECT);
DefineHPPump(1, 1, 25, 300);
Define402Dilutor(2, 0, 10000, 1000, 10000);
```

```
CheckConfiguration;
InitializeSystem;
SetSensitivity(1, 3);
```

```
{*****Start Method*****}
```

```
ClrEol;
REPEAT
Writeln('N° de replicas para cada muestra (1-5)');
InputN(nveces);
Writeln;
UNTIL (nveces>=1) and (nveces<=5);
```

```
ClrEol;
REPEAT
Writeln('N° de muestras');
InputN(nmuestra);
Writeln;
UNTIL (nmuestra>=1) and (nmuestra<=5);
```

```

writeln ('PON LA PRIMERA MUESTRA EN EL CANAL d');

BEGIN

FOR i := 1 To nmuestra DO
BEGIN
ClrEol;
Write('volumen de la muestra ',i,' (ml)');
LinputN(vmuestra[i]);
Writeln;
END;

ClrEol;
Writeln('flujo de acond/carga del cartucho en ml/min? ');
linputN(facond);
Writeln;

END;

q:=1;
p:=1;
l:='d';

ClrEol;
Write('Cuanto tiempo transfiero ');
InputN(ttransf);
Writeln;

ClrEol;
Write('Cuanto tiempo espero ');
InputN(tespera);
Writeln;

REPEAT

```

REPEAT

acondicionar;

canal:='p'+i;

BufCmd(20, canal);

SwitchValve(1, RIGHT, INJECT);

WriteLn('purga del canal de la muestra ',i);

DispenseToInjectionPort(1,1, RIGHT, 5000,5,5);

SwitchValve(1, RIGHT, LOAD);

WriteLn('preconcentracion de ',vmuestra[p],' ml de muestra del canal ',i);

DispenseToInjectionPort(1, 1, RIGHT, 1000*vmuestra[p], facond, facond);

SwitchValve(1, LEFT, INJECT);

SetElectricalContact(1, 1, PULSE);

SetElectricalContact(1, 3, PULSE);

BufCmd(20, 'pc');

WaitTime(tttransf);

SwitchValve(1, LEFT, LOAD);

WaitTime(tespera);

q:=q+1;

UNTIL (q>nveces);

q:=1;

l:=succ(l);

p:=p+1;

UNTIL (p>nmuestra);

AbortApplication;

End.