TESIS DOCTORAL

ESTRATEGIAS PARA EL ESTUDIO DE PLAGUICIDAS ESPECÍFICOS Y SUS METABOLITOS MEDIANTE ESPECTROMETRÍA DE MASAS DE ALTA RESOLUCIÓN

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STRATEGIES FOR THE STUDY OF SPECIFIC PESTICIDES AND THEIR METABOLITES USING HIGH RESOLUTION MASS SPECTROMETRY

Rosalia López Ruiz

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TESIS DOCTORAL

Estrategias para el estudio de plaguicidas específicos y sus metabolitos mediante espectrometría de masas de alta resolución

Memoria presentada por Rosalía López Ruiz
para optar al grado de Doctor en Ciencias Químicas
con Mención de Calidad Internacional por el
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Fdo. Rosalía López Ruiz

Directora de Tesis	Codirector de Tesis
Fdo. Antonia Garrido Frenich	Fdo. Roberto Romero González

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Resumen

La presente Tesis Doctoral aporta nueva información relacionada con el comportamiento de plaguicidas específicos en alimentos y medioambiente, así como el estudio de la presencia de los metabolitos derivados de ellos. Para ello se han empleado técnicas de extracción genéricas como el método QuEChERS (*Quick, Easy, Cheap, Effective, Rugged y Safe*), la extracción sólido-líquido (*Solid-Liquid Extraction*, SLE) y la extracción en fase sólida (*Solid Phase Extraction*, SPE). Posteriormente se ha llevado a cabo el análisis mediante dos técnicas cromatográfícas ampliamente utilizadas como son la cromatografía de gases (*Gas Chromatography*, GC) y la cromatografía de líquidos (*Liquid Chromatography*, LC) acopladas a analizadores de espectrometría de masas de alta resolución (*High Resolution Mass Spectrometry*, HRMS). Se han usado dos modos de trabajo, dirigido y no dirigido, aplicándose en este último, las variantes de análisis de sospechosos (*suspect screening*) y análisis de desconocidos (*unknown analysis*).

La Tesis se ha estructurado en cuatro partes. Mientras que la primera de ellas se basa en la revisión de los avances de la espectrometría de masas en el ámbito alimentario y medioambiental, las tres restantes están relacionadas con el estudio de plaguicidas específicos y sus metabolitos. De esta forma, la primera parte se centra en una revisión bibliográfica de las técnicas analíticas empleadas en la presente Tesis doctoral, así como sus modos de análisis y/o trabajo. Los trabajos incluidos son los siguientes:

- Revisión bibliográfica de los avances en cromatografía de líquidos de ultra alta resolución (*Ultra High Performance Liquid Chromatography*, UHPLC) acoplada a MS (UHPLC-MS) en la última década para el análisis de una gran variedad de compuestos orgánicos (plaguicidas, micotoxinas, fármacos y compuestos fenólicos) en medioambiente y alimentos. Esta revisión incluye la descripción de las ventajas y

desventajas del uso de la espectrometría de masas de baja resolución (*Low Resolution Mass Spectrometry*, LRMS) frente a la de HRMS.
- Revisión bibliográfica de las aplicaciones más importantes en el campo de análisis de contaminantes en alimentos mediante el uso de herramientas metabolómicas aplicadas a datos de LC-MS y GC-MS. En la misma se discuten los modos de trabajo que la HRMS permite como son el análisis dirigido y no dirigido, incluyendo *suspect screening* y *unknown analysis*.

La segunda parte se centra en el estudio de dos insecticidas, desarrollados en la última década y de amplio interés como son flonicamida y tiociclam, así como sus metabolitos. La técnica de análisis empleada fue la UHPLC-HRMS. Los estudios desarrollados en esta parte incluyen:

- Desarrollo y validación de un procedimiento analítico basado en el método QuEChERS para la extracción de flonicamida y sus metabolitos en pimiento mediante UHPLC-HRMS.
- Estudio del comportamiento en campo y laboratorio del insecticida flonicamida en naranja mediante UHPLC-HRMS. La técnica de extracción empleada fue el método QuEChERS seguida de una etapa de purificación mediante extracción dispersiva en fase sólida (*dispersive Solid-Phase Extration*, d-SPE) con amina primaria secundaria (*Primary Secondary Amine*, PSA). Además se monitorizaron sus metabolitos conocidos, ácido 4-trifluorometilnicotínico (TFNA), 4-(trifluorometil)nicotinoil glicina (TFNG) y 4-trifluorometilnicotinamida (TFNA-AM). También se llevaron a cabo estudios de rutas metabólicas empleando la técnica de resonancia magnética nuclear (*Nuclear Magnetic Resonance*, NMR).
- Estudio del comportamiento en laboratorio del pro-insecticida tiociclam en tomate mediante UHPLC-HRMS, monitorizando su metabolito más conocido, nereistoxina. Se identificaron de manera tentativa otros cuatro metabolitos mediante análisis no dirigido. La técnica de extracción empleada fue el método QuEChERS en su versión tamponada AOAC 2007.

La tercera parte se centra en el estudio del comportamiento de cuatro herbicidas y sus metabolitos en matrices medioambientales usando la UHPLC-HRMS como técnica de análisis. Los herbicidas estudiados fueron quizalofop-p-etil, quizalofop-p-tefuril, propaquizafop y dimetacloro. A tal fin se desarrollaron los siguientes trabajos de investigación:

- Estudio del comportamiento en laboratorio de los herbicidas quizalofop-p-etil, quizalofop-p-tefuril y propaquizafop en suelos y aguas mediante UHPLC-HRMS. Además se llevó a cabo la separación quiral de su metabolito más conocido, (*RS*)-quizalofop-p, usando para ello una fase estacionaria quiral que permitió la separación de los enantiómeros.

- Estudio del comportamiento en laboratorio del herbicida dimetacloro en suelos y aguas mediante UHPLC-HRMS. Se llevó a cabo un estudio de sus metabolitos desconocidos mediante análisis no dirigido. La técnica de extracción empleada fue el método QuEChERS, incluyendo una etapa de d-SPE con C_{18} para suelo, mientras que en agua se empleó únicamente SPE. Durante la etapa de SPE fue necesario adicionar a la muestra un modificador de par iónico, tetrabutilamonio, para mejorar la retención de los analitos, en especial los metabolitos, los cuales tenían una mayor polaridad.

La cuarta parte está centrada en la determinación de varios fungicidas como famoxadona, propamocarb y fenamidona, en alimentos y matrices ambientales. Se desarrollaron los siguientes artículos científicos:

- Estudio del comportamiento en invernadero del fungicida famoxadona en calabacín, pepino y tomate mediante UHPLC-HRMS. Paralelamente se llevó a cabo un estudio de sus metabolitos desconocidos mediante análisis no dirigido, conduciendo a la identificación tentativa de un nuevo metabolito de famoxadona.
- Estudio del comportamiento en laboratorio del fungicida famoxadona en suelos y aguas mediante UHPLC-HRMS. Para ello se llevó a cabo un análisis no dirigido de tipo *suspect screening,* usando como base de datos la creada a partir del anterior trabajo en calabacín, pepino y tomate, identificándose tres metabolitos de famoxadona.
- Estudio del comportamiento en invernadero de los fungicidas propamocarb y fenamidona en calabacín, pepino y tomate mediante UHPLC-HRMS y GC-HRMS. Esta fue la primera vez que se empleó la GC-Q-Orbitrap para este tipo de estudios. También se realizó un estudio de sus metabolitos desconocidos, identificando tentativamente cuatro nuevos compuestos, no descritos hasta la fecha para propamocarb y uno en el caso de fenamidona.
- Estudio del comportamiento en laboratorio de los fungicidas propamocarb y fenamidona en suelos y aguas mediante UHPLC-HRMS. Se llevó a cabo una búsqueda de metabolitos mediante *suspect screening* (metabolitos detectados en trabajos previos en matrices vegetales) y mediante *unknown analysis* detectando de forma tentativa tres nuevos metabolitos de propamocarb.

Objetivos

La presente Tesis Doctoral tiene como principal objetivo conocer la disipación de plaguicidas tras su aplicación, así como la aparición de metabolitos, tanto conocidos como no, en matrices de origen alimentario y medioambiental. Con el fin de estudiar el comportamiento de los plaguicidas y sus metabolitos se han llevado a cabo estudios en campo y/o laboratorio, simulando condiciones climatológicas, estudiando diferentes cultivos y/o diferentes tipos de suelos. Además, se ha desarrollado una metodología de procesado de datos, para identificar metabolitos desconocidos hasta la fecha de realización de esta Tesis de los plaguicidas objeto de estudio. A continuación, se exponen los objetivos específicos desarrollados, para poder cumplir el objetivo general.

1. Revisión bibliográfica de los avances analíticos en los últimos años en el campo de la UHPLC-MS **(artículo científico I)** y revisión crítica de la aplicación de herramientas metabolómicas en la determinación de contaminantes en alimentos mediante técnicas cromatográficas acopladas a HRMS **(artículo científico II)**.
2. Desarrollo de un método analítico mediante UHPLC-HRMS para flonicamida y sus metabolitos en pimiento y naranja **(artículos científicos III y IV)**. Evaluación del comportamiento en campo y laboratorio de flonicamida y sus metabolitos en naranja **(artículo científico IV)**.
3. Desarrollo de un método analítico mediante UHPLC-HRMS para determinar tiociclam y su metabolito principal, nereistoxina, en tomate y monitorizar su disipación **(artículo científico V)**. Procesado de datos analíticos para identificar tentativamente nuevos metabolitos de este plaguicida.

4. Desarrollo de métodos analíticos mediante UHPLC-HRMS para derivados del quizalofop, dimetacloro, famoxadona, fenamidona y propamocarb y sus respectivos metabolitos en suelos **(artículos científicos VI, VIII, X y XII)** y aguas **(artículos científicos VII, VIII, X y XII)**. Estudio del comportamiento de dichos compuestos en condiciones de laboratorio e identificación de nuevos metabolitos mediante procesado de datos analíticos.
5. Desarrollo de métodos analíticos mediante UHPLC-HRMS y GC-HRMS para famoxadona, fenamidona y propamocarb y sus respectivos metabolitos en tomate, calabacín y pepino **(artículos científicos IX y XI)**. Estudios de disipación en condiciones de campo y evaluación de la presencia de nuevos metabolitos mediante técnicas de análisis de desconocidos.

Abstract

The current Thesis provides new knowledge related to the behaviour of selected pesticides in food and environment, as well as the monitoring of both known and unknown metabolites. For this purpose, generic extraction techniques as QuEChERS (*Quick, Easy, Cheap, Effective, Rugged and Safe*), Solid-Liquid Extraction (SLE) and Solid Phase Extraction (SPE) were used. After that, determination was carried out using two chromatographic techniques widely applied, as gas chromatography (GC) and liquid chromatography (LC) coupled to high resolution mass spectrometry (HRMS) analysers. Two working modes, targeted and non-targeted, were employed using the last one to perform suspect screening and unknown analysis.

The Thesis was divided in four sections. The first one was focused on the review of the recent trends of the application of MS in the field of food and environmental analysis, whereas the other sections were focused on the study of specific pesticides and their metabolites. Thus, the first part includes a review of the analytical techniques employed in the Thesis, as well as their applications and/or working modes. The included studies were:

- Review of the development of ultra high performance liquid chromatography (UHPLC) coupled to MS in the last decade for the analysis of a wide range of organic compounds (pesticides, mycotoxins, drugs and phenolic compounds) in food and environmental samples. This review included the advantages and disadvantages of low resolution mass spectrometry (LRMS) in comparison with HRMS.
- A review discussing the most important applications developed in the field of contaminant analysis in food applying metabolomics

tools, using LC-MS and GC-MS data. A discussion about the workflows that can be applied when HRMS is used, as targeted and non-targeted analysis including suspect screening and unknown analysis, was performed.

The second part was based on the study of two insecticides, widely used in the last decade, as flonicamid and thiocyclam, as well as their metabolites. The analytical technique employed was UHPLC coupled to HRMS. The developed studies included in this section were:

- Development and validation of an analytical procedure based on QuEChERS method for the extraction of flonicamid and its metabolites in bell pepper by UHPLC-HRMS.
- Field and laboratory studies of flonicamid in orange using UHPLC-HRMS. The extraction method used was the QuEChERS procedure followed by a clean-up step using dispersive solid-phase extraction (d-SPE) with primary secondary amine (PSA). Its known metabolites, 4-(trifluoromethyl)nicotinic acid (TFNA), 4-(trifluoromethyl) nicotinoyl glycine (TFNG) and 4-trifluoromethylnicotinamide (TFNA-AM), were also monitored. In addition, metabolomics studies were carried out using the Nuclear Magnetic Resonance technique (NMR).
- Laboratory study of the behaviour of the proinsecticide thiocyclam in tomato by UHPLC-HRMS, monitoring its metabolite nereistoxin. Four new metabolites of nereistoxin were tentatively identified by non-targeted analysis. The selected extraction method was the buffered QuEChERS version, AOAC 2007.

The third section includes the study of the behaviour of four herbicides and their metabolites in environmental matrices using UHPLC-HRMS. The studied herbicides were quizalofop-p-ethyl, quizalofop-p-tefuryl, propaquizafop and dimethachlor. The following research studies were carried out:

- Laboratory study of the behaviour of the quizalofop-p-ethyl, quizalofop-p-tefuryl and propaquizafop herbicides in soils and water by UHPLC-HRMS. In addition, a chiral separation of (*RS*)-quizalofop-p was carried out using a chiral stationary phase that allowed the enantiomeric separation of both isomers.
- Laboratory study of the behaviour of the dimethachlor herbicide in soils and water by UHPLC-HRMS. An unknown metabolite study was carried out by non-targeted approach. For the extraction of compounds from soils a QuEChERS based method was employed,

including a d-SPE step with C_{18}, meanwhile in water, SPE was used. During the SPE step, the addition of tetrabutylammonium was necessary to improve the retention of the analytes, specially the metabolites with a higher polarity.

The fourth section was based on the determination of several fungicides as famoxadone, propamocarb and fenamidone in food and environmental matrices. The following studies were developed:

- Greenhouse study of the behaviour of the famoxadone fungicide in courgette, cucumber and tomato by UHPLC-HRMS. Additionally, a study of the unknown metabolites was carried out by non-targeted analysis, performing a tentative identification of a new metabolite of famoxadone.
- Laboratory study of the behaviour of the famoxadone fungicide in soils and water by UHPLC-HRMS. A suspect screening was performed using a home-made database, identifying three famoxadone metabolites.
- Greenhouse study of the behaviour of the propamocarb and fenamidone fungicides in courgette, cucumber and tomato by UHPLC-HRMS and GC-HRMS. This was the first time that GC-HRMS was employed for this type of studies. In addition, a study of unknown metabolites was done, and four new metabolites, not previously described for propamocarb, and one of fenamidone, were tentatively identified.
- Laboratory study of the propamocarb and fenamidone fungicides in soils and water using UHPLC-HRMS. A suspect screening (metabolites detected in previous studies in vegetable matrices) and unknown analysis studies were carried out, and three new metabolites of propamocarb were tentatively detected.

Objectives

This Thesis has as general objective focused on the evaluation of the dissipation of pesticides after their application, as well as, the detection of targeted and non-targeted metabolites in food and environmental matrices. With this aim, the behaviour of pesticides and their metabolites was carried out in field and/or laboratory studies, simulating environmental conditions, studying different crops and/or different types of soils. A processing data methodology was also developed to identify unknown metabolites of selected pesticides. The specific objectives developed are set out below, in order to fulfil the general objective.

1. Reviewing of the analytical advances in the last decade in the field of UHPLC-MS (**Publication I**) and reviewing of the application of metabolomics tools for the determination of contaminants in food by chromatographic techniques coupled to HRMS (**Publication II**).
2. Development of an analytical method by UHPLC-HRMS for the determination of flonicamid and its metabolites in bell pepper and orange (**Publications III and IV**). Evaluation of the behaviour of flonicamid and its metabolites in orange (**Publication IV**)
3. Development of an analytical method by UHPLC-HRMS for the determination of thiocyclam and its main metabolite, nereistoxin, in tomato, monitoring their dissipation (**Publication V**). Additionally, tentative identification of new metabolites of this pesticide was carried out.
4. Development of analytical methods based on UHPLC-HRMS for quizalofop derivates, dimethachlor, famoxadone, fenamidone and propamocarb and their metabolites in soils (**Publications VI, VIII, X and XII**) and water (**Publications VII, VIII, X y XII**). Study of

the behaviour of these compounds in laboratory conditions and identification of new metabolites by data processing.

5. Development of analytical methods by UHPLC-HRMS and GC-HRMS for famoxadone, fenamidone and propamocarb and their metabolites in tomato, courgette and cucumber (**Publication IX and XI**). Dissipation studies under field conditions and evaluation of the presence of new metabolites by unknown approaches.

Lista de publicaciones

A continuación, se muestra la lista de publicaciones derivadas de la presente Tesis:

I. **López-Ruiz, R.**; Romero-González, R.; Garrido Frenich, A. Ultrahigh-pressure liquid chromatography-mass spectrometry: An overview of the last decade. *TrAC Trends Anal. Chem.* 2019, *118*, 170–181.
II. **Lopez-Ruiz, R.**; Romero-González, R.; Garrido Frenich, A. Metabolomics approaches for the determination of multiple contaminants in food. *Curr. Opin. Food Sci.* 2019, *28*, 49–57.
III. **López-Ruiz, R.**; Romero-González, R.; Martínez-Vidal, J.; Garrido Frenich, A. Determination of flonicamid and its metabolites in bell pepper using ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (Orbitrap). *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.* 2016, *33*, 1685–1692.
IV. **López-Ruiz, R.**; Ruiz-Muelle, A.; Romero-González, R.; Fernández, I.; Martínez-Vidal, J.; Garrido Frenich, A. The metabolic pathway of flonicamid in oranges using an orthogonal approach based on high-resolution mass spectrometry and nuclear magnetic resonance. *Anal. Methods* 2017, *9*, 1718–1726.
V. **López-Ruiz, R.**; Belmonte-Sánchez, E.; Romero-González, R .; Martinez Vidal, J; Ramirez-Cassinello, J. J.; Garrido Frenich, A. Dissipation study of the proinsecticide thiocyclam and nereistoxin in tomato using liquid chromatography high resolution mass spectrometry. *Food Chem.* 2020. Submitted.

VI. **López-Ruiz, R.**; Romero-González, R.; Martínez-Vidal, J.; Fernández-Pérez, M.; Garrido-Frenich, A. Degradation studies of quizalofop-p and related compounds in soils using liquid chromatography coupled to low and high resolution mass analyzers. *Sci. Total Environ.* 2017, *607–608*, 204–213.

VII. **López-Ruiz, R.**; Romero-González, R.; Martínez Vidal, J.L.; Garrido Frenich, A. Behavior of quizalofop-p and its commercial products in water by liquid chromatography coupled to high resolution mass spectrometry. *Ecotoxicol. Environ. Saf.* 2018, *157*, 285–291.

VIII. **Lopez-Ruiz, R.**; Romero-González, R.; Ortega-Carrasco, E.; Garrido-Frenich, A. Degradation studies of dimethachlor in soils and water by UHPLC-HRMS: putative elucidation of unknown metabolites. *Pest Manag. Sci.* 2020, *76*, 721–729.

IX. **Lopez-Ruiz, R.**; Romero-Gonzalez, R.; Ortega-Carrasco, E.; Garrido-Frenich, A. Dissipation studies of famoxadone in vegetables under greenhouse conditions using liquid chromatography coupled to high-resolution mass spectrometry: putative elucidation of a new metabolite. *J. Sci. Food Agric.* 2019, *99*, 5368–5376.

X. **Lopez-Ruiz, R.**; Romero-González, R.; Serra, B.; Garrido-Frenich, A. Dissipation kinetic studies of fenamidone and propamocarb in vegetables under greenhouse conditions using liquid and gas chromatography coupled to high-resolution mass spectrometry. *Chemosphere* 2019, *226*, 36–46.

XI. **Lopez-Ruiz, R.**; Romero-González, R.; Garrido-Frenich, A. Residues and dissipation kinetics of famoxadone and its metabolites in environmental water and soil samples under different conditions. *Environ. Pollut.* 2019, *252*, 163–170.

XII. **López-Ruiz, R.**; Romero-González, R.; Garrido-Frenich, A. Dissipation kinetics of fenamidone, propamocarb and their metabolites in ambient soil and water samples and unknown screening of metabolites. *J. Environ. Manage.* 2020, *254*, 109818.

Contribución del autor en cada publicación

Artículos científicos III, IV, V, VI, VII, VIII, IX, X, XI y XII: La autora participó en el diseño de los experimentos, realizó la parte experimental y redactó los manuscritos.

Revisión bibliográfica I y II: La autora participó en la redacción de ambos trabajos de revisión y discusión bibliográfica.

Abreviaturas y acrónimos

ADI	Ingesta diaria admisible (*Aceptable Daily Intake*)
AIF	Fragmentación de todos los iones *(All-Ion-Fragmentation)*
AOAC	Asociación de Químicos Analíticos Oficiales (*Association of Official Analytical Chemists)*
ARfD	Dosis de referencia aguda *(Acute Reference Dose)*
d-SPE	Extracción dispersiva en fase sólida (*dispersive Solid-Phase Extration)*
DT_{50}	Vida media *(Half-lives)*
EFSA	Autoridad Europea para la Seguridad Alimentaria (*European Food Safety Authority*)
EMRL	Límite máximo de residuos extraños (*Extraneous Maximum Residue Limit)*
EPA	Agencia para la Protección del Medioambiente (*Enviromental Protection Agency*)
EQS	Normas de calidad ambiental *(Environmental Quality Standards)*
EU	Union Europea (*European Union*)
FAO	Organización para la Agricultura y la Alimentación (*Food and Agriculture Organization*)

FWHM	Anchura a media altura (*Full Width at Half Maximum)*
GAP	Buenas prácticas agrícolas (*Good Agricultural Practice*)
GC	Cromatografía de gases (*Gas Chromatography)*
GCB	Carbón negro grafitizado *(Graphitized Carbon Black)*
HILIC	Cromatografía de interacción hidrofílica *(Hydrophilic Interaction Chromatography)*
HPLC	Cromatografía de líquidos de alta eficacia (*High Performance Liquid Chromatography)*
HRMS	Espectrometría de masas de alta resolución *(High Resolution Mass Spectrometry)*
ICA	Análisis por componentes independientes *(Independent Component Analysis)*
IEX	Cromatografía de intercambio iónico *(Ion-Exchange Chromatography)*
IPs	Puntos de identificación *(Identification Points)*
LC	Cromatografía de líquidos (*Liquid Chromatography)*
LLE	Extracción líquido-líquido *(Liquid-Liquid Extraction)*
LOD	Límite de detección (*Limit of Detection)*
LOQ	Límite de cuantificación (*Limit of Quantification)*
LRMS	Espectrometría de masas de baja resolución (Low *Resolution Mass Spectrometry)*
MRLs	Límites máximos de residuos (*Maximum Residue Limits)*
MS	Espectrometría de masas (*Mass Spectrometry*)
MSPD	Dispersión de la matriz en fase sólida *(Matrix Solid Phase Dispersion)*
NMR	Resonancia magnética nuclear *(Nuclear Magnetic Resonance)*
OECD	Organización para la Cooperación y el Desarrollo Económico *(Organization for Economic Co-operation and Development)*

PAHs	Hidrocarburos aromáticos policíclicos (*Polycyclic Aromatic Hydrocarbons)*
PCA	Análisis por componentes principales (*Principal Component Analysis)*
PCBs	Bifenilos policlorados (*Polychlorobiphenyls)*
PLE	Extracción con líquidos presurizados (*Pressurised Liquid Extraction)*
PLS-DA	Análisis discriminante por mínimos cuadrados parciales (*Partial Least Square Discriminant Analysis)*
POPs	Contaminantes orgánicos persistentes (*Persistent Organic Pollutants)*
PSA	Amina primaria secundaria *(Primary Secondary Amine)*
QqQ	Analizador triple cuadrupolo *(Triple Quadrupole mass spectrometer)*
S/N	Relación Señal Ruido *(Signal to Noise ratio)*
SEC	Cromatografía de exclusión por tamaño *(Size-Exclusion Chromatography)*
SFE	Extracción con fluidos supercríticos *(Supercritical Fluid Extraction)*
SFO	Modelo de primer orden *(Single First-Order Rate)*
SLE	Extracción sólido-líquido *(Solid-Liquid Extraction)*
SPE	Extracción en fase sólida *(Solid-Phase Extraction)*
SPME	Microextracción en fase sólida *(Solid-Phase Microextraction)*
TBA	Acetato de tetrabutilamonio *(Tetrabutylammonium acetate)*
TOF	Tiempo de vuelo *(Time of Flight)*
TPs	Productos de transformación (*Transformation Products*)
UHPLC	Cromatografía de líquidos de ultra alta eficacia (*Ultra-high Performance Liquid Chromatography)*
WHO	Organización Mundial de la Salud (*World Health Organization*)

CAPÍTULO

1

Introducción

1. PLAGUICIDAS Y SU IMPORTANCIA

1.1. PLAGUICIDAS: uso y tipos

En la actualidad, y a pesar del creciente desarrollo de la agricultura ecológica, el uso de productos fitosanitarios o plaguicidas está ampliamente establecido en el cultivo intensivo de frutas y hortalizas. Su aplicación representa uno de los tipos más importantes de protección frente a plagas y conlleva un aumento de la producción [1]. Los plaguicidas tras su aplicación pueden permanecer en las frutas y hortalizas en forma de residuo, posibilitando mediante esta vía su entrada en la cadena alimentaria. Dichos residuos deben de estar a concentraciones bajas para evitar daños perjudiciales en el ser humano durante la ingesta de estos productos.

Existen diversas definiciones de producto fitosanitario o plaguicida. Una de ellas se recoge en el documento titulado "Código Internacional de Conducta sobre la Distribución y Uso de Plaguicidas" [2], de la Organización para la Agricultura y la Alimentación (*Food and Agriculture Organization*, FAO) de las Naciones Unidas. La FAO define el término "plaguicida" como «cualquier sustancia o mezcla de ellas destinadas a prevenir, destruir o controlar plagas, incluyendo los vectores de enfermedades humanas o animales, las especies no deseadas de plantas o animales que ocasionan un daño duradero u otras que interfieren con la producción, procesado, almacenamiento, transporte y comercialización de alimentos, los productos agrícolas de consumo, la madera y sus productos, los piensos para animales o los productos que pueden administrárseles para el control de insectos, arácnidos u otras plagas corporales. Incluyéndose además las sustancias usadas como reguladores del crecimiento, defoliantes y desecantes, agentes para reducir la densidad de la fruta, agentes para evitar la caída prematura de la fruta y las sustancias aplicadas a los cultivos antes o después de la cosecha, para proteger el producto contra el deterioro, durante el almacenamiento y transporte».

Otra definición es la establecida por la Agencia para la Protección del Medioambiente (*Environmental Protection Agency*, EPA) [3] de Estados Unidos, que establece el término "producto fitosanitario" como una mezcla que contiene una materia activa y otras inertes. La materia activa se define como aquella que previene, destruye, repele o mitiga una plaga, mientras que la materia inerte se establece como aquella que es importante para el rendimiento del producto y la usabilidad del mismo.

La materia activa debe identificarse por su nombre en la etiqueta del producto fitosanitario junto con su porcentaje en peso o volumen, existiendo varias categorías [4]:

- Convencional, que son todos los ingredientes que no sean plaguicidas biológicos y plaguicidas antimicrobianos.
- Antimicrobianos, que son sustancias o mezclas de sustancias utilizadas para destruir o suprimir el crecimiento de microorganismos dañinos, ya sean bacterias, virus u hongos en objetos y superficies inanimadas.
- Bioplaguicidas, que son aquellos procedentes de ingredientes derivados de ciertos materiales naturales.

La materia inerte se adiciona intencionadamente a fin de aumentar la eficacia de la sustancia activa y el rendimiento del producto fitosanitario. Los ingredientes inertes son sustancias químicas, como disolventes o surfactantes, pero también están incluidos los productos alimenticios comunes (por ejemplo, ciertos aceites comestibles, especias, hierbas) y algunos materiales naturales (por ejemplo, cera de abejas, celulosa).

Existen una gran variedad de plaguicidas que se pueden clasificar atendiendo a diversos criterios, tales como según el organismo frente al que actúan, según su familia química o por su modo de acción. El modo más común de clasificarlos es el que se refiere al organismo al que hacen frente, diferenciando insecticidas, herbicidas, acaricidas, fungicidas, bactericidas, antibióticos, rodenticidas y molusquicidas. Los más empleados [5], y que han sido estudiados en la presente Tesis, son los insecticidas, herbicidas y fungicidas.

❖ *Insecticidas*

Los insecticidas son empleados en el control de plagas de insectos indeseados como mosca blanca, pulgón, etc. En cuanto al modo de acción pueden actuar mediante contacto, ingestión o inhalación, como las cipermetrinas [6]. Además, se encuentran los llamados insecticidas sistémicos, como flonicamida o imidacloprid, que cuando son aplicados por pulverización foliar penetran y se reparten por toda la planta de modo que ésta se vuelve venenosa para el insecto en todas sus partes. Aparentemente este tipo de insecticidas poseen grandes ventajas por su sistemia, pero cuando comenzaron a utilizarse provocaron numerosos problemas imprevistos como intoxicación aguda y crónica de aplicadores, trabajadores agrícolas e incluso consumidores; mortandad de peces, aves y otros animales salvajes; alteración del control biológico natural y polinización; contaminación extensiva del agua subterránea, y generación de resistencia a plaguicidas en poblaciones de plagas [7]. Actualmente la mayoría de los insecticidas son sistémicos. En la presente Tesis se han estudiado dos insecticidas, como son flonicamida y tiociclam (**Figura 1.1**).

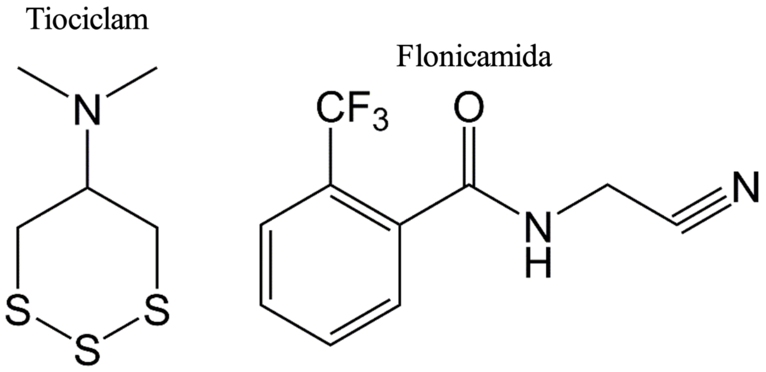


Figura 1.1. Insecticidas estudiados en la presente Tesis.

❖ *Herbicidas*

Se define como herbicida aquel producto que es empleado para inhibir o interrumpir el desarrollo de plantas indeseadas, como malas hierbas [8], que pueden perjudicar a los cultivos o bien afectar a la recolección, dificultando la recogida de las cosechas. Los herbicidas se pueden clasificar atendiendo a sus propiedades químicas, a su modo de acción o a su modo de uso. Atendiendo a este último criterio se pueden distinguir herbicidas de [9,10]:

- Pre-plantación: aquellos que se aplican antes de la siembra.
- Pre-emergencia: se aplican siempre antes de la emergencia de las malezas.
- Post-emergencia: se aplican después que el cultivo y las malezas han emergido.

Dentro de los herbicidas, el más usado a nivel mundial es el glifosato, aunque ha sido prohibido en muchos países debido a su poder cancerígeno y elevada persistencia [11]. En esta Tesis se han incluido para su estudio cuatro herbicidas: dimetacloro, quizalofop-p-etil, quizalofop-p-tefuril y propaquizafop (**Figura 1.2**)

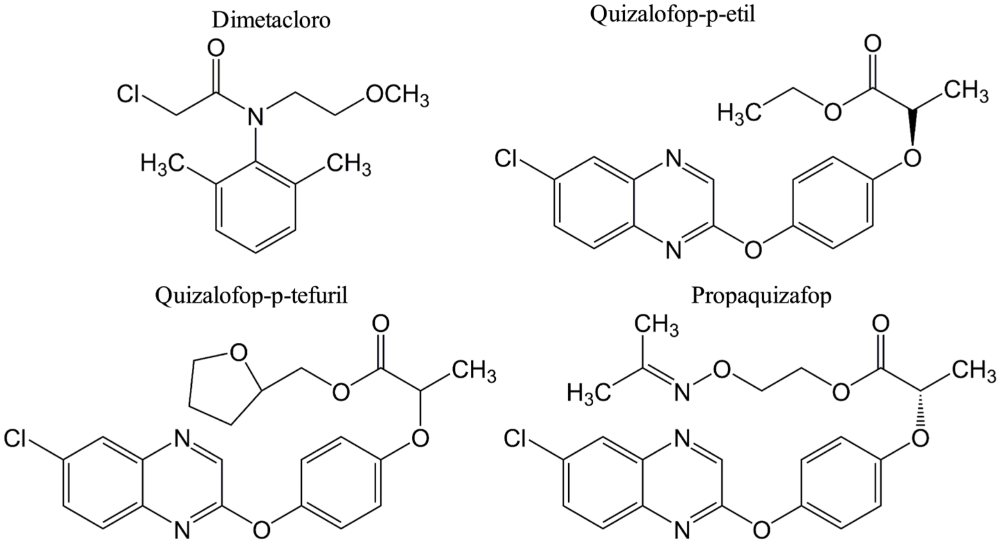


Figura 1.2. Herbicidas estudiados en la presente Tesis.

❖ *Fungicidas*

El principal uso de los fungicidas, como su nombre indica, es evitar el crecimiento de hongos tanto en cultivos, como en semillas, grano o frutos durante su transporte o almacenamiento. Son muy empleados después de la cosecha (post-cosecha) para evitar pérdidas por plagas que pueden afectar a los productos cosechados durante el almacenamiento. Estos últimos se suelen aplicar en el agua de lavado de los frutos o vegetales, creando una película protectora en la piel de éstos e impidiendo la proliferación de hongos. Los fungicidas han sido también utilizados para reducir la contaminación por micotoxinas en cereales [12].

Además de los fungicidas post-cosecha, existen gran variedad de ellos usados durante el cultivo, aplicándose en forma de polvo o mediante pulverización foliar, y en muchas ocasiones se necesitan diversas aplicaciones para eliminar la plaga. De este último grupo, en la presente Tesis se han estudiado tres fungicidas: famoxadona, fenamidona y propamocarb (**Figura 1.3**).

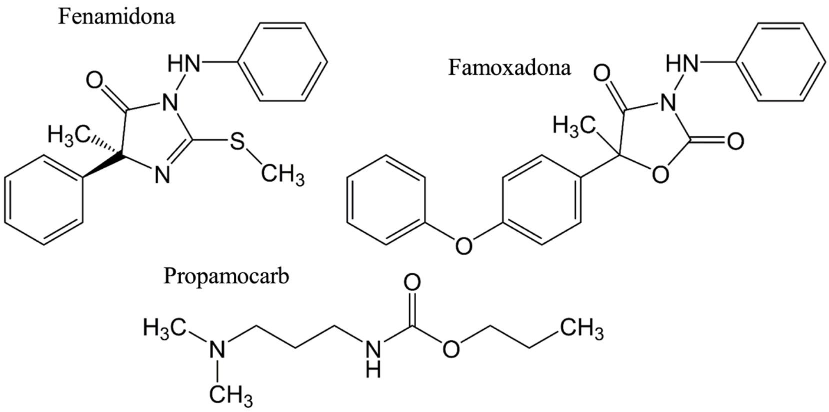


Figura 1.3. Fungicidas estudiados en la presente Tesis.

1.2. PROBLEMAS ASOCIADOS AL USO DE PLAGUICIDAS

Sin obviar la importancia de los plaguicidas, tanto en la agricultura como en las actividades de salud pública, son innegables los efectos tóxicos que generan en el ser humano y en el medioambiente. En este último ámbito, tanto agua, aire o suelo son los principales recursos que el ser humano requiere para obtener de ellos el alimento. El agua puede contaminarse de forma indirecta con el uso de plaguicidas, ya que, tras su aplicación a plantas o suelo, pueden ser transferidos a las aguas superficiales o subterráneas, provocando riesgos potenciales para la salud humana y medioambiental.

En relación con el ser humano, su biodisponibilidad en el organismo depende de la toxicocinética (absorción, distribución, metabolismo y eliminación) de cada compuesto. Dichos procesos están influenciados tanto por factores externos, relacionados con los patrones de exposición y con las sustancias químicas (modo de empleo, temperatura ambiental, tipo de plaguicida, frecuencia, intensidad y duración de la exposición, etc.) [13], como por factores inherentes al individuo (edad, sexo, dotación genética, estado de salud y nutricional, estilos de vida, vía principal de absorción, etc.) [14].

La absorción depende de las propiedades del formulado del plaguicida y de la vía de entrada que determinan que un producto cruce las barreras del cuerpo hasta alcanzar la sangre u otro fluido o tejido diana.

La vía aérea representa una ruta importante de transmisión debido a la frecuente aplicación de plaguicidas en zonas de cultivo por esta vía, su arrastre por el viento hacia zonas aledañas y el uso común en el hogar de productos en aerosol, nebulizaciones, bombas de humo, etc. Todo esto favorece la presencia del producto en el ambiente de forma continua y en pequeñas cantidades [15].

A nivel mundial la industria agroquímica está preocupada por los daños que pueden causar los plaguicidas a edades tempranas en el ser humano y señalan que los límites máximos de residuos (*Maximum Residue Limits*, MRLs) no son un nivel de seguridad, sino más bien que están relacionados con los residuos que se espera encontrar cuando los plaguicidas se aplican siguiendo unas Buenas Prácticas Agrícolas (*Good Agricultural Practice*, GAP). Su opinión es que exceder los MRLs ocasionalmente no constituye un riesgo apreciable para la salud, a menos que se excedan los límites convencionales basados en la salud, como la Ingesta Diaria Admisible (*Aceptable Daily Intake*, ADI) o la dosis de referencia aguda (*Acute Reference Dose*, ARfD) [4].

Sin embargo, un estudio del Consejo Nacional de Investigación de los Estados Unidos realizado en 1998, señaló que la exposición a compuestos neurotóxicos, tales como el plaguicida clorpirifos, a niveles considerados seguros para adultos podrían resultar en una pérdida permanente de la función cerebral cuando la exposición se produce en la infancia temprana. Estas preocupaciones fueron relevantes cuando se tuvo en cuenta la exposición a residuos de plaguicidas en dietas infantiles [16].

En otras palabras, los bajos niveles de plaguicidas hallados rutinariamente en alimentos pueden dañar nuestra salud en el medio o largo plazo. En este estudio fue fundamental el establecimiento del Acta de 1996 para la Protección de la Calidad de Alimentos de los Estados Unidos, que requiere el establecimiento de niveles mucho más estrictos para proteger a bebés y niños [17].

Además de los riegos que los plaguicidas pueden causar en los seres humanos, diversos estudios se centran en los riesgos para la flora y la fauna. Es bien conocido como el uso de ciertos insecticidas neurotóxicos, como los organofosforados o neonicotinoides (nuevos nicotinoides), han causado graves problemas en la población de abejas a nivel mundial. Por lo general, las abejas se encuentran expuestas a plaguicidas continuamente, ya que su hábitat y modo de alimentación está cerca de entornos agrícolas donde se aplican plaguicidas [18]. Los insecticidas neurotóxicos tienen especial importancia a niveles subletales en las abejas, produciendo cambios de comportamiento que interfieren con la búsqueda de alimento, el éxito del recorrido, el rendimiento de navegación y la comunicación social entre ellas [19]. Actualmente los neonicotinoides y organofosforados están parcialmente restringidos en Europa por este tipo de daños causados, pero todavía son aplicados en muchas partes del mundo [20].

1.3. METABOLITOS: conceptos básicos

El término "metabolito" se define como aquel intermedio, producto del metabolismo, que es de un tamaño inferior a 1 kDa [21]. Cabe destacar la diferencia entre el término "metabolito" y el de "producto de transformación" (*transformation product*, TP). Un TP es un derivado de molécula degradada por procesos químicos, como oxidación o fotólisis, entre otros, es decir reacciones en las que no está implicado el metabolismo de los seres vivos. Por otro lado, los derivados que surgen de las reacciones en las que interviene el metabolismo de los seres vivos son los metabolitos. En lo sucesivo, y por simplificar, en la presente Tesis Doctoral a los compuestos degradados de plaguicidas, ya sean metabolitos o TPs, se denominarán con el término "metabolito".

Los plaguicidas en plantas, animales o en el medioambiente se pueden transformar en una amplia gama de metabolitos a través de procesos químicos, biológicos o físicos. En este campo, los estudios metabolómicos son necesarios para estudiar el comportamiento de los plaguicidas, identificar posibles metabolitos y dar información acerca del riesgo de ingesta en humanos [22].

Un metabolito se considera relevante si hay una razón para suponer que tiene propiedades intrínsecas comparables a la sustancia original en términos de su actividad diana biológica, plantea un riesgo para los organismos mayor o comparable al de la sustancia original, o tiene ciertas propiedades toxicológicas que se consideran inaceptables [23].

Los metabolitos pueden ser mucho más tóxicos que el propio compuesto progenitor, ya que suelen ser más polares que aquel [6]. Estos se generan por reacciones metabólicas simples, que en muchos casos son comunes entre las familias de plaguicidas. Un ejemplo es el de flonicamida, un insecticida sistémico del grupo de los nicotinoides, que posee gran variedad de metabolitos descritos por la Autoridad Europea para la Seguridad de los Alimentos (*European Food Safety Authority*, EFSA) en su informe de riesgos [24], y que se generan por reacciones simples como es la de oxidación. En la **Figura 1.4** se pueden observar distintos metabolitos de flonicamida. Así el ácido 6-hidroxi-4-trifluorometilnicotínico (TFNA-OH) se genera por la oxidación del ácido 4-trifluorometilnicotínico (TFNA), o el 6-hidroxi-4-trifluorometilnicotinamida (OH-TFNA-AM) a partir del 4-trifluorometilnicotinamida (TFNA-AM), en el que se produce la oxidación de la molécula con la introducción de un grupo –OH.

Los metabolitos de flonicamida son conocidos, y dado que algunos de ellos (TFNA y 4-(trifluorometil)nicotinoil glicina (TFNG)) son toxicológicamente relevantes, actualmente se incluyen en la definición de

MRL siendo necesario su control. Sin embargo, este caso es algo muy poco común aún, ya que la degradación de los plaguicidas después de su aplicación puede originar varios metabolitos (no tóxicos o desconocidos) en las plantas tratadas, que pueden ser igualmente importantes que los tóxicos. Estos pueden considerarse, en cierto sentido, como "marcadores" del uso de plaguicidas [25].

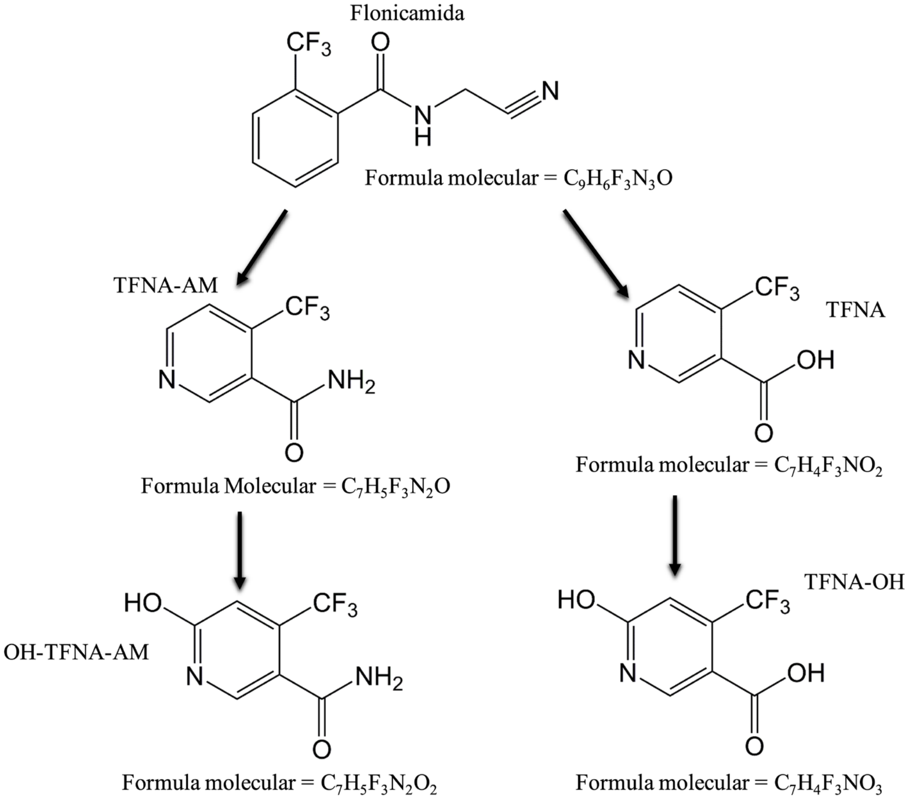


Figura 1.4. Flonicamida y algunos de sus metabolitos.

Por este motivo, el análisis de los metabolitos de los plaguicidas es igual de importante que el análisis de los compuestos progenitores en sí, aunque puede ser más difícil por varias razones [26]:

- La concentración suele ser muy baja, por lo que es difícil de detectar, ya que a partir de un plaguicida original se originan varios metabolitos.
- Se desconoce la dinámica del origen de los metabolitos en cultivos específicos, ya que hay más estudios generales que los desarrollados en un medio específico, como un solo tipo de cultivo o condición ambiental.

- La mayoría de los metabolitos son más polares que el compuesto original, como se ha comentado anteriormente, por lo que no pueden incorporarse directamente en métodos multirresiduo comunes. Por lo tanto, se deben desarrollar e implementar nuevos procedimientos analíticos.
- Los estándares analíticos solo están disponibles comercialmente para unos pocos metabolitos, por lo que la cuantificación de la mayoría de ellos no es posible.

Debido a esta problemática, en la presente Tesis se han desarrollado métodos de análisis para plaguicidas y sus correspondientes metabolitos con el objetivo de llevar a cabo un control más eficaz en este ámbito.

1.4. DEL PLAGUICIDA AL METABOLITO: TRANSPORTE Y TRANSFORMACIONES

Es muy probable que la lucha química siga siendo la estrategia central para el control de plagas en cultivos y tierras no agrícolas en el futuro. Sin embargo, la creciente preocupación por el impacto ambiental de los plaguicidas ha provocado la implantación de unas medidas más restrictivas, que requieren un conocimiento más extenso del destino ambiental, la toxicología y la persistencia de los plaguicidas, así como los niveles de residuos de metabolitos en los alimentos. Estos factores requieren un estudio adicional de las vías de degradación, las actividades enzimáticas, la regulación y expresión de las enzimas y la genética del organismo frente al que actúan, especialmente a medida que se desarrollan nuevos plaguicidas. La mayoría de los plaguicidas presentan una gran degradación en las plantas y medioambiente. Las transformaciones que sufren los plaguicidas pueden ser biológicas (metabolismo) o químicas y físicas, como fotodegradación (fotólisis), autolisis, oxidación, reducción, reordenamiento e inactivación debido a procesos de unión a suelos o macromoléculas. La fotodegradación es uno de los factores más importantes implicados en la descomposición de los plaguicidas y depende de las condiciones climáticas, la presencia de fotosensibilizadores, etc. Sin embargo la principal vía de eliminación de plaguicidas es la degradación biológica, por el metabolismo de plantas y organismos [27,28].

En la degradación biológica, la naturaleza de los residuos de plaguicidas en las plantas y los animales depende de los siguientes procesos [6]:

1. Absorción: El movimiento del plaguicida a través de las membranas. Los plaguicidas pueden ser transferidos dentro y fuera de las células de un sistema biológico por difusión pasiva, ósmosis o mecanismos de

transporte activo. Las propiedades fisicoquímicas de los plaguicidas, como la lipofilicidad (log P) y la acidez (pKa), influyen en el proceso de absorción después de su aplicación en la planta, junto con los tipos de membrana celular y el potencial electroquímico en las células.

2. Distribución: Transporte dentro del sistema biológico. La distribución de plaguicidas en la planta depende de su entrada al sistema de transporte de la planta que utiliza una red de conductos vasculares, xilema y floema, para transferir nutrientes y agua. El paso y la retención de plaguicidas en el floema también están influenciados por sus características físico-químicas, principalmente su log P y pKa. En el caso de los animales, los plaguicidas que entran en la circulación sistémica se distribuyen en los tejidos mediante los mismos procesos mecánicos que se mencionaron anteriormente. La distribución depende de la dinámica del tejido sanguíneo y de la tendencia de los plaguicidas a unirse con las proteínas plasmáticas.

3. Metabolismo: Transformación biológica o química de plaguicidas resultantes de procesos naturales en los sistemas biológicos. El metabolismo de los plaguicidas puede involucrar un proceso de tres fases o etapas [29]:

    - Fase I. Las propiedades iniciales de un compuesto original se transforman a través de la oxidación, reducción o hidrólisis para producir generalmente un producto más soluble en agua y generalmente menos tóxico que el principal.
    - Fase II. Involucra la conjugación de un plaguicida o metabolito de plaguicida a un azúcar, aminoácido o glutatión, lo que aumenta la solubilidad en agua y reduce la toxicidad en comparación con el plaguicida parental. En general, los metabolitos de la Fase II tienen poca o ninguna fitotoxicidad y pueden almacenarse en orgánulos celulares.
    - Fase III. Implica la conversión de los metabolitos de la Fase II en conjugados secundarios, los cuales no son tóxicos.

- Eliminación: el plaguicida y sus metabolitos se eliminan a través de procesos celulares activos (transporte, excreción).

2. ESTUDIOS DE DISIPACIÓN DE PLAGUICIDAS Y SUS METABOLITOS

Conocer el comportamiento de un plaguicida en el medioambiente es una tarea de gran interés. Toda la información generada en los estudios de degradación de plaguicidas es crucial para conocer las cantidades residuales de cada uno de los principios activos aplicados y evaluar el destino medioambiental de los mismos [30,31]. Cabe destacar que existen dos términos que hacen referencia a estos estudios: degradación y disipación. Cuando se habla de estudios de degradación se hace referencia a los cambios que sufre un plaguicida, transformándose la molécula progenitora en algún metabolito o metabolitos. Cuando se hace referencia a estudios de disipación, éstos incluyen todos los cambios que puede sufrir un plaguicida para llegar a la eliminación general de éste, bien sea transformándose en metabolitos o eliminándose por procesos como volatilización o escorrentía [32]. Sus resultados determinan una constante de degradación/disipación definida para un plaguicida en determinadas circunstancias. En lo sucesivo, y debido a que la mayoría de los trabajos evalúan sobre todo la disipación de un plaguicida y no tanto cómo éste se transforma en metabolitos, en la presente Tesis Doctoral a los estudios de disipación/degradación, nos referiremos como estudios de disipación.

Durante la disipación de plaguicidas, la vía de eliminación predominante viene determinada principalmente por sus propiedades químicas y las condiciones ambientales. En la **Figura 1.5** se pueden observar todas las interacciones planta-ambiente posibles. Por ejemplo, la absorción que se lleva a cabo por difusión a través de las raíces (en el caso del suelo y agua) y hojas (en el caso del agua y aire), mientras que la eliminación es llevada a cabo por los procesos de fotodegradación o volatilización, difusión en agua o por sorción de las raíces en el suelo.

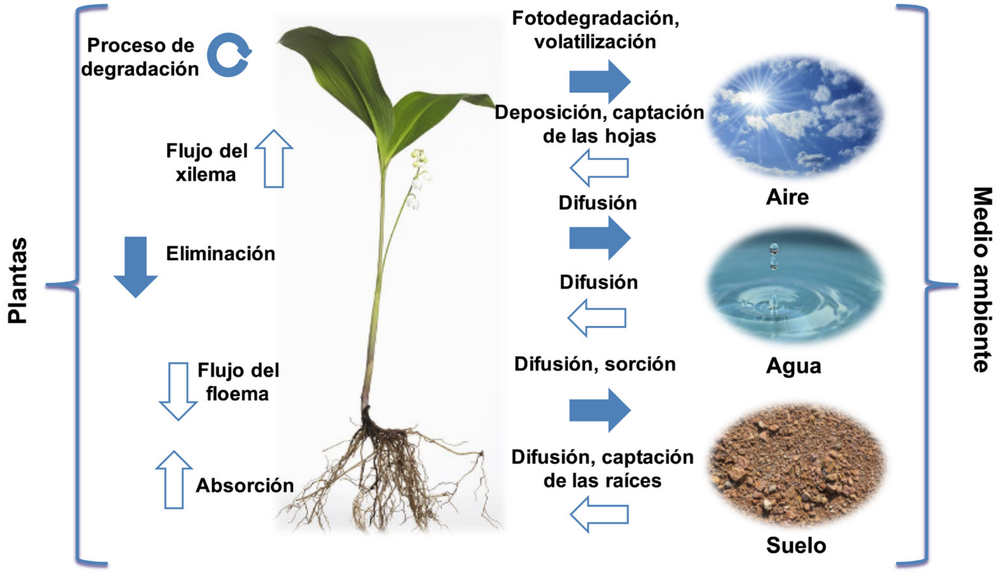


Figura 1.5. Representación de las interacciones planta-ambiente con procesos que contribuyen directamente a la absorción o transporte de plaguicidas en plantas (⇨) y procesos que contribuyen directamente a la disipación de plaguicidas de las plantas (➡). Fuente: [30].

La evaluación del ajuste de los resultados experimentales obtenidos a una serie de modelos cinéticos permite conocer la vida media de un plaguicida o su velocidad de disipación [31]. La constante de disipación de un plaguicida es el parámetro que marca toda la información para determinar su comportamiento medioambiental. Esta se interpreta en función de tres variables: la vía de disipación, las causas de la disipación y la velocidad de disipación [33,34]. Además de estas variables, la constante de disipación de un plaguicida está determinada por otros factores como el tipo de especie cultivada (características del cultivo, tasa de crecimiento, dependencia del pH, etc...), condiciones climáticas, parámetros de la aplicación (tipo de formulado, número de aplicaciones, volumen de agua en el caldo, tipo de boquilla de aplicación, altura, presión de aplicación, etc...) [35,36], además de las reacciones abióticas, que causan una reducción de contaminantes. Por lo tanto, el destino final de un plaguicida depende de una serie de factores que deben de tenerse en cuenta y que se señalan a continuación [33].

- Condiciones medioambientales:
    - Fotodegradación
    - Temperatura
    - Lluvia
    - Humedad
    - Variabilidad espacial
    - Tipo de suelo

- Condiciones en los cultivos
    - Tasa de crecimiento
    - Volatilización
    - Acidez en las plantas
    - Estructura fisiológica

- Condiciones químicas
    - Tipo de formulado
    - Polaridad del plaguicida
    - Dependencia del pH
    - Descomposición química
    - Condiciones físicas
    - Estereoquímica del plaguicida

En la **Tabla 1.1** se presenta un resumen de los modelos cinéticos establecidos para el estudio de la disipación de plaguicidas.

Estos modelos determinan valores de constante de disipación y tiempo de vida media (tiempo que tarda un plaguicida en disiparse a la mitad de su concentración inicial ($t_{1/2}$ o DT_{50})). Con ellos se planean estudios para conocer el comportamiento del plaguicida, donde el valor de DT_{50} será fundamental para conocer la persistencia de un plaguicida en el medio. Se ha estipulado que, si un plaguicida posee una DT_{50} menor que 15 días, éste no es persistente; si se encuentra entre 15 días < DT_{50} < 30 días, es ligeramente persistente; entre 30 días < DT_{50} < 60 días, es medianamente persistente; entre 60 días < DT_{50} < 120 días, es muy persistente; y si DT_{50} > 120 días, es extremadamente persistente. Estos valores determinarán, sobre todo en matrices alimentarias, el tiempo necesario que hay que esperar antes de recolectar los frutos para su consumo (plazo de seguridad) [15].

Tabla 1.1. Modelos de ajuste de la concentración residual de plaguicidas y estimación de vida media[a]

Modelo	*Curva de concentración residual (mg/kg)*	*Vida media (días)*
Orden cero	$C(t)=C_0-kt$	$t_{1/2}=\frac{C_0}{2k}$
Orden medio	$C(t)=\left(\sqrt{C_0}-\frac{kt}{2}\right)$	$t_{1/2}=\frac{\left(2-\sqrt{2}\right)\sqrt{C_0}}{k}$
Primer orden	$C(t)=C_0-e^{kt}$	$t_{1/2}=\frac{\ln 2}{k}$
Orden uno y medio	$C(t)=\left(\frac{1}{\sqrt{C_0}}+\frac{kt}{2}\right)^{-2}$	$t_{1/2}=\frac{2\sqrt{2}-2}{k\sqrt{C_0}}$
Segundo Orden	$C(t)=\frac{C_0}{1+ktC_0}$	$t_{1/2}=\frac{1}{kC_0}$
Hockey Stick	$C(t)=C_0e^{k_1t}$ *para* $t \leqslant t_b$	$t_{1/2}=\frac{\ln 2}{k_1}$ si $t_{1/2} \leqslant t_b$
	$C(t)=C_0e^{-k_1t}e^{-k_2(t-t_b)}$ *para* $t > t_b$	$t_{1/2}=t_b+\frac{\ln 2-k_1t_b}{k_2}$ si $t_{1/2} > t_b$

[a] Abreviaturas: C_0: concentración inicial de plaguicidas; C(t): concentración residual de plaguicidas a un tiempo *t*; *k*: constante de disipación; t: tiempo (días); $t_{1/2}$: tiempo de vida media; t_b: tiempo al que la constante *k* cambia.

En la mayoría de los trabajos publicados con anterioridad a la presente Tesis Doctoral, la disipación de plaguicidas se ajusta a modelos de *Primer orden* (**Tabla 1.1**). Ello indica que el comportamiento de la mayoría de ellos está determinado por una curva de disipación de primer orden. Ejemplos de este comportamiento son algunos plaguicidas como propaquizafop en suelos [37], clotianidina [38] y espiromesifen [39] en repollo y suelos o fomesafen en suelos [40]. Aunque todos ellos se comporten siguiendo el mismo patrón, los valores de constante de disipación y vida media son muy diferentes, ya que éstos dependen de las propiedades físico-químicas del plaguicida, del tipo de matriz, condiciones ambientales en las que se lleve a cabo el estudio o la cantidad de plaguicida en ellas. De hecho, en algunos trabajos se llevan a cabo estudios de disipación en diferentes matrices y a diferentes concentraciones de aplicación. Por ejemplo, Zhong et al. [41] estudiaron el comportamiento de tres herbicidas en suelo y plantas de maíz de diferentes localizaciones, observando valores de DT_{50} diferentes: 0.7 y 1.4 días para fluroxipir, 1.1 y 2.1 días para florasulam y finalmente 0.4 y 1.3 días para fluroxipir-meptil. Tsochatzis et al. [42] llevaron a cabo estudios de disipación de tres plaguicidas en agua y suelo, bajo condiciones de campo y de laboratorio, obteniendo por ejemplo para penoxulam un valor de DT_{50} de 6 días en aguas y de 1.5 días en suelo, para profoxidim 0.5 y 0.6 días en aguas y suelos respectivamente, y de 16.4 y 197 días para triciclazol.

En otros trabajos se realizó una evaluación de las diferentes curvas de disipación, según los modelos representados en la **Tabla 1.1**, determinando como modelo óptimo aquel para el que el valor del ajuste por mínimos cuadrados de la representación gráfica de la ecuación fuese más próximo a 1 ($r^2 \approx 1$). Por ejemplo, Diez et al. [43] llevaron a cabo la evaluación de cinco modelos cinéticos de disipación diferentes para doce herbicidas en cebada, concluyendo como óptimo el modelo de Hockey Stick ya que presentaba un valor de r^2 más próximo a 1 para todos los compuestos ($r^2 = 0.9824$).

La necesidad de conocer el comportamiento de un plaguicida es primordial debido a que éstos, en muchos casos, se pueden degradar en metabolitos que pueden o no estar descritos con anterioridad, y que pueden estar presentes en la matriz de estudio y no ser detectados. A pesar de que la identificación de estos metabolitos es muy importante, hasta la fecha de realización de esta Tesis Doctoral hay pocos trabajos en los que se realiza de manera simultánea un estudio de la disipación de un plaguicida y la presencia de sus metabolitos. Uno de los pocos estudios encontrados es el de Chen et al. [44] que evaluaron la disipación de fenoxaprop-p-etil y su metabolito fenoxaprop-p en trigo y suelo. Se observó como a medida que disminuía la concentración de fenoxaprop-p-etil, se iba detectando e incrementando la concentración del metabolito fenoxaprop-p, hasta que llegado un tiempo (5 días) el metabolito comenzaba a disiparse hasta el final del estudio (35 días).

2.1. DISIPACIÓN EN FRUTAS Y HORTALIZAS

El estudio de disipación de plaguicidas permite conocer el comportamiento de éstos en la matriz en la que se encuentren. Los estudios de disipación en matrices alimentarias como frutas y hortalizas se pueden realizar en invernadero o campo abierto. Estos estudios se basan en la aplicación del producto técnico o producto fitosanitario del plaguicida a estudiar en el cultivo de interés bajo condiciones ambientales reales. Las dosis en las que el producto fitosanitario se aplica normalmente son las establecidas por el fabricante y una dosis más alta, cuyo fin es poder detectar los metabolitos que se generan a partir de la molécula progenitora. En ambos tipos de estudios se recolectan porciones representativas de los frutos durante cierto tiempo (de una semana a 30 días) y se analizan en modo de análisis dirigido (*target)*, monitorizando la variación de la concentración del compuesto progenitor, mientras que, en modo de análisis de desconocidos (*unknown*), en su variante de rutas metabólicas y *suspect* se lleva a cabo la identificación de los metabolitos generados. Entre los trabajos más relevantes, relacionados con este tipo de estudios, se encuentra el de Chen et al. [45] que estudiaron la disipación de fenamidona y propamocarb en cultivos de pimiento bajo condiciones de campo abierto en tres zonas diferentes de China, a una dosis 1.5 veces la indicada por el fabricante. La recolección y el análisis se realizó a las 2 h y 1, 3, 7, 14 y 28 días después de la aplicación, obteniendo valores de DT_{50} entre 6 y 16 días. Otro estudio, ha sido el llevado a cabo por Wu et al. [46], evaluando la disipación de flonicamida en calabacín y manzana bajo condiciones de campo abierto, aplicando el producto comercial a un valor 1.5 veces mayor a la indicada por el fabricante. Los frutos se recolectaron para su análisis a las 2 h y 1, 2, 3, 5, 7, 10 y 14 días para calabacín, y 2 h, 1, 2, 3, 5, 7, 10, 14, 21 y 28 días para manzana, después de la aplicación foliar del producto técnico. Los valores de DT_{50} obtenidos fueron 3 y 6 días para calabacín y manzana respectivamente. Al-Rahman et al. [47] estudiaron la disipación de seis plaguicidas con diferente modo de acción (fungicidas, insecticidas y acaricidas) en tomate bajo condiciones de campo abierto a diferentes dosis de aplicación. Estas fueron las recomendadas por el fabricante y el fruto se recolectó a 1, 2, 3, 5, 7, 9, 12 y 15 días después de la aplicación del producto comercial. Para todos los plaguicidas los valores de DT_{50} variaron entre 3 y 9 días. Finalmente, Siddamallaiah et al. [39] estudiaron la disipación de espiromesifen en repollo, aplicando el producto comercial dos veces, la segunda vez diez días después de la primera aplicación, a la dosis recomendada por el fabricante. La recolección de las muestras se llevó a cabo a las 2 h y 1, 2, 3, 5, 7, 10, 15, 20, 25 y 30 días después de la segunda aplicación, llegando a la conclusión que es un plaguicida poco persistente con valores de DT_{50} menores que 4.5 días. En todos estos trabajos no se estudiaron los posibles metabolitos de los plaguicidas, conocidos o no, y por lo tanto, no evalúan el comportamiento de éstos. Por lo tanto, la presente Tesis introduce como novedad la búsqueda de metabolitos conocidos y su monitorización, para conocer su comportamiento, así como la de posibles metabolitos no conocidos hasta la fecha en los estudios de disipación.

2.2. DISIPACIÓN EN SUELO Y AGUA

Los estudios de disipación de plaguicidas en suelo y agua se llevan a cabo de manera similar a los de matrices alimentarias. Se pueden realizar estudios *in situ*, aunque los más comunes, sobretodo, en el caso de agua, son los realizados en laboratorio, por la facilidad de controlar las condiciones y la dificultad que supone estudiar la disipación de plaguicidas en agua o en campo. A tal fin se fortifican con el producto técnico distintas alícuotas de agua en recipientes transparentes o topacio (cuando se quieren simular condiciones de oscuridad como son las aguas subterráneas). Las concentraciones a las que se realizan los estudios son normalmente de 1 mg/L y alguna dosis más alta. En los estudios de campo en suelo el producto técnico se aplica en el mismo, mientras que en los estudios de laboratorio se toman porciones de suelo sobre las que se aplica el producto técnico. Al igual que en el apartado anterior, los estudios se suelen realizar a las concentraciones que estima el fabricante y a una dosis superior.

En relación a los trabajos encontrados en bibliografía, Zhong et al. [41] estudiaron en campo abierto la disipación de florasulam, fluroxipir y fluroxipir metil en campos de maíz a una dosis 1.5 veces mayor a la determinada por el fabricante. La toma de muestra de suelo, llevada a cabo a profundidades de 0-10 cm, se realizó a las 2 h y 1, 2, 3, 5, 7, 10, 14, 21 y 30 días después de la aplicación del producto comercial de todos ellos. El estudio concluyó que los tres herbicidas no eran persistentes en suelo, ya que los valores de DT_{50} fueron menores que 1.3 días. Hou et al. [48] evaluaron la disipación de afidopiropen en campos de algodón, a dos dosis, la recomendada por el fabricante y a una dosis 5 veces mayor. Se tomaron porciones de suelo (entre 1-2 kg) a 1 h y 3, 5, 7, 14, 21, 28, 35, 42 y 49 días después de la aplicación del producto comercial. Para ambas dosis se obtuvieron valores de DT_{50} entre 1 a 3 días. Chen et al. [44] estudiaron la disipación de fenoxaprop-p-etil y sus metabolitos en suelos, a la dosis normal y a una dosis 1.5 mayor. Se tomaron diferentes porciones de suelo a las 2 h y 1, 2, 3, 5, 7, 14, 21, 28 y 35 días después de la aplicación del producto comercial, obteniendo valores de DT_{50} entre 1.8 y 2.4 días para fenoxaprop-p-etil. En relación con fenoxaprop-p, éste se disipa por completo a los 15 días de aplicación del producto comercial. Cabe destacar que éste es uno de los pocos trabajos que incluye metabolitos de plaguicidas específicos en su determinación.

Por otro lado, Tsochatzis et al. [42] evaluaron la disipación de penoxsulam, triciclazol y profoxidim en agua de campos de arroz bajo condiciones de campo y en laboratorio. En campo, el producto comercial se aplicó a la dosis estimada por el fabricante en pequeñas cubículas donde crecía el arroz y se tomaron 200 mL de muestra a los 21, 35 y 150 días. En el caso del estudio en laboratorio se trataron porciones de 250 mL de agua desionizada con las disoluciones acuosas de los productos comerciales y posteriormente se incubaron a 30-33°C y a un 75% de humedad relativa. El compuesto profoxidim fue el menos persistente (DT_{50} de 0.8 días en suelo y 1.2 días en agua), seguido por penoxsulam (DT_{50} de 1.4 días en suelo y 5 días en agua) que persistió más tiempo especialmente en agua y finalmente triciclazol (DT_{50} de 60.2 días en suelo y 4.5 días en agua). Al igual que se ha comentado para el caso de matrices alimentarias, solo en el trabajo de Chen et al. [44] se incluyó el estudio de metabolitos de plaguicidas específicos, lo cual pone de manifiesto la escasez de estudios en este ámbito.

3. MARCO NORMATIVO

3.1. SEGURIDAD ALIMENTARIA: Plaguicidas

La seguridad alimentaria es un concepto que preocupa a nivel mundial. La exposición humana a plaguicidas a través de la cadena alimentaria como resultado del uso extensivo de éstos en la agricultura puede representar un gran riesgo en la salud pública. Es por ello que autoridades nacionales e internacionales han desarrollado normativa y medidas estrictas para garantizar la seguridad alimentaria. Dentro de estas medidas están incluidas una serie de normas para la aprobación de las sustancias activas usadas en los productos fitosanitarios, el establecimiento de MRLs en alimentos y piensos y el estricto control de plaguicidas en alimentos y medioambiente [1].

3.1.1. Autorización de los plaguicidas

Antes de llevar a cabo la comercialización de plaguicidas es necesario realizar una serie de ensayos biológicos, químicos o toxicológicos para garantizar su seguridad durante su uso. Estos procedimientos están establecidos internacionalmente y de acuerdo con las directrices marcadas por la Organización para la Cooperación y el Desarrollo Económico (*Organization for Economic Co-operation and Development*, OECD), la Organización para la Agricultura y la Alimentación (*Food and Agriculture Organization*, FAO) y Organización Mundial de la Salud (*World Health Organization*, WHO) [49].

En la Unión Europea (*European Union*, EU) el procedimiento de solicitud, la evaluación de las sustancias activas y su aprobación están descritos en el Reglamento (CE) 1107/2009 [50] del Parlamento y Consejo Europeo que define el marco legal para la comercialización de productos fitosanitarios. Tras éste, los reglamentos de la Comisión más recientes especifican los datos necesarios para que las sustancias activas y los productos fitosanitarios se aprueben y se comercialicen [51,52].

3.1.2. Límites máximos de residuos

El MRL se define como la concentración máxima de residuo de plaguicida permitida en un alimento para el consumo humano, o pienso para el consumo animal [53]. En este sentido 'residuo' se define como una o más sustancias presentes en las plantas o vegetales, frutas, piensos, agua potable o en cualquier parte del medioambiente como resultado del uso intencionado de un plaguicida, incluyendo sus metabolitos y TPs de importancia toxicológica. El MRL siempre se expresa en mg de compuesto/kg de alimento o pienso.

Los MRLs se establecieron por primera vez en 1961 por la comisión formada por el Codex Alimentarius de la FAO y WHO. Desde entonces, esta comisión es la encargada de establecer los MRLs y los límites máximos de residuos extraños (*Extraneous Maximum Residue Limits*, EMRLs) a nivel mundial para garantizar la inocuidad de los alimentos para el consumo humano y el comercio internacional justo.

Los MRLs son establecidos y actualizados por diversos organismos como el Codex Alimentarius [54] a nivel internacional, o la Dirección General de Sanidad y de los Consumidores (SANTE) [55] de la Comisión Europea, entre otros, con el fin de minimizar la exposición y evitar riesgos para la salud. Estos MRLs se van actualizando con el transcurso de los años, ya que en 2005, el Reglamento (CE) 396/2005 [56] estableció MRLs para un total de 500 sustancias activas en 378 productos, mientras que en la 39ª Reunión del Codex Alimentarius en julio de 2016, se establecieron un total de 4844 MRLs para diferentes combinaciones plaguicida/alimento.

Los MRLs se establecen según una serie de ensayos y datos determinados por la FAO en su manual de evaluación de plaguicidas [49,57]:

- El adecuado uso de un plaguicida en el cultivo, por ejemplo, cantidad, frecuencia, etapa de crecimiento de la planta según establece el manual de GAP.
- Datos experimentales sobre los residuos esperados cuando el plaguicida se aplica de acuerdo con las GAPs.
- Valores de referencia toxicológicos para el plaguicida. La toxicidad crónica se mide con la ADI y ARfD.

Junto con esto, hay que tener en cuenta que los MRLs no son límites toxicológicos máximos, ya que éstos se basan en las GAPs, pero se entiende que los niveles generados en estas prácticas agrícolas que cumplen con los MRLs deben de ser toxicológicamente aceptables.

En la EU se han establecido MRLs para más de 1000 sustancias activas utilizadas en todo el mundo, en una gran variedad de alimentos de origen vegetal y animal (frutas, hortalizas, frutos secos, leche, carne, etc.), así como en piensos para alimentación animal. Estos valores pueden ser consultados en la base de datos gestionada por la EU [58]. Asimismo, la normativa establece a qué partes del alimento vegetal o animal se aplican los MRLs.

Actualmente en la EU el organismo encargado de llevar a cabo los ensayos de riesgos es la EFSA, que verifica que un residuo es seguro para todos los grupos de consumidores europeos, incluidos los grupos vulnerables como bebés, niños y vegetarianos [59]. Las normas de la EU cubren los plaguicidas que se usan o se han usado en la agricultura dentro o fuera de la UE. Si una combinación plaguicida/matriz no está específicamente en la lista de la EU, se aplica un MRL por defecto de 0.01 mg/kg. En general, los MRLs en el Reglamento (CE) 396/2005 [56] están en el rango de 0.01-10 mg/kg.

En el caso de alimentos destinados a la alimentación infantil, se aplica un MRL de 0.01 mg/kg para la mayoría de plaguicidas. Para el caso de plaguicidas cuya toxicidad es acusada, como clorpirifos, se establecen MRLs por debajo de 0.01 mg/kg mientras que para otros plaguicidas muy tóxicos su uso está prohibido en alimentos infantiles según las Directivas (CE) 2003/13 [60] y (CE) 2006/125 [61].

3.2. INCORPORACIÓN DE LOS METABOLITOS EN EL ÁMBITO DE LA SEGURIDAD ALIMENTARIA

Según la definición de residuo de plaguicida, citada en el apartado anterior, los metabolitos forman parte del residuo, pero ello no significa necesariamente que éstos deban incorporarse siempre en la definición de residuo con fin de incluirlos en los MRLs o para estimar la ingestia alimentaria. La inclusión de los metabolitos en la definición de residuos depende de una serie de factores, como toxicidad, la velocidad de disipación del compuesto progenitor, etc. Hay que destacar que actualmente cada vez son más los plaguicidas en cuyo MRL se incluye la suma de uno o varios de sus metabolitos. La decisión sobre si han de incluirse los metabolitos es compleja y depende de los organismos oficiales como la FAO o la WHO [62,63]. En Europa, para que estos metabolitos sean incluidos en los MRLs, EFSA ha desarrollado un protocolo [64], mostrándose en la **Figura 1.6** el esquema de trabajo empleado por dicho organismo.

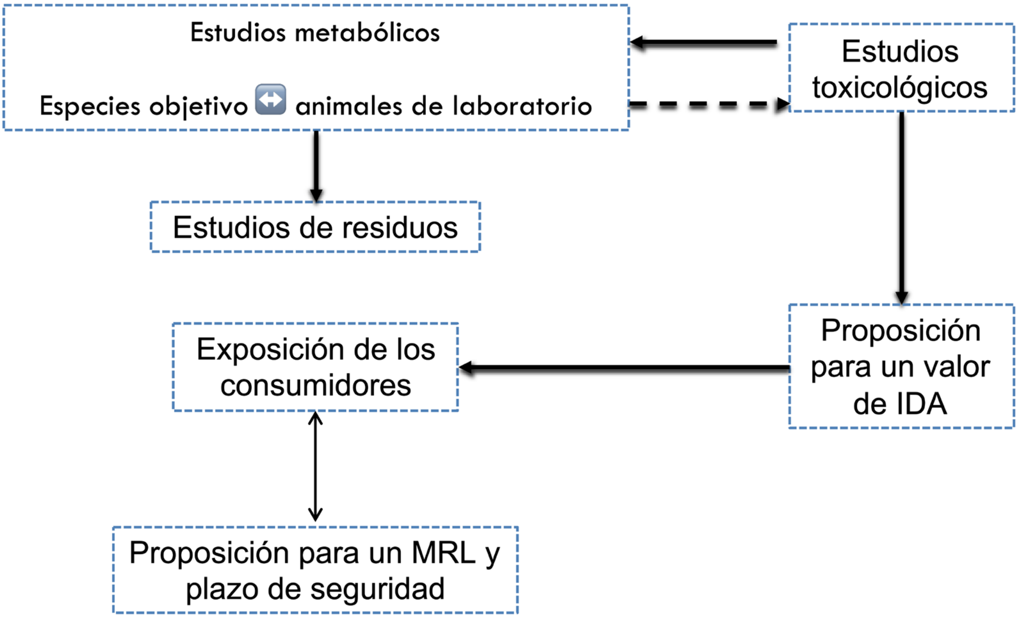


Figura 1.6. Esquema propuesto por la EFSA para el establecimiento de MRLs en estudios metabólicos de plaguicidas. Fuente [64].

Un ejemplo que en la presente Tesis es objeto de estudio es el caso de flonicamida, cuyo MRL inicialmente no incluía metabolitos. Sin embargo, tras numerosos estudios y debido a los avances científicos desarrollados en el campo de los plaguicidas, EFSA emitió un informe en 2010, indicando la necesidad de incluir dos metabolitos de flonicamida (TFNG y TFNA) en el MRL del compuesto progenitor, debido a la alta toxicidad y persistencia que éstos presentaban (**Tabla 1.2**) [65]. Otro ejemplo es el de propamocarb, cuyo MRL se establece como la suma de éste, y dos de sus metabolitos, propamocarb N-óxido y propamocarb N-desmetil (**Tabla 1.2**) [66].

A modo de resumen, en la **Tabla 1.2** se incluyen las definiciones de MRLs para cada uno de los plaguicidas estudiados en la presente Tesis, además de sus valores en las matrices objeto de estudio.

Tabla 1.2. Definición y valores de MRLs de los plaguicidas estudiados en la presente Tesis en frutas y hortalizas.[a]

Analitos	*Definición MRL*	*MRL*
Flonicamida	Suma de flonicamida, TFNG y TFNA	0.15 mg/kg (naranja) 0.3 mg/kg (pimiento)
Fenamidona	Fenamidona	1 mg/kg (tomate) 0.2 mg/kg (cucurbitáceas)
Propamocarb	Suma de propamocarb, propamocarb N-óxido y propamocarb N-desmetil	4 mg/kg (tomate) 5 mg/kg (cucurbitáceas)
Famoxadona	Famoxadona	2 mg/kg (tomate) 0.2 mg/kg (cucurbitáceas)

[a]Abreviaturas: MRL: límite máximo de residuo.

3.3. LEGISLACIÓN AMBIENTAL

Además de la legislación de plaguicidas en alimentos y piensos, existen una serie de normativas y/o reglamentos dedicados al control ambiental. En el caso de suelos no existe normativa establecida acerca de las concentraciones máximas de residuos de plaguicidas en ellos. Solo existe un Real Decreto 9/2005 [67,68] en el que se establecen una serie de niveles genéricos de referencia para un número limitado de sustancias (unas 50-60), casi todas ellas contaminantes orgánicos muy persistentes como es el caso del diclorodifeniltricloroetano (DDT) o diclorodifenildicloroetileno (DDE). Estos niveles genéricos de referencia desde el punto de vista de la protección humana, es la máxima concentración de una sustancia en el suelo que permite tener la garantía de que la contaminación del suelo no supone un riesgo inaceptable para el ser humano. Mediante comparación de éstos con los valores de concentración obtenidos en los estudios de suelo se puede determinar si un suelo está contaminado o no. Sin embargo, desde el punto de vista de la protección de los ecosistemas, este criterio no es suficiente. Así, para clasificar el suelo como no contaminado, además de cumplir con los niveles genéricos de referencia, es necesario determinar mediante ensayos toxicológicos que estas sustancias no presentan riesgo para el ecosistema.

En cuanto al control de la calidad de las aguas, a nivel europeo citar la Directiva 2013/39/EC [69] que establece una serie de directrices para el control de la contaminación en aguas superficiales e incluye una lista de sustancias prioritarias, que deben ser controladas. Además, introduce las normas de calidad ambiental (*Environmental Quality Standards*, EQS) para las sustancias prioritarias, estableciendo una concentración máxima admisible por año y toxicidad según la familia de contaminantes y tipo de masa de agua (superficial o continental). A nivel nacional, el Real Decreto 817/2015 [70] establece una serie de criterios básicos y homogéneos para el diseño e implantación de los programas de seguimiento de las masas de agua. Además, dicho Real Decreto transpone las EQS para las sustancias prioritarias y fija el procedimiento para calcular las EQS de los contaminantes específicos con objeto de conseguir un buen estado ecológico de las aguas superficiales o un buen potencial ecológico. Se incluyen una serie de sustancias prioritarias objeto de estudio, entre las que se encuentran metales y plaguicidas como endosulfan o las cipermetrinas, entre otros. Sin embargo no incluye o hace referencia a ningún metabolito de plaguicida que deba de ser controlado.

3.4. MÉTODOS DE ANÁLISIS Y VALIDACIÓN

Para garantizar la calidad y la comparabilidad de los resultados es necesaria la validación de los métodos analíticos empleados para el control de residuos de plaguicidas y sus metabolitos. A la hora de desarrollar y validar un método analítico es indispensable considerar dos documentos: la Decisión de la EU 2002/657/EC [71] y la Guía SANTE, documento nº SANTE/12682/2019 [55]. El primero, establece una serie de requisitos que deben de cumplir los métodos analíticos para lograr un alto nivel de seguridad en la identificación y confirmación de los analitos. Esta Decisión introduce el concepto de puntos de identificación (*identification points*, IPs), que implica el número mínimo de iones que deben de ser monitorizados para una correcta identificación de los analitos. Se establecen 3 IPs para las sustancias autorizadas y 4 IPs para las sustancias prohibidas, durante la identificación/confirmación de un compuesto. La Guía SANTE, está principalmente enfocada en el análisis de residuos de plaguicidas en alimentos y piensos, siendo una referencia en el análisis de contaminantes a niveles traza y propone junto con la Decisión de la EU 2002/657/EC, los iones necesarios para identificar un compuesto dependiendo del tipo de analizador de espectrometría de masas (*Mass Spectrometry*, MS) empleado (**Tabla 1.3**). Además, recomienda el uso de la MS acoplada a la cromatografía como técnica de identificación/ confirmación de residuos de plaguicidas y establece los parámetros de validación, como son linealidad, efecto matriz, veracidad, precisión y límites de cuantificación (*limits of quantification*, LOQs) que deben de ser cumplidos.

La guía SANTE clasifica los criterios de identificación de analitos en función del tipo de analizador, diferenciando analizadores de MS de baja resolución (*Low Resolution Mass Spectrometry*, LRMS) y de alta resolución (*High Resolution Mass Spectrometry*, HRMS). Para analizadores de LRMS, se requieren como mínimo 3 iones como criterio de identificación si se usan analizadores de cuadrupolo simple y 2 iones producto si son de triple cuadrupolo. Además, deben ser detectados con una señal ruido (*Signal to Noise*, S/N) mayor o igual que 3 y una relación de iones de ±30% (**Tabla 1.3**).

Para los analizadores de HRMS, en modo híbrido (Q) o no, como (Q)-Orbitrap o (Q)-TOF (*Time of Flight*, TOF), se establece que se deben de detectar 2 iones (uno de ellos debe de ser el ion molecular) con exactitud de masa inferior a 5 ppm y una S/N mayor o igual que 3. Sin embargo, en este tipo de analizadores el ruido a veces es nulo, por lo que se establece que para estos casos el ion monitorizado debe de estar presente en al menos 5 scans consecutivos (**Tabla 1.3**)

Tabla 1.3. Iones necesarios para la identificación establecidos mediante espectrometría de masas en la guía SANTE.[a]

Detector de MS		***Requerimientos***		
Resolución	*Analizador*	*Modo de adquisición*	*Número mínimo de iones*	*Otros*
LRMS	Cuadruplo, trampa de iones, TOF	Barrido completo (*full scan*), rango de *m/z* limitado, SIM	3 iones	S/N⩾3 Relación iones ± 30%
	Triple cuadrupolo, Q-trap, Q-TOF	Barrido completo (*full scan*), SRM/MRM	2 iones producto	
HRMS	(Q)-Orbitrap (Q)-TOF	Barrido completo (*full scan*), rango de *m/z* limitado, SIM, fragmentación con y sin selección de ion precursor	2 iones (uno de ellos debe de ser el ion molecular) con exactitud de masa < 5 ppm, al menos un fragmento	S/N⩾3 Cuando la S/N no sea posible calcularla, la señal debe de estar representada por al menos 5 scans

[a] Abreviaturas: HRMS: espectrometría de masas de alta resolución; LRMS: espectrometría de masas de baja resolución; MRM: monitorización de reacciones múltiples; SIM: monitorización de iones selectivos; S/N: señal-ruido; SRM: monitorización de reacciones seleccionadas; TOF: tiempo de vuelo; Trap: trampa de iones.

Sin embargo, el desarrollo de estos métodos es una tarea compleja debido a una serie de dificultades y problemas prácticos, relacionados con: (a) los valores tan bajos para algunos MRLs de contaminantes, que implican la necesidad de disminuir los límites de detección (*Limits of Detection*, LODs) y los LOQs de los métodos analíticos hasta valores inferiores y/o iguales que los límites legales; (b) los requisitos impuestos por la legislación, relacionados con la selectividad y seguridad en la identificación/confirmación de los analitos; (c) la complejidad de algunas de las matrices objeto de estudio; (d) el gran número de compuestos que deben de ser controlados, provenientes en muchos casos de familias diferentes y con propiedades físico-químicas distintas; (e) el uso de la calibración en matriz para compensar el efecto de muchos de los componentes de la matriz (efecto matriz) y poder garantizar la calidad y comparabilidad de los resultados, y (f) los criterios de aceptación de los parámetros de validación que deben de cumplirse para garantizar la calidad y comparabilidad de los resultados.

4. DETERMINACIÓN ANALÍTICA DE PLAGUICIDAS Y SUS METABOLITOS

4.1. TRATAMIENTO DE LA MUESTRA

La etapa de preparación de la muestra es una de las más importantes en el proceso analítico. Generalmente dicha etapa incluye tres procesos claramente definidos, como son:

- Homogeneización de la muestra
- Extracción de los analitos de interés
- Proceso de limpieza o *clean up* para eliminar los posibles interferentes de la matriz

Actualmente existen diversos métodos de tratamiento de muestra para el análisis de plaguicidas y sus metabolitos en alimentos y medioambiente. La selección de una técnica de extracción u otra va a estar determinada fundamentalmente por la naturaleza y/o propiedades químicas de los analitos de interés, como por ejemplo polaridad, miscibilidad etc…, por la naturaleza de la matriz y por la técnica de detección que se pretende aplicar. Principalmente las técnicas de extracción más usadas son la extracción sólido-líquido (*solid-liquid extraction*, SLE), extracción líquido-líquido (*liquid-liquid extraction*, LLE), extracción en fase sólida (*solid-phase extraction*, SPE), microextracción en fase sólida (*solid-phase microextraction*, SPME), dispersión de la matriz en fase sólida (*matrix solid phase dispersion*, MSPD) o la extracción con fluidos supercríticos (*supercritical fluid extraction*, SFE) [72–74].

Estas técnicas se han empleado para la determinación de un número limitado de plaguicidas, generalmente pertenecientes a la misma familia o compuestos cuyas propiedades físico-químicas son muy similares. Sin embargo, debido a la gran variedad de compuestos que hay, en los últimos años las técnicas de extracción se han ido orientando hacia el desarrollo de métodos multi-familia. Además, debido a la diversidad de matrices alimentarias y/o ambientales, también se intentan desarrollar métodos aplicables a un gran número de matrices. En este campo, tienen especial importancia en el tratamiento de la muestra, la eliminación de interferentes, la automatización del proceso, la disminución del uso de disolventes orgánicos, y en especial los organoclorados, la reducción del tiempo de extracción, de costes y por supuesto el desarrollo de métodos orientados hacia la química verde. En este ámbito, el método basado en la metodología SLE empleado por excelencia es el método QuEChERS (*Quick, Easy, Cheap, Rugged and Safe*) [75]. Dicho método con sus dos modificaciones oficiales, la versión EN15662 [76] y la versión AOAC 2007 [77] es ampliamente usado en el campo del análisis de residuos de plaguicidas y sus metabolitos.

A continuación, se presenta una breve descripción de las técnicas de extracción más empleadas en la actualidad para la determinación de plaguicidas y sus metabolitos en muestras alimentarias y ambientales.

4.1.1. Muestras alimentarias

La principal técnica empleada para la extracción de plaguicidas y sus metabolitos de muestras de origen alimentario es la SLE [74,78,79], debido a su simplicidad, robustez, eficiencia y bajo coste. Esta se basa en usar un disolvente lo más adecuado posible para separar o aislar los analitos de la muestra. De este modo, la SLE se basa en la afinidad que los analitos de interés tienen por la muestra o por el disolvente empleado. El disolvente rompe las interacciones de los analitos con los componentes de la matriz hasta que queden disueltos en él. La elección del disolvente es la etapa más crítica de este procedimiento y normalmente es elegido según la naturaleza del analito a extraer. Los disolventes más empleados suelen ser acetonitrilo o metanol, y en muchos casos, sobre todo cuando las matrices presentan un bajo contenido en agua como por ejemplo té, harina o frutos secos, en combinación con agua (**Tabla 1.4**). Por ejemplo, se ha utilizado acetonitrilo para la extracción de flonicamida y sus metabolitos en pepino y espinaca [80] con recuperaciones entre el 80-94% o en frutas y vegetales diversos [81] con recuperaciones entre 70-120%. El metanol se ha empleado para la extracción de fenamidona y propamocarb en pimiento, patata, tomate y repollo [45], obteniendo valores de recuperación de los compuestos entre el 75-108%. Asimismo la mezcla acetonitrilo:agua (1:1, *v/v*) se ha usado en la extracción de flonicamida y sus metabolitos, obteniendo recuperaciones aceptables (66-110%) en una muestra seca como es el lúpulo [82].

En algunas ocasiones se suelen usar disolventes tales como diclorometano o *n*-hexano en combinación con agua, acetonitrilo o metanol. Este hecho, hace que los componentes apolares/polares de la muestra se dirijan a una u otra fase, debido a la separación en dos fases de los disolventes por su inmisciblidad, seleccionando una fase u otra en función de la polaridad de los analitos objeto de estudio. Un ejemplo es el empleo de una disolución acidificada de agua (0.1% ácido fórmico) y 5 mM de formiato de amonio con diclorometano, para la extracción de cartap y sus metabolitos en té, obteniéndose recuperaciones entre el 87-120% [83] (**Tabla 1.4**). En el estudio fue necesaria una etapa de limpieza con diclorometano para eliminar componentes de la matriz que interferían en la determinación de los analitos.

Además del disolvente, otro de los factores que afectan durante la etapa de extracción es el pH. Este debe de ser optimizado para facilitar la extracción de los analitos, y sobre todo los metabolitos, empleándose ácidos como el fórmico o el acético, bases como el amoniaco o el hidróxido de sodio, o disoluciones tamponadas, tales como ácido fórmico y formiato de amonio o ácido acético y acetato de amonio. Por ejemplo, el tampón acético/acetato (pH = 3) se ha empleado para la determinación de cartap y sus metabolitos en té, favoreciendo las recuperaciones de los metabolitos más polares como es la nereistoxina. (**Tabla 1.4**).

Una variante de la SLE muy aplicada en los últimos años, sobre todo para la extracción de residuos de plaguicidas y metabolitos en frutas y hortalizas, es el método QuEChERS. Este fue desarrollado por Anastassiades et al. [77] en el año 2003 y rápidamente se hizo muy popular especialmente en alimentos, debido a su rapidez, bajo coste, posibilidad de determinar compuestos en un amplio rango y flexibilidad. Este se basa principalmente en el uso del acetonitrilo como disolvente de extracción seguido de una etapa de adición de sales, como son el cloruro de sodio y el sulfato de magnesio y centrifugación **(Figura 1.7)**.

En la **Tabla 1.4** se reúnen diversos estudios en los que se aplica el QuEChERS para la extracción de plaguicidas y metabolitos en muestras alimentarias. Como se puede observar se obtienen valores adecuados de recuperación en la extracción de neonicotinoides y sus metabolitos (98-101%) en pepino [84], de famoxadona, fenamidona, fenhexamida e iprodiona (75-105%) en tomate [85] o de pirifluquinazon y metabolitos ((71-106%) en gran variedad de frutas y verduras [86].

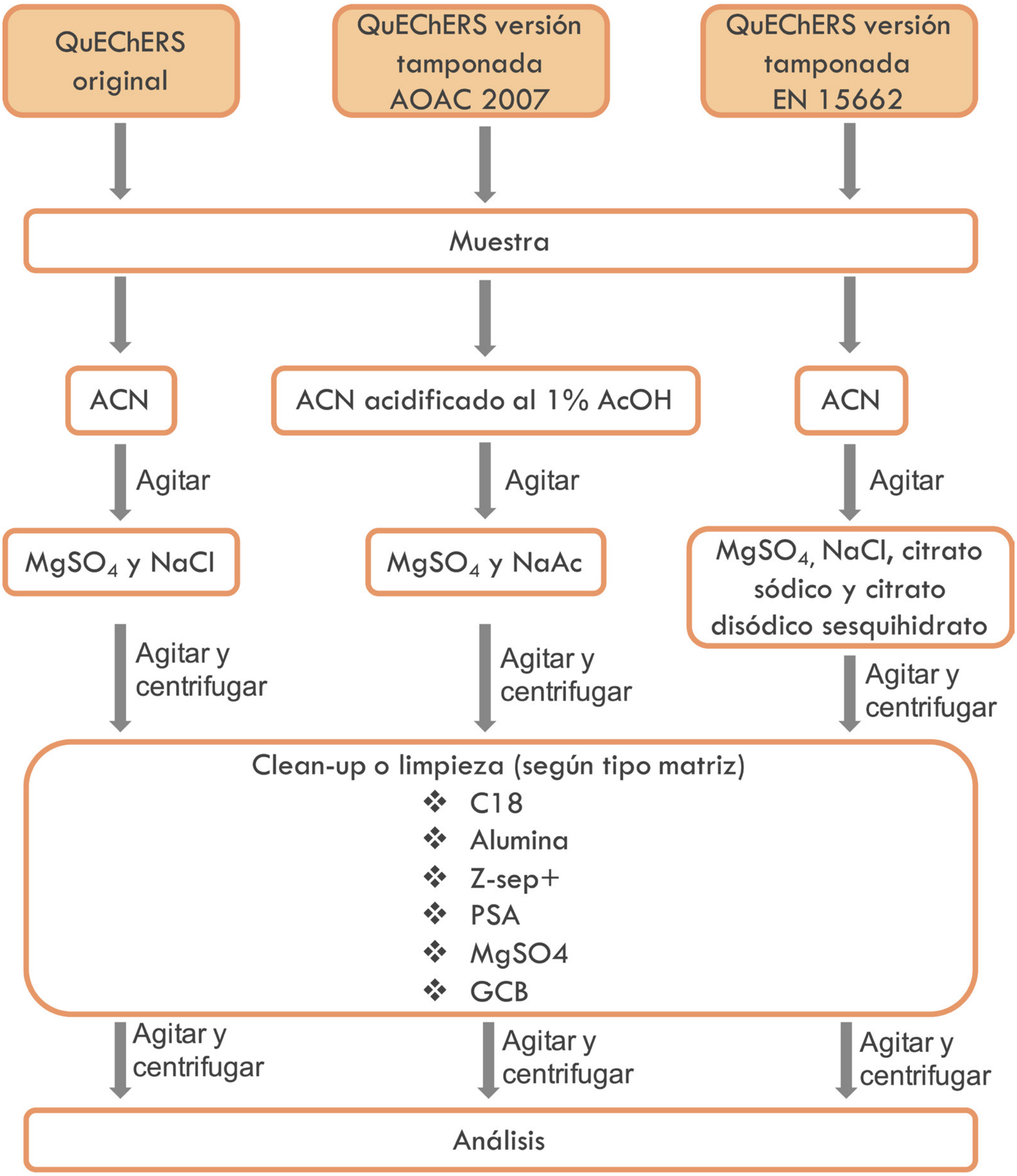


Figura 1.7. Esquema del método QuEChERS original y sus dos versiones, AOAC 2007 y EN 15662.

Tabla 1.4. Principales métodos de extracción de plaguicidas y sus metabolitos en muestras alimentarias.[a]

Analitos	***Matriz***	***Tipo de extracción***	***Etapa de limpieza***	***Recuperaciones (%)***	***Referencia***
Flonicamida, TFNA, TFNA-AM y TFNG	Pepino y espinaca	SLE; acetonitrilo, homogeneización con polytron	PSA, GCB y $MgSO_4$	80-94	[80]
Flonicamida, TFNA, TFNA-AM y TFNG	Frutas y vegetales	SLE; acetonitrilo	SPE	70-120	[81]
Fenamidona y propamocarb	Pimiento, patata, tomate y repollo	SLE; metanol, homogeneización con polytron y $MgSO_4$	PSA y $MgSO_4$	75-108	[45]
Flonicamida, TFNA, TFNA-AM y TFNG	Lúpulo seco	SLE; acetonitrilo: agua (1:1, *v*/*v*) y ácido clorhídrico concentrado	-	68-110	[82]
Cartap y nereistoxina	Té	SLE; agua 1% ácido fórmico 5 mM formiato de amonio y diclorometano (1:1, *v*/*v*)	C_{18} y SPE (Oasis HLB)	87-120	[83]
Neonicotinoides y metabolitos	Pepino	QuEChERS; acetonitrilo y sales $MgSO_4$ y NaCl	C_{18} y $MgSO_4$	98-101	[84]

Analitos	Matriz	Tipo de extracción	Etapa de limpieza	Recuperaciones (%)	Referencia
Famoxadona, fenamidona, fenhexamida and iprodiona	Tomate	QuEChERS; acetonitrilo y sales $MgSO_4$ y NaCl	-	75-105	[85]
Pyrifluquinazon y metabolitos	Frutas y verduras	QuEChERS; acetonitrilo y $MgSO_4$ y NaCl	PSA y $MgSO_4$	71-106	[86]
Fipronil y metabolitos	Maiz	QuEChERS; agua/ acetonitrilo y sales $MgSO_4$ y NaCl	C_{18}, PSA y GCB	87-105	[87]
Quizalofop-p-ethyl y tres hebicidas más	Cacahuete	QuEChERS; agua/ acetonitrilo y sales $MgSO_4$ y NaCl	-	70-95	[88]
Flonicamida	Pepino y manzana	QuEChERS; acetonitrilo:metanol (4:1, v/v) y sales $MgSO_4$ y NaCl	PSA y $MgSO_4$	80-109	[46]
Tres fungicidas y metabolitos	Frutas y verduras	QuEChERS; acetonitrilo acidificado 1% ácido acético y sales $MgSO_4$ y NaCl	PSA, C18 y $MgSO_4$	55-95	[92]

Analitos	***Matriz***	***Tipo de extracción***	***Etapa de limpieza***	***Recuperaciones (%)***	***Referencia***
Nereistoxina, tiosultap y tiociclam	Pimiento	QuEChERS; agua 2N ácido clorhídrico/acetonitrilo acidificado 1% ácido acético y sales $MgSO_4$ y acetato de sodio	$MgSO_4$	40-90	[89]
Fenamidona (método multirresiduo)	Lechuga	QuEChERS; acetonitrilo y sales tipo $MgSO_4$, NaCl, citrato de sodio y citrato de disodio sesquihidrato	PSA, GCB y $MgSO_4$	70-120	[90]
Flonicamida, TFNA y TFNG	Pimentón	QuEChERS; acetonitrilo y sales $MgSO_4$, NaCl, citrato de sodio y citrato de disodio sesquihidrato	C_{18} y $MgSO_4$	84-110	[91]
Propamocarb (método multirresiduo)	Tomate	QuEChERS; acetonitrilo acidificado 1% ácido acético y sales $MgSO_4$, NaCl, citrato de sodio y citrato de disodio sesquihidrato	PSA y $MgSO_4$	83-94	[47]

[a] Abreviaturas: GCB: carbón negro grafitizado; PSA: amina primaria secundaria; SLE: extracción solido-liquido; SPE: extracción en fase solida

En algunas ocasiones, la muestra presenta un bajo porcentaje de agua, por lo que es necesario el empleo de agua para hidratarla y mejorar la difusión de los analitos de interés. Es por ello que previa a la adición de acetonitrilo, se suele añadir un volumen de agua a la muestra, como por ejemplo para la extracción de fipronil y sus metabolitos en maíz, obteniéndose resultados de recuperaciones entre 87-105% [87] o para la determinación de un grupo de herbicidas, donde se encuentra quizalofop-p-etil en cacahuete (recuperaciones 70-95%) [88]. Además se puede emplear una mezcla de acetonitrilo con metanol, como por ejemplo en la proporcion 4:1 (*v/v*), para la extracción de flonicamida en pepino y manzana con recuperaciones entre 80-109% [46].

A partir de las condiciones iniciales del método QuEChERS sus autores han realizado diversas modificaciones (**Figura 1.7)**. La primera modificación fue el denominado QuEChERS tamponado AOAC 2007, empleado para plaguicidas sensibles en medio básico. Se basa en el empleo de acetonitrilo acidificado al 1% con ácido acético y la adición de acetato de sodio en lugar de cloruro de sodio para formar el medio tamponado. Esta versión, fue seleccionada como método oficial por la Asociación de Químicos Analíticos Oficiales (*Association of Official Analitycal Chemists*, AOAC) en 2007. Por ejemplo se ha utilizado para la extracción de nereistoxina, tiosultap y tiociclam en pimiento [89]. La segunda modificación, desarrollada por Anastassiades, es la versión tamponada EN15662, en la cual se usa el tampón citrato para obtener un medio débil en términos de fuerza iónica. Este método fue empleado para la determinación de fenamidona en un método multirresiduo en lechuga [90], para la extracción de flonicamida y sus metabolitos en pimentón [91] o para la determinación de propamocarb en otro método multirresiduo en tomate [47].

Además, como se ha comentado previamente, en ocasiones es necesario ajustar el pH del medio para favorecer la extracción de los metabolitos de la matriz. En el método QuEChERS es muy común el empleo de acetonitrilo acidificado al 1% con ácido acético o ácido fórmico para analitos con polaridad alta. Esta mezcla se ha empleado para la extracción de tres fungicidas de polaridad alta como son pirimetanil, ciprodinil, mepanipirim y sus metabolitos en frutas y hortalizas con recuperaciones aceptables entre 55-95% [92].

En cualquiera de las variantes del método QuEChERS, tras la etapa final de centrifugación, los extractos se someten a una etapa de limpieza rápida y sencilla como es la extracción dispersiva en fase sólida (*dispersive Solid-Phase Extration*, d-SPE). Esta implica la adición de una pequeña cantidad de un adsorbente al extracto final de acetonitrilo. Los adsorbentes más empleados suelen ser amina primaria secundaria (*Primary*

Secondary Amine, PSA), sulfato de magnesio, el C_{18} y carbón negro grafitizado (G*raphitized Carbon Black*, GCB). PSA es el adsorbente más empleado debido a su capacidad para retener los ácidos grasos, azúcares y otros componentes vegetales. C_{18} es empleado para la eliminación de lípidos y GCB para la eliminación de clorofilas. Además, el $MgSO_4$ se emplea para la eliminación de posibles restos de agua y en ocasiones para eliminar componentes de la matriz a través de la formación de quelatos. Como se puede observar en la **Tabla 1.4**, todos ellos son empleados para la determinación de plaguicidas y sus metabolitos en diversas matrices alimentarias obteniéndose recuperaciones entre el 70-120% para todos ellos.

Finalmente, la SLE y el método QuEChERS ofrecen excelentes resultados de recuperación para la determinación de plaguicidas y sus metabolitos en matrices de origen alimentario, por lo que están implantados en gran variedad de laboratorios de rutina.

4.1.2. Muestras ambientales

Las **Tabla 1.5 y 1.6** muestran un resumen de los principales métodos de extracción de plaguicidas y sus metabolitos de muestras de origen ambiental, suelos y aguas, respectivamente.

En relación a suelos, los métodos de extracción más empleados son la extracción con líquidos presurizados (*Pressurised Liquid Extraction*, PLE), la SLE y la variante QuEChERS [93]. Así por ejemplo, se ha aplicado la PLE para la extracción de metribuzina y sus metabolitos de suelo, empleando una mezcla de metanol y agua (75:25, *v/v*) como disolventes de extracción y obteniendo recuperaciones entre el 75-85% para los analitos estudiados [94].

En relación a la SLE (**Tabla 1.5**), hay que destacar el uso de acetonitrilo y agua (60:40, *v/v*) para la extracción de metabolitos derivados de ácidos oxalínicos y sulfónicos, con recuperaciones entre el 70-120% [95]. Se ha usado una mezcla de diclorometano y agua (50:50, *v/v*) para la extracción de glufosinato y sus metabolitos. La utilización de diclorometano permitía la extracción de los componentes más apolares del suelo y minimizar en la fase acuosa (la que se analizará) los posibles interferentes. Las recuperaciones variaron entre el 88-94% [96]. Por último, la mezcla diclorometano y acetonitrilo (8.3:16.7, *v/v*) se ha usado para la extracción de insecticidas, neonicotinoides y sus metabolitos de suelo, con recuperaciones entre el 98-101%. Además fue necesario aplicar una etapa de limpieza con el adsorbente C_{18}, para eliminar posibles interferentes [84].

En relación con el método QuEChERS, tanto el método original como la versión tamponada EN15662 se han empleado para la extracción de residuos de plaguicidas y metabolitos de suelos. El método original ha sido aplicado para la extracción de afidopiropen y sus metabolitos, previa adición de agua para hidratar la matriz. Además se utilizó una etapa de limpieza con C_{18} y PSA, obteniendo recuperaciones entre el 85-100% [48]. El mismo método se aplicó, sin etapa de limpieza, para la extracción de fenoxaprop-p-etil y sus metabolitos, obteniendo recuperaciones entre el 70-120% [44]. Finalmente, la versión original del método QuEChERS ha sido empleada para la extracción de ciantraniliprol y sus metabolitos. En este caso no se realizó la hidratación previa de la muestra, pero se empleó una etapa de limpieza con PSA y $MgSO_4$. Las recuperaciones oscilaron entre el 85-101% [97].

Tabla 1.5. Principales métodos de extracción de plaguicidas y sus metabolitos en suelos[a]

Analitos	***Tipo de extracción***	***Etapa de limpieza***	***Recuperaciones (%)***	***Referencia***
Metribuzina y metabolitos	PLE; metanol y agua (75:25, *v*/*v*)	-	75-85	[94]
Metabolitos derivados de ácidos oxalínicos y sulfónicos	SLE; acetonitrilo y agua (60:40, *v*/*v*)	-	70-120	[95]
Glufosinato y metabolitos	SLE; diclorometano y agua (50:50, *v*/*v*)	-	88-94	[96]
Insecticidas neonicotinoides y metabolitos	SLE; diclorometano y acetonitrilo (8.3:16.7, *v*/*v*), NaCl	C_{18}	98-101	[84]
Afidopiropen y metabolitos	QuEChERS; agua/acetonitrilo y sales $MgSO_4$ y NaCl	C_{18} y PSA	85-100	[48]

Analitos	**Tipo de extracción**	**Etapa de limpieza**	**Recuperaciones (%)**	**Referencia**
Fenoxaprop-p-etil y sus metabolitos	QuEChERS; agua/acetonitrilo y NaCl	-	70-120	[44]
Ciantraniliprol y metabolitos	QuEChERS; acetonitrilo y $MgSO_4$ y NaCl	PSA y $MgSO_4$	85-101	[97]
Quizalofop-p-etil, metazacloro, oxifluorfen y quinmerac	QuEChERS; agua/acetonitrilo acidificado 1% ácido acético y sales $MgSO_4$, NaCl, citrato sódico y citrato disódico sesquihidrato.	C_{18} y $MgSO_4$	84-110	[98]
Ácido 4-cloro-2-metilfenoxiacetico y metabolito	SLE y SPE; elución con metanol y agua	-	75-91	[99]

[a] Abreviaturas: PLE: extracción con líquidos presurizados; PSA: amina primaria secundaria; SLE: extracción sólido-líquido; SPE: extracción en fase sólida

La versión tamponada EN15662 ha sido empleada para la extracción de quizalofop-p-etil, metazacloro, oxifluorfen y quinmerac, previa hidratación de la muestra con agua, y aplicando una etapa de limpieza con C_{18} y $MgSO_4$. Las recuperaciones oscilaron entre el 75-108% [98] (**Tabla 1.5**).

En relación con la extracción de plaguicidas y sus metabolitos específicos en aguas, el método usado por excelencia es la SPE. Este es uno de los métodos más importantes en preparación de la muestra, sobre todo para la determinación de residuos de plaguicidas y sus metabolitos en matrices líquidas. Se basa en la retención selectiva de los analitos de interés en un adsorbente para su posterior elución con un disolvente adecuado. Los analitos son retenidos mediante una fase estacionaria apropiada que se encuentra generalmente dentro de un tubo, denominado cartucho. Esta fase estacionaria está constituida por unas pequeñas partículas que presentan gran afinidad por los analitos de interés. La SPE combina dos procesos, por un lado, la extracción de los analitos de interés y por otro su pre-concentración. De forma general, el proceso de SPE consta de las siguientes etapas: acondicionamiento o activación de la fase estacionaria, carga de la muestra, elución de los interferentes y elución de los analitos de interés con el disolvente adecuado, tras una etapa de secado del cartucho.

La elección del adsorbente es crítica en SPE ya que la selectividad, capacidad y afinidad que presente frente al analito es crucial para el éxito de la extracción, aunque éste también depende del tipo de matriz y de sus interacciones con el adsorbente. Casi todos los adsorbentes son similares a los empleadas en d-SPE, como los de sílice, C_{18} y $C_{8,}$ adsorbentes polares como florisil (silicato de magnesio activado), GCB, los de intercambio iónico con grupos funcionales como los iónicos, catiónicos y aniónicos o los de materiales poliméricos como los OASIS HLB y Strata X, ampliamente usados en la determinación de residuos de plaguicidas y sus metabolitos en los últimos años [100]. Los cartuchos de C_{18} han sido empleados para la extracción del ácido 4-cloro-2-metilfenoxiacético y sus metabolitos en agua y suelos, eliminando los interferentes y pre-concentrando los analitos, mejorando así la sensibilidad y selectividad del método, con recuperaciones entre el 75-91% [99] y usando como disolvente de elución metanol seguido de agua. Los cartuchos de GCB, como el caso de los cartuchos Envi-Carb®, se han empleado para la extracción de herbicidas clorados y sus metabolitos, entre los que se encuentra dimetacloro, en agua de consumo. GCB es empleado por su capacidad para eliminar las sustancias húmicas y fúlvicas que se encuentran en el agua de consumo, habiéndose desarrollado un método para determinar 535 plaguicidas en este tipo de aguas con recuperaciones entre el 75-108%

[101]. Finalmente, los cartuchos con materiales poliméricos, como los OASIS HLB, han sido empleados para la extracción de quizalofop-p-etil, metazacloro, oxifluorfen y quinmerac en agua, usando metanol acidificado como eluyente y obteniendo recuperaciones entre el 87-120% [98]. Los cartuchos Strata X han sido aplicados para la extracción de glifosato y sus metabolitos en agua, con recuperaciones entre el 70-120% y usando metanol como eluyente [102] (Véase **Tabla 1.6**).

En los últimos años la SPE ha sufrido un proceso de automatización, llevándose a cabo esta etapa en línea (*on-line*) en el mismo cromatógrafo. De esta forma se reduce el tiempo de análisis y también de costes, ya que los cartuchos empleados para la SPE *on-line*, son reutilizables a diferencia de los de la SPE convencional. Los adsorbentes usados son los mismos que los de la metodología convencional. Cada vez es más empleada en la determinación de plaguicidas y sus metabolitos en muestras de agua. Así, la SPE *on-line* con cartuchos de OASIS HLB se ha utilizado para la extracción de más de 500 contaminantes en aguas, con recuperaciones entre el 70-120% [103] o con cartuchos de C_{18} para la extracción de plaguicidas y sus metabolitos en métodos multirresiduo, todos ellos con recuperaciones aceptables entre 75-116% [104,105] (Véase **Tabla 1.6**).

Finalmente, mencionar otros dos métodos usados para la extracción de plaguicidas específicos y sus metabolitos en muestras de agua. Por un lado el método QuEChERS original se ha utilizado para determinar oxatiapiprolin en agua con recuperaciones aceptables (65-113%) [106]; y por otro lado se ha empleado la concentración de los analitos por evaporación a vacío para la extracción de plaguicidas en un método multirresiduo en agua subterránea, obteniendo recuperaciones entre el 70-120% [107].

Tabla 1.6. Principales métodos de extracción de plaguicidas y sus metabolitos en aguas.[a]

Analitos	***Tipo de extracción***	***Recuperaciones (%)***	***Referencia***
Ácido 4-cloro-2-metilfenoxiacetico y metabolito	SPE; Cartuchos C_{18} elución con metanol y agua	75-91	[99]
Dimetacloro (535 herbicidas clorados)	SPE; Cartuchos Envi-carb carbón elución con metanol 10 mM de acetato amónico	75-108	[101]
Quizalofop-p-etil, metazacloro, oxifluorfen y quinmerac	SPE; Cartuchos Oasis HLB elución con metanol acidificado	87-120	[98]
Glifosato y metabolitos	SPE; Cartuchos Strata X elución con metanol	70-120	[102]
Multirresiduo (500 compuestos)	SPE *on-line*; cartuchos Oasis HLB	70-120	[103]
Multirresiduo	SPE *on-line*; Hypersil gold (C_{18})	89-116	[104]
Plaguicidas y sus metabolitos	SPE *on-line*; Cartucho C_{18}	75-115	[105]
Oxatiapiprolin y metabolitos	QuEChERS; acetonitrilo y NaCl: Limpieza; $MgSO_4$	65-113	[106]
Plaguicidas (300 compuestos) y metabolitos (1100 compuestos)	Concentración de la muestra	70-120	[107]

[a] Abreviaturas: SPE: extracción en fase sólida

4.2. TÉCNICAS CROMATOGRÁFICAS

En la actualidad las dos técnicas cromatográficas más importantes son la cromatografía de gases (*Gas Chromatography*, GC) y la cromatografía de líquidos (*Liquid Chromatography*, LC). La GC es empleada en el análisis de ciertos plaguicidas específicos y sus metabolitos de polaridad media/baja, aunque principalmente se utiliza en el análisis de plaguicidas volátiles, bifenilos policlorados (*Polychlorobiphenyls,* PCBs), contaminantes orgánicos persistentes (*Persistent Organic Pollutants*, POPs) o hidrocarburos aromáticos policíclicos (*Polycyclic Aromatic Hydrocarbons*, PAHs), tanto en muestras alimentarias como ambientales [108].

La LC es la técnica más usada para la determinación de plaguicidas y sus metabolitos debido a su alta selectividad y sensibilidad. Esta es una técnica de separación ampliamente usada para analitos de polaridad media a alta. Fue desarrollada con el objetivo de resolver problemas analíticos y en los últimos años ha experimentado notables mejoras a fin de hacer frente a la determinación de numerosos tipos de analitos y complejidad de las muestras. La mejora más importante ha sido el desarrollo de columnas con tamaño de partícula inferior a 2 µm, pasando a denominarse esta técnica como cromatografía de líquidos de ultra alta eficacia (*Ultra-high Performance Liquid Chromatography*, UHPLC), en lugar de cromatografía de líquidos de alta eficacia (*High Performance Liquid Chromatography*, HPLC). Este avance fue totalmente necesario para el desarrollo de los métodos multirresiduo, permitiendo la reducción del tiempo de análisis, así como un incremento de la señal analítica y de la capacidad de separación, pudiéndose analizar gran variedad de analitos en tiempos de análisis menores que 30 minutos. Un ejemplo es el método desarrollado por Meng et al. [109] que emplearon un columna con tamaño de partícula de 1.9 µm para la determinación de una gran variedad de contaminantes y sus metabolitos (plaguicidas, fármacos, aditivos plásticos y surfactantes) en agua, y el tiempo de análisis fue de 25 min.

La fase estacionaria más comúnmente empleada para la determinación de plaguicidas y sus metabolitos es la de C_{18}. Esta fase estacionaria presenta una alta eficacia, permitiendo trabajar en amplios rangos de pH y aplicable a una gran variedad de familias de plaguicidas. La más usada es aquella con un tamaño de partícula inferior a 2 µm [63,109–112], aunque también son usadas aquellas con tamaño de partícula de 5 µm [113,114] (**Tabla 1.7**). Además de la fase estacionaria de C_{18}, en algunos casos si la polaridad de los analitos y/o metabolitos a determinar es elevada, se suelen emplear columnas de tipo cromatografía de líquidos de interacción hidrofílica (*Hydrophilic Interaction Liquid Chromatography*, HILIC). Estas son apropiadas para compuestos muy polares, teniendo la peculiaridad de poder trabajar con ellas en fase normal, pero empleando fases móviles que se usan en fase reversa. Este es el caso de la determinación de cartap y nereistoxina, un insecticida y su metabolito muy polares, en té [83]. En este trabajo, se empleó una columna de tipo HILIC con tamaño de partícula de 5 µm, usando acetonitrilo y agua 0.1% ácido fórmico y 5 mM formiato de amonio, como fase móvil.

En relación a las fases móviles, las más empleadas son acetonitrilo [63,83,107,109,113] y metanol como disolventes orgánicos [110–112,114] en combinación con agua. Además, se suelen emplear modificadores de la fase móvil para mejorar la retención de los compuestos, la forma de pico o la ionización de los mismos, especialmente en el caso de los metabolitos. Se suelen emplear ácidos, como el ácido fórmico o acético, o disoluciones reguladoras como el formiato y el acetato de amonio, o bien combinaciones de estos dos para dar lugar a una disolución tamponada a un pH determinado. Las más comunes en el estudio de plaguicidas y sus metabolitos son la combinación de ácido fórmico y formiato de amonio, o bien el uso de solo ácido fórmico como modificador de la fase acuosa o también de la fase orgánica para mejorar la ionización de los plaguicidas y/o metabolitos. Por ejemplo, para la determinación de imazalil, tiabendazol y sus metabolitos se ha empleado como fase móvil metanol y agua, ambos con 10 mM formiato de amonio, mientras que para la determinación de clorantranipol y ciantraniliprol, tanto en la fase orgánica (metanol) como en la acuosa, se adicionó tampón formiato de amonio y ácido fórmico (véase en **Tabla 1.7**).

Tabla 1.7. Principales métodos de análisis de plaguicidas y sus metabolitos usando LC-HRMS.[a]

Analitos	*Matriz*	*Columna/Flujo*	*Fase móvil*	*Tipo de analizador*	*LOD//LOQ*	*Referencia*
Multirresiduo (1400 plaguicidas y metabolitos)	Frutas y vegetales	XDB-C_{18} (50 mm x 4.6 mm, 1.8 µm) / 0.5 mL/min	Acetonitrilo Agua 0.1% ácido fórmico	Q-TOF	-// 0.02 µg/L	[63]
Cartap y nereistoxina	Té	ZIC-HILIC (150 mm × 2.1 mm, 5 µm) / 0.3 mL/min	Acetonitrilo Agua 0.1% ácido fórmico 5 mM formiato de amonio	Q-Exactive	1 µg/L// 10 µg/L	[83]
Glufosinato y metabolitos	Suelos	CROWNPAK CR(+) chiral (150 mm x 4.6 mm, 5 µm) / 0.3 mL/min	Agua 1.5% ácido fórmico	Q-Exactive	1 µg/L// 50 µg/L	[96]
Multirresiduo (plaguicidas y metabolitos)	Agua	Atlantis T3 (150 mm x 3 mm, 3 µm) / 0.3 mL/min	Acetonitrilo 0.08% ácido fórmico Agua 0.08% ácido fórmico	Q-Exactive	-//0.01 µg/L	[107]

Analitos	**Matriz**	**Columna/Flujo**	**Fase móvil**	**Tipo de analizador**	**LOD//LOQ**	**Referencia**
Multirresiduo (plaguicidas y metabolitos)	Agua	Hypersil Gold C_{18} (50 mm x 2.1 mm, 1.9 µm) / 0.3 mL/min	Acetonitrilo Agua 0.1% ácido fórmico	Q-Exactive	// 0.2 µg/L	[109]
Multirresiduo (2000 contaminantes)	Agua	Acquity BEH C_{18} (100 mm × 2.1 mm, 1.7 µm) / 0.3 mL/min	Metanol 0.01% ácido fórmico Agua 0.01% ácido fórmico	Q-TOF	-//0.02, 0.1 y 0.5 µg/L	[110]
Multirresiduo (450 plaguicidas y metabolitos)	Agua	Acquity BEH C_{18} (100 mm × 2.1 mm, 1.7 µm) / 0.3 mL/min	Metanol 0.01% ácido fórmico Agua 0.01% ácido fórmico	Q-TOF	-	[111]
Imazalil, tiabendazol y sus metabolitos	Manzana y pera	Acquity BEH C_{18} (150 mm × 2.1 mm, 1.7 µm) / 0.2 mL/min	Metanol 10 mM formiato amónico Agua 10 mM formiato de amonio	Q-TOF	// 0.05-1 µg/kg	[112]
Biocidas y metabolitos	Agua	NUCLEO- DUR® RP-C_{18} (125 mm x /2.1 mm, 3 µm) / 0.4 mL/min	Acetonitrilo Agua 0.01% ácido fórmico	Q-Exactive	-	[113]

Analitos	*Matriz*	*Columna/ Flujo*	*Fase móvil*	*Tipo de analizador*	*LOD//LOQ*	*Referencia*
Clorantranipol y ciantraniliprol	Lechuga y naranja	Aqua C_{18} (50 mm × 2.0 mm, 5 µm) / 0.25 mL/min	Metanol 5 mM formiato amónico 0.1% ácido fórmico Agua 5 mM formiato amónico 0.1% ácido fórmico	Q-Exactive	// 0.01 mg/kg	[114]
Multirresiduo (plaguicidas y metabolitos)	Aire	Hypersil Gold aQ (100 mm x 2.1 mm, 1.9 µm) / 0.3 mL/min	Metanol 4 mM formiato amónico 0.1% ácido fórmico Agua 4 mM formiato amónico 0.1% ácido fórmico	Exactive	// 2.6 a 75 pg/m3	[117]
Multirresiduo (plaguicida, drogas y micotoxinas)	Pienso	Hypersil Gold (100 mm x 2.1 mm, 1.9 µm) / 0.4 mL/min	Metanol:acetonitrilo (90:10, *v/v*) 0.1 ácido acético Agua 0.1% ácido acético	Exactive	// 12.5 a 20 µg/kg	[118]

[a] Abreviaturas: LOD: límite de detección; LOQ: límite de cuantificación; Q: cuadrupolo; TOF: tiempo de vuelo.

4.3. ESPECTROMETRIA DE MASAS

Se pueden diferenciar dos tipos de analizadores de espectrometría de masas, los de baja resolución (LRMS) y los de alta resolución (HRMS), siendo esta última la principal técnica empleada en la presente Tesis Doctoral, debido a su capacidad de análisis en modo no dirigido, muy adecuada para el estudio de metabolitos de plaguicidas. La LRMS es usada principalmente en métodos de análisis dirigido (*target*), donde los analitos a determinar son conocidos. Por otro lado, la HRMS se emplea para la determinación de compuestos tanto en modo dirigido como no dirigido, pudiendo ser compuestos conocidos o bien desconocidos. La HRMS está siendo cada vez más empleada por su modo de trabajo *full scan* y su elevada resolución. Permite la identificación y/o caracterización de una gran cantidad de analitos de interés con valores de masa exacta, entre 4 y 6 cifras decimales, tanto de los iones precursores como de sus fragmentos. A pesar de que muchos autores identifican esta técnica como poco sensible en comparación con la LRMS, con los desarrollos instrumentales que se están llevando a cabo en los últimos años, este parámetro se ha visto considerable mejorado. Hay dos tipos de analizadores de HRMS usados principalmente para la determinación de plaguicidas y sus metabolitos, tanto en métodos multirresiduo como en métodos monorresiduo: Orbitrap o Q-Orbitrap y el analizador de tiempo de vuelo (*Time Of Flight*, TOF) o acoplado también a un simple cuadrupolo (Q-TOF) (**Tabla 1.7**).

En relación con el analizador Orbitrap, desde su desarrollo en 2005, se han comercializado varias versiones. La primera de ellas, era una versión que acoplaba una trampa de iones lineal con un espectrómetro de masas Orbitrap a través de un cuadrupolo que actuaba como celda de colisión (LTQ-Orbitrap) y cuyo principal campo de aplicación era la proteómica. A continuación, se pasó a una versión simple (de una sola etapa) (Exactive), y en la actualidad hay diversos modelos de analizadores híbridos, Q-Exactive. La principal diferencia de este último es que es más sensible y con capacidad de trabajo tanto en modo *full scan* como en modo MS/MS dirigido. Presentan una capacidad de resolución de hasta 100000 de anchura a media altura (*Full Width at Half Maximum*, FWHM) (para una *m/z* 200), en la variante Exactive, y de hasta 240000 FWHM en la variante híbrida, y una alta precisión (< 3 ppm) en la medida de masa [115]. Esto permite una discriminación adecuada entre interferentes isobáricos y los analitos de interés en un gran rango dinámico sobre el que se determinan las masas exactas.

La HRMS suele ir acoplada a una etapa previa de separación cromatográfica, como es la GC o la LC. En relación con la GC, hasta el momento de redacción de esta Tesis Doctoral no se ha publicado ningún artículo científico que aplique esta técnica cromatográfica junto con la HRMS en el estudio de familias de plaguicidas y sus metabolitos. Sin embargo, sí se ha utilizado en métodos multirresiduo de análisis no dirigido en modo *full scan* para la detección de gran cantidad de plaguicidas de polaridad media/baja. Por ejemplo, Della-Flora *et al*. [116] han desarrollado un método de análisis para la determinación de 60 plaguicidas en aguas superficiales de Brasil, usando GC-Q-TOF con valores de LOQ de 0.5 μg/L. Chiesa *et al.* [108] realizaron la determinación de contaminantes ambientales y otros plaguicidas en mejillones y almejas con valores de LOQ entre 0.005 y 0.02 μg/kg.

LC-HRMS es la técnica usada por excelencia para la determinación de plaguicidas específicos y sus metabolitos, empleándose principalmente el analizador Q-Exactive (**Tabla 1.7**), debido a su capacidad de trabajo en modo MS/MS dirigido. Sin embargo con el analizador Exactive, al presentar un modo de fragmentación MS/MS no dirigido, todos los iones que entran en la celda se fragmentan por lo que no es posible la selección de un único ion para fragmentar, dificultando el proceso de confirmación a través de los fragmentos en relación a analizadores híbridos (Q-Exactive o Q-TOF). El analizador Exactive se ha utilizado para determinar alrededor de 300 plaguicidas y metabolitos en aire, obteniendo LOQs entre 2.6 y 75 pg/m^3 [117], o para la determinación de 77 plaguicidas, micotoxinas y medicamentos veterinarios en piensos con valores de LOQs aceptables entre 12.5 y 20 μg/kg [118]. El analizador Q-Exactive se ha empleado para determinar glufosinato y sus metabolitos en suelos, obteniendo unos valores de sensibilidad aceptables con un LOD de 1 μg/L y un LOQ de 50 μg/L [96]. También ha sido empleado para determinar clorantranipol y ciantraniliprol en lechuga y naranja, con LOQs de 10 μg/kg [114], para la determinación de biocidas y sus metabolitos en aguas [113], y finalmente en gran variedad de métodos multirresiduo en aguas, con LOQs de 0.01 μg/L [107] y 0.2 μg/L [109], empleando en estos dos últimos estudios técnicas de análisis dirigido y no dirigido (**Tabla 1.7**).

El analizador híbrido Q-TOF presenta modos de trabajo similares al analizador Q-Exactive, permitiendo trabajar en modo *full MS* y MS/MS. Presentan un poder de resolución de 30000 FWHM (para una *m/z* 200) o superiores, y una exactitud en la medida de masa exacta de 3 a 5 ppm. Se ha empleado para la determinación de plaguicidas y sus metabolitos en métodos multirresiduo en frutas y vegetales con LOQs de 0.02 μg/L [63], en aguas con LOQs entre 0.02 y 0.5 μg/L [110,111] y para la determinación de imazalil y tiabendazol junto con sus metabolitos en manzana y pera con LOQ de 0.05-1 μg/kg, respectivamente [112], aplicando en todos ellos análisis dirigido y no dirigido (Véase **Tabla 1.7**).

4.4. USO DE HRMS PARA LA DETERMINACIÓN DE METABOLITOS

El desarrollo de la HRMS ha supuesto una revolución en el campo del análisis de plaguicidas y sus metabolitos. Hasta la fecha, con los analizadores de LRMS solo se podían analizar plaguicidas y metabolitos de los que se disponía de patrón comercial para llevar a cabo su determinación en matrices alimentarias o ambientales, bien por LC-LRMS o GC-LRMS. Sin embargo, con la incorporación de la HRMS en la última década, ha sido posible el estudio del comportamiento de los plaguicidas y llevar a cabo la determinación de sus metabolitos, tanto conocidos como no conocidos previamente, o también llamados desconocidos, junto con el empleo de *software* específicos. Además, permite la identificación tentativa de metabolitos previamente descritos, pero de los que no hay patrón analítico disponible, lo cual hace imposible su análisis mediante LRMS usando bases de datos. Para ello son dos los modos de trabajo desarrollados para el análisis de plaguicidas y sus metabolitos: análisis de sospechosos o *suspect screening,* y análisis de desconocidos o *unknown*. En la **Figura 1.8** se representa un esquema de los modos de trabajo posibles usando HRMS, tanto para compuestos conocidos como desconocidos.

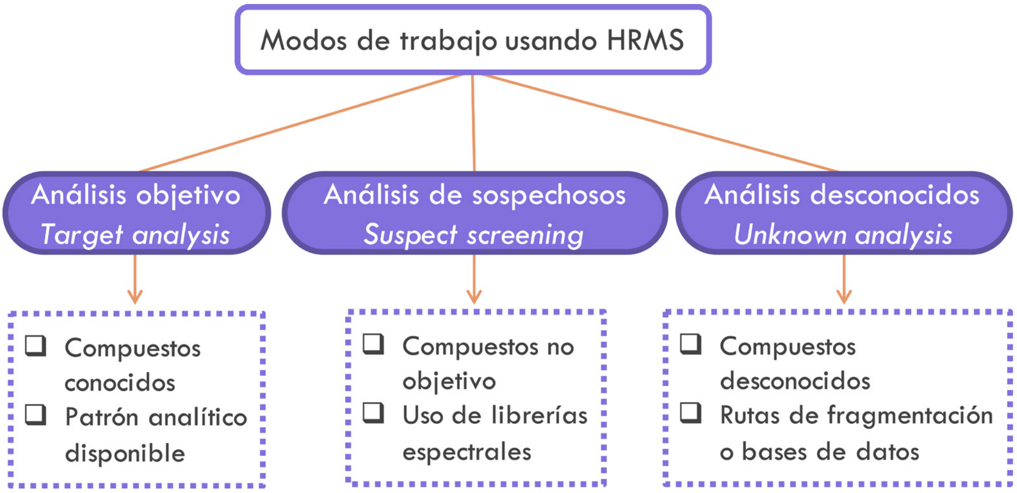


Figura 1.8. Modos de trabajo usando HRMS para la determinación de plaguicidas y sus metabolitos.

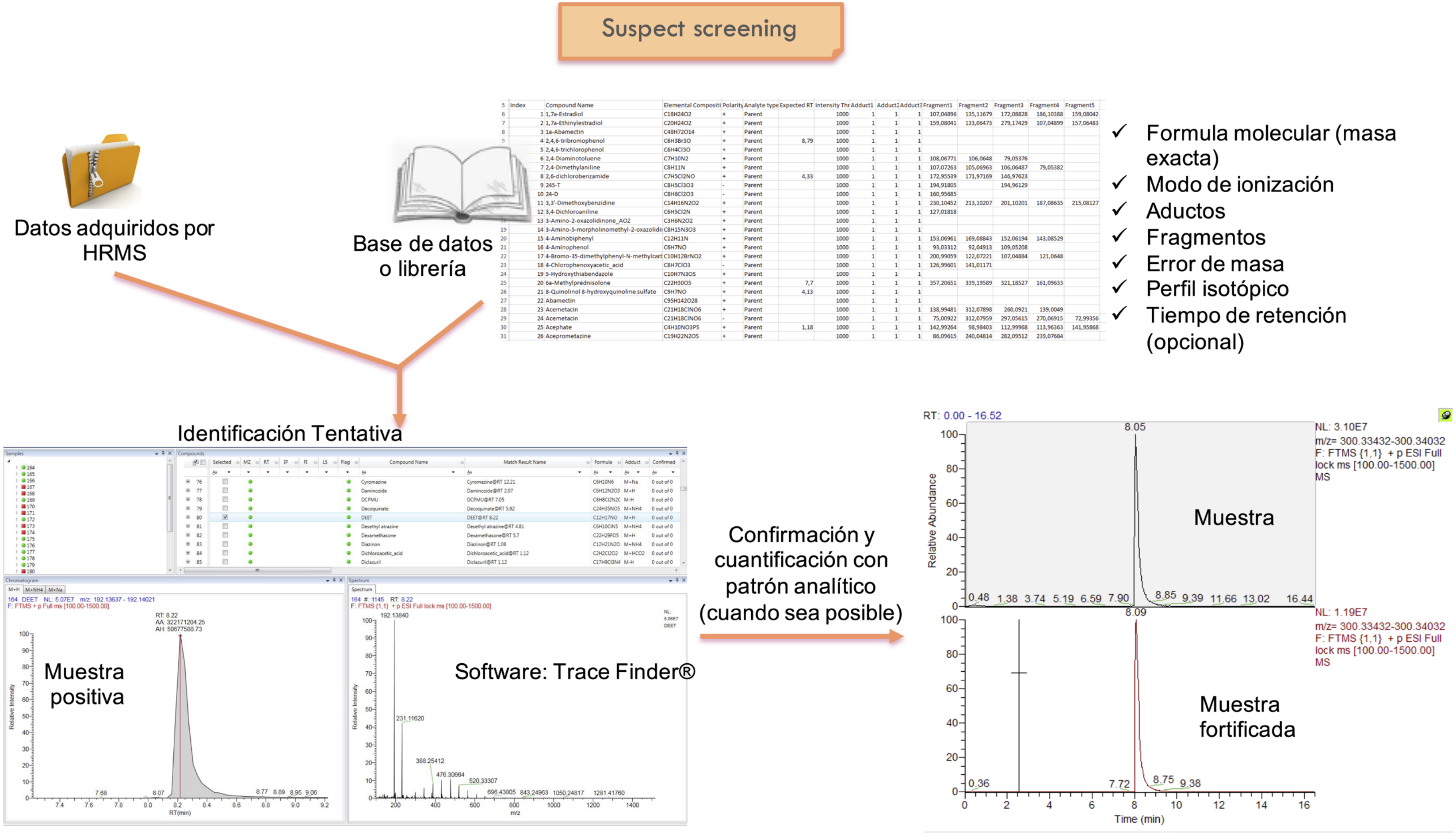
Ambos métodos de análisis (*suspect screening* y *unknown*) llevan a cabo la identificación tentativa del compuesto de interés en base a la masa exacta y los fragmentos que deriven de ese ion precursor. Sin embargo, la identificación completa no se puede llevar a cabo sin el correspondiente patrón analítico o empleo de una técnica ortogonal. Por ello en función de la clasificación desarrollada por Schymanski et al. [119] para la identificación de compuestos, en función de la información extraída de ellos y de la veracidad de los mismos, se puede expresar la identificación tentativa de cada compuesto según una serie de niveles de confianza. Los niveles, ordenados de menor a mayor fiabilidad son:

- **Nivel 5:** Es aquel que está determinado solo por el valor de la masa exacta del compuesto (*m/z*) y no puede asignársele ninguna fórmula molecular asociada.
- **Nivel 4:** Aquel donde es posible la asignación inequívoca de una fórmula molecular a un ion precursor (*m/z*) con los datos espectrales tales como aductos, isótopos o fragmentos.
- **Nivel 3:** Es posible asignar una(s) estructura(s) molecular(es) tentativa(s) para el ion precursor de interés con los datos experimentales tales como MS y MS^2, pero no una definitiva.
- **Nivel 2:** Identificación de una estructura muy probable mediante comparación de los datos experimentales (MS, MS^n) con: a) librería espectral o b) por evidencia, ya que no hay ninguna otra estructura posible.
- **Nivel 1:** Confirmación definitiva de la estructura mediante la comparación de las señales del analito de interés con las de su patrón analítico.

4.4.1. Suspect screening

El *suspect screening* se basa en el empleo de librerías o bases de datos de una gran cantidad de compuestos, plaguicidas, metabolitos, drogas o fármacos entre otros. Estas bases de datos son elaboradas previamente bien por una casa comercial que las vende o bien por los usuarios. Estas pueden ser desarrolladas a partir de datos procedentes de bibliografía, como son fórmula molecular o modo de ionización. Por ejemplo, la creación de una base de datos con los metabolitos de interés de un plaguicida previamente descritos en bibliografía o bien realizándose la caracterización de los compuestos de interés por HRMS. Cabe destacar que aspectos como tiempo de retención, solo serán equiparables cuando se emplee el mismo sistema cromatógrafo y condiciones cromatográficas (columna, fase móvil, flujo, etc...). De lo contrario, éste no podrá ser empleado en la identificación de los compuestos de interés. El ion precursor o característico, obtenido en condiciones de masa exacta, se determina a partir de la fórmula molecular de cada uno de los compuestos, y sí será comparable entre distintos analizadores de masas. En cambio, los fragmentos dependerán de la energía de colisión empleada durante la fragmentación del ion precursor. Por ello muchas bases de datos se han creado con diferentes energías de colisión para que sean útiles en diferentes condiciones de análisis. Otro parámetro que las bases de datos incluyen es el modo de ionización, indicando si el compuesto es ionizado en modo positivo o negativo, además de indicar si se forman aductos con el ion hidrógeno o bien con el ion amonio, formiato, acetato..., etc., según el modo de ionización y fase móvil empleada.

En resumen, una base de datos está compuesta por la masa exacta del ion precursor, iones fragmento, modo de ionización (polaridad), aductos y tiempo de retención en los casos que éstos sean equiparables. Dichas bases de datos se generan normalmente en archivos de datos tipo ".csv" y son incorporadas a los software de análisis tipo TraceFinder® (Thermo Scientific) o Metaboscape® (Bruker) que, en función, en primer lugar, del error de masa entre el ion precursor de la base de datos y el ion precursor experimental determinará si hay presencia de este compuesto o no en la muestra. En el caso de que el error de masa sea menor que 5 ppm, esta masa experimental será aceptada como buena y a continuación se compararán los fragmentos para realizar una identificación tentativa de la presencia del analito o no en la muestra, siempre y cuando el error de masa de los fragmentos sea menor que 10 ppm [120]. Cabe indicar que esto dará lugar a una identificación tentativa del analito de interés, y que solo podrá ser confirmada totalmente cuando se empleen patrones analíticos. En la **Figura 1.9**, a modo de resumen se refleja la metodología de trabajo para el *suspect screening*.



Index	Compound Name	Elemental Compositi	Polarity	Analyte type	Expected RT	Intensity Thr	Adduct1	Adduct2	Adduct3	Fragment1	Fragment2	Fragment3	Fragment4	Fragment5
1	1,7a-Estradiol	C18H24O2	+	Parent		1000	1	1	1	107,04896	135,11679	172,08828	186,10388	159,08042
2	1,7a-Ethinylestradiol	C20H24O2	+	Parent		1000	1	1	1	159,08041	133,06473	279,17429	107,04899	157,06483
3	1a-Abamectin	C48H72O14	+	Parent		1000	1	1	1					
4	2,4,6-tribromophenol	C6H3Br3O	+	Parent	8,79	1000	1	1	1					
5	2,4,6-trichlorophenol	C6H4Cl3O	+	Parent		1000	1	1	1					
6	2,4-Diaminotoluene	C7H10N2	+	Parent		1000	1	1	1	108,06771	106,0648	79,05376		
7	2,4-Dimethylaniline	C8H11N	+	Parent		1000	1	1	1	107,07263	105,06963	106,06487	79,05382	
8	2,6-dichlorobenzamide	C7H5Cl2NO	+	Parent	4,33	1000	1	1	1	172,95539	171,97169	146,97623		
9	245-T	C8H5Cl3O3	-	Parent		1000	1	1	1	194,91805		194,96129		
10	24-D	C8H6Cl2O3	-	Parent		1000	1	1	1	160,95685				
11	3,3'-Dimethoxybenzidine	C14H16N2O2	+	Parent		1000	1	1	1	230,10452	213,10207	201,10201	187,08635	215,08127
12	3,4-Dichloroaniline	C6H5Cl2N	+	Parent		1000	1	1	1	127,01818				
13	3-Amino-2-oxazolidinone_AOZ	C3H6N2O2	+	Parent		1000	1	1	1					
14	3-Amino-5-morpholinomethyl-2-oxazolidi	C8H15N3O3	+	Parent		1000	1	1	1					
15	4-Aminobiphenyl	C12H11N	+	Parent		1000	1	1	1	153,06961	169,08843	152,06194	143,08529	
16	4-Aminophenol	C6H7NO	+	Parent		1000	1	1	1	93,03312	92,04913	109,05208		
17	4-Bromo-35-dimethylphenyl-N-methylcarb	C10H12BrNO2	+	Parent		1000	1	1	1	200,99059	122,07221	107,04884	121,0648	
18	4-Chlorophenoxyacetic_acid	C8H7ClO3	-	Parent		1000	1	1	1	126,99601	141,01171			
19	5-Hydroxythiabendazole	C10H7N3OS	+	Parent		1000	1	1	1					
20	6a-Methylprednisolone	C22H30O5	+	Parent	7,7	1000	1	1	1	357,20651	339,19589	321,18527	161,09633	
21	8-Quinolinol 8-hydroxyquinoline sulfate	C9H7NO	+	Parent	4,13	1000	1	1	1					
22	Abamectin	C95H142O28	+	Parent		1000	1	1	1					
23	Acemetacin	C21H18ClNO6	+	Parent		1000	1	1	1	138,99481	312,07898	260,0921	139,0049	
24	Acemetacin	C21H18ClNO6	-	Parent		1000	1	1	1	75,00922	312,07959	297,05615	270,06915	72,99356
25	Acephate	C4H10NO3PS	+	Parent	1,18	1000	1	1	1	142,99264	98,98403	112,99968	113,96363	141,95868
26	Aceprometazine	C19H22N2OS	+	Parent		1000	1	1	1	86,09615	240,04814	282,09512	239,07684	

- ✓ Formula molecular (masa exacta)
- ✓ Modo de ionización
- ✓ Aductos
- ✓ Fragmentos
- ✓ Error de masa
- ✓ Perfil isotópico
- ✓ Tiempo de retención (opcional)

	Selected	MZ	RT	IP	FI	LS	Flag	Compound Name	Match Result Name	Formula	Adduct	Confirmed
76	☐	●					●	Cyromazine	Cyromazine@RT 12.21	C6H10N6	M+Na	0 out of 0
77	☐	●					●	Daminozide	Daminozide@RT 2.07	C6H12N2O3	M+H	0 out of 0
78	☐	●					●	DCPMU	DCPMU@RT 7.05	C8H8Cl2N2C	M-H	0 out of 0
79	☐	●					●	Decoquinate	Decoquinate@RT 5.92	C24H35NO5	M+NH4	0 out of 0
80	☑	●					●	DEET	DEET@RT 8.22	C12H17NO	M+H	0 out of 0
81	☐	●					●	Desethyl atrazine	Desethyl atrazine@RT 4.81	C6H10ClN5	M+NH4	0 out of 0
82	☐	●					●	Dexamethasone	Dexamethasone@RT 5.7	C22H29FO5	M+H	0 out of 0
83	☐	●					●	Diazinon	Diazinon@RT 1.08	C12H21N2O	M+NH4	0 out of 0
84	☐	●					●	Dichloroacetic_acid	Dichloroacetic_acid@RT 1.12	C2H2Cl2O2	M+HCO2	0 out of 0
85	☐	●					●	Diclazuril	Diclazuril@RT 1.12	C17H9Cl3N4	M-H	0 out of 0

Figura 1.9. Esquema del proceso de *suspect screening*.

Son diversos los estudios que han empleado este modo de trabajo para determinar plaguicidas y metabolitos junto con otros contaminantes en muestras de diferente origen. López et al. [121] utilizaron una base de datos con 263 metabolitos de plaguicidas para su determinación en orina. Se identificaron 26 de ellos con la base de datos, siendo 8 confirmados con patrones analíticos. León et al. [118], a partir de una base de datos desarrollada por ellos con 425 contaminantes (plaguicidas, metabolitos, drogas y toxinas) y con el software TraceFinder, determinaron 77 compuestos en piensos, usando como criterio que el error de masa del ion precursor de los analitos de interés fuese inferior que 5 ppm

Polgár et al. [40] emplearon una base de datos con 1396 compuestos (850 compuestos padre, 447 fragmentos y 99 metabolitos) para realizar un análisis de 29 muestras de alimentos (frutas y verduras), detectando plaguicidas como propamocarb, iprodiona y fenhexamida, así como sus metabolitos. De Dominics et al. [102] desarrollaron una base de datos para determinar plaguicidas, aflatoxinas y antibióticos en productos derivados de pan, comprobando que éstos podían ser detectados a niveles traza mediante la adición directa de los contaminantes a las muestras. López et al. [103] emplearon una base de datos de 240 metabolitos de plaguicidas para su identificación en aire, identificando 34 metabolitos de plaguicidas, confirmando y cuantificando 11 de ellos con patrones analíticos en rangos de concentración entre 6.8 y 198.3 pg/m^3.

4.4.2. Análisis unknown

El análisis *unknown* permite, además de un análisis de desconocidos, un análisis mediante rutas metabólicas. El empleo de rutas metabólicas es un modo de análisis muy empleado sobre todo cuando es de interés descubrir posibles metabolitos de compuestos de interés, como pueden ser los plaguicidas (**Figura 1.10**). En él se emplean softwares característicos como Compound Discoverer® (Thermo Scientific) o MassChemsite® (Molecular Disocovery Ltd.), que a partir de la estructura del compuesto progenitor o precursor y una librería de reacciones simples como metilación, desmetilación, oxidación, reducción, sulfonación, etc..., generan unas potenciales estructuras de los metabolitos desconocidos, que se buscan mediante la masa exacta (m/z) derivada de la fórmula molecular en el espectro de masas experimental generado del análisis previo de la muestra. Esa masa exacta llevará asociado un error de masa que deberá ser inferior que 5 ppm. Esta sistemática de identificación, derivada de las estructuras del compuesto progenitor, ha sido escasamente aplicada en estudios previos, habiéndose encontrado solo dos trabajos en el ámbito de residuos de plaguicidas. Uno de ellos identifica el metabolito de un carbamato (metil-N-fenilcarbamato) en orina [121], mientras que en el otro se detecta un metabolito de malatión en aire [122]. A pesar de su escasa aplicación, en la presente Tesis Doctoral se utilizará para la determinación de metabolitos desconocidos de todos los plaguicidas analizados, debido a la gran capacidad de detección/identificación que presenta en relación a otros métodos de análisis.

El modo de análisis de desconocidos o *unknown* surgió en la última década del siglo XX, junto con el termino metabolómica, entendida como la parte de la ciencia (-ómica) que estudia pequeñas moléculas o metabolitos provenientes de alimentos, organismos, plantas y humanos [123]. En los últimos años cada vez son más los trabajos que hacen uso del análisis *unknown*, debido a su capacidad para determinar gran variedad de compuestos o metabolitos mediante un solo análisis y en una gran variedad de muestras, mostrándose en la **Figura 1.10** un esquema de dicha metodología. El análisis *unknown* engloba dos procedimientos: *fingerprinting* (huella característica de la muestra) y *profiling* (perfilado). El *fingerprinting* es una metodología rápida, efectiva y conveniente para clasificar muestras según su perfil metabolómico, identificando las regiones más significativas del espectro de cada muestra. Esta se basa en el empleo de herramientas de análisis discriminante para obtener los elementos diferenciadores entre un conjunto de muestras y las muestras control o de referencia. Estas herramientas estadísticas, se distinguen entre no supervisadas, como el análisis por componentes principales (*Principal Component Analysis*, PCA) o supervisadas, como el análisis discriminante por mínimos cuadrados parciales (*Partial Least Square Discriminant Analysis*, PLS-DA) [124]. El *fingerprinting* se emplea principalmente para generar la huella dactilar de una muestra y poder así hacer una clasificación según tipo, origen, naturaleza, etc… Por ejemplo, se ha utilizado para clasificar variedades de aceite de oliva de diferentes lugares del mundo por sus componentes y poder clasificar muestras desconocidas por comparación [125]. Sin embargo, este modo de trabajo no es muy útil si el objetivo es determinar contaminantes o sus metabolitos, por lo que se suele emplear el otro procedimiento, el perfilado. Este se basa en la identificación de los contaminantes o metabolitos más discriminatorios detectados en las muestras usando bases de datos o software analíticos [126]. Entre las bases de datos más conocidas se encuentran MassBank, Chemspider, Metlin, Molbank, m/z cloud, NIST® o Human Metabolome Database (HMDB). Todas ellas se encuentran disponibles *online* y con acceso gratuito, pero para hacer uso de ellas es necesario un tratamiento previo de los datos.

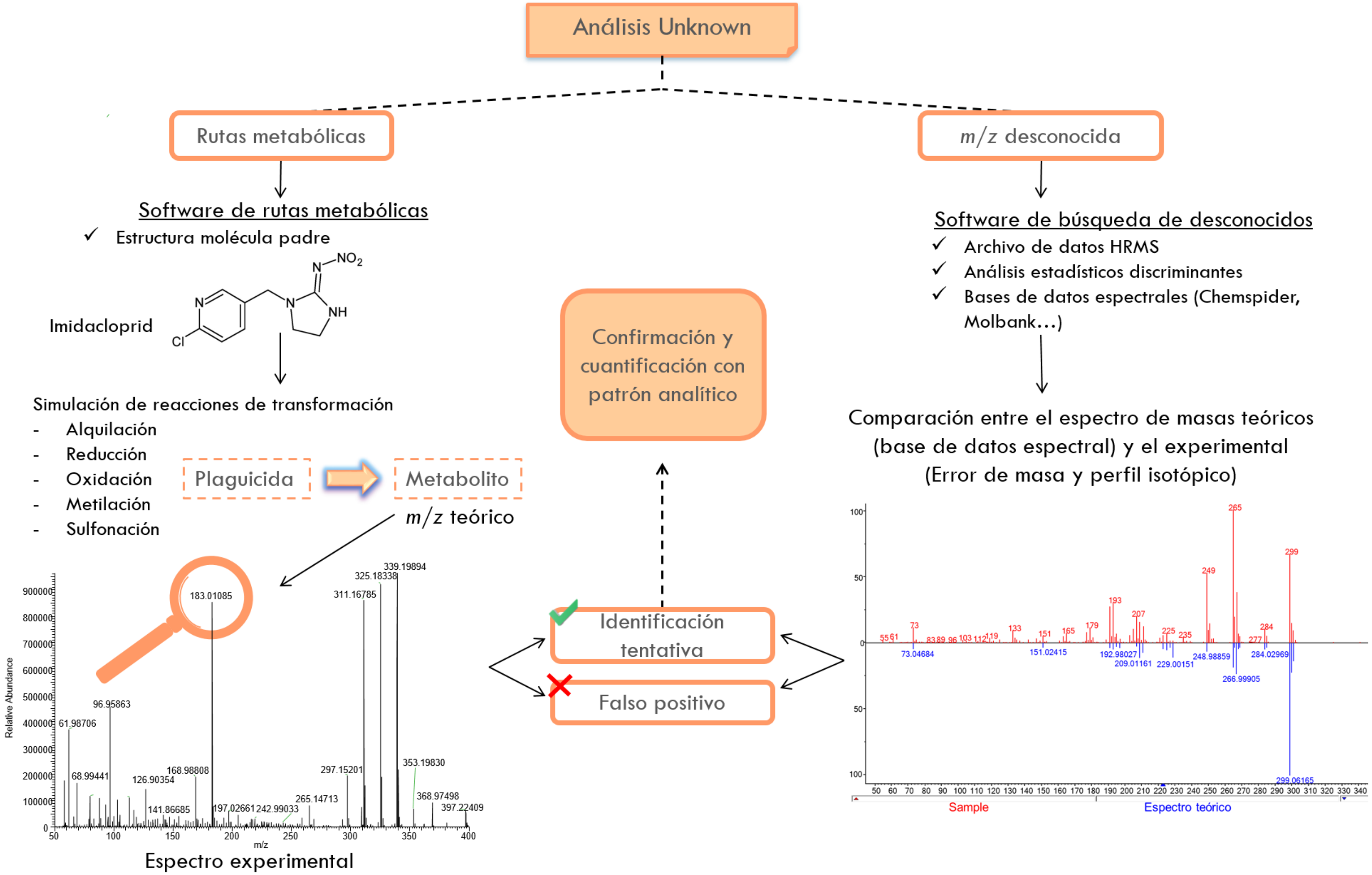


Figura 1.10. Esquema del proceso de análisis *unknown*

Además de estas bases de datos, hay softwares analíticos que llevan a cabo el tratamiento de los datos y automáticamente asocian el resultado con las bases de datos que el usuario selecciona entre una gran variedad. Ejemplos de este tipo de software es Compound Discoverer®, Metaboscape®, MassHunter profiling® (Agilent Technologies) o XCMS (The Scripps Research Institute). Alygizakis et al. [127] emplearon bases de datos como Chemspider y Pubchem, además de usar herramientas de espectros de masas predictivos o in-silico como Metfrag. Esta herramienta in-silico genera espectros de MS/MS teóricos y los compara con los espectros de MS/MS generados experimentalmente, llevando a cabo la identificación tentativa de 14 contaminantes. Schollée et al. [128] llevaron a cabo un análisis de los fragmentos de los compuestos detectados usando bases de datos y después una base de datos in-silico, "RMassBank", para la identificación de los compuestos.

Vanryckeghem et al. [129] realizaron el análisis de compuestos desconocidos en agua de mar de Bélgica usando la base de datos Chemspider, usando como criterio que el error de masa fuese inferior a 5 ppm, además del empleo de perfiles isotópicos de MS. Otro trabajo similar, es el desarrollado por Wang et al. [130] que usaron la base de datos espectral NIST para determinar compuestos tóxicos y sus metabolitos en sangre y orina humana, detectando glifosato, metanfetamina y clonazepam, en alguna de las muestras. Finalmente Meng et al. [109] emplearon el software *Compound Discoverer* unido a *m/zcloud* para determinar contaminantes en agua superficial, detectando 5 plaguicidas, 6 fármacos y distintos derivados plásticos. El uso de estas metodologías es relativamente reciente por lo que aún hay pocos trabajos que se centren en la determinación de nuevos metabolitos, aunque sin duda es de un gran interés científico. Es por ello que en la presente Tesis Doctoral se aplicarán técnicas de *profiling* para determinar metabolitos desconocidos de plaguicidas específicos.

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CAPÍTULO

2

Tendencias de la espectrometría de masas en el ámbito de la seguridad alimentaria y ambiental

1. INTRODUCCIÓN

En los últimos años los avances en el campo de LC-MS han sido de gran relevancia científica. Hace más de una década se produjo el desarrollo de columnas con tamaños de partícula inferiores a 2 µm, que hizo que la cromatografía pasara de denominarse de HPLC a UHPLC. Como consecuencia fue posible la separación de una gran variedad de compuestos con una considerable reducción del tiempo de análisis, así como la mejora en la capacidad de resolución y reducción de los flujos de trabajo, llevando ello a una reducción en el coste de los análisis [1].

Por otro lado, con el desarrollo de los analizadores de HRMS a comienzo de siglo, surgió el término de "masa exacta", motivado por la medida de masa de estos analizadores, pudiendo detectar masas con una exactitud de masa de hasta la sexta cifra decimal [2]. Esto supuso una gran revolución en el campo del análisis químico, ya que cualquier masa detectada mediante estos analizadores de HRMS, era inequívocamente una formula molecular, a diferencia de los de LRMS, cuya masa detectada podría corresponder a varias fórmulas moleculares. Esto soluciono la desventaja principal de los analizadores de LRMS, como es la presencia de interferentes de la matriz con los analitos de interés [3]. Además de esto, al modo de análisis comúnmente conocido (dirigido), se le unió el modo de análisis no dirigido. Este permitía a su vez dos modos de trabajo, *suspect screening* y *unknown*, ya que mediante la adquisición en *full scan*, todos los componentes de una muestra son registrados, pudiendo ser tentativamente identificados sin presencia de patrón analítico. Para ello es necesario el empleo de bases de datos [4] (*suspect screening*) o de software especializados de búsqueda de desconocidos (*unknown*) [5]. Este último modo de trabajo es muy utilizado en estudios metabolómicos [6], que en los últimos años están siendo ampliamente usados. Básicamente consisten en el empleo de la HRMS junto con herramientas de análisis estadístico para caracterizar o buscar diferencias entre diferentes muestras, ya sea por su composición endógena como exógena (como plaguicidas, drogas, metabolitos, etc…). Paralelamente permite llevar a cabo la identificación de metabolitos procedentes de contaminantes, no descubiertos hasta la fecha.

Por ello, se ha llevado a cabo una revisión bibliográfica de los últimos avances existentes en la UHPLC acoplada tanto a analizadores de LRMS como de HRMS (**Artículo científico I)** en el campo del análisis alimentario y ambiental. De igual modo, se han revisado los avances metabolómicos en el campo de análisis de contaminantes en alimentos usando como herramienta principalmente la HRMS (**Artículo científico II)**.

- **Artículo científico I.** Ultrahigh-pressure liquid chromatography-mass spectrometry: An overview of the last decade

- **Artículo científico II.** Metabolomics approaches for the determination of multiple contaminants in food

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Publicación I

ULTRAHIGH-PRESSURE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY: AN OVERVIEW OF THE LAST DECADE

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Ultrahigh-pressure liquid chromatography-mass spectrometry: An overview of the last decade

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ABSTRACT

In the last years, advances in ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS) have been increased exponentially. This technique has been widely used by both research and routine laboratories, decreasing the cost and time of analysis, and increasing sample throughput. This review is focused on the main advances of UHPLC-MS in the last decade, describing the pros and cons of its use coupled to both Low Resolution Mass Spectrometry (LRMS) and High Resolution Mass Spectrometry (HRMS), and the main applications. Targeted analyses have been carried out by UHPLC-LRMS, but other approaches, screening or unknown analyses, have also been performed with HRMS. New advances in UHPLC-MS are also discussed, introducing techniques as two dimensional chromatography (2D-LC), ion mobility separation (IMS) and supercritical fluid chromatography (SFC). The coupling of LC with automated extraction techniques ,as on-line solid phase extraction (SPE) and turbulent flow chromatography (TurboFlow®), has also been evaluated.

1. Introduction

Ultra-High Performance Liquid Chromatography coupled to mass spectrometry (UHPLC-MS) has increased exponentially in the last decade as it can be observed in Fig. 1, where the number of published articles increased from 32 in 2009 to 862 in 2018. UHPLC can be coupled to Low Resolution Mass Spectrometry (LRMS) or High Resolution Mass Spectrometry (HRMS) analyzers, and the number of studies focused on both analyzers has steadily increased. Nevertheless, the use of UHPLC-LRMS is still much higher (up to 700 published papers in 2018) than the applications based on UHPLC-HRMS (111 articles published in 2018).

The increase of UHPLC-MS can be explained because current MS analyzers have ideal characteristics to be coupled to UHPLC, as high full scan acquisition rates, dwell times of 1 ms and polarity switching of 30 ms or less [1]. When UHPLC is used, resolving power increased, and ion suppression can be minimized because the coelution of matrix interferences can be avoided, as well as peak shape can be improved, allowing better peak definition, and more reproducible and accurate peak integration. In addition, UHPLC reduces the volume of chemicals (for example mobile phases) and waste, and it is more environmentally friendly [1]. Furthermore, the sensitivity improves, obtaining methods with lower limits of quantification (LOQs) in comparison to conventional high performance liquid chromatography (HPLC) [2].

Despite the advantages of UHPLC-MS, this also has several shortcomings as frictional heating effects, narrow analyte peaks (short dwell times are needed to obtain enough points per peak) and column blockage [1]. The last one is a critical point in UHPLC analysis, because samples and mobile phases have to be filtered prior chromatographic analyses to remove any solid material that can provoke blockage in the column frits (0.2 μm particle size), increasing the back pressure and therefore, damages in the analytical column. In relation to mobile phases, solvents of UHPLC grade are preferable to filtration step, but the cost is higher in comparison to conventional solvents. In addition, higher flow rates can have a negative impact on sensitivity, which can be more pronounced when electrospray ionization is used, reducing the ionization efficiency. Rodriguez-Aller et al. [3] indicated that technical solutions have been provided by manufacturers of UHPLC–MS instruments as Jet Stream thermal gradient focusing technology proposed by Agilent Technologies®, heated Electrospray Ionization (HESI-II) provided by Thermo Fisher Scientific® or Vacuum-Insulated Probe (VIP) heated electrospray by Bruker Daltonics®. In

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Abbreviations

CCS	Collision Cross Section
dd-MS2	data-depended MS2 analysis
HILIC	Hydrophilic Interaction Chromatography
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
IEX	Ion-Exchange Chromatography
IMS	Ion Mobility Separation
LOQ	Limit of Quantification
LRMS	Low Resolution Mass spectrometry
MS	Mass Spectrometry
SEC	Size-Exclusion Chromatography
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
SFC	Supercritical Fluid Chromatography
TOF	Time Of Flight
QqQ	Triple Quadrupole
QTrap	Triple Quadrupole Linear Ion Trap
TurboFlow®	Turbulent Flow Chromatography
2D-LC	Two Dimensional Liquid Chromatography
UHPLC	Ultra-High Performance Liquid Chromatography
UHPSFC	Ultra-High Performance Supercritical Fluid Chromatography

relation to short dwell times, a reduction of the sensitivity could be observed [4] and several strategies can be used, as reducing the flow rate during peak elution [5], or multiple time windows can be set during acquisition [6].

UHPLC-MS allows the detection and quantification of as many chemicals as possible, increasing sample throughput and keeping data quality. In the last few years, the coupling of UHPLC and MS analyzers has resulted in a seismic shift away from traditional chromatographic techniques in the field of food contaminant analysis, towards multi-class and multi-residue methods, with short injection cycle times and minimal sample preparation [1]. UHPLC-MS has been commonly used in several research fields, as multiresidue determination in food, environmental or biological matrices, including bioanalysis, drug metabolism and metabolomics [3,7,8] and the main analyzers are triple quadrupole (QqQ) and triple quadrupole linear ion trap (QTrap).

Because the advantages provided by UHPLC-MS, it is well-implemented, as routine technique, in research and routine assay laboratories, increasing the productivity. For instance, in pesticide residue analysis, the use of UHPLC-MS allows for the analysis of more than 18,000 samples per year, so the amortization of the equipment is usually six years [9]. Whereas LRMS analyzers, mainly QqQ, is used in routine laboratory analysis, HRMS analyzers as time of flight (TOF) or Orbitrap, as well as hybrid analyzers (Q-TOF or Q-Orbitrap) are commonly used in research laboratories. The capabilities of these analyzers considerably increase the scope of the analysis, and in addition to performing targeted analysis, non-targeted or unknown analysis can also be carried out. Thus, retrospective analysis or identification of new compounds can be accomplished.

Bearing in mind that the number of compounds analyzed by UHPLC-MS considerably increases, a critical step is the application of generic extraction methods that allows the extraction of as many compounds as possible. The use of QuEChERS (acronym of Quick, Easy, Cheap, Effective, Rugged and Safe) [10] or "dilute and shoot" [11] approaches has been commonly applied when multiresidue or multi-class methods have been developed, minimizing sample handling and reducing the use of organic solvents and the extraction step.

Therefore, the coupling of these generic extraction methods with UHPLC-MS is nowadays one of the most common approaches during the development of reliable analytical methods in several fields as food safety and quality, environmental analysis, metabolomics, doping control, etc …, as it is described in the following sections, where the main application of UHPLC-MS (both LRMS and HRMS) in the last few years will be highlighted, paying special attention to new advances in this field.

2. UHPLC coupled to LRMS: applications

UHPLC-LRMS, where QqQ or QTrap are the analyzers most commonly used, has a wide range of applications. It is mainly used to solve analytical problems related to food safety, followed by metabolomics, environmental studies and food quality, whereas

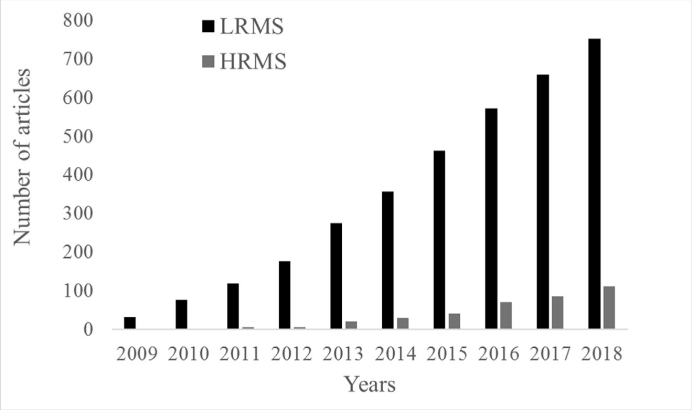


Fig. 1. Number of articles published in the last decade of UHPLC-LRMS and UHPLC-HRMS. Source: Scopus, search from 2009 to 2018 with keywords 'UHPLC', 'LRMS' and 'HRMS'. Date of information gathering: March 2019.

fewer applications have been found in biomedical field (see Fig. 2). In the field of food safety, many studies based on multiresidue methods with hundreds of pesticides and other contaminants (as pharmaceuticals, veterinary drugs, metabolites) in vegetables, fruits, baby food and other matrices have been developed. Table 1 highlights the main multiresidue methods developed in several matrices as food [12–20], oils [21], water [22,23], muscle [24–26] and baby food [27].

At the beginning of the decade the number of multiresidue methods related to food safety was only one, meanwhile in 2018, the number increased to 41 articles, analyzing different types of contaminants as pesticides, veterinary drugs, mycotoxins (Table 1). In addition, one advantage of UHPLC was shorter analysis time in multiresidue methods than with conventional HPLC. For instance Evans et al. [28], demonstrated how the UHPLC method reduces the inject-to-inject time from 32 min to 12 min (Fig. 3A, B). In addition, two peaks were fully resolved applying UHPLC (Fig. 3C), meanwhile only one peak was observed when HPLC was used (Fig. 3D).

UHPLC-LRMS is commonly used in pesticide residue analysis in food (fruits, vegetables, and other matrices), using QuEChERS as extraction method. In this field, the use of LRMS is needed, due to the fact that high sensitivity is required in order to detect concentration levels at μg/kg to ensure food safety [8]. For example, Carneiro et al. [12] determined 128 pesticides in banana using a ODS II column and a QqQ as analyzer, achieving 13 min as a total running time. For most of the compounds, limit of quantification (LOQ) was set at 10 μg/kg, which was lower than the maximum residue limit (MRL) for these compounds in banana (Table 1). In the same way, Wang et al. [13] determined 270 pesticides in foods (vegetables, fruits, tea, oil, cereals and eggs), using a HSS T3C18 column and a QTRAP analyzer, with a total run time of 13 min, obtaining LOQ values ranging from 2 to 10 μg/kg. Dias et al. [21] determined 165 pesticides in edible oils applying UHPLC-QqQ-MS/MS. Because the complexity of the sample, a new generation of dispersive solid phase extraction clean-up (Bond Elut QuEChERS Enhanced Matrix Removal-Lipid (EMR-Lipid)) was tested, showing advantages in comparison with other clean-up procedures as primary-secondary amine (PSA) and Z-sep. This approach allows the extraction of pesticides with recoveries between 70 and 120%.

In the field of veterinary drugs (Table 1), Geis-Asteggiante et al. [24] determined more than 100 veterinary drugs in bovine muscle samples using UHPLC-LRMS with a High Strength Silica (HSS) UHPLC column and a QqQ as analyzer. In relation to the determination of mycotoxin analysis, Arroyo-Manzanares et al. [20] developed a sensitive, simple and rapid method for the determination of 14 mycotoxins in nuts and seeds (including almonds, peanuts, sunflower seeds, pumpkin seeds, walnuts, macadamia nuts, pistachios, hazelnuts and pine nuts) using UHPLC-QqQ-MS/MS, with a run time of 8 min.

There are multiresidue methods that simultaneously analyzed different families of contaminants. Zhan et al. [14] developed a generic multi-class and multiresidue method that detected 255 pesticides and veterinary drugs in raw milk by UHPLC-QqQ-MS/MS, with a run time of 12 min.

In environmental analysis, there are various studies focused on the determination of contaminants or drugs in matrices as water, sediments or soils. For example, Marin et al. [22] have developed a method for the monitoring of 37 pesticides in three different environmental water samples (running time: 10 min), obtaining low LOQs (0.025 μg/L). In addition, López-Serna et al. [23] developed a fast method for the analysis of 74 pharmaceuticals in different types of waters (groundwater, river and wastewater from influent and effluent). In this case, LOQ values were established between 0.1 ng/L and 1 μg/L.

In baby food, multiclass method was developed by Zhang et al. [27] that determined 220 veterinary drugs and pesticides in infant formula samples using a HSS T3 column, with a run time of 12 min. Good validation results were achieved taking into account the difficulties of a multi-class, multi-residue method for these extremely complicated matrices.

Other applications focused on metabolomics (including lipidomics) and biomedical applications have also been developed [29–32]. For instance, Zhao et al. [29] described the main applications of lipidomics, as determination of biomarkers for investigating diseases or drug research, and the lipidomics of plants. They conclude that UHPLC is more suitable for untargeted lipidomics than HPLC. In the same way, Chen at al [30]. performed the simultaneous determination of metabolome and lipidome from a single tissue sample (liver and muscle) with UHPLC-MS. The analytical column used in the study was an ACQUITY T3 column (100 mm × 2.1 mm, 1.8 μm) and run time of 30 min. In the field of proteins, Zhang et al. [32] developed a method for the analysis of modifications in therapeutic monoclonal antibodies, using peptide mapping. For that purpose, an ACQUITY BEH C4 column (2.1 × 250 mm, 1.7 μm) was used for the separation of antibodies in a total run time of 15 min. Kochling et al. [33] developed an analytical platform for protein analysis that combined UHPLC-UV and UHPLC-MS, reducing the overall cost of the analysis. In conclusion, UHPLC-MS allows the detection of polar compounds, as metabolites, and that is why, this is commonly used in metabolomics studies, where most of the main metabolites are polar [34].

Otherwise, UHPLC-MS has also been used to characterize typical products of the traditional Chinese medicine (TCM), including *in vivo* studies of herbal medicines [35]. In addition, it has been used for the determination of polyphenols in food. For instance, Alberts et al. [36] determined 121 anthocyanins and derived pigments in red wine using a UHPLC-MS/MS equipped with C18 column (running time: 30 min).

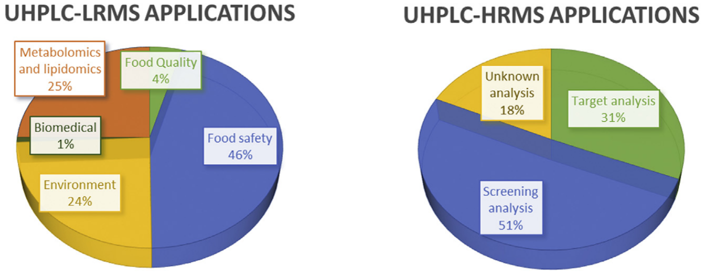


Fig. 2. Main applications in UHPLC-LRMS and UHPLC-HRMS classified by fields of application.

Table 1
Main applications in the field of multiresidue methods using UHPLC-LRMS.

Matrix	Type of compounds (number)	Analytical column	Analysis time (min)	Analyzer	References
Banana	Pesticides (128)	Shim-pack XR-ODSII (100 mm × 2.0 mm, 2.2 μm)	13.0	SCIEX 5500 Triple Quadrupole mass spectrometer	[12]
Foods	Pesticides (270)	HSS T3C18 (100 mm x 2.1, 1.8 μm)	15.0	SCIEX QTRAP 4500 system	[13]
Milk	Pesticides and veterinary drugs (255)	HSS T3 (150 mm × 2.1 mm, 1.8 μm)	12.0	Xevo TQ™ MS/MS triple quadrupole	[14]
Mango	Pesticides (113)	Waters Acquity UPLC® BEH C18 (50 mm × 2.1 mm, 1.7 μm)	11.0	Xevo TQ™ MS/MS triple quadrupole	[15]
Golden berry	Pesticides (42)	Waters Acquity UPLC® BEH C18 (50 mm × 2.1 mm, 1.7 μm)	10.0	Xevo TQ™ MS/MS triple quadrupole	[16]
Soy beverages	Pesticides (39)	Waters Acquity UPLC® BEH C18 (50 mm × 2.1 mm, 1.7 μm)	10.0	Xevo TQ™ MS/MS triple quadrupole	[17]
Crops	Pesticides (72)	Waters Acquity UPLC® BEH C18 (50 mm × 2.1 mm, 1.7 μm)	10.0	Xevo TQ™ MS/MS triple quadrupole	[18]
Peanut	Pesticides (113)	Shim-pack XR-ODSII (100 mm × 2.0 mm, 2.2 μm)	13.0	SCIEX 5500 Triple Quadrupole mass spectrometer	[19]
Edible nuts and seeds	Mycotoxins (14)	Zorbax Eclipse Plus C8 (50 mm × 2.1 mm, 1.8 μm)	8.0	SCIEX 3200 Triple Quadrupole mass spectrometer	[20]
Edible oils	Pesticides (165)	Zorbax Eclipse Plus C8 (100 mm × 2.1 mm, 1.8 μm)	19.5	Agilent Technologies 6490 TripleQuad	[21]
Water	Pesticides (37)	HSS T3 (100 mm × 2.1 mm, 1.8 μm)	10.0	Waters Acquity TQD	[22]
Water	Drugs (74)	Waters Acquity UPLC® BEH C18 (100 mm × 2.1 mm, 1.7 μm)	8.0	Waters Acquity TQD	[23]
Bovine muscle	Veterinary drugs (130)	HSS T3 (100 mm × 2.1 mm, 1.8 μm)	9.5	Waters Acquity TQD	[24]
Porcine and bovine muscle	Veterinary drugs, pesticides and mycotoxins (226)	HSS T3 (100 mm × 2.1 mm, 1.8 μm)	12.0	Xevo TQ™ MS/MS triple quadrupole	[25]
Bovine muscle	Veterinary drugs (30)	Waters Acquity UPLC® BEH C18 (50 mm × 2.301 mm, 1.7 μm)	5.0	Quattro Premier XE Triple Quadrupole	[26]
Infant Formula	Veterinary drugs and pesticides (220)	HSS T3 (100 mm × 2.1 mm, 1.8 μm)	12.0	Xevo TQ™ MS/MS triple quadrupole	[27]

Although reversed phase was commonly used as stationary phase in UHPLC, other phases as chiral stationary phases, size-exclusion chromatography (SEC), ion-exchange chromatography (IEX) and hydrophilic interaction chromatography (HILIC) [3] have been used and UHPLC improved the efficiency of the elution of the compounds in comparison to HPLC [37]. For instance, HILIC allows the determination of polar compounds, as pesticides like glyphosate or polar pesticide metabolites (metabolomics). Thus, Yoshida et al. [38] determined the pesticide nitenpyram and three metabolites in agricultural products as vegetables, fruits or rice. The use of HILIC allowed the determination of the three metabolites, which are more polar than the parent compound. In addition, the use of chiral chromatography has been used applying UHPLC conditions, reducing the particle size from conventional 5 μm–3 μm allowing benefits in terms of throughput and efficiency [37]. Marin-Sáez et al. [39] compared two chiral columns (one of 5 μm particle size and other of 3 μm particle size) for the enantioseparation of atropine, obtaining the best peaks shapes and resolution when the 3 μm particle size chiral column was used (Fig. 4). In the same way, Baranowska et al. [40] simultaneously determined naringenin and hesperetin enantiomers in plant material using Chiralpak AD-3R column for the first time.

3. UHPLC coupled to HRMS analyzers: applications

The main difference between HRMS and LRMS is that LRMS provides measurements in single digit mass units, whereas HRMS provides exact mass measurements with four to six decimal digits, that allows the differentiation between compounds with the same nominal mass [41].

In the last years, recent studies focused on two main HRMS analyzers, Orbitrap or TOF, both coupled to Q or not, are mainly used in routine and research laboratories, although other analyzers, as ion cyclotron, have also been used [42]. The main characteristics of HRMS are mass range, efficiency, speed, linear dynamic range, sensitivity, resolution and mass accuracy [41,43]. Orbitrap technology is able to operate at acquisition rates of 12 Hz. However, this parameter depends on the resolution. For instance, resolving power of 17,500 FWHM was achieved at 12 Hz, whereas the maximum resolution that can be theoretically attained, 140,000 FWHM, can be set at 1 Hz, achieving few points per peak when UHPLC is used (narrow peaks). However, if (Q)TOF analyzer is used, this problem can be minimized, due to (Q)TOF allows 50 or more scans per second [42]. In consequence, (Q)TOF analyzers provide a comparable performance at faster acquisition rates, lower space requirements and cost than Orbitrap [44]. Nevertheless, if a technical solution can be found for the Orbitrap technology to maintain the exceptional resolution at elevated acquisition rate, this analyzer will probably become the most efficient one to be coupled with UHPLC technology [3].

One disadvantage of (Q)TOF analyzers is that polarity switching is not possible in comparison to Orbitrap analyzers [8]. Nevertheless, the use of UHPLC-HRMS provides faster methods in relation to conventional HPLC-HRMS procedures, and this permits the implementation of two sequential injections of the sample, one in positive and another in negative ion mode. In this case, the ionization parameters can be optimized for both methods, increasing the number of detected compounds [28].

UHPLC-HRMS is a perfect combination between the ultra-high capacity of liquid chromatography and the high mass resolution of mass spectrometry, and the types of applications are different compared with LRMS. This can be explained because in HRMS, targeted analysis was in the background and other types of analysis as screening or unknown analyses are commonly performed (Fig. 2).

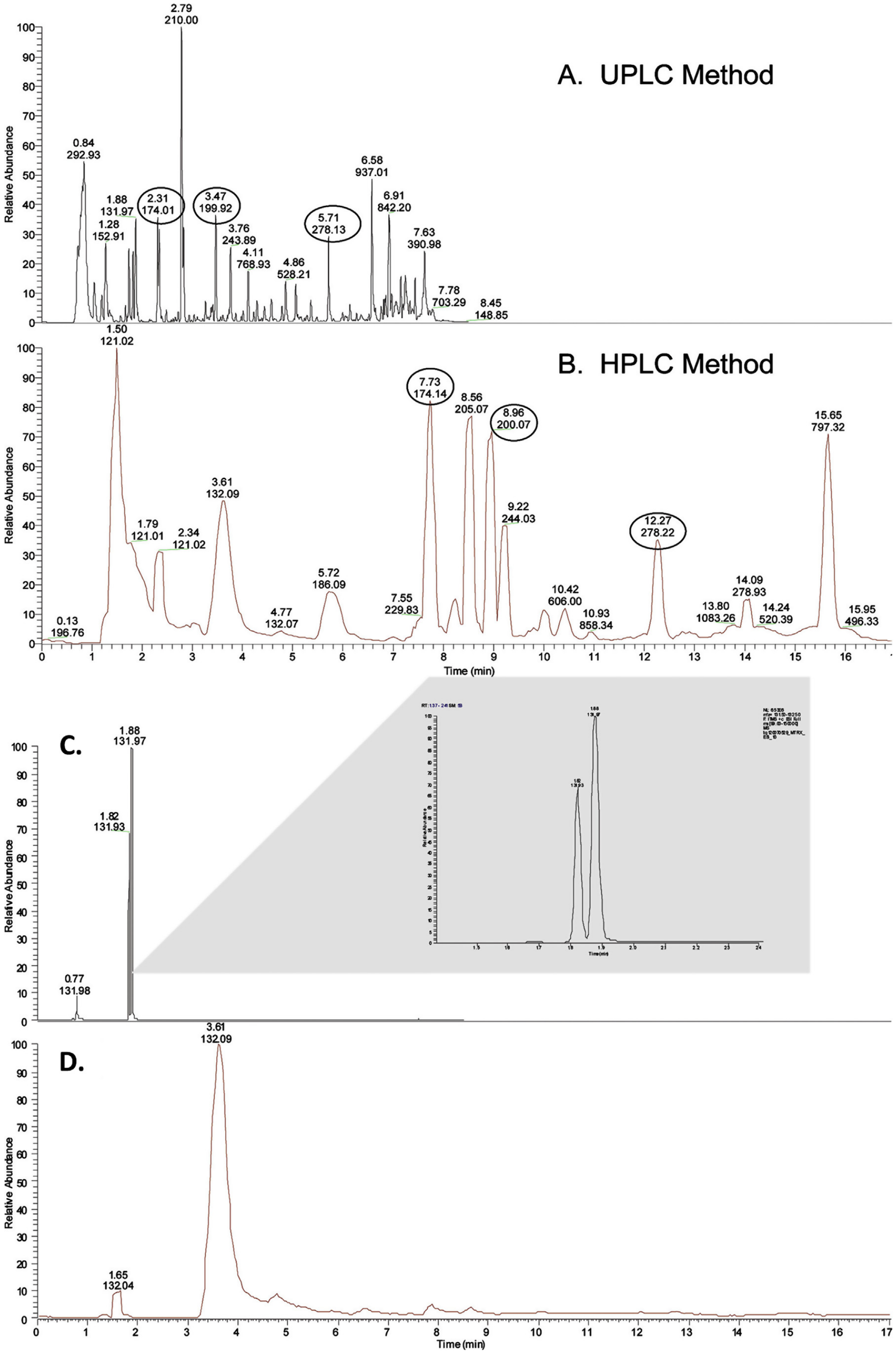


Fig. 3. Total ion chromatogram of: a) UHPLC method, b) HPLC method, c) two peaks were fully resolved applying UHPLC meanwhile d) with the use of HPLC only one peak was observed. Evans et al. [28]. Copyright with Permission of American Chemical Society.

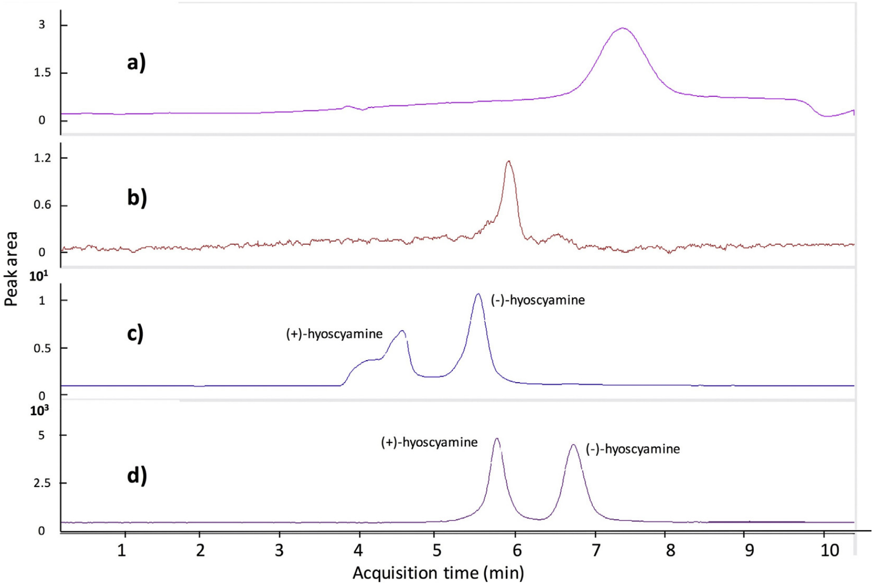


Fig. 4. Enantioseparation of atropine with different columns and chromatography conditions: a) Chirobiotic V ((A) water/acetonitrile (90/10 v/v) and (B) methanol/acetonitrile (50/50 v/v) both with 10 mM of ammonium formate); b) Chirobiotic V (methanol/acetic acid/triethylamine (A) 100/0.05/0.04, v/v/v and (B) 100/0.05/0.1, v/v/v); c) Chiralpak AY3 (hexane/ethanol/diethanolamine (80/20/0.1 v/v/v)); d) Chiralpak AY3 (ethanol/DEA (100/0.1 v/v). Marin et al. [39]. Copyright with Permission of Elsevier.

In UHPLC-HRMS, the main purpose is the development of generic methods to detect the largest number of analytes, as contaminants, drugs, metabolites, natural products, etc. [8]. Other approaches are screening and unknown analyses, searching for example, the parent compound (i. e. contaminants or drugs) and its transformation products [45,46] using databases or metabolomics, applying software tools (i. e., Compound Discoverer®) for the identification of characteristic components or unknown metabolites of contaminants searching possible transformations from the parent compound molecule. Furthermore, in-silico fragmentation tools (i.e., MetFrag®) have been used for the characterization of unknown compounds comparing simulated mass spectra with the experimental spectrum obtained [47]. Table 2 shows the main applications of UHPLC-HRMS in different matrices.

For instance, Wang et al. determined 166 pesticides in fruits and vegetables using UHPLC-Q-Orbitrap [48] (Table 2). UHPLC-Q-Orbitrap-MS in full MS scan mode was used for quantification, meanwhile UHPLC/ESI Q-Orbitrap Full MS/dd-MS2 (data-depended MS2 analysis) was performed to obtain product ion spectra for identification purposes. The scope has been increased and two years later, Wang et al. [49], determined more than 450 pesticide residues in fruits and vegetables using Q-Orbitrap analyzer. For that purpose, a simple and generic method based on full MS scan was used for high method accuracy, repeatability, and sensitivity during quantification, and dd-MS2 provided product ion spectra with accurate mass measurement that allowed unambiguous confirmation of pesticides (Table 2).

The use of UHPLC coupled to HRMS is also a powerful tool in environmental analysis, allowing the detection of known and unknown compounds in complex matrices at low concentrations. For instance, Gosetti et al. [50] discussed the differences between mass spectrometry analyzers in the field of unknown analysis, and the different modes of screening these analyzers provided. However, unknown analysis presented important drawbacks, as difficulties

Table 2
Main applications in the field of UHPLC-HRMS.

Matrix	Type of compounds (number)	Aim of study	Analyzer	References
Ambient air	Pesticides, metabolites and other pollutants (35 target + 300 post-target)	Targeted and screening analysis	Orbitrap MS (Exactive™)	[45]
Sediments	Micropollutants (>100)	Screening and unknown analysis	Quadrupole Orbitrap (Q-Exactive^{TM})	[46]
Rum	Rum components (>100)	Screening applying chemometrics	Orbitrap MS (Exactive™)	[47]
Fruits and vegetables	Pesticides (116)	Targeted analysis	Quadrupole Orbitrap (Q-Exactive^{TM})	[48]
Fruits and vegetables	Pesticides (451)	Targeted analysis	Quadrupole Orbitrap (Q-Exactive^{TM})	[49]
Ambient air	Pesticide and metabolites (>100)	Screening and unknown analysis	Orbitrap MS (Exactive™)	[52]
Urine	Pesticide and metabolites (>100)	Screening and unknown analysis	Orbitrap MS (Exactive™)	[53]
Urine	Veterinary drugs (87)	Screening analysis	Orbitrap MS (Exactive™)	[54]
Meat	Pesticides and veterinary drugs (>350)	Screening analysis	Orbitrap MS (Exactive™)	[55]
Tomato (organic and conventional)	Pesticides (>100)	Unknown analysis applying chemometrics	Quadrupole Orbitrap (Q-Exactive^{TM})	[56]
Dried blood	Prohibited drugs (>100)	Targeted analysis	Quadrupole Orbitrap (Q-Exactive^{TM})	[57]
Wastewater	Polar organic contaminants (>100)	Screening and unknown analysis	QTOF-MS	[58]
Products	Drugs (>100)	Unknown analysis	XEVO G2 hybrid QTOF-MS	[59]

for compound detection at low concentrations and very few possibilities during the elucidation of new compounds. Therefore Gosseti et al. highlighted that an effort should be made to speed the development of more intelligent software for screening unknown compounds.

In the same way, HRMS has been applied for the characterization of polyphenols in food using Q-TOF or Orbitrap mass analyzers with negative electrospray ionization mode. In this sense, Lucci et al. [51] described recent advances in LC-HRMS for the characterization of polyphenols in food, focusing on the most relevant applications published in the last years.

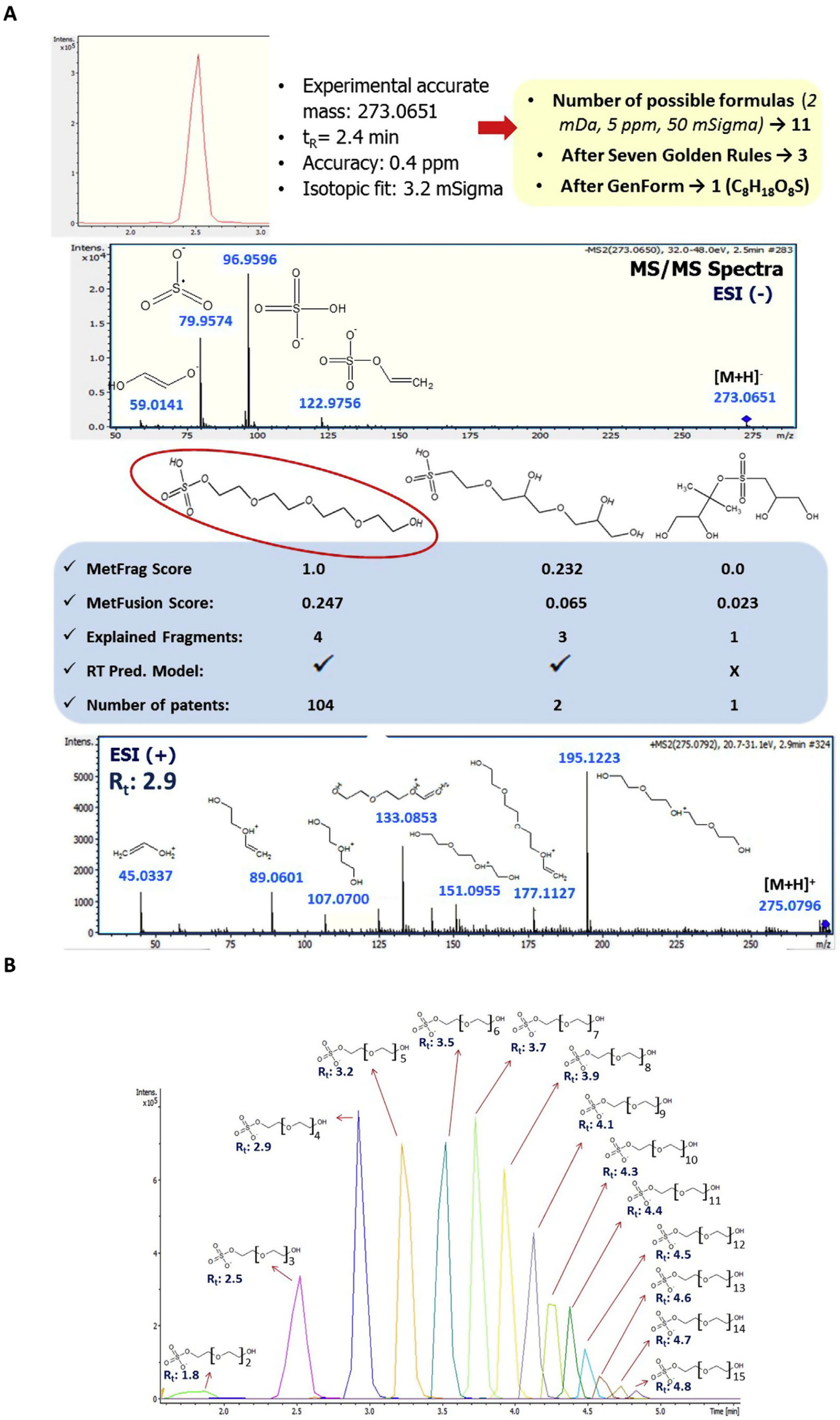
In addition, UHPLC-HRMS has introduced a wide range of new applications as suspect screening and unknown analysis that cannot be possible with LRMS analyzers (Table 2). In the case of suspect screening, several applications have been developed. For instance, Lopez et al. [52,53] has built a customized database with 250 pesticide metabolites, including the theoretical mass and their main fragments for the screening of ambient air and urine samples and also to elucidate new pesticide metabolites using a fragmentation-degradation tool. In the same way, Leon et al. [54] determined 87 banned veterinary drugs in bovine urine applying UHPLC-Orbitrap-MS by screening analysis; targeted concentrations between 0.2 μg/L and 20 μg/L were obtained, demonstrating the usefulness of UHPLC—HRMS as an ideal tool for compliance monitoring in regulatory laboratories. Coscollà et al. [45] combined targeted analysis of currently used pesticides with screening, using a customized theoretical database that allowed the tentative identification of new substances present in ambient air, including metabolites, without the need of reference standards or additional analysis. In the field of sediments [46], the strategy to search for micropollutants with a clear isotopic pattern (Cl, Br) and mass defects facilitated the search of unknown compounds. Also the use of software tools as Metfrag and Molgen allowed the identification of three suspect candidates, two unknown compounds (hexachlorophene and flucofuron), and one metabolite of a suspect compound (bromochlorophene). They were confirmed with reference standards, while a further metabolite was tentatively identified. An UHPLC-Orbitrap-MS method was developed for the identification of more than 350 pesticides, biopesticides and drugs in meat [55] using a compound database. Despite good results were obtained, authors revealed that several difficulties were found when Exactive Orbitrap was used. Thus, fragments were not easy to find due to the utilization of full scan MS/MS libraries with variable collision energy were not suitable, and they usually had low sensitivity or the signal was not reproducible.

For unknown analyses using HPLC-HRMS (Table 2), specific software is needed as Compound Discoverer® in the case of ThermoScientific, Metaboscape® for Bruker data, MassHunter unknown analysis for Agilent Technologies® or XIC Manager for Sciex. These software tools require an additional knowledge of the data, and in some cases, statistical analysis was also needed. Applications in forensic analysis, doping control and metabolomics have grown up in the last years, due to the development of UHPLC-HRMS and software. For example, in the case of forensic or doping, the number of publications has increased from one to twenty in the last ten years, whereas for metabolomics, 1 reference was published in 2009, whereas 78 references were published in 2018. For instance, Martinez-Bueno et al. identified food markers to discriminate between organic and conventional tomatoes using UHPLC-Q-Orbitrap-MS methods and the software tool, Compound Discoverer®, was used to automatically detect the analytes, monitoring precursor ions with a mass tolerance of 5 ppm [56]. UHPLC-Orbitrap-MS was used for rum authentication, also using Compound Discoverer®, and the compounds that had higher discrimination capacity were identified. In addition, the data obtained by Compound Discoverer® was treated by statistical analysis in order to identify rum categories, providing suitable information of the metabolomics chemistry of golden rums, as well as to avoid potential fraud [47]. In the field of doping control, Thomas et al. [57] developed a method to identify prohibited drugs in dried blood spots using UHPLC-Q-Orbitrap-MS. This method has several advantages due to the analysis of dried blood spots is discreet, stable, cost-effective and fast testing protocol. Single-product ion mass spectra are acquired using the data-dependent analysis mode (employing an inclusion list) for previously selected precursors of known prohibited compounds with fixed retention time ranges and besides, a sensitive screening in a targeted approach as well as unknown analysis for retrospective data evaluation are possible. Gago-Ferrero et al. [58] carried out suspect and unknown strategies to characterize polar organic contaminants in wastewater. A surfactant was tentatively identified using in-silico software tools as MetFrag or MetFusion. Fig. 5 shows an illustration of the identification mode, where the number of possible formulas for the same mass was represented (a total of 11). The use of different rules and scores determined by MetFrag and MetFusion allowed the selection of only one as the best option. Fabregat-Safon et al. [59] identified and characterized a new psychoactive substance in products from internet purchased as drugs. UHPLC-QTOF was used for the elucidation of these new molecules and other techniques as nuclear magnetic resonance was used as orthogonal approach. Finally Rathahao et al. [60] discussed the advantages and disadvantages of the use of HRMS for the structural identification of metabolites, and the principal problem is that mass spectral databases, as well as reference standard compounds for all metabolites are not available.

4. New advances in UHPLC-MS

In the last years, several advances in the field of UHPLC-MS have been carried out. The most important can be 2D chromatography (2D-LC), ion mobility separation (IMS), supercritical fluid chromatography (SFC) or the development of fully automated methods, including the extraction, separation and detection of compounds.

The application of the 2D-LC technique has grown with the analysis of samples with large numbers of chemical groups, and compounds as polymers or triacyl glycerides, because the use of a single column in conventional UHPLC-MS methods is not enough to achieve a suitable separation of these compounds. When the number of components exceeds 37% of the peak capacity of the method, the peak resolution is statistically reduced, and in this case, the use of 2D-LC is a good option [61]. When 2D-LC is used, the speed of the second column is critical. This speed should be as fast as possible, and UHPLC stationary phases are suitable to achieve this goal. 2D-LC has two approaches, comprehensive (LCxLC) and heart cutting (LC-LC). In LCxLC, the complete effluent from the first column is transferred to the second one. On the other hand, when LC-LC is performed, one or multiple peaks or parts of the first chromatogram are transferred to the second column. Compared to LCxLC, first and second dimension run times are de-coupled, meaning that there are no time constraints on the second dimension separation [62]. Several applications have been developed using 2D-UHPLC-MS as the determination of fatty alcohol surfactants, utilizing a ZIC®-HILIC column in the first dimension and a Reprosphere 100 C8-Aqua column in the second dimension, showing in Fig. 6 the two-dimensional chromatogram, where five substance classes were separated from each other [63]. Other applications allows the determination of epoxy resins, coupling SEC and Liquid Chromatography at Critical Conditions (LCCC) [64] or the comparison of LC and 2D-LC to optimize the separation of complex peptide samples [65]. Finally, it can be highlighted that up to know, most of the 2D-LC applications have been focused on targeted



✓ MetFrag Score	1.0	0.232	0.0
✓ MetFusion Score:	0.247	0.065	0.023
✓ Explained Fragments:	4	3	1
✓ RT Pred. Model:	✓	✓	X
✓ Number of patents:	104	2	1

Fig. 5. (A) Nontarget identification of the unknown compound diglycol ether sulfate. (B) Extracted ion chromatograms of the corresponding homologous series, glycol ether sulfate (GES) surfactants. Gago-Ferrero et al. [58]. Copyright with Permission of American Chemical Society.

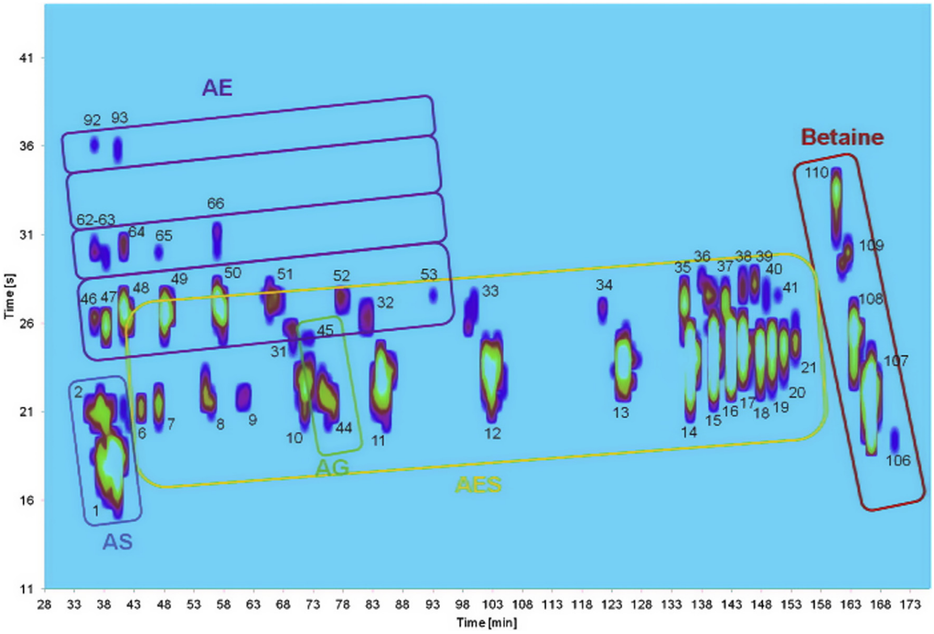


Fig. 6. Two-dimensional chromatogram of the surfactant mixture; first dimension: ZIC®-HILIC, ACN: NH4OAc, 0.025 mL/min, 1 min sampling rate; second dimension: Reprospher C8–Aqua, MeOH: NH4OAc, 3 mL/min. Elsner et al. [63]. Copyright with Permission of Elsevier.

analysis using LRMS or HRMS [66], but the field of unknown screening is not developed yet but it will become a powerful tool in the future.

Ion mobility separation (IMS) provides a new dimension in addition to chromatography and MS. The use of ion mobility is applied for the separation of isobaric compounds, minimizing the background noise or separating endogenous matrix interferences [67]. It has been used for the separation of flavonoids in seven analytical standards, which is a difficult task by conventional UHPLC-MS because the presence of isomers that usually coeluted, requires longer chromatographic methods, and they cannot be “spectrometrically resolved” by LRMS or HRMS [68]. IMS adds a complementary separation dimension without increasing cycle time, and with minimal added experimental complexity; thus the compounds can be separated on the millisecond time scale. One of the benefits of IMS is the ability to measure collision cross section (CCS) values of ions. That value is a robust and precise property related to the chemical structure, adding an additional dimension during the confirmation process. IMS is reproducible and transferable between instruments due to reduced mobility and CCS of ions are physical quantities in comparison with the retention times and reproducibility in UHPLC-MS [68]. Thus, CCS robustness has been demonstrated in a recent inter-laboratory evaluation, obtaining low relative standard deviation values (0.29%) for all the tested compounds [69].

The last new advance that recently has gained importance in UHPLC-MS is SFC or ultra-high performance supercritical fluid chromatography (UHPSFC), due to the new instruments using supercritical fluids as mobile phase have been improved in terms of performance, reliability and robustness [37]. UHPLC stationary phases have become available for SFC and it has been demonstrated that supercritical fluids have a low viscosity, which results in fast diffusion of the compounds in the mobile phase, similarly to the results observed when high temperature liquid chromatography is used, but without the risk of thermal degradation [70]. The majority of applications of that technique have been developed in biological studies, as quantification of neurotransmitters of melatonin and N-acetyl-serotonin in human serum [71]. Both SFC-MS/MS and UHPLC-MS/MS were compared, and it is shown that SFC-MS/MS is a useful method for the analysis of polar analytes in biological samples because of the ionization regime established under CO_2 conditions (same MS conditions as those used when conventional UHPLC system was utilized), making it potentially complementary to UHPLC-MS/MS. Other application that compares UHPLC and SFC is the screening of beta-agonist in feeding stuff [72]. SFC offered enhanced retention for polar compounds [72], the selectivity was improved and some coeluting issues could be overcome using SFC as can be observed in Fig. 7. Fig. 7A shows the separation based on LC, where compounds 3,4,5,8 and 9 coeluted meanwhile in Fig. 7B the use of SFC allowed the separation of all compounds except compound 5.

Finally, automated methods, such as on-line solid phase extraction (SPE) or turbulent flow chromatography (TurboFlow®), have been used to increase the speed of analysis as well as to minimize the errors associated to sample handling, increasing the number of applications in the last few years. However an important constraint of these automated methods is pressure capability of on-line SPE cartridges. Conventional UHPLC columns generate high column back-pressures, which is not compatible with on-line SPE systems that commonly operate at low back pressures. An on-line SPE coupled to microUHPLC-MS/MS has been developed for the determination of 10 nonsteroidal anti-inflammatory drugs in drinking water, wastewater and surface water in 4.5 min [73]. Another application allows the determination of 22 metabolites of diethylphthalate and alternative plastizers from PVC medical devices in urine samples using SPE-UHPLC-MS/MS [74]. In the field of

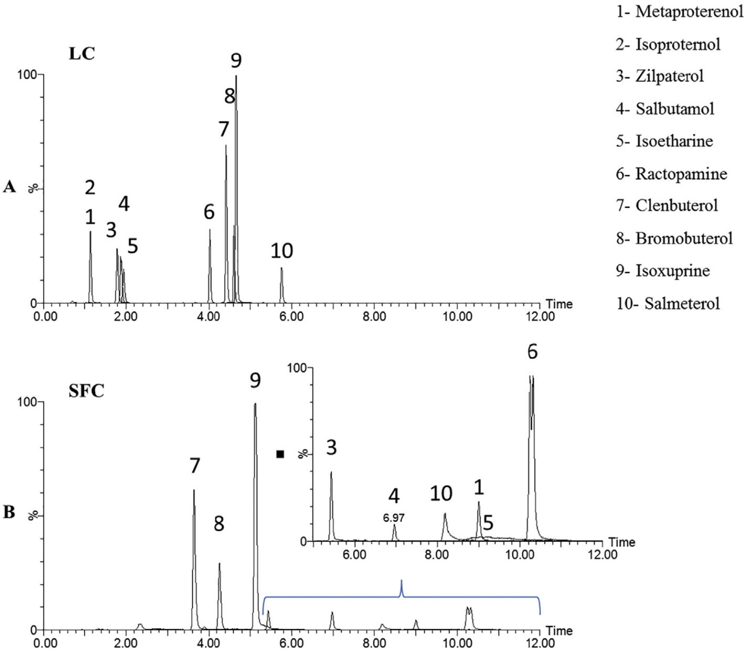


Fig. 7. Extracted Ion chromatogram of sample spiked with 10 β-agonists at 25 μg/kg in (A): LC using PFP column and (B): SFC Torus 2-PIC column. Herpin et al. [72]. Copyright with Permission of Elsevier.

HRMS, Cotton et al. [75] developed a multiresidue method for the determination of 539 pesticides and drugs in water, using SPE-UHPLC-Q-Orbitrap-MS, with a total running time of 36 min.

In the case of Turboflow®, most of the publications were focused on the determination of contaminants in urine, as the determination of bisphenol A [76] or pesticides and metabolites [77]. Nevertheless there are other applications focused on food quality, as the determination of polyphenols in royal jelly [78] where the use of TurboFlow® reduces the extraction time, allowing a fast an easy sample preparation, eliminating matrix components that produce matrix effect and interference in complex samples as royal jelly.

5. Conclusions and future perspectives

In this review, advantages of UHPLC-MS in comparison to HPLC-MS have been highlighted, describing applications in several fields as food safety, environmental and bioanalysis. In addition to UHPLC-LRMS methods, advantages and disadvantages of UHPLC-HRMS instruments in relation to UHPLC-LRMS have been discussed. The new capabilities provided by HRMS allow for the development of new approaches as screening and unknown analysis, detecting new transformation products of contaminants or other compounds. Two new relevant fields where HPLC-HRMS is being used are metabolomics and putative elucidation of new molecules, emphasizing that UHPLC-HRMS is a reference technique when new analytical methods with a wide scope analysis must be developed. In addition, for screening applications, the implementation of software tools combined with chemometrics is necessary. Data treatment requires a high time consuming that can be solved partially with these tools.

In the future UHPLC-LRMS technology may be replaced by UHPLC-HRMS, due to the wide range of applications that can be developed with HRMS. However, these analyzers will be worth for routine laboratory analysis when sensitivity, dynamic range and overall quantitative characteristics improve, as well as the cost of them will be reduced.

Future outlooks of UHPLC-MS can be related with the new advances described in this review as 2D-LC, IMS or UHPSFC. For instance, the use of UHPSFC can minimize the use of solvents (mobile phase), and if it is coupled to simple or miniaturized methods, can provided “green methods”, which will be considered as environmentally friendly methodologies.

In addition, automated methods as on-line SPE will be interesting in the future in order to minimize sample handling. This allows for a fast method development, reducing analysis time as Solid Phase Microextraction (SPME) in gas chromatography.

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Publicación II

METABOLOMICS APPROACHES FOR THE DETERMINATION OF MULTIPLE CONTAMINANTS IN FOOD

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Current Opinion in Food Science

Metabolomics approaches for the determination of multiple contaminants in food

Rosalía López-Ruiz, Roberto Romero-González and Antonia Garrido Frenich

In the last decade metabolomics has become an important methodological approach in the field of food science. It combines analytical chemistry tools employed in food safety, as liquid chromatography and gas chromatography coupled to mass spectrometry, with sophisticated data analysis as multivariate statistics. This combination provides numerous applications in the study of contaminants and metabolites in food, and these approaches have been widely used for several topics as food safety or food frauds.

This review is focused on the main applications of metabolomics in the field of food safety, describing the separation and detection strategies commonly used and the workflow approaches performed. In addition, different modes of data acquisition (targeted and non-targeted) and data analysis that involved both acquisition modes (chemometrics and compound identification) are discussed.

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Introduction

Metabolomics is one the most important part of –omics techniques that studies small molecules or metabolites within food, organisms, plants or humans [1]. Together with genomics, transcriptomics and proteomics, metabolomics is involved in the study of foodomics approaches among others [2,3]. It has become an important tool in many research areas in the last few years [4], as food science, that involved food quality, food safety and food traceability [5]. Therefore, metabolomics approach is a suitable solution to ensure food safety, which has become a major issue worldwide, and contaminants (i.e. organic pollutants) or toxins should be monitored in order to satisfy the consumer demand [6]. Food safety involves several issues, including the legislation regarding the presence of selected compounds in foods that may be present below certain limits (maximum residue limits (MRLs)), the detection of microbial spoilage, environmental contaminants as well as banned external compounds, or natural toxins [7••].

In the field of food safety, liquid chromatography (LC) and gas chromatography (GC) coupled to mass spectrometry (MS) are the most important techniques used. They are powerful tools for the detection, assignment, quantification and elucidation of compounds/structures in complex matrices [8]. GC-MS is used for the analysis of volatile metabolites whereas LC-MS is suitable for the analysis of semi-polar and polar compounds [9]. When MS is used as detection system, low resolution mass spectrometry (LRMS) and high resolution mass spectrometry (HRMS) can be used. LRMS provides information about the nominal mass of the analyte and HRMS gives the m/z with four to six decimal digits [10]. Although both LRMS and HRMS can be used for metabolomics studies, information provided by LRMS was limited, because only analytes included in a list are usually monitored. In metabolomics studies, MS coupled to LC and GC used two analysis strategies: targeted or untargeted. In the first case a list of known compounds are firstly selected, for example pesticides and their known metabolites in multiresidue methods [11]. In contrast, untargeted analysis focuses on the detection of as many groups of metabolites as possible to obtain patterns or fingerprints without identifying nor quantifying specific compounds [4,12]. Untargeted analyses are most interesting and important in the field of metabolomics, and two strategies can be applied: fingerprinting and profiling. Fingerprinting is a fast, convenient and effective tool to classify samples based on metabolite patterns and it identifies the most important regions of the spectrum for further analysis [13]. This approach utilizes chemometric tools as principal component analysis (PCA) or partial least square discriminant analysis (PLS-DA) to classify the components obtained in the samples in different regions to obtain a typical fingerprint of the sample. In contrast, profiling is based on the identification of the most discriminant metabolites detected in samples using databases or software tools [14]. In addition, profiling can also include targeted analysis, monitoring a very limited number of metabolites, such as analytes or products of biochemical reactions [15].

Other technique recently used in the field of metabolomics is nuclear magnetic resonance (NMR) spectroscopy. When it is associated with chemometric techniques, this has the potential to elucidate the interactions between targeted analytes and the environment. NMR has several advantages as straightforward sample preparation, high sample throughput, stable chemical shifts, quantification without standards, and reliable identification of isolate metabolites [16••]. However, it has several shortcomings compared with chromatographic techniques coupled to MS [14]. For example, NMR has a limited sensitivity (compounds with concentration lower than mg/kg are not detected) and the identification in complex mixtures is difficult.

The aim of this review is the discussion of recent advances in the field of metabolomics applied to food contaminants as organic contaminants and toxins, to ensure food safety. Sample preparation as solid–liquid extraction or 'dilute and shoot', analytical modes of separation and detection as LC and GC coupled to MS, NMR, data processing and analysis (targeted and untargeted) will be discussed in this review. In addition, recent applications will be commented.

Sample treatment

Nowadays, sample treatment in metabolomics is a great concern. Metabolomics involves the determination of a large number of different compounds with different physico-chemical properties in one simple analysis. In addition, a simple and quick sample treatment is commonly sought in order to increase sample throughput. The most common sample treatments include an extraction step with organic solvents, clean-up and an additional pre-concentration step, when it is needed. Solid-phase extraction (SPE) [17], 'dilute and shoot procedure' [18], and solid–liquid extraction are commonly applied [19]. Nevertheless, QuEChERS (acronym of Quick, Easy, Cheap, Effective, Rugged and Safe) extraction procedure is usually employed in the majority of the studies in food samples when targeted and non-targeted approaches are used [7••]. For instance, Konak *et al.* [20] used the AOAC QuEChERS extraction version for the determination of sulphonamides and acetylated metabolites in baby foods. A clean-up step before ultrahigh-performance liquid chromatography (UHPLC) coupled to Orbitrap-MS analysis with PSA (primary secondary amine) and C18 was applied in order to reduce the matrix effect produced by baby food. In the same way, De Dominicis *et al.* [21] used the same QuEChERS version for the determination of pesticides, antibiotics and mycotoxins in bakery products. Instead of anhydrous sodium acetate and anhydrous magnesium sulfate, they only used anhydrous sodium sulfate as extraction salts. In contrast, in the field of fruits and vegetables, Xie *et al.* [22] used non-buffered QuEChERS extraction version for the behaviour study of one pesticide (afidopyropen) and its metabolite (M440I007). Acetonitrile (ACN) as extraction solvent and sodium chloride and anhydrous magnesium sulfate as extraction salts were used, followed by clean-up step with a mixture of PSA, anhydrous magnesium sulfate and GBC.

In the case of liquid samples, 'dilute and shoot' is used, especially when samples do not contain many matrix interferents. This is a quick and easy procedure with a small use of solvents and materials. Tengstrand *et al.* [23] used 'dilute and shoot' for the non-targeted extraction of contaminants from juice. Samples were diluted with ACN and shaken during 1 min before UHPLC coupled to time of flight (TOF) analyser. Mung *et al.* [24] used a mixture of ACN/water as extraction solvent for the quantitative metabolomics based on chemical isotope labelling to detect adulterants in milk.

SPE procedure is also employed when an important matrix effect is observed or a preconcentration step is required. SPE procedure is usually used in liquid samples as juice or milk, but also in solid samples after a previous solid–liquid extraction. This step is important when matrix effect is significant (<±20%), and interferents were reduced after the application of this clean-up step [25]. In the case of drugs, SPE procedure provided acceptable concentration factors as clean-up step [26].

Other extraction technique that is common when GC-MS is used, is solid-phase microextraction (SPME). This technique is quick and solvent-free, reducing drastically matrix effect. Bearing in mind that an SPME-fibre is used to catch the analytes and later desorb them in the injector, the selection of the fibre is crucial when a wide range of analytes-metabolites should be determined. Zhang *et al.* [27] reviewed the advantages and disadvantages of SPME in the study of environmental organic contaminants in '*in vivo*' fish. Authors indicate that the application of SPME in metabolomics methodologies would greatly improve our understanding of how fish adapt to contaminants. Future developments in material science may lead to fibre coatings that allow the simultaneous extraction of a wide range of compounds. In addition, the improvements in chromatography and MS, may yield more robust and accurate approaches for quantification as well as to reduce matrix effects. For example, in the last year, Roszkowska *et al.* [28] developed an SPME method combined with metabolomics for monitoring the exposome to contaminants in '*in vivo*' fish tissue. They used a polyacrylonitrile-C_{18} thin-film (60 μm) in the SPME step, previously prepared using spray coating method.

Separation and detection modes

Separation is an important step in metabolomics analysis. LC and GC have been the two main techniques used in the last years in this field. When these separation techniques are coupled to MS, the hyphenated technique takes the advantages of providing an important knowledge in the field of metabolomics as speed, efficiency, sensitivity, reproducibility and detection of a wide range of compounds.

Targeted and untargeted analyses can be carried out using these techniques. Both allow metabolomics studies but it is important to highlight that untargeted analyses provide more information in metabolomics studies, since targeted analysis was defined only for a group of compounds that are known previously. In targeted analysis, Konak *et al.* [20] developed a method for the detection of 11 sulphonamides and 5 acetylated metabolites in baby food using UHPLC-HRMS (Orbitrap) analyser, applying the method in 47 samples. Xie *et al.* [22] used UHPLC-LRMS (triple quadrupole (QqQ)) for the detection of afidopyropen and one metabolite in fruits (nectarine) and vegetables (cucumber), concluding that the mixed residues of afidopyropen and its metabolite at the recommended dose do not affect the human health. Gaweł *et al.* [29] determined 207 pesticide residues in honey by UHPLC–LRMS (QTRAP) and GC–LRMS (QqQ). Residues of 21 pesticides were determined in honey, being cyano-substituted neonicotinoids quantified in 77% of the samples. Table 1, reveals the main studies published in this field (targeted analysis), where HRMS [20,30,31] or LRMS [19,22,29,32] are used.

In the field of untargeted analysis, several papers were published (Table 1) [21,23,31,33,34•,35••,36–38]. As an example, Tengstrand *et al.* [23] studied different brands of orange juice to detect potential contaminants as pharmaceuticals, mycotoxins and pesticides using the technology of TOF-HRMS analyser. Few false positives were obtained and authors concluded that the proposed method can be used to identify new or unexpected chemical hazards in food as new contaminants or metabolites. In the same way, Cotton *et al.* [36] used HRMS to address three goals (i) targeted analyses of pollutants, (ii) detection of untargeted and unknown xenobiotics, and (iii) detection of metabolites useful for the characterization of food matrices. In this case Orbitrap-MS was used as analyser and 76 honey samples was studied detecting one pesticide banned in France in the samples.

Other technique recently used was NMR-based metabolomics. It has been successfully applied for the assessment of health and safety aspects of food and food processes, as well as to provide valuable information on the quality status and authenticity of food products [39] (Table 1). For instance, Søfteland *et al.* [40] utilized NMR for the identification of contaminants (pesticides and polycyclic aromatic hydrocarbons (PAHs)) in salmon feeds, evaluating how phenanthrene, benzo(a)pyrene, endosulphan and chlorpyriphos affected in the salmon health, whereas Cappello *et al.* [39] evaluated the presence of contaminants in tuna and they concluded that according to the European legislation on maximum levels of contaminants in seafood, the consumption of bluefin tuna does not represent a risk for human health.

Data acquisition and quality assessment in metabolomics

Quality assurance (QA) and quality control (QC) are two quality management processes that are necessary to achieve reliable results, and in the context of metabolomics, they are crucial for the acquisition of high quality data in high-throughput analytical chemistry laboratories [41]. QA consists of a set of planned procedures, protocols and quality criteria implemented by the laboratory quality system to ensure that the laboratory fulfils quality requirements, whereas QC involves the steps selected to monitor and control the quality of the analysis during data generation, ensuring that the results are representative of the samples tested in the study [42]. Those procedures ensure that the data obtained in the analysis of samples are consistent and reliable across different days, batches or even laboratories. In addition, improvements provided by QC and QA processes have a synergistic effect on the statistical power of metabolomics studies. Tolerances between QC/QA have been defined by the metabolomics community [41,43]. They are necessary to obtain general protocols in this field and the most widely QC checkpoints are summarized by Quintas *et al.* [42]. QA samples are prepared using reference mixtures of several analytes related to the study. QC samples consisted in a mixture of all samples in the study, and equal portions of each sample were collected and mixed. In an analytical batch related to metabolomics, QA samples were injected at the beginning of the batch (three times); between the samples (at the beginning of sample injection, in the middle and at the end); and finally at the end of the batch (three times), as it is shown in Figure 1 [44]. QC samples were injected at the beginning and at the end of the batch, as QA samples, and in addition, every 5–10 samples. The frequency depends on the number of samples included in the study and the mass analyser reproducibility. Because analysis time, resolution and raw data size, MS/MS data is not possible to obtain for all samples of the study, and therefore, QC pooled samples were also included in the analytical batch, and MS/MS were acquired for these samples.

In addition to QC and QA samples, blank extract was also injected. It was used to minimize the number of undesirable interferents from the sample collection, handling and processing. Moreover, it is used to check the carryover. Blank extract was injected at the beginning of the run, between QC and QA, before sample injection, after

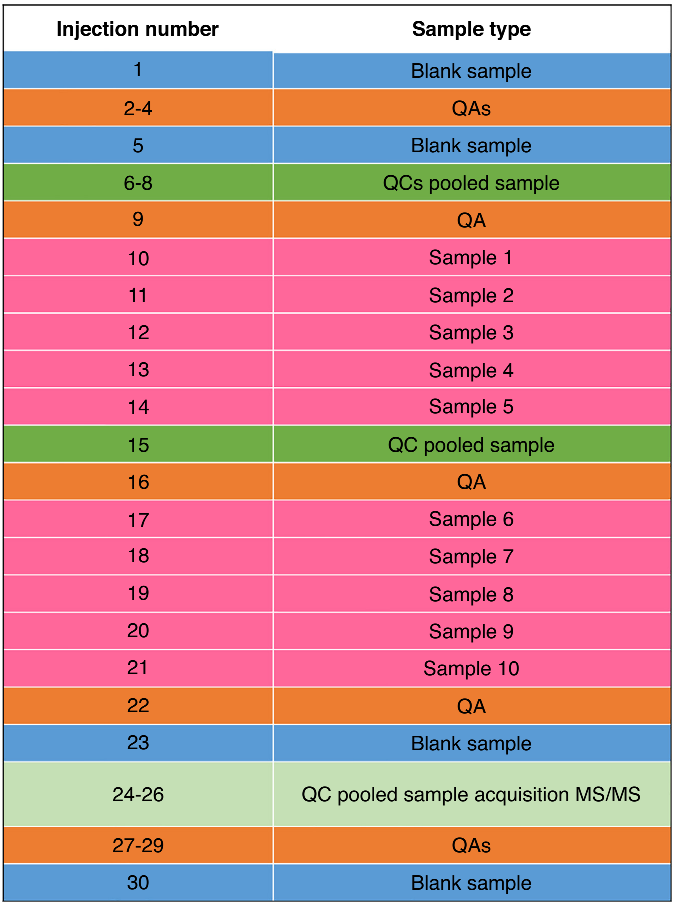
Table 1

Recent advances in metabolomics food analysis[a]

Analytes	Matrices	Sample treatment	Separation and detection	Analysis mode	Data treatment	References
Dimethenamid, saflufenacil and metabolites	Maize	QuEChERS	UHPLC-QqQ-MS	Targeted	Compound detection	[19]
Sulfonamides and metabolites	Baby food	QuEChERS	UHPLC-Orbitrap-MS	Targeted	Compound detection	[20]
Pesticides, antibiotics and mycotoxins	Bakery products	QuEChERS	UHPLC-Orbitrap-MS	Non-targeted	Chemometrics (PCA)	[21]
Afidopyropen and one metabolite	Nectarine and cucumber	QuEChERS	UHPLC-QqQ-MS	Targeted	Compound detection	[22]
Pesticides and mycotoxins	Orange juice	Dilute and shoot	UHPLC-TOF-MS	Non-targeted screening	Chemometrics (PCA)	[23]
Pesticides	Honey	QuEChERS	LC-QTRAP-MS GC-QqQ-MS	Targeted	Compound detection	[29]
Fenamidone and propamocarb and metabolites	Cucumber, tomato and courgettes	QuEChERS	UHPLC-Orbitrap-MS	Targeted and non-targeted	Compound identification	[30]
Deoxyvalenol and metabolites	Crackers, biscuits and bread	Solid-liquid	UHPLC-Q-Orbitrap-MS	Targeted and non-targeted	^{13}C labelling study	[31]
Carboxin and metabolites	Penauts	QuEChERS	UHPLC-QqQ-MS	Targeted	Compound detection	[32]
Sulfur compounds	Garlic	No data	UHPLC-HRMS	Non-targeted	Chemometrics (PCA)	[33]
Pesticides	Enfant food	QuEChERS and dilute and shoot	UHPLC-Q-Orbitrap-MS	Non-targeted	Compound identification	[34•]
Pesticides, antibiotics and mycotoxins	Honey	Dilute and shoot	UHPLC-Orbitrap-MS	Non-targeted	Chemometrics (PCA and PLS-DA)	[36]
Contaminants	Infant formula	SPE	UHPLC-TOF-MS	Non-targeted	Chemometrics (PCA and PLS-DA)	[37]
Contaminants	Tea	QuEChERS and dilute and shoot	UHPLC-QTOF-MS	Non-targeted	Chemometrics (ICA)	[38]
Contaminants	Fish	No data	NMR	Non-targeted	Chemometrics (PCA and PLS-DA)	[39]
Contaminants and contaminant mixtures	Salmon feeds	Solid-liquid	NMR FT-ICR	Non-targeted	Chemometrics (PCA)	[40]

[a] Abbreviations: FT-ICR, Fourier-Transform-Ion Cyclotron Resonance; GC, gas chromatography; HRMS, high resolution mass spectrometry; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; PCA, principal component analysis; PLS-DA, partial least square discriminant analysis; QqQ, triple quadrupole; SPE, solid phase extraction; TOF, time of flight; UHPLC, ultrahigh-performance liquid chromatography.

Figure 1



Injection number	Sample type
1	Blank sample
2-4	QAs
5	Blank sample
6-8	QCs pooled sample
9	QA
10	Sample 1
11	Sample 2
12	Sample 3
13	Sample 4
14	Sample 5
15	QC pooled sample
16	QA
17	Sample 6
18	Sample 7
19	Sample 8
20	Sample 9
21	Sample 10
22	QA
23	Blank sample
24-26	QC pooled sample acquisition MS/MS
27-29	QAs
30	Blank sample

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Typical analysis batch for metabolomics studies.

sample injection and at the end of the batch. Figure 1 summarizes a typical analytical batch in metabolomics for an untargeted analysis of food contaminants.

These methodologies are implemented in several studies, especially when statistical analysis workflow is used, as Knolhoff *et al.* [45] revealed in their study. QA and QC minimized the effects of any experimental or instrumental variability that may be present due to performance differences over time or a change in LC solvents. QC is included in the acquisition sequence to ensure the suitability of the collected data. Cladiere *et al.* [18] analyzed pesticides, mycotoxins and other contaminants using quality controls. They also included blank samples and standards in the analytical run, and bearing in mind the information obtained when blanks were injected, it was observed that no cross-contamination was detected. Finally, Bonnefoy *et al.* [44] employed QA samples, QC pooled samples and blank samples across the analytical run to ensure a correct data in *Gammarus fossarum* after *in vivo* exposure to pharmaceuticals.

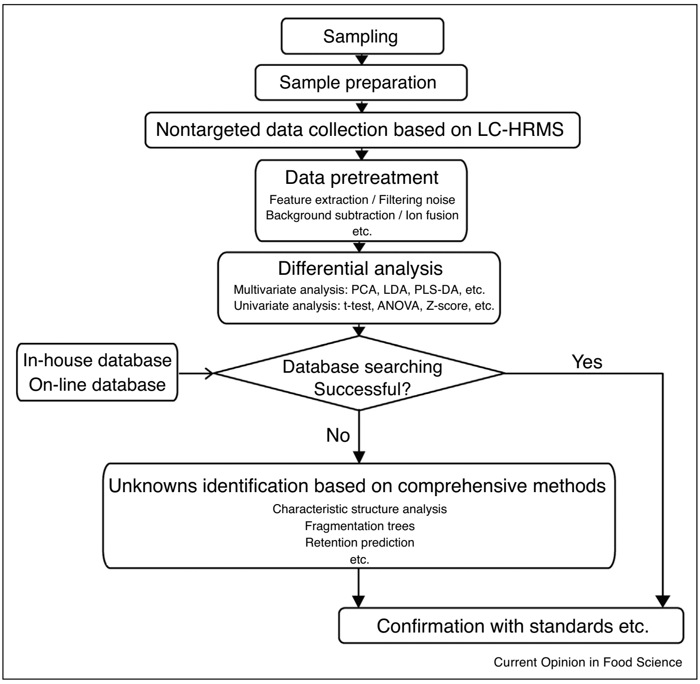
Data analysis

Before data analysis, processing raw data has to be performed. The basic steps that are mandatory in data processing are peak alignment, peak filtering, peak identification and metabolite identification. All these steps depend on the quality control followed in metabolomics batch [46•]. In targeted metabolomics, data processing only involves noise subtraction, reduction and scaling. However, non-targeted metabolomics is more time-consuming and the information remains unknown until the end of processing. This stage can be minimized because baseline correction, normalization, peak picking, alignment and integration [6].

When the data were processed, they can be studied using chemometrics followed by compound identification, showing in Figure 2 a typical workflow used in metabolomics studies [47].

Chemometrics involves statistical methods to distinguish groups of samples and is often performed in metabolomics. Chemometric methods have been applied to the analysis of food using a variety of techniques, as PCA or PLS-DA [45], which are the most used in metabolomics studies. PCA belongs to unsupervised statistics and PLS-DA to supervised statistics [48]. One of the most important parts of supervised statistics is the validation of the models built. This validation is performed to avoid false positives, and to assess the ability of the models to predict unknown samples [6,49]. Chemometrics has become an important tool for the metabolomics analysis and some instrument vendors provided different software tools that allow multivariate analysis. Software as Compound Discoverer® (Thermo Scientific), Metaboscape® (Bruker Daltonics) or XCMS (The Scripps Research Institute) are used. Recent papers as De Dominics *et al.* [21] and Tengstrand *et al.* [23] used chemometrics (PCA) for the detection of possible contaminants in bakery products (pesticides, aflatoxins, zearalenone, trichothecenes, sulphonamides and phenicol antibiotics) and orange juice (pesticide, mycotoxins and pharmaceuticals) respectively, comparing blank samples with spiked samples. Diretto *et al.* [33] used PCA to identify different distribution patterns of the sulfur and saponin metabolites between the studied ecotypes. They demonstrated that garlic should be consumed without discarding the clove tunics since they contain the most important molecules responsible for the properties of garlic. Cotton *et al.* [36] determined contaminated honey samples using mining tools as PCA and PLS-DA, detecting at least one of the 89 pollutants included in the study in 74 of the 76 samples of honey, observing a clear discrimination between

Figure 2



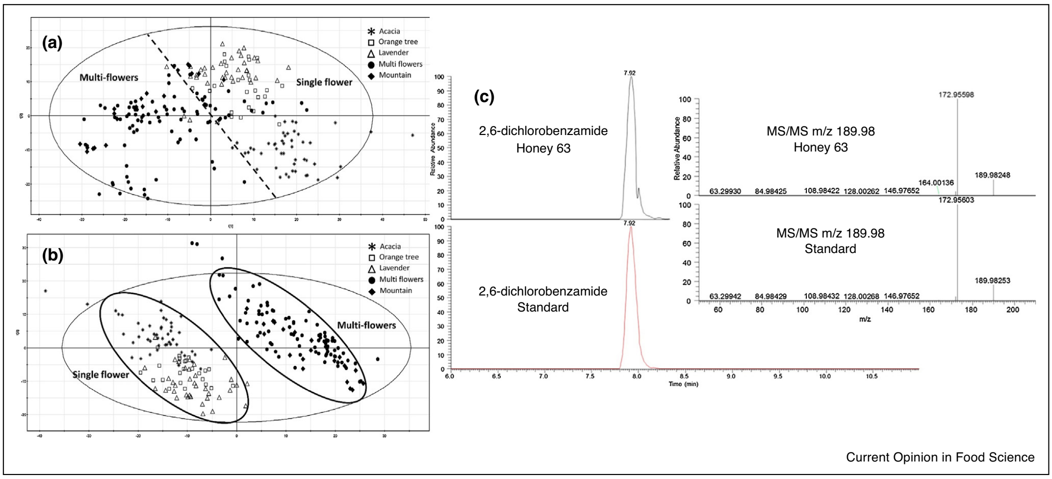
Typical workflow for food metabolomics analysis. Fu *et al.* [47]. Copyrights with permission of Elsevier.

single-flower (acacia, lavender and orange) and multifloral (multiple flowers and mountain) honeys, as it can be observed in Figure 3a. In addition, acacia honey samples were well separated from orange and lavender honey samples. Figure 3b shows the results when PLS-DA was used to confirm previous results obtained with PCA. In NMR studies, Søfteland *et al.* [40] and Cappello *et al.* [39] used the advantages of chemometrics in NMR studies (potential value to elucidate interactions between organisms and environment) to determine how the contaminants affect Mediterranean sea fish, such as salmon and tuna respectively.

The next step in data analysis was compound identification, an important issue in metabolomics analysis. In some cases, it has been performed after chemometrics stage, but when chemometrics are not possible, compound identification was carried out using raw data. As indicated in the previous section, several software as Compound Discoverer® or Metaboscape® have the ability to generate possible formulas from the parent molecule structure and also to compare the experimental full-MS and MS/MS spectra with the theoretical spectra of the different spectral databases as *m/z* Cloud, Metlin, Human Metabolome Database (HMDB) or *m/z* vault. The workflow for compound identification was as follow: several formulas were proposed according to the exact mass and the mass error set by the researcher (normally lower than 5 ppm). Once one formula was selected, if experimental MS/MS spectrum was provided, it could be compared with the theoretical MS/MS spectra (if they are available) from the databases. If the MS/MS spectra are similar and the correlation between theoretical and experimental spectra is close to 100%, the compound was putatively elucidated using databases. Moco *et al.* [8] revealed the number of metabolite records present in MS and NMR studies, pathway and chemical databases available for compound identification. Once identification is correct, confirmation with standards is required in order to give a complete and successful elucidation. For example, Delaporte *et al.* [38] carried out chemometrics using XCMS followed by compound annotation or identification using data from several databases as Metlin, *m/z* cloud and Pesticides Properties Database (PPDB). Using this procedure, 24 pesticides in tea were identified. In Figure 3c, Cotton *et al.* [36] identified a pollutant in honey by matching its retention time, accurate measured mass, isotopic pattern, and Collision-induced dissociation (CID) spectrum to those obtained with the purchased compound. Surprisingly, that compound was the metabolite of dichlobenil, an herbicide which was widely used in lavender and lavendin plantations until it was banned in France in 2010. In addition Inoue *et al.* [37] used chemometrics followed by compound databases as KEGG or Chemical Entities of Biological Interest (ChEBI) to identify two markers, one related with the increased of the concentration of nicotinic acid and other with the decreased of the concentration of nicotinamide in infant food.

Finally, other studies do not use chemometrics for the determination of metabolites. For instance, López-Ruiz *et al.* [30] putatively identified new metabolites of

Figure 3



Multivariate statistical analyses of the data set of 76 honey samples. Multifloral (●), mountain (⧫), acacia (*), orange tree (□), and lavender (△) honey samples. **(a)** PCA score plot of honey samples (excluding eucalyptus), **(b)** PLS-DA score plot of honey samples. **(c)** Identification of 2,6-dichlorobenzamide in honey ($[M + H]^+ = 189.9821$, *m/z* ±8 ppm). Cotton *et al.* [36]. Copyrights with permission of ACS publications.

fenamidone and propamocarb in vegetables. For that, the determination of possible metabolites of a contaminant was elucidated considering different reactions in the parent molecule as oxidation, reduction or methylation that can originate metabolites, using different software tools, as Compound Discoverer® and MassChemSite® [30].

Future outlook and conclusions

As it has been shown in this review, metabolomics has become an important topic for food safety in the last few years. However, there are some disadvantages in this field. Databases are still in progress and some compounds are not included yet and their elucidation is not straightforward. In contrast, the use of chemometrics is a complex stage, where a previous knowledge is required, so this type of studies required qualified researches. In addition, most of published studies used LC-MS meanwhile GC-MS is not used a lot yet, and HRMS is the best detection system for metabolomics. However, HRMS analysers are more expensive compared with LRMS, so it is difficult that this technology is implemented in routine analysis for metabolomics studies.

In the future, the development of metabolomics can be increased due to the introduction of new techniques as ion mobility separation (IMS). IMS provides a new dimension in addition to chromatography and MS. One of the benefits of IMS is allowed the monitoring of quality attributes during food processing. Because IMS allows *in situ* automatic sampling, it can be used for determining the completion of certain processes assuring standard quality based in a group of metabolites. Evaluation of food quality during food processing is a really important step to evaluate losses of food properties and to study the behaviour of contaminants and toxic substances during this process. For example, Marín-Sáez *et al.* [50] determined the effect of the boiling process in tea and pasta samples in tropane alkaloids concentration. They determined that tropane alkaloids mainly degraded into tropinone and tropine after boiling process and another tropane alkaloid (scopine) was also detected by non-targeted studies. In addition, other advances such as the orthogonal approach of HRMS with NMR technologies can be useful to increase the scope of the analysis as well as the reliability of the identification processes. All data generated with the coupling of both techniques and the introduction of all these data into databases or libraries can improve the advances in the field of compound identification.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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CAPÍTULO

3

Estudios de disipación de insecticidas e identificación de sus metabolitos en matrices alimentarias

1. INTRODUCCIÓN

El estudio del comportamiento de insecticidas en los alimentos es de gran interés. Se requiere conocer como éstos se degradan o se transforman en otros compuestos para que esté garantizada la seguridad alimentaria. Son diversos los estudios que determinan el comportamiento de insecticidas mediante estudios de disipación y/o degradación en alimentos [1]. Estos se encargan de determinar la persistencia de los insecticidas en el fruto, con el fin de establecer el tiempo de espera (plazo de seguridad) que es necesario para asegurar un consumo seguro de los mismos. La técnica de análisis más empleada para su determinación suele ser LC o GC acopladas a MS [2], usando como métodos de extracción la SLE [3] o el QuEChERS [4].

Asimismo los insecticidas pueden transformarse en metabolitos, que pueden permanecer en el fruto incluso más tiempo que el compuesto progenitor y ser más tóxicos que el mismo [5]. Para algunos insecticidas como flonicamida se conocen algunos de sus metabolitos generados tras su aplicación en campo, y de hecho algunos de ellos se incluyen en la definición de MRL [6]. Sin embargo, para muchos insecticidas este campo aún no ha sido estudiado. Es por ello que resulta interesante llevar a cabo estudios del comportamiento de insecticidas en fruto, evaluando no solo la eliminación del compuesto progenitor, sino también comoéste se degrada en metabolitos. Para ello es necesario el empleo de la HRMS, debido a su modo de trabajo en *full scan* para determinar los residuos en los frutos y posteriormente, mediante análisis *unknown,* identificar posibles nuevos metabolitos provenientes del compuesto progenitor y no descritos previamente.

Por consiguiente, los trabajos incluidos en este capítulo se han focalizado en el desarrollo de métodos analíticos para el estudio del comportamiento de insecticidas específicos en matrices alimentarias, tales como naranja, pimiento, tomate, calabacín y pepino (**Artículos científicos III, IV y V**). Por otro lado, también se ha realizado la búsqueda de metabolitos procedentes de cada uno de los compuestos objetivo mediante análisis *unknown,* haciendo uso tanto de software que permiten establecer rutas metabólicas, así como de búsqueda de desconocidos (**Artículos científicos IV y V)**.

- **Artículo científico III.** Determination of flonicamid and its metabolites in bell pepper using ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (Orbitrap)

- **Artículo científico IV.** The metabolic pathway of flonicamid in oranges using an orthogonal approach based on high-resolution mass spectrometry and nuclear magnetic resonance

- **Artículo científico V.** Dissipation study of the proinsecticide thiocyclam and nereistoxin in tomato using liquid chromatography high resolution mass spectrometry

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Publicación III

DETERMINATION OF FLONICAMID AND ITS METABOLITES IN BELL PEPPER USING ULTRA-HIGHPERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO HIGH-RESOLUTION MASS SPECTROMETRY (ORBITRAP)

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Determination of flonicamid and its metabolites in bell pepper using ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (Orbitrap)

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ABSTRACT

The development and validation of a method to determine flonicamid and its metabolites as TFNA (4-trifluoromethylnicotinic acid), TFNG (N-(4-trifluoromethylnicotinoyl) glycine) and TFNA-AM (4-trifluoromethylnicotinamide) in bell pepper samples by ultra-high-performance liquid chromatography coupled to Orbitrap mass spectrometry (UHPLC-Orbitrap-MS) was performed. A fast and simple extraction procedure with acidified acetonitrile and salts (magnesium sulfate and sodium chloride) was used. The methodology was validated, checking for specificity, recovery, precision, limits of detection (LODs) and limits of quantification (LOQs). The recoveries ranged from 84% to 98%, and precisions were lower than 17%. Finally, LODs ranged from 1 μg kg^{-1} (flonicamid) to 6 μg kg^{-1} (TFNA-AM), while LOQs ranged from 10 μg kg^{-1} (flonicamid) to 30 μg kg^{-1} (TFNA-AM). Bell pepper samples were analysed and concentrations up to 98 μg kg^{-1} (flonicamid) were detected, although the sum of flonicamid and metabolites did not exceed the maximum residue limit (MRL) set by the European Union.

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KEYWORDS
Flonicamid; metabolites; Orbitrap; UHPLC; HRMS; QuEChERS; TFNA; TFNG; TFNA-AM

Introduction

Flonicamid (N-(cianomethyl)-4-(trifluoromethyl)nicotinamide), a pyridinecarboxamide compound, is a relatively novel systemic pesticide that kills aphid pest (aphids) and Aleyrodidae (whiteflies). This compound shows selective activity against similar insecticides with cross-resistance (Seo et al. 2015). When this pesticide is applied to crops, it reaches the inner parts of the plant and turns into a variety of metabolites. Many of them are biologically active, and they are retained to a considerable extent by plants, exhibiting high toxicity (Sabatino et al. 2013; Son et al. 2013). Flonicamid's degradation usually occurs at normal conditions (EFSA 2010), and the main metabolites detected in fruits and vegetables are 4-(trifluoromethyl)nicotinol glycine (TFNG), 4-trifluoromethylnicotinic acid (TFNA) and 4-trifluoromethilnicotinamide (TFNA-AM) (Hengel & Miller 2007; Xu et al., 2011) (Figure 1), and the MRL may be exceeded due to the appearance of metabolites (Jung et al. 2016).

The MRL of flonicamid in fruits and vegetables is the total sum of the parent compound, flonicamid and its metabolites, TFNA and TFNG, according to European Commission Regulation (EU) No. 67/2016), which is 0.3 mg kg^{-1}. The other metabolite TFNA-AM has not been included in the MRL for fruit and vegetables, but this has been included in the MRL of flonicamid in samples from animal origin (Pesticides Data Base 2015; European Commission Regulation (EU) No. 67/2016).

Concerning the sample treatment for the extraction of flonicamid and its metabolites from fruit and vegetables, mainly a QuEChERS approach (Xu et al. 2011; Chen et al. 2013; Ko et al. 2014; EURL-SRM 2015; Seo et al. 2015; Jung et al. 2016) and solid–liquid extraction with solvents such as methanol or acetonitrile, followed by SPE as a clean-up step (Hengel & Miller 2007; Xu et al. 2011) have been used previously.

For the separation and detection of the target compounds, LC-MS, using low-resolution analysers, has been mainly used (Hengel & Miller 2007; Szczesnieswski et al. 2009; Xu et al. 2011; Chen et al. 2013; EURL-SRM 2015; Seo et al. 2015; Jung et al. 2016). To our knowledge, no method uses high-resolution mass spectrometry (HRMS) for the

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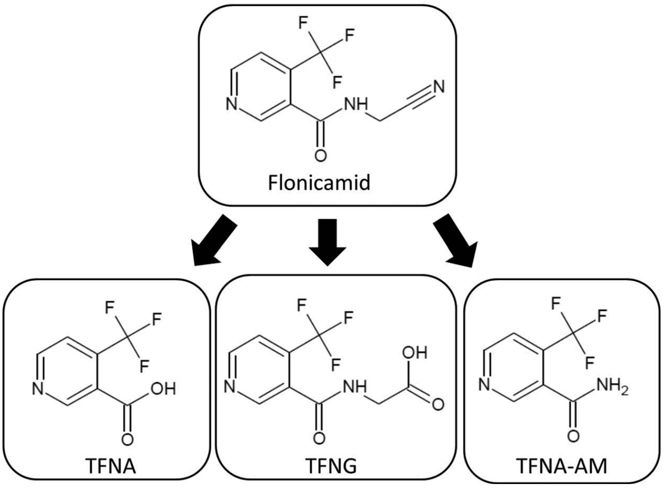

Figure 1. Structure of flonicamid and its related metabolites.

detection of flonicamid and its metabolites in vegetables and/or related matrices, and only multiresidue methods are used that include flonicamid (Zomer & Mol 2015). HRMS is the most suitable technique for the identification of compounds, bearing in mind that several parameters such as retention time window (RTW), accurate mass error (< 5 ppm) and isotopic pattern are the main tools used for the reliable identification of the compounds. The use of full-scan ultra-high-performance liquid chromatography coupled to Orbitrap-MS (UHPLC-Orbitrap-MS) (Makarov et al. 2006) allows for the detection of a wide range of compounds at low concentration levels in complex sample matrices with high mass accuracy. HRMS analysers have several important features for the detection and identification of compounds and their metabolites, and target and non-target analysis modes can be performed (Senyuva et al. 2015).

The aim of this study is the development and validation of an analytical method for the quantitative determination of flonicamid and its metabolites, TFNA, TFNG and TFNA-AM, in bell pepper samples using a fast and simple extraction method, and by applying UHPLC coupled to Orbitrap-MS for the identification of the compounds. Moreover, validation of a method was carried out by studying the parameters determined by SANTE guidelines (Document No. SANTE/11945/2015) such as specificity, recovery, precision, LODs and LOQs. The developed method was also successfully applied to the analysis of real samples collected from greenhouses.

Materials and methods

Equipment, material and reagents

Flonicamid (CAS Registry No. 158062-67-0, purity > 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). TFNA (CAS Registry No. 158063-66-2, ≥ 98% purity) and TFNG (CAS Registry No. 207502-65-6, > 99% purity) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany), while TFNA-AM (CAS Registry No. 158062-71-6, > 99% purity) was obtained from Apollo Scientific Ltd (Manchester, UK).

Stock solutions (1000 mg l^{-1}) were prepared by dissolving 50 mg of the pure compound in methanol (50 ml). An intermediate solution of the four compounds (10 mg l^{-1}) was prepared by taking 100 µl of each stock solution and diluting up to 10 ml with methanol in a volumetric flask. All stock and intermediate solutions were stored at 4ºC. Stock solutions were stable for a year and intermediate solution for 2 months.

Acetonitrile (ACN) and methanol (MeOH) (both LC-MS grade) were acquired from Fluka (St. Louis, MO, USA). Water (LC-MS grade) was acquired from J.T. Baker (Deventer, the Netherlands) and formic acid was obtained from Fisher Scientific (Erembodegem, Belgium). Magnesium sulfate (Sigma-Aldrich), sodium chloride (J.T. Baker), primary secondary amine (PSA) (Scharlab, Barcelona, Spain) and graphitised carbon black (GCB) (Scharlab) were used during the extraction procedure.

A mixture of acetic acid, caffeine, Met-Arg-Phe-Ala-acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrat and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative) from Thermo-Fisher (Waltham, MA, USA) were employed for accurate mass calibration of the Orbitrap analyser.

For the treatment and preparation of samples, an analytical balance AB204-S (Mettler Toledo, Greifensee, Switzerland), a vortex mixer WX from Velp Scientifica (Usmate, Italy), a Reax 2 rotatory agitator from Heidolph (Schwabach, Germany), a Centronic BL II centrifuge (J.P. Selecta, Barcelona, Spain) and a Polytron PT2100 (Kinematica A.G., Littan/Luzern, Switzerland) were used.

UHPLC-Orbitrap-MS analysis

A Thermo Fisher Scientific Transcend^{TM} 600 LC (Thermo Fisher Scientific, San Jose, CA, USA) was used for chromatographic analysis.

A Zorbax Eclipse Plus C_{18} column (100 × 2.1 mm, 1.8 µm particle size) supplied by Agilent Technologies (Santa Clara, CA, USA) was used. A flow rate of 0.2 ml min^{-1} was set for separation of the selected compounds in the UHPLC system. The mobile phase consisted of eluent A, which was a water solution of 0.1% formic acid and eluent B, formed by an organic solution of MeOH:ACN (80:20 v/v) containing 0.1% formic acid.

The step gradient was as follows: 0–4 min 95% B; then it was linearly decreased to 10% in 30 s, and remained constant during 1 min. The total running time was 5.5 min. The column temperature was set at 25°C and the injection volume at 10 µl.

The chromatographic system is coupled to a single mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive^{TM}, Thermo Fisher Scientific, Bremen, Germany) using an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose) in positive mode. ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N_2, > 95%), 35 (adimensional); auxiliary gas (N_2, > 95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305°C; capillary temperature, 300°C. The mass spectra were acquired employing two alternating acquisition functions: (1) full MS, ESI+, without fragmentation (higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 FWHM; scan time = 0.25 s; and (2) all-ion fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy = 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s.

The chromatograms were acquired using the external calibration mode and they were processed using Xcalibur^{TM} version 2.2, with Quanbrowser and Qualbrowser, and Mass Frontier^{TM} 6.0 (Thermo Fisher Scientific, Les Ulis, France).

Sample extraction

Samples were treated as follows: 1 kg of sample was crushed and homogenised, without neglecting any part thereof, skin or outer shell or bones as established by current regulations (Document No. SANTE/11945/2015), storing it in the freezer at −21°C. Then, 10 g of homogenised sample were introduced in a 50 ml plastic centrifuge tube. After that, 10 ml of acidified acetonitrile (0.1% formic acid, v/v) were added and shaken for 1 min in a Polytron. The sample was then stirred for 15 min in a rotary shaker and 1 g of NaCl and 4 g of $MgSO_4$ were added, and the mixture was shaken vigorously for 1 min in a vortex. After that the samples were centrifuged for 10 min at 5000 rpm (4136*g*) and 1 ml of the supernatant was collected and injected into the UHPLC-Orbitrap.

Samples

Fresh fruit (bell pepper) was donated by greenhouse farmers located in Almeria, Spain. The samples were collected at least 10 days after the last application of technical product (Teppeki®) by foliar application. Approximately 100 g of technical product were applied per hectare twice, waiting at least 1 week between the first and second applications.

Method validation

The validation of the analytical method was carried out according to SANTE guidelines (Document No. SANTE/11945/2015), calculating the following parameters: matrix effect, linearity and working range, trueness (% recovery), precision (intra- and inter-day), and LOQs and LODs.

The matrix effect was carried out by analysing standards at different concentrations in solvent (MeOH) and standards prepared in matrix extract. For flonicamid and TFNG, the concentrations were 10, 60, 100, 200 and 300 µg l^{-1}, for TFNA 20, 30, 60, 100, 200 and 300 µg l^{-1}, and for TFNA-AM 30, 60 100, 200 and 300 µg l^{-1}.

Linearity was performed using matrix-matched calibration, spiking blank extracts at five concentration levels of flonicamid and its metabolites (from 10 to

300 μg l^{-1}). The linearity of the calibration curves was evaluated by the determination coefficients (R^2).

Precision was estimated by performing intra- and inter-day studies, expressed as RSD %. Intra-day precision (repeatability) was studied at two different concentration levels for each analyte within the same day. For flonicamid and TFNG at 10 and 100 μg kg^{-1}, for TFNA at 20 and 100 μg kg^{-1}, and for TFNA-AM at 30 and 100 μg kg^{-1}, performing five replicates at each level. Inter-day precision (reproducibility) was assessed by fortifying samples at the same concentration levels of intra-day precision, and they were analysed on 10 different days. Trueness was evaluated in terms of recovery by spiking blank matrix (previously checked) at the same concentration levels selected for precision studies.

LODs and LOQs were estimated by injecting extracted blank samples spiked at low concentrations (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 and 30 μg kg^{-1}). LODs were assigned as the minimum concentration at which the characteristic ion is monitored with a mass error lower than 5 ppm. For the estimation of LOQ, in addition to the characteristic ion, one fragment should be monitored at the same retention time and chromatographic shape than the characteristic one, being the mass error lower than 5 ppm.

Moreover, to verify if the applied method is sensitive enough, the SANCO/12574/2014 document that focused on complex residue definitions was applied (Document No. SANCO/12574/2014 30/11-01/12 2015 rev. 5), and it was checked if the sum of the LOQs of the individual analytes obtained was equal to or lower than the MRL set at the LOQ. For this, the following equation was applied, where Mm is the molecular mass:

$$\begin{aligned} Sum\ of\ LOQs = {} & LOQ_{Flonicamid}\frac{Mm_{Flonicamid}}{Mm_{Flonicamid}} \\ & + LOQ_{TFNA}\frac{Mm_{Flonicamid}}{Mm_{TFNA}} \\ & + LOQ_{TFNG}\frac{Mm_{Flonicamid}}{Mm_{TFNG}} \end{aligned}$$

If the sum of LOQs is ≤ MRL (0.3 mg kg^{-1}), the sensitivity check is good. TFNA-AM was not included in the sum of LOQs because it is not included in the current MRL for vegetables.

Results and discussion

UHPLC-Orbitrap-MS optimisation

First, the spectrometric characterisation of the compounds was carried out. For that purpose, an intermediate solution of 1 mg l^{-1} was injected into the UHPLC-Orbitrap-MS system. The protonated ion of each compound was monitored according to its molecular formula, and the exact masses were selected with the criterion that mass error was lower than 5 ppm for the target compounds. Then fragments of each analyte were studied according to the procedure described by Gómez-Pérez et al. (2014). In order to identify the compounds, spectra acquired after applying a fragmentation step by HCD were studied for each compound. These fragments were produced in the collision cell (based on a straight multipole mounted inside a metal tube), and all the ions generated in the ion source were fragmented (all ion fragmentation). Note that the ions and the fragments generated in the HCD were monitored in the Orbitrap at 10,000 FWHM because it is the most suitable value for the identification of fragment ions (Gómez-Pérez et al. 2014), as well as to obtain enough points per peak to perform a reliable quantification of the detected compounds.

The procedure used for the identification of the fragments involved two different approaches. On the one hand, the application of software tools available on the market, and herein, Mass Frontier™ version 6.0 was chosen. From the chemical structure of the compound it is possible to determine the fragmentation mechanisms and the corresponding fragment ions, with their corresponding exact masses. Using this approach, looking for common fragments of the target compounds is also possible. On the other hand, by comparison of MS and HCD (MS/MS) spectra at the retention time of the target compound in order to determine different ions is another approach. The most intense ions observed in the HCD spectra were selected in both traces (with and without HCD). Thus, if one ion was observed in both spectra but the intensity was higher in the HCD spectrum, it might indicate that this ion had been fragmented from the parent compound. In this case, experimental formula, obtained using the software Qualbrowser™, was evaluated to determine if it comes from the parent structure. Table 1 shows that there are two common fragments for the compounds included in this study corresponding to $C_7H_3NOF_3$ and $C_6H_5NF_3$. Figure 2 shows theoretical, experimental and fragment mass spectra of the parent compound, flonicamid and one of its metabolites, TFNA.

Secondly, chromatographic conditions were studied. Mobile phase formed by MEOH:acidified water (0.1% formic acid) was evaluated. Poor results were obtained for TFNA-AM, which shows bad peak shape, and bearing in mind that acetonitrile was also used as organic phase for the separation of flonicamid and related compounds (Makarov et al. 2006; Hengel & Miller 2007; Szczesnieswski et al. 2009), different mixtures of

Table 1. HRMS parameters used for identification of the target compounds.

	Precursor ion		Fragments			
Compound	Exact mass (*m/z*)	Mass error (ppm)	Exact mass (*m/z*)	Molecular formula	Mass error (ppm)	Retention time window (min)
Flonicamid	230.05357	−0.013	203.04267	$C_8H_6ON_2F_3$	−2.630	4.57–4.67
			174.01612	$C_7H_3ONF_3$	−0.086	
			148.03686	$C_6H_5NF_3$	−1.016	
TFNA	192.02669	0.002	174.01612	$C_7H_3ONF_3$	−1.810	4.65–4.75
			148.03686	$C_6H_5NF_3$	−0.678	
TFNG	249.04815	−0.013	203.04267	$C_8H_6ON_2F_3$	−0.512	4.45–4.55
			174.01612	$C_7H_5ONF_3$	−0.937	
			148.03686	$C_6H_5NF_3$	−1.448	
TFNA-AM	191.04267	−0.021	174.01612	$C_7H_5ONF_3$	3.880	4.10–4.20
			148.03686	$C_6H_5NF_3$	−0.250	

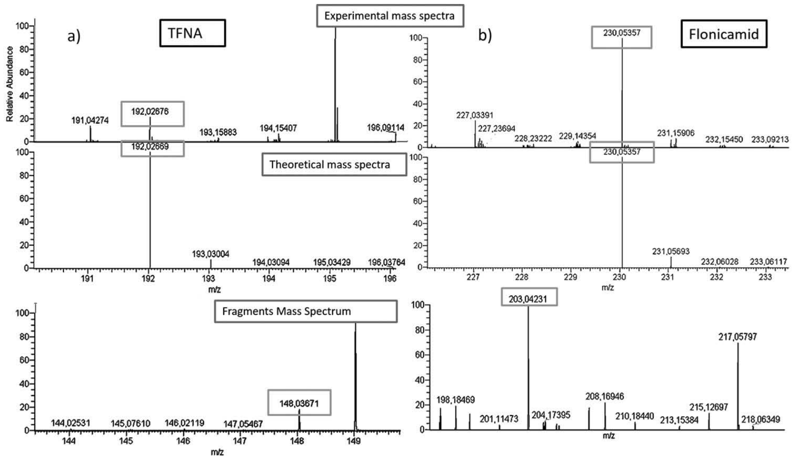

Figure 2. Experimental, theoretical and fragments mass spectra of (a) TFNA and (b) flonicamid (tested concentration = 1 mg l^{-1}).

MeOH:ACN containing formic acid (0,1% v/v) were evaluated (80:20, 60:40, 40:60, 20:80, 0:100, v/v). The best results were obtained when a mixture of MeOH: ACN 80:20 (v/v) containing formic acid (0.1% v/v) was used. Gradient elution was optimised in order to improve the peak shape of TFNA-AM and three different gradients from bibliography (EURL-SRM 2015; Xu et al. 2011) were tested, but poor results (bad peak shape) were obtained. Therefore, and using the mixture of MeOH:ACN optimised previously, several gradient profiles were evaluated obtaining suitable results with the conditions described in the 'UHPLC-Orbitrap-MS analysis' section. Finally, Figure 3 shows the chromatograms of the target compounds spiked at 300 μg kg^{-1} in blank bell pepper.

Optimisation of the extraction procedure

The extraction procedure was based on the methodology developed by the EURL-SRM method (EURL-SRM 2015). This is based on acidified QuEChERS, using acetonitrile containing 1% (v/v) formic acid and a mixture of salts (magnesium sulfate and sodium chloride). When this method was evaluated, at low (10, 20 and 30 μg kg^{-1}) and high concentrations (100 μg kg^{-1}), recoveries ranged from 50% to 70%, and RSD values higher than 25% were obtained. In consequence, a stirring step with a polytron was tested before a rotary shaker step in order to improve recovery as well as the repeatability of the method. Recoveries improved from 84% (for TFNG) to 98% (for TFNA), with RSDs < 20%. Afterwards, to check if the addition of cleaning sorbents improved the results, as it has been indicated previously (Xu et al. 2011), a clean-up step based on dispersive solid-phase extraction (d-SPE) was evaluated. PSA and GCB were tested sorbents for this clean-up step. First, 50 mg of PSA were used, but the results were not improved and recoveries ranged from 65% (TFNG) to 85% (flonicamid). Then a mixture of 50 mg GCB and 50 mg PSA was evaluated, but recoveries decreased and they ranged from 60% (TFNA-AM) to 80% (flonicamid). Therefore, this cleaning

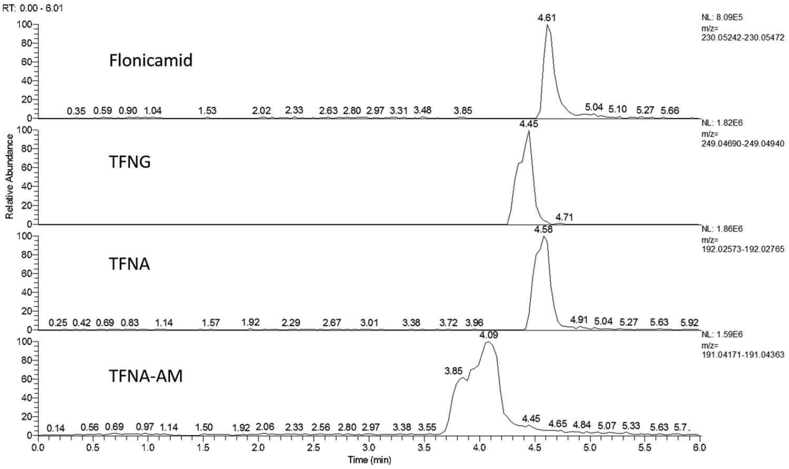

Figure 3. Chromatograms of a blank bell pepper spiked with the target compounds (0.3 mg kg^{-1}) applying the optimal conditions.

step was eliminated, and it was considered as not necessary in matrices with a high water content because the analytes would be retained in the binding sites of PSA and GCB.

Method validation

First, the matrix effect was evaluated, and several concentrations (from 10 to 300 μg l^{-1}) were analysed in pure solvent (MeOH) and in a blank extracted sample of bell pepper. The slopes obtained in the calibration with matrix-matched standards were compared with those obtained with solvent-based standards. If the ratio of the slopes was lower than 0.8, there was a suppression effect, whereas if it was higher than 1.2, an enhancement effect occurred. The matrix significantly suppresses the response for TFNG (Table 2), whereas the matrix induced a chromatographic response enhancement effect for TFNA. Therefore, matrix-matched calibration was used for quantification purposes, although for the other compounds (flonicamid and TFNA-AM) a matrix effect could be considered as negligible (Table 2).

Then linearity and working range were evaluated by spiking blank bell pepper samples with different concentrations of flonicamid and its metabolites. The linearity range was from 10 to 300 μg l^{-1} for flonicamid and TFNG, from 20 to 300 μg l^{-1} for TFNA, and from 30 to 300 μg l^{-1} for TFNA-AM. Good linearity was found, with determination coefficients > 0.99. The working range performed like linearity and ranged from 10 to 300 μg kg^{-1} for flonicamid and TFNG, from 20 to 300 μg kg^{-1} for TFNA, and from 30 to 300 μg kg^{-1} for TFNA-AM, allowing the quantification of the analytes at trace levels.

In order to evaluate the trueness of the proposed method, blank samples were spiked at two different concentration levels for each analyte (see the 'Samples' section). Table 2 shows the obtained results when each concentration level was extracted five times. It can be observed that recoveries ranged from 88% to 98% at the lowest concentration tested. As expected, adequate recoveries (in the range 84–91%) were also obtained at high concentration (100 μg kg^{-1}).

Intra-day precisions ranged from 6% to 16%, obtaining the highest value for TFNA and the lowest value for

Table 2. Validation parameters of the optimised method.

	R^2	Matrix effect	Concentration (μg kg^{-1})	Recovery (%)	Intra-day precision (% RSD)	Inter-day precision (% RSD)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
Flonicamid	0.9951	0.86	10	88	12	17	1	10
			100	91	8	7		
TFNG	0.9930	0.62	10	89	11	15	1	10
			100	84	11	7		
TFNA	0.9996	1.18	20	98	16	5	5	20
			100	88	7	7		
TFNA-AM	0.9999	0.95	30	88	6	4	6	30
			100	87	10	3		

TFNA-AM, whereas inter-day precision ranged from 3% (TFNA-AM) to 17% (flonicamid).

Individual LODs ranged from 1 to 6 μg kg^{-1}, whereas LOQ values varied from 10 to 30 μg kg^{-1} (Table 2). These limits are below the MRL of flonicamid in bell pepper (0.3 mg kg^{-1}) (Pesticides Data Base 2015), which takes into account flonicamid and its metabolites. Moreover, the sum of LOQs was calculated (Document No. SANCO/12574/2014 30/11-01/12 2015 rev. 5) as described previously, and this value was 43.3 μg kg^{-1}, which is much lower than the MRL set for flonicamid in bell pepper, (0.3 mg kg^{-1}), so the developed method is sensitive enough for the determination of this compound and related metabolites in bell pepper.

Analysis of samples

The validated method was applied for the simultaneous determination of flonicamid and its metabolites in 20 bell pepper samples collected in greenhouses from Almeria province.

In order to guarantee the quality of the results, an internal quality control was carried out in every group of samples. This quality control means the analysis in each set of samples of a matrix-matched calibration, a reagent blank and a spiked blank sample at 100 μg kg^{-1}.

The results are shown in Table 3. It can be observed that the concentration of the sum of flonicamid is lower than the MRL set for flonicamid in all the positive bell pepper samples (Pesticides Data Base 2015). Flonicamid and/or its metabolites were detected in seven samples at concentrations higher than their LOQs at concentrations ranging from 11.5 to 98.2 μg kg^{-1}. In relation to the metabolites, TFNA was detected in only one sample at 20.1 μg kg^{-1}. TFNG was found in all the positive flonicamid samples at concentrations from 19.8 to 61.9 μg kg^{-1}. Moreover, it was also detected in the other three samples at concentrations between 12.7 and 34.0 μg kg^{-1}, where flonicamid was not detected. This fact highlights the need for the inclusion of these metabolites in routine analysis in order to fulfil European Union regulations regarding MRL. Moreover, the TFNA-AM metabolite was detected in three samples, but below its LOQ. Figure 4 shows the chromatogram of sample 1 where flonicamid was detected at 98.2 μg kg^{-1}, TFNA at

Table 3. Concentration (μg kg^{-1}) of the target compounds in positive bell pepper samples.

Sample	1	2	3	4	5	6	7	8	9	10
Flonicamid	98.2	12.6	< LOD	< LOD	< LOD	97.7	< LOD	< LOD	11.5	< LOD
TFNA	20.1	< LOQ	< LOQ	< LOD	< LOD	< LOD	< LOD	< LOQ	< LOD	< LOD
TFNG	58.0	19.8	< LOD	22.2	34.0	42.2	< LOQ	< LOQ	61.9	12.7
Flonicamid sum[a]	175.9	30.9	< LOQ	20.5	31.4	136.7	< LOQ	< LOQ	68.7	11.8
TFNA-AM	< LOQ	< LOD	< LOD	< LOD	< LOD	< LOQ	< LOD	< LOD	< LOQ	< LOD

Note: [a]Flonicamid sum $= 1C_F + \frac{Mm_F}{Mm_{TFNA}} C_{TFNA} + \frac{Mm_F}{Mm_{TFNG}} C_{TFNG}$, where C_F, C_{TFNA} and C_{TFNG} are the concentration of flonicamid, TFNA and TFNG respectively; and Mm_F, Mm_{TFNA} and Mm_{TFNG} correspond to the molecular mass of flonicamid, TFNA and TFNG respectively.

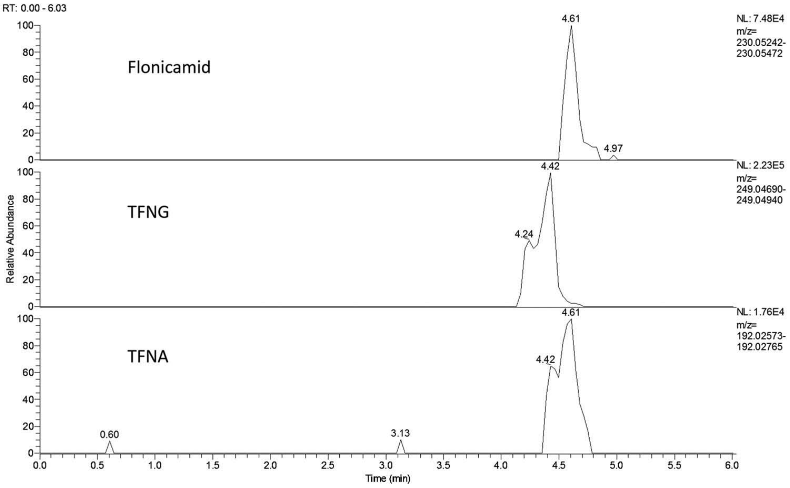

Figure 4. Extracted ion chromatograms of flonicamid (98.2 μg kg^{-1}), TFNA (20.1 μg kg^{-1}) and TFNG (58.0 μg kg^{-1}) detected in sample 1.

20.1 μg kg^{-1} and TFNG at 58.0 μg kg^{-1}, whereas TFNA-AM was not detected above its LOQ.

So far it is not possible to compare the current results with previous studies, bearing in mind that this is the first study where positive samples of flonicamid were detected, because in previous studies degradation studies were performed, where flonicamid was spiked with blank samples to evaluate the degradation of this compound (Xu et al. 2011; Seo et al. 2015).

Conclusions

The presence of pesticide residues in vegetables requires powerful analytical methodologies capable of their identification and quantification, including their metabolites, at low concentration levels. Therefore, the analytical capabilities of the platform integrated by UHPLC-HRMS Orbitrap have been evaluated for the implementation of target analysis of current pesticides. For the quantitative target analysis of flonicamid and its metabolites, the developed method presents appropriate linearity, recoveries and precision, with LOQs < MRLs. On the other hand, the study of real samples has revealed that the concentration of flonicamid and its metabolites is in some cases high, although the MRLs of the sum of flonicamid has not been exceeded. According to the results included in this study, the addition of flonicamid metabolites in routine multiresidue analytical methods is mandatory due to metabolites that can be detected, although the parent compounds were not found in some samples.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Publicación IV

THE METABOLIC PATHWAY OF FLONICAMID IN ORANGES USING AN ORTHOGONAL APPROACH BASED ON HIGHRESOLUTION MASS SPECTROMETRY AND NUCLEAR MAGNETIC RESONANCE

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The metabolic pathway of flonicamid in oranges using an orthogonal approach based on high-resolution mass spectrometry and nuclear magnetic resonance†

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An orthogonal approach has been used to perform a metabolic profiling study of flonicamid in orange fruits. Nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) have been applied to monitor the degradation pathway of flonicamid into its metabolites in field and laboratory studies. 4-Trifluoromethylnicotinic acid (TFNA) and 4-(trifluoromethyl)nicotinol glycine (TFNG) were detected after 15 days of flonicamid application in field studies, whereas in laboratory trials, TFNG was found in oranges after 70 days of flonicamid application. The results were confirmed through the use of one-dimensional ^{1}H, ^{2}H and ^{19}F NMR, observing the formation of TFNG as well as the detection of *N*-(4-trifluoromethylnicotinoyl)glycinamide (TFNG-AM) as a transient species, which is also a known flonicamid metabolite. The deuteration of the methylenic carbon of both flonicamid and TFNG was also observed, and as a matter of a fact, exploited for the first time as a route for selective isotope labeling. Orthogonal techniques, such as high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) have been applied for the first time in the study of the metabolic pathway of flonicamid in oranges. Thus, a comprehensive view of the transformation process has been achieved taking advantage of the possibilities of both techniques.

Introduction

In the intensive farming of fruits and vegetables, a variety of pesticides are applied to prevent or eliminate harmful pests from plants. These pesticides can remain in the edible parts as residues entering into the food chain.[1,2] In most of the cases, pesticides can be transformed into metabolites, which are intermediate products of metabolism formed either in plants or animals. According to the Regulation (EC) no. 1107/2009[3] degradation products formed in the environment should also be considered for the evaluation and authorization of the parent compound, bearing in mind that degradation products or metabolites can be more toxic than the parent compound itself.[4,5]

In general, metabolites are generated by metabolic and simple reactions, which in many cases are common among several families of pesticides. In these cases, the metabolite profiling strategy, defined as the identification and quantification of a selected number of pre-defined metabolites, which is generally related to a specific metabolic pathway(s),[6] is a good choice to establish the metabolic behavior and identification of both endogenous and exogenous metabolites, and suitable techniques able to monitor these compounds and identify metabolic pathways are needed to perform a comprehensive study of the degradation of the parent compound.

Flonicamid (*N*-cyanomethyl-4-trifluoromethylnicotinamide) is a systemic insecticide to control aphids, and it is mainly applied by foliar spraying. Its degradation usually occurs under normal conditions,[7] 4-(trifluoromethyl)nicotinol glycine (TFNG), 4-trifluoromethylnicotinic acid (TFNA) and 4-trifluoromethylnicotinamide (TFNA-AM) being the main metabolites detected in fruits and vegetables.[8–10] According to the Regulation EU no. 67/2016 (ref. 11) the maximum residue level (MRL) of flonicamid in fruits and vegetables is defined as the total sum of flonicamid and its metabolites TFNA and TFNG. The TFNA-AM metabolite has not been included in the MRL set for fruits and vegetables, but this shows up in the MRL of flonicamid for animals,[12,13] which has been set as the sum of

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flonicamid and TFNA-AM. As far as we are aware, there are several studies focused on the simultaneous determination of flonicamid and its metabolites in food commodities.[8–10,13–15] Among them, only two studies[16,17] evaluate the dissipation of flonicamid in vegetables with high water content such as bell-peppers and cucumbers, but no metabolic pathways have been proposed so far, and in none of them the inclusion of nuclear magnetic resonance (NMR) methodologies has been explored. Moreover, and as far as we know, degradation of flonicamid has not been evaluated in matrices with high acid and water content such as oranges.

Advances in analytical techniques with increased sensitivity have led to the detection of a growing number of metabolites at low concentrations. Nowadays, mass spectrometry (usually coupled with liquid chromatography, LC-MS) is the most used analytical technique for the determination of metabolites.[18,19] This has been favored by the increased availability of high-resolution mass spectrometry (HRMS) analyzers, such as time-of-flight (TOF) or Orbitrap.[20] These analyzers have several important features for the detection and identification of compounds, and target and non-target analysis modes can be performed. However, unequivocal identification of metabolites by HRMS is achieved only when reference compounds are available.[21–23] Otherwise, it is necessary to confirm them by a complementary or orthogonal technique, NMR spectroscopy being the most widely used,[21,22,24] for definitive metabolite identification. In fact, there is an increased interest in combining NMR and MS methods to establish structures for numerous unknown metabolites, to better interpret biological functions and mechanisms, and to improve biomarker discovery.[25] NMR spectroscopy exhibits numerous unique and favorable characteristics such as it is a non-destructive technique, can analyze intact samples with no need for sample preparation or separation, it is highly reproducible and quantitative, where a single internal reference is sufficient for absolute metabolite quantitation over a wide dynamic range, and most importantly, it contains a plethora of NMR experiments which enables the unambiguous identification of structures for unknown metabolites including isotope-labeled or isotopically enriched species.[26] Among its weaknesses one has to mention its lack of sensitivity compared with HRMS.[24] In consequence, the combined use of LC-HRMS and NMR could be envisaged as a synergic combination towards comprehensive route fragmentations.[27]

In this study, the metabolic profiling, intended as the analysis of a group of metabolites either related to a specific metabolic pathway or a class of compounds,[28] of flonicamid in a matrix with high acid and water content as oranges has been investigated for the first time. For this purpose, LC-HRMS, with an Orbitrap analyzer, and NMR, as orthogonal techniques, have been used. Therefore, this manuscript aims at: (i) applying an efficient LC-MS-Orbitrap method to simultaneously determine flonicamid, TFNG, TFNA and TFNA-AM in oranges; (ii) assessing the dissipation of flonicamid in oranges, both in field and in laboratory trials, and finally (iii) study the conditions where these flonicamid metabolites are generated in solution through the use of ^{1}H, ^{2}H and ^{19}F one-dimensional NMR methods, and confirmation by HRMS, evaluating the metabolic pathway of flonicamid's degradation.

Materials and methods

Materials and reagents

Flonicamid (CAS registry no. 158062-67-0, purity >99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Trifluoromethylnicotinamide (TFNA-AM, CASregistry no. 158062-71-6, >99% purity) was obtained from Apollo Scientific Limited (Manchester, UK). 4-Trifluoromethylnicotinic acid (TFNA, CAS-registry no. 158063-66-2, ≥98% purity) and 4-(trifluoromethyl) nicotinol glycine (TFNG, CASregistry no. 207502-65-6, >99% purity) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Water (LC-MS grade) was acquired from J.T. Baker (Deventer, The Netherlands) and formic acid was obtained from Fisher Scientific (Erembodegem, Belgium). Acetonitrile and methanol (both LC-MS grade) were acquired from Fluka (St. Louis, MO, USA). A mixture of acetic acid, caffeine, Met–Arg–Phe–Ala–acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrate and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-hybrid ESI negative) obtained from Thermo-Fisher (Waltham, MA, USA) were employed for the accurate mass calibration of the Orbitrap analyzer.

Magnesium sulfate (Sigma-Aldrich), sodium chloride (J.T. Baker) and primary secondary amine (PSA) (Scharlab, Barcelona, Spain) were used during the extraction procedure.

Stock solutions (1000 mgL^{-1}) of the four compounds were prepared in methanol by weighing 50 mg of the pure compound in a 50 mL volumetric flask. An intermediate solution of the four compounds (10 mgL^{-1}) was prepared by taking 100 μL of each stock solution and diluting up to 10 mL with methanol in a volumetric flask. All stock and intermediate solutions were stored at 4°C. The stock solutions were stable for a year and intermediate solution for 2 months.

The deuterated solvents such as deuterium oxide ($D \geq$ 99.90%) and chloroform-d$_1$ ($D \geq 99.50\%$) were purchased from Eurisotop (St-Aubin Cedex, France) and were stored under 3 Å molecular sieves. All other reagents and solvents were of commercial quality and were used without further purification.

Apparatus

For the treatment and preparation of samples, a vortex mixer WX from VelpScientifica (Usmate, Italy), a Reax 2 rotatory agitator from Heidolph (Schwabach, Germany), a Centronic BL II centrifuge (J.P. Selecta, Barcelona, Spain) and a Polytron PT2100 (Kinematica A.G.,Littan/Luzern, Switzerland) were used.

UHPLC-MS-Orbitrap analysis

Chromatographic analysis was carried out using a liquid chromatograph Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA). The analytical column was a Zorbax Eclipse Plus C$_{18}$ (100 mm × 2.1 mm and 1.8 μm particle size) supplied by Agilent Technologies (Santa Clara, CA, USA).

The chromatographic system is coupled to a single mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen, Germany) using an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA) in positive mode. The ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N_2, >95%), 35 (adimensional); auxiliary gas (N_2, >95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305°C; capillary temperature, 300°C. The mass spectra were acquired employing two alternating acquisition functions: (1) full MS, ESI+, without fragmentation (higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25 000 FWHM; scan time = 0.25 s; (2) all-ion fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy = 30 eV), and mass resolving power = 10 000 FWHM; scan time = 0.10 s.

The chromatograms were acquired using the external calibration mode and they were processed using Xcalibur™ version 2.2, with Qualbrowser and Quanbrowser, and Mass Frontier™ 6.0 (Thermo Fisher Scientific, Les Ulis, France).

Chromatographic conditions

Chromatographic separation was carried out by gradient elution developed previously[29] using a mobile phase formed by an aqueous solution of 0.1% formic acid as eluent A and an organic solution of methanol : acetonitrile (80 : 20 v/v) containing 0.1% formic acid as eluent B.

NMR spectroscopy

NMR spectra were recorded using Bruker AvanceIII 300 (^{1}H, 300.13 MHz; ^{2}H, 46.10 MHz; ^{13}C, 75.47 MHz; ^{19}F, 282.40 MHz), Bruker AvanceIII 500 (^{1}H, 500.13 MHz; ^{13}C, 125.76 MHz; ^{19}F, 564.63 MHz), and a Bruker AvanceIII 600 (^{1}H, 600.13 MHz; ^{13}C, 150.92 MHz) spectrometers (Bruker, Bremen, Germany), using a 5 mm BBFO^{1}H/BB(^{19}F) probe, an indirect 5 mm TBI 1H/^{31}P/BB triple probe, and a quadruple QCI (^{1}H, ^{13}C, ^{15}N, and^{31}P) cryoprobe, respectively. The spectral references used were internal tetramethylsilane for ^{1}H and ^{13}C and an external reference of CF_3CO_2H for ^{19}F. Coupling constants (*J*) are given in hertz as positive values regardless of their real individual signs. ^{2}H NMR spectra were recorded without proton decoupling, although in most cases splitting of the signals could not be observed due to quadrupolar line broadening. All reactions were monitored by ^{1}H or ^{19}F NMR spectroscopy using Topspin 3.2 on a Bruker AvanceIII operating at 500 MHz. The experimental temperature was set to the temperature described further below (25, 40, 60 or 80°C) and maintained with a Bruker BCU II-80/60 temperature control unit. Initially, the temperature was equilibrated with a standard sample containing pure flonicamid in the same concentration as that in the actual sample. The shim was optimized using "topshim". The samples were inserted into the spectrometer and the acquisition was started. Sequential ^{1}H or ^{19}F NMR spectra were measured every 30 s in the case of full monitoring experiments, and every 300 s in the case of slow rate processes. The data obtained were processed (ef, phc0, phc1, pk, and abs) using the batch processing command "multicmd" with phasing parameters determined for the last spectrum measured. Then, for every measured point the integrals for the signals of flonicamid, the intermediates as well as products formed were determined and exported with the help of the AU program "multi_integ3". The exported data were then imported into an excel datasheet, and a time scale was generated by multiplying the data row number by 30 s or 300 s, respectively. The concentrations of substrates and products were determined by the relative integration of growing or decreased signals of product and substrate peaks. The NMR experiment array gives better precision for both concentration (*via* integrations) and reaction time, because each measurement is conducted under almost identical shimming and temperature conditions. ^{1}H and ^{13}C NMR spectra for commercial flonicamid, TFNG and TFNA-AM metabolites are given in Fig. S1 to S6 (see ESI).†

Field and laboratory studies

To appropriately cover the identification of metabolites (known or unknown) generated in the process, oranges were treated with flonicamid at the dose recommended (field trials), and at high concentration levels (laboratory trials).

The field dissipation experiment was conducted in an orange farm (300 m^2). The orange variety was *Navelate* and organic farming was used before flonicamid application. Flonicamid, whose commercial formulation (Teppeki®) was a water dispersive granule (WG) with *ca.* 50% of flonicamid as an active ingredient, was applied at the recommended dose rate of 0.15 g of the commercial product per L of water using a compressed sprayer of low volume. Six samples of approximately 250 g each were randomly collected at 24 hours, 7, 15, 21, 30, 40 and 50 days. The samples were pooled, crushed and homogenized and frozen at −21°C until analysis.

For laboratory trials, 60 oranges were taken from the previous orange farm before flonicamid application; each of them was weighed, referenced and spiked at a concentration level of 1 mgkg^{-1} of flonicamid (spiking volume between 2 to 3 mL of the commercial product Teppeki®, depending on the oranges' weight) using an hypodermic syringe to inject the compound into the sample. The spiked samples were conserved at room temperature. The samples composed of six pieces were taken at 24 hours, 7, 15, 21, 30, 40, 50, 60 and 70 days after the addition of flonicamid. They were chopped and homogenized and frozen at −21°C until analysis.

Sample extraction

Fresh fruits (*Navelate* oranges) were treated as follows: 1.5 kg of pooled collected samples was crushed and homogenized, without neglecting any part thereof, skin or outer shell or bones as established by current regulations,[30] storing it in the freezer at−21°C. Some of these samples, were blank for the pesticides under study, and they were subsequently used for the preparation of fortified samples and the preparation of matrix-matched calibration points.

The orange samples were treated as follows: 10 g of the homogenized sample was introduced in a 50 mL plastic centrifuge tube. Then, 10 mL of water and 10 mL of acidified acetonitrile (0.1% formic acid, v/v) were added and shaken for 1 min in a Polytron. After that, the sample was stirred for 15 minutes in a rotary shaker and 1 g of NaCl and 4 g of $MgSO_4$ were added and the mixture was shaken vigorously for 1 min in a vortex. Then, the samples were centrifuged for 10 min at 5000 rpm (4136*g*). 3 mL of the supernatant were collected and added in a 15 mL centrifuge tube with 50 mg of PSA. Subsequently, the samples were centrifuged for 5 min at 5000 rpm (4136*g*) and 1 mL of the supernatant was collected and injected into the UHPLC-MS-Orbitrap.

UHPLC-MS-Orbitrap method validation

In order to obtain reliable quantitative results, the UHPLC-MS-Orbitrap analytical method was validated according to SANTE guidelines,[30] calculating the matrix effect, linearity, trueness (% recovery), precision (intra and inter-day), and limits of quantification (LOQs) and detection (LODs).

The matrix effect was investigated by analyzing standards in a solvent (methanol) and standards prepared in an extracted blank matrix. The calibration curves were obtained for different concentrations. For flonicamid and TFNG, the concentrations ranged from 10 to 300 μgkg^{-1}, for TFNA from 20 to 300 μgkg^{-1}, and for TFNA-AM from 30 to 300 μgkg^{-1}.

The linearity of the method was studied using the matrix-matched calibration using the same levels as those utilized previously to evaluate the matrix effect. Linearity was checked by the determination coefficients (R^2). Accuracy and precision (intra and interday) were established across the specified range of the analytical procedure by analyzing samples spiked with known amounts of the compounds (2 concentrations/5 replicates each). For flonicamid and TFNG 10 and 100 μgkg^{-1}, for TFNA 20 and 100 μgkg^{-1} and for TFNA-AM 30 and 100 μgkg^{-1}. Accuracy is reported as the percent of mean recovery ± the relative standard deviation (RSD) in %, and precision is reported as RSD in %. Interday precision was studied on ten different days.

LODs and LOQs were estimated by injecting the extracted blank samples spiked at low levels. LODs were assigned as the minimum concentration at which the characteristic ion is monitored with a mass error lower than 5 ppm. For the estimation of LOQ, in addition to the characteristic ion, one fragment should be monitored at the same retention time and chromatographic shape than the characteristic one, the mass error being lower than 5 ppm. In addition, the recovery and precision at LOQ should be within 70–120% and lower than 20% respectively.

Results and discussion

Development and validation of the UHPLC-MS-Orbitrap method

In a previous study, an UHPLC-MS-Orbitrap method was optimized for the determination of flonicamid and metabolites in bell-peppers,[29] the LC-MS characteristics of the monitored compounds are shown in Table S1†.

However, the extraction procedure optimized for bell-peppers did not provide suitable results in oranges, and therefore, it has been re-evaluated. As the starting point, the EURL-SRM method,[9] based on acidified QuEChERS, was checked but poor recoveries were obtained (<10–70%). Therefore, 10 mL of MilliQ water were added before the addition of acidified acetonitrile (0.1% formic acid, v/v), in order to enhance phase separation. This addition improved the recoveries, ranging from 30% (TFNA) to 84% (TFNA-AM). Then, a cleaning step was tested, using 3 mL of the final extract, in order to decrease the presence of interferents. Experiments with 50 mg PSA, 50 mg GBC, and a mixture of both sorbents (50 mg each) were performed, obtaining a better peak shape and area when only PSA was used. Recoveries ranged from 40% (TFNA) to 90% (TFNA-AM), and therefore, the cleaning step was added. Finally, a stirring step with a Polytron was used because of the problem of crushing the fruit. This step was performed before shaking the mixture in the rotary shaker, and the cells of the matrix were suitably broken, improving the contact of the analytes with the extraction solvent. The recoveries improved considerably, ranging from 76% (TFNG) to 90% (flonicamid).

Finally, the optimized method was validated in oranges, studying the parameters previously mentioned. The matrix effect was evaluated comparing the slopes of the calibration curves of each analyte prepared in the blank matrix and in the solvent. For TFNA and flonicamid, matrix suppression was observed and the matrix-matched calibration was used for quantification purposes.

Table 1 Validation parameters of the UHPLC-MS-Orbitrap optimized method

Compound	Concentration (μgkg^{-1})	R^2	Matrix effect	% *R*	Intra-day precision (% RSD)	Inter-day precision (% RSD)	LOD (μgkg^{-1})	LOQ (μgkg^{-1})
Flonicamid (F)	10	0.9951	27%	102	15	7	1	10
	100			84	8	5		
TFNG	10	0.9930	8%	84	4	8	1	10
	100			76	16	7		
TFNA	20	0.9996	47%	82	19	7	5	20
	100			74	16	4		
TFNA-AM	30	0.9999	7%	79	9	6	6	30
	100			79	7	2		

The linearity of the method was studied ranging from 10–300 μgkg^{-1} for flonicamid and TFNG, 20–300 μgkg^{-1} for TFNA and 30–300 μgkg^{-1} for TFNA-AM. It can be highlighted that the determination coefficients (R^2) were higher than 0.99 for all target compounds. If the concentration of the target compounds in the samples were higher than the upper level of the linear range, dilution was necessary and it was performed using an extracted blank sample.

Adequate recoveries were obtained, ranging from 74% to 102% at the two concentration levels evaluated (see Table 1). The intra-day precision ranged from 4–19%, obtaining the highest value for TFNA as it can be observed in Table 1. On the other hand, the inter-day precision (n = 5) ranged from 2% (TFNA-AM) to 8% (TFNG). In comparison with other studies, this method has similar recoveries and intra-day and inter-day precision than those provided in other matrices such as dried hops,[8] spinach and cucumbers,[10] paprika,[14] and bell peppers.[16,29]

The lower limits of the method (LOD and LOQ) are also shown in Table 1. The LODs ranged from 1 μgkg^{-1} to 6 μgkg^{-1}, whereas the LOQ values varied from 10 μgkg^{-1} to 30 μgkg^{-1}. These limits are below the maximum residue limit of flonicamid in oranges (0.1 mgkg^{-1}),[10] which takes into account flonicamid and its metabolites. Also, these values were lower or similar than those obtained in previous studies.[10,14,16]

Analysis of samples

Degradation of flonicamid and its metabolites was studied in field and laboratory trials. The UHPLC-MS-Orbitrap method was used to perform qualitative and quantitative analysis of the incurred samples, whereas NMR was applied to confirm the qualitative results obtained by HRMS as well as to elucidate the metabolic pathway of the flonicamid into its metabolites.

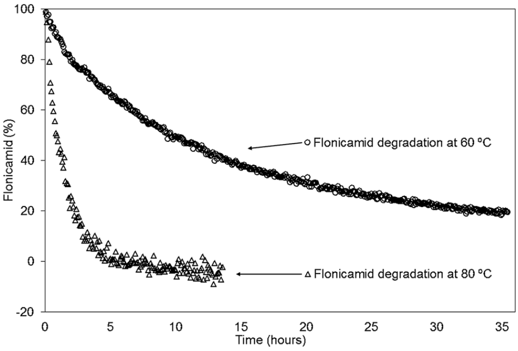


Fig. 2 ^{1}H NMR (300.13 MHz, D_2O) monitoring of the methylenic deuteration of flonicamid (F) as a function of temperature.

Field sample analysis. First, oranges from an orange farm treated with Teppeki® (commercial formulation of flonicamid), were randomly collected and analyzed for 24 hours, 7, 15, 21, 30, 40 and 50 days after application, *via* the UHPLC-MS-Orbitrap method previously developed. The results are shown in Fig. 1a where the initial concentration of flonicamid was obtained at 116 μgkg^{-1}. This concentration slightly increased up to 15 days after application (135 μgkg^{-1}) and after that, the concentration decreased, observing a behavior similar to that by Jung *et al.*[16] The detected concentration after 50 days of application was 19 μgkg^{-1}, observing that the flonicamid content decreased 84% in relation to the initial concentration. Regarding its metabolites, TFNA (31 μgkg^{-1}) and TFNG (32 μgkg^{-1}) were detected after 15 days of the application of flonicamid. Moreover, TFNA-AM was also detected 15 days after application, but the concentration was lower than the established LOQ (30 μgkg^{-1}). After that, TFNG concentration increased up to 68 μgkg^{-1}, while TFNA concentration was lower than the LOQ (20 μgkg^{-1}) and TFNA-AM was not detected after this time. As it can be deduced, the

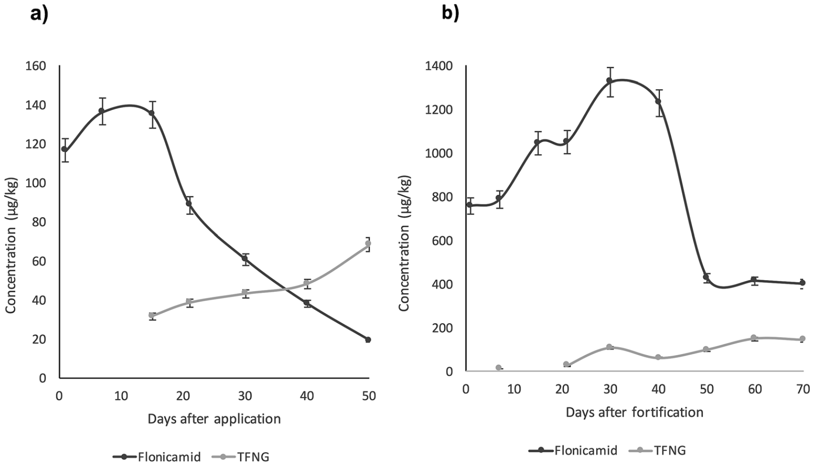
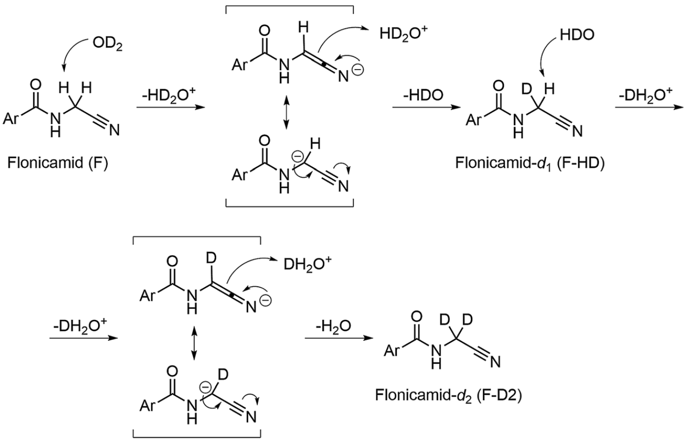


Fig. 1 Evaluation of flonicamid and TFNG concentrations in incurred oranges during field (a) and laboratory (b) trials.



Scheme 1 Proposed mechanism for the water-catalyzed perdeuteration of flonicamid (F) at the methylenic position (Ar = 4-((trifluoromethyl) pyridine)-3-il).

concentration of TFNG could have increased after 50 days of application, but unfortunately no more oranges could be collected after that time. In order to confirm the latter statement, laboratory trials were planned and eventually performed.

Laboratory sample analysis. In the laboratory study, three replicates were analysed at the aforementioned time intervals after the injection of flonicamid, as it has been described in the "Field and Laboratory Studies" section (Fig. 1b). During the monitoring period, only flonicamid and TFNG were detected above their LOQ as it was observed in the field study, although a different behavior was noted. For example, the initial concentration of flonicamid was 758 μgkg^{-1}, but it was increased up to 1325 μgkg^{-1} after 30 days of the injection of flonicamid. Although the spiked concentration was only 1000 μgkg^{-1}, the found concentration was higher than this value, and it could be explained considering the water loss during orange storage. After that, the concentration decreased down to 430 μgkg^{-1} (50 days), which remained constant until the end of the monitoring period (70 days). In relation to TFNG, it was detected at concentrations close to the LOQ (10 μgkg^{-1}) but it increased after 21 days, yielding concentrations higher than 140 μgkg^{-1} after 70 days of the injection of the parent compound in the orange samples. This result is in accordance with that obtained in the field study, where it was observed that the concentration of TFNG increases after 40 days.

NMR studies: metabolic pathways of flonicamid. In order to confirm these results and evaluate the degradation routes of this pesticide, multinuclear NMR (^{1}H, ^{2}H, ^{13}C and ^{19}F) was applied as a tool to measure kinetics, to characterize the final product composition, and in some cases deuterium incorporation.

The first variable studied was the temperature. An oven-dried 5 mm NMR tube was filled with 0.5 mL of a D$_2$O extract of Teppeki® (*ca.* 4.1 mg of pure flonicamid, see Fig. S7†), and heated at 60°C. A second sample was also studied at 80°C in order to find the differences in temperature behavior. The reaction crudes were monitored through ^{1}H NMR and Fig. 2 shows the two degradation curves. The concentration of *ca.* 25 mM (4.1 mg) in flonicamid in both samples was determined by NMR analysis, using the sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP) as the internal standard. To our surprise, the monitored methylene signal in the ^{1}H NMR spectra located at δ_H 4.4 ppm progressively disappeared within the reaction time (see below). When the ^{13}C NMR spectra of both samples were analyzed at the beginning and at the end of the heating process, it was found that the nitrile signal (δ_C 116.5 ppm) remained over the whole period of time, indicating that the flonicamid carbon skeleton persisted unaltered, and only deuteration of the methylenic carbon occurred. The latter statement was supported through two different experiments. The first one was based on the acquisition of a ^{13}C DEPT-135

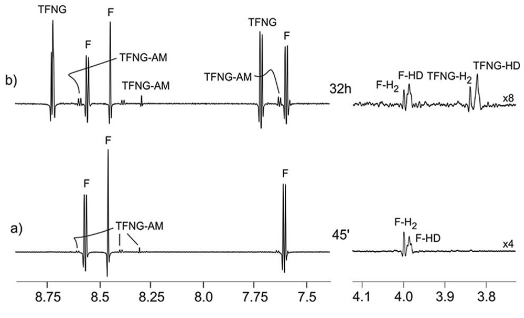


Fig. 3 ^{1}H NMR spectra (500.13 MHz, D$_2$O) after (a) 45 minutes and (b) 32 hours, of treatment of flonicamid (F) with NaOH 5 M at 25°C. From the methylenic region the two isotopomers of F and TFNG are observed, both showing coupling with the deuterium nucleus. The spectra are obtained after the Gaussian multiplication of the FID in order to obtain enough resolution and coupling constants.

NMR spectrum, that showed no signal for this specific methylene (located at δ_C 28.1 ppm) since no transfer of polarization could occur from ^{1}H to ^{13}C when there are no attached protons. Then, the acquisition of a ^{2}H NMR spectrum, evidenced a broad singlet at δ_D 4.41 ppm, due to deuteration at this position. The ^{2}H nucleus is NMR active with spin $I = 1$ and its analysis can be performed on standard spectrometers by utilizing the deuterium lock channel as the observation channel. In addition, when the ^{1}H NMR spectrum is acquired in anhydrous THF, an extra signal located at δ_D 8.56 ppm assigned to the exchangeable ND signal was observed (Fig. S8†), confirming that trideuteration of flonicamid has occurred.[31]

Based on these results, a reaction mechanism can be proposed which is induced by the acidity of the methylenic hydrogens which are *alpha* with respect to the nitrile (Scheme 1).

The presence of sodium hydroxide and therefore the existence of basic conditions was the second variable under study. When the flonicamid extract is treated with 10 equivalents of NaOH 5 M (basic media), the reaction outcome was significantly different. At room temperature, we could observe changes in the ^{1}H spectra within the reaction time. During the first 45 minutes, the ^{1}H NMR spectra evidenced the same pattern previously observed, *i.e.*deuteration of the methylenic carbon (Fig. S9a†), in this case catalyzed by the hydroxide anion. Interestingly, in the region of δ_H 4.0 ppm, together with the protonated singlet of flonicamid (F), a new signal ascribed to the partially deuterated species F-HD is observed with a nice two bond coupling constant of 3.2 Hz between proton and deuterium, (Fig. S9b†).

At longer reaction times, the aromatic region changed with the appearance of two sets of signals. A first set at δ_H 8.59 (*J* 5.8 Hz), 8.29 and 7.62 (*J* 5.8 Hz) ppm that increased at the beginning, and progressively decreased down to a noise after 61 hours of reaction time. The second set was located at δ_H 7.71 (*J* 5.4 Hz), 8.72 (*J* 5.4 Hz) and 8.73 ppm, which continuously increased until becoming the main product of the reaction after 61 hours. Fig. 3a shows the ^{1}H NMR spectrum after 45 minutes of reaction time, where the main signals of flonicamid (F-d_3) and the transient species TFNG-AM-d_5 as an incipient species can be observed. Fig. 3b depicts the ^{1}H NMR spectrum after 32 hours, where it is clearly observed that the signals arising from the reaction product (TFNG-d_4) as well as from the short-lived TFNG-AM-$d_5$finally disappeared as it was observed after 61 hours of the reaction (see below). At the same time, the two partially deuterated TFNG and flonicamid were also observed in the region of *ca.* 3.8 and 4.0 ppm, respectively, in the ^{1}H NMR spectrum (Fig. 3b), as previously mentioned. To the best of our knowledge, this is the first time that TFNG-AM was detected as transient species during the flonicamid's metabolic pathway. Bearing in mind these results, retrospective analysis was performed in order to detect the presence of TFNG-AM in the samples analysed previously by LC-HRMS. Because this is a transient species, this compound was only detected in one laboratory sample (mass error = 0.05 ppm) 40 days after treatment, the chromatogram, as well as the experimental and theoretical spectra are shown in Fig. S10.†

The presence of the CF$_3$ group in flonicamid offers the opportunity to monitor the reactions where this compound is involved. The ^{19}F nucleus has the advantage of 100% natural abundance and a high gyromagnetic constant of about 0.94 times that of ^{1}H. The chemical shift range is about twenty times that of hydrogen, so that the resonances of different fluorine nuclei are usually well separated. Furthermore, spectral positions are sensitive to the environments of fluorine atoms.[32] In our case, three signals were observed in the ^{19}F NMR spectrum located at δ_F−61.90,−61.75, and −61.71 ppm, assigned to flonicamid-d_3, TFNG-AM-d_5 and TFNG-d_4, respectively (Fig. 4). As in the case of ^{1}H NMR, the former progressively disappeared at the same time that the latter increased. Compound TFNG-AM-d_5 is again transitorily detected and finally disappears at a reaction time of about 90 hours.

Bearing in mind these results, a kinetic profile of the transformation of flonicamid (F) into TFNG-d_4 was obtained by following the evolution of the ^{19}F NMR signals as a function of time, as depicted in Fig. 5. It is important to mention that all the fluorine and ^{1}H spectra for monitoring purposes were acquired using a 30° pulse and a delay between scans of 10 seconds. The

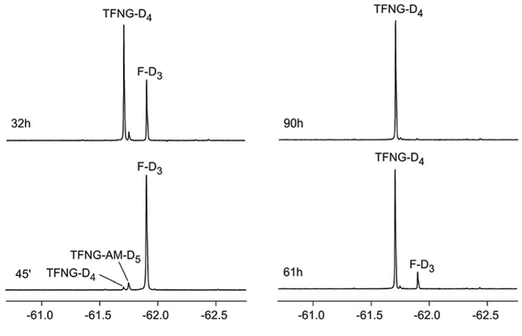


Fig. 4 ^{19}F NMR spectra (282.40 MHz, D$_2$O) after 45 minutes, 32, 61 and 90 hours of treatment of flonicamid (F) with NaOH 5M at 25°C.

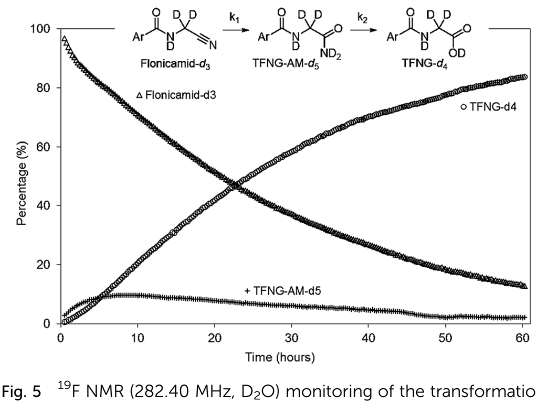
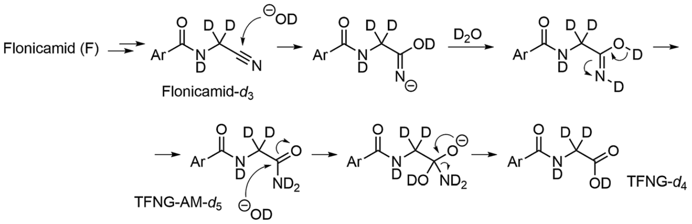


Fig. 5 ^{19}F NMR (282.40 MHz, D$_2$O) monitoring of the transformation of flonicamid (F) after its treatment with NaOH 5M at 25°C. The three fluorine signals at δ_F−61.71 (TFNG-d_4),−61.75 (TFNG-AM-d_5), and −61.90 ppm (flonicamid-d_3) were monitored.



Scheme 2 Proposed mechanism for the hydroxide-catalyzed deuteration of flonicamid (F) and its further transformation into TFNG (Ar = 4-((trifluoromethyl)pyridine)-3-il).

observations are consistent with a consecutive reaction pathway, in which TFNG-d_4 is formed only from TFNG-AM-d_5 and is not due to an alternative route. Simulations based on this two-step consecutive reaction were performed for the collected 244 spectra, corresponding to a k_1 value of 0.034 min^{-1}, a k_2 value of 0.25 min^{-1} and an initial percentage of species F of 99.6%.

The treatment of flonicamid with sodium hydroxide was also performed at higher temperatures and the kinetics monitored through ^{19}F NMR spectroscopy. Fig. S11† shows how at 60°C the reaction needs less than a couple of hours to quantitatively proceed, and about 12 hours to quantitatively transform flonicamid into TFNG-d_4. Finally, Scheme 2 shows the basic hydrolysis of flonicamid, in which the experimental concentration profile given in Fig. 5 applies when the intermediate TFNG-AM-d_5 species is highly reactive.[33]

Finally, acidic conditions were also assayed as a variable that could have affected flonicamid degradation. Heating of flonicamid at pH *ca.* 4 in a mixture of ascorbic and citric acids in a 0.7 : 1 ratio at several temperatures during 72 hours, produced no transformation of flonicamid, evidencing great stability under these protic conditions (Fig. S12 and S13).†

Conclusions

This is the first time that HRMS and NMR have been combined to monitor the metabolic transformation of flonicamid. The parent compound and metabolites were first monitored in oranges (a matrix with high acid and water content), and only flonicamid and TFNG were detected in the incurred samples. Both compounds were persistent, being detected after 70 days of pesticide application, proposing a metabolic pathway which transforms flonicamid into its main metabolite (TFNG). To get a comprehensive view of the transformation process, the combination of HRMS and multinuclear NMR was needed, taking advantage of the possibilities of both techniques. HRMS has been mainly used to identify and quantify the compounds included in this study at low concentrations, whereas NMR has emerged as a powerful tool to elucidate the metabolic pathway of flonicamid including the new isotopic derivatives coming from selective deuteration of the methylenic moiety, and observing the presence of transient species such as TFNG-AM during flonicamid's transformation, which was confirmed by retrospective analysis using HRMS. The acquisition of ^{2}H NMR has proved to be the key to their full characterization. The results obtained *via* both orthogonal approaches (MS and NMR) are in concordance, which reinforce the synergistic effect that can be achieved when both techniques are used.

Acknowledgements

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Publicación V

DISSIPATION STUDY OF THE PROINSECTICIDE THIOCYCLAM AND NEREISTOXIN IN TOMATO USING LIQUID CHROMATOGRAPHY HIGH RESOLUTION MASS SPECTROMETRY

López-Ruiz, R.; Belmonte-Sánchez, E.; Romero-González, R.;
Martinez Vidal, J; Ramirez-Cassinello, J. J.; Garrido Frenich, A.
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Abstract: The reduction of the use of pesticides with the increasing of organic farming activity, gives to the pest market new biological products against pests as thiocyclam, a biological proinsecticide. It is scarcely studied, being necessary the study of its degradation in vegetables as tomato. Dissipation study in tomato, monitoring the main metabolites of thiocyclam, was carried out using a method based on ultra-high-performance liquid chromatography-Orbitrap mass spectrometry (UHPLC-Orbitrap-MS. It was studied in a period of 1 to 60 days after foliar application of the commercial product, monitoring also, nereistoxin, its main metabolite. Thiocyclam was a non-persistent insecticide (DT50 < 15 days), but nereistoxin remains in vegetables more than 60 days. Four metabolites of nereistoxin were monitored and detected at low concentration (>100 µg/kg). The study provided for the first time the dissipation pattern of thiocyclam and nereistoxin and demonstrated that a revision of legislation of these compounds is required.

Cover Letter

UNIVERSIDAD DE ALMERÍA
Department of Chemistry and Physics

Almería, April 09th 2020

Dear Editor,

Please find enclosed the manuscript entitled "*Dissipation study and risk assessment of the proinsecticide thiocyclam and nereistoxin in tomato using liquid chromatography high resolution mass spectrometry"* by López-Ruiz et al., for consideration of publication in Food Chemistry, as an original research paper.

Currently, the fast development of the organic products industry in the European Union forces to use less pesticide and introduce natural pesticides to control pests by conventional agricultural practices. As a case study, thiocyclam is monitored in this work due to its potential use in current agricultural practices, where dissipation and control of natural insecticides as thiocyclam, is required. Thiocyclam is a proinsecticide that comes from the precursor molecule, nereistoxin, which is the main degradation product of thiocyclam. This compound is mainly applied into crops in an insecticide form, which quickly degrades into the precursor molecule, a natural toxin (nereistoxin). In this paper dissipation of thiocyclam was evaluated in tomato, obtaining low persistence values. However, nereistoxin appeared quickly and remains in tomato more than 60 days. Despite of this persistence, neither thiocyclam nor nereistoxin have been regulated, and there are not maximum residue limits for these compounds. It can be considered that thiocyclam has maximum residue limit (MRL) of 0.01 mg/kg (default value set by the European Union) meanwhile its main metabolite, which is really persistent, is not controlled or included in the MRL definition of thiocyclam, despite it can cause respiratory problems in humans. According to the results obtained and the risk assessment in this study, a review of the regulation of these substances is required in order to protect the consumer health.

By submitting the manuscript to Food Chemistry, the authors understand that the work described has not been published previously and it is not under consideration for publication elsewhere. This publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will

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I hope that the reviewing process finds the manuscript acceptable for publication in the journal.

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Yours Sincerely,
Prof. Antonia Garrido Frenich

Highlights

- Degradation of thiocyclam was evaluated in tomato using LC-HRMS
- Nereistoxin was detected in incurred samples during monitoring period.
- Putative elucidation was performed, detecting new metabolites of nereistoxin
- Risk assessment studies required an evaluation of nereistoxin toxicity

Dissipation study and risk assessment of the proinsecticide thiocyclam and nereistoxin in tomato using liquid chromatography high resolution mass spectrometry

Rosalía López-Ruiz[1], Eva Belmonte-Sánchez[1], Roberto Romero-González[1], José Luis Martínez Vidal[1], Juan José Ramírez-Cassinello[2] and Antonia Garrido Frenich[1]*

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Abstract

The reduction of the use of pesticides with the increasing of organic farming activity, gives to the pest market new biological products against pests as thiocyclam, a biological proinsecticide. It is scarcely studied, being necessary the study of its degradation in vegetables as tomato. Dissipation study in tomato, monitoring the main metabolites of thiocyclam, was carried out using a method based on ultra-high-performance liquid chromatography-Orbitrap mass spectrometry (UHPLC-Orbitrap-MS). It was studied in a period of 1 to 60 days after foliar application of the commercial product, monitoring also, nereistoxin, its main metabolite. Thiocyclam was a non-persistent insecticide (DT_{50} < 15 days), but nereistoxin remains in vegetables more than 60 days. Four metabolites of nereistoxin were monitored and detected at low concentration (>100 µg/kg). The study provided for the first time the dissipation pattern of thiocyclam and nereistoxin and demonstrated that a revision of legislation of these compounds is required.

KEYWORDS: Thiocyclam, nereistoxin, metabolites, dissipation, HRMS, UHPLC, tomato

1. INTRODUCTION

Currently, the fast development of the organic product industry in the European Union forces conventional agricultural practices to use less pesticides and introduce natural pesticides to control pests. One evidence of that is the consumers' belief about synthetic pesticides, which are perceived as a serious health risk, in comparison with organic farming, where the use of pesticides is zero or low (Schusterova et al., 2019). In this sense, new biopesticides are being highly used as thiocyclam. It was classified as a proinsecticide, so it is applied in its manufactured form but it degrades to a specific active component (nereistoxin) very quickly (Ferrer et al., 2010; Lee, Tomizawa, & Casida, 2003). Cartap, bensultap or thiosultap belong to the same group of proinsecticides and all of them are obtained by synthesis processes from nereistoxin. This is a natural toxin originated from a marine annelid worm, with a strong insecticidal activity, blocking the nicotinic acetylcholine receptor (Cheng, Yu, Chen, & Li, 2008). Because of this, thiocyclam is not considered as a synthetic pesticide, and therefore, it was extensively used in organic crops.

Despite the wide use of these proinsecticides (Lee et al., 2003), toxicity of nereistoxin was not considered, and this is an important issue due to these compounds are quickly degraded into it. However nereistoxin has significant neuromuscular toxicity, resulting in respiratory failure in mammals (Park et al., 2015). In this field, dissipation studies can be an option to clarify these issues, providing suitable information that can be used in toxicity studies. For instance, the knowledge about the behaviour of nereistoxin and when it appears during thiocyclam dissipation in crops is really important. Nereistoxin can be generated from thiocyclam hydrolysis, when it is dissolved in water previous the application in the field, as it happens with bensultap or thiosultap (Park et al., 2015), or it can be generated by the plant metabolisms by simple reactions as oxidation

(Takahashi, Yamamoto, Todoriki, & Jin, 2018). These factors should be studied in order to elucidate the possible risk of thiocyclam when it is applied to crops.

In relation to the legislation, thiocyclam is not included as active substance in the Regulation (EC) 1107/2009 (European Union, 2009), and therefore there is not a defined Maximum Residue Limit (MRL) in Europe for it and for the other proinsecticides. Thus the generic MRL of 0.01 mg/kg defined by the Regulation (EC) 396/2005 (European Comission, 2005) should be used for these compounds. Additionally, no information about acceptable daily intake was reported (European Commission., 2016). Nereistoxin was not mentioned in any regulation, because it was a natural toxin and was not used as an active substance, so legislation about its used and possible risk was not defined yet.

There are few analytical methods that determined these substances (thiocyclam and nereistoxin), and they do not study their behaviour or dissipation kinetics, only one study was focused in the dissipation kinetics, but for cartap (Dai et al., 2020). Only one method employed liquid chromatography coupled to mass spectrometry (LC-MS) for the determination of thiocyclam and nereistoxin in peppers (Ferrer et al., 2010) and another one determined nereistoxin and cartap in tea using LC-MS (Dai et al., 2019). Nevertheless, LC coupled to high resolution mass spectrometry (LC-HRMS) has not been employed yet. The advantages of the use of exact mass, resolution power and full scan acquisition mode (targeted or untargeted analysis) (López-Ruiz, Romero-González, & Garrido Frenich, 2019) can be really interesting in the determination of both molecules and other potential compounds that could be generated from the dissipation of thiocyclam or nereistoxin. Extraction methods used for the determination of these compounds were based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology employing acidified acetonitrile (ACN), extraction salts (magnesium sulfate and trihydrated sodium acetate). In addition, a clean-up step with magnesium sulphate (Ferrer et al., 2010), or solid phase extraction (SPE) procedures with strong anion exchanger cartridges (Dai et al., 2019) or mixed-mode cationic exchange cartridges are needed (Park et al., 2015).

The aim of this work was the study of the behaviour of thiocyclam in tomato, its degradation in nereistoxin and other metabolites, which has not been studied before. This provides more information about the nature and behaviour of this family of compounds that is recently used. For that purpose, this is the first time that an analytical method based on LC-HRMS, and using targeted and untargeted analyses, has been developed for the dissipation study of thiocyclam and nereistoxin, detecting several metabolites in tomato.

2. MATERIALS AND METHODS

2.1. Materials, reagents and apparatus

Analytical standards of nereistoxin (>97% purity) and thiocyclam (>87% purity) were purchased from Dr Ehrenstrofer (LGC standards, Middledsex, UK). Information related with other materials, reagents and apparatus are shown in supplementary material *Section 1.*

2.2. Tomato studies

To monitor the dissipation of thiocyclam in tomato, 5 kg of sample were collected from an ecological greenhouse located in Almería (Spain). Thiocyclam dissipation studies were performed under laboratory conditions at two doses. Trials were developed as follows: 2 kg of tomato were placed into two bowls and sprayed with commercial product of thiocyclam at theoretical concentrations of 1 mg/kg (low dose) and 20 mg/kg (high dose) respectively. Samples were placed in shelf and kept under natural sunlight and temperature (max temp: 25ºC, min temp: 20ºC) conditions. For analysis, approximately 150 g of tomato were collected, crushed and homogenised prior to sample extraction. Samples were collected at 3h, 6h, 12h, and 1, 2, 4, 7, 9, 11, 15 and 23 days in the case of low dose, and 1, 7, 15, 22, 30, 37, 45 and 60 days at high dose. Quality control was carried out in order to obtain a control sample during the dissipation study. The same quantity of tomato (2 kg) was sprayed with an analytical standard of thiocyclam at the same doses and conditions described above collecting and analysing samples with the same periodicity as in the previous study. This allows the determination of any background from the environmental matrix, as well as increasing the knowledge of the overall dissipation of this compound and the confidence in the results.

2.3. Sample extraction

Tomato extraction was based on QuEChERS procedure (Lehotay et al., 2010). Firstly, 10 g of samples was weighed in a 50 mL Falcon® tube and 10 mL of ACN containing 1% acetic acid were added. Samples were vortexed 1 min and sonicated at room temperature during 10 min. After that, 4 g of $MgSO_4$, 1 g of sodium chloride, 1 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogencitrate sesquihydrate were added and vortexed 1 min. Then, the samples were centrifuged for 10 min at 3700 rpm and the supernatant (1mL) was injected into UHPLC-Orbitrap-MS.

2.4. Method validation

The analytical method was checked in order to provide valuable results using SANTE guidelines (European Commission, 2017), calculating matrix effect, linearity, precision (intra and inter-day), limits of quantification (LOQs) and trueness (% recovery).

Matrix effect was tested by analysing standards in ACN and standards prepared in an extracted blank matrix (tomato), meanwhile linearity was studied calculating the determination coefficients (R^2) of the calibration curves, which ranged from 10 to 100 µg/kg. Precision (intra e interday) studies were performed analysing samples spiked at the lowest and the highest concentration of the calibration curves (10 and 100 µg/kg), using 5 replicates per each level, evaluating relative standard deviation (RSD (%)). Trueness was studied at the same levels selected for precision in terms of recovery (%).

Limits of quantification (LOQs) were estimated by injecting extracted blank samples spiked at low levels. LOQ, was defined as the lower concentration that provides recovery within 70-120 % and precision lower than 20 %.

3. RESULTS AND DISCUSSION

3.1. Optimization and validation of the analytical method

Characterization of spectrometric conditions was carried out following the procedure described by Lopez-Ruiz et al (Lopez-Ruiz, Romero-Gonzalez, Ortega-Carrasco, & Garrido-Frenich, 2019). Table 1 shows the parameters obtained. Once spectrometric conditions were optimized, chromatographic conditions were tested, studying the type of column and elution gradient. First, several types of columns were tested according to previous studies. Firstly, a C18 column (Zorbax Eclipse plus C_{18}) was checked, obtaining a quick elution of the compounds, between 1 and 1.5 min. Because the polar characteristics of the compounds (log P < 1.3), a Hilic column was evaluated based on the study performed by Dai et al. (Dai et al., 2019). Zorbax Hilic plus was tested but peak shape and separation of the compounds were not suitable, deciding to couple both columns (Hilic + C_{18}), as in previous studies for quizalofop-p and its metabolites (López-Ruiz, Romero-González, Martínez Vidal, & Garrido Frenich, 2018), taking advantage of C18 columns (separation and peak shape) and Hilic columns (retention time of polar compounds) properties. Using this coupling, peak shape of both compounds was good and retention time was 2.55 min for thiocyclam and 2.65 for nereistoxin.

Once the columns were selected, elution gradient was evaluated. First, elution gradient developed by Lopez-Ruiz et al. (López-Ruiz et al., 2018) using coupled columns was tested. Retention times were 2.75 min (thiocyclam) and 2.85 (nereistoxin). In order to minimize potential matrix effect, the gradient profile was modified despite longer retention times could be achieved. Thus, the percentage of ACN was increased from 40% to 65% and compounds eluted at 3.89 min (thiocyclam) and 4.08 min (nereistoxin) so it was decided to use the modified gradient described in "Apparatus" section.

Finally, extraction method was studied. QuEChERS citrate-buffered (Lehotay et al., 2010) version was tested, obtaining good recoveries for thiocyclam (95%) but not for nereistoxin (49%) (Supporting Information, Table S1). Acidification of the extraction solvent (1% acetic acid) was tested in order to improve recoveries for nereistoxin. They increased up to 71% but in the case of thiocyclam decreased until 65%. For this reason, a sonication step after addition of extraction solvent was tested as in previous studies (Ferrer et al., 2010), in order to improve the migration of the compounds to the solvent extraction. Better recoveries were obtained and for instance, the recovery for thiocyclam increased till 77% (Table S1), whereas the recovery for nereistoxin was 70 %.

The optimized method was validated and the results obtained for thiocyclam and nereistoxin in tomato were in the ranges established by SANTE guidelines (European

Commission, 2017) (see Table S2). Recoveries were between 72% and 82%, precision values were lower than 20% and determination coefficients (R^2) were greater than 0.99. LOQs were 10 µg/kg for both compounds.

3.2. Laboratory trials

As indicated in the introduction section, thiocyclam degrades in nereistoxin, its principal metabolite. For this reason, dissipation kinetics of thiocyclam was studied in tomato samples under laboratory conditions and a "Single First-Order Rate" (SFO) model was used (Eq. 1.),

$$C_t = C_0\, e^{-k\mathrm{t}} \qquad \text{Eq. 1}$$

where C_0 is the initial concentration, k is the rate constant (k) and C_t is the concentration at time t (Lopez-Ruiz, Romero-González, & Garrido-Frenich, 2019). In addition to this model, other models were studied in order to obtain the best fit. Zero order, one-and-a-half-order, first order and second order were tested, achieving the best results (lowest value for the residual sum of squares) using SFO model (Table S3).

3.2.1. Dissipation study of thiocyclam at low dose

The first experience carried out was the dissipation kinetics of thiocyclam in tomato at low dose (1 mg/kg). After applying SFO model (Table 2 and Figure S1) it was observed that thiocyclam dissipation into nereistoxin was really fast. After 2 days of the commercial product application, concentration of thiocyclam was approximately 10 times lower than the application dose. In addition, half-lives of thiocyclam (DT_{50}) was 0.5 days, which indicates that the persistence was really low, being a non-persistent pesticide (DT_{50}<15 days) (Ortiz, 2008). Nereistoxin appearance was significant, and its concentration increased quickly. Three hours after application, nereistoxin was present at 0.2 mg/kg (Figure 1a) and two days later, the maximum concentration (1.6 mg/kg) was achieved. Even at the end of the monitoring period (23 days) concentration of nereistoxin was still really high (1 mg/kg), and comparing with thiocyclam behaviour, nereistoxin was more persistent in tomato. In addition, mass balance was carried out and it was noted that thiocyclam was mainly degraded into nereistoxin, observing that 7 hours after the application, there was an equimolar mixture of both components, and after that, the amount of nereistoxin was higher, due to the dissipation of thyocyclam.

It can be concluded that thiocyclam persistence was low meanwhile nereistoxin persistence was really high and probably its metabolites could be detected in vegetables (Namera, Watanabe, Yashiki, Kojima, & Urabe, 1999; Roberts, Hutson, Philip, Lee, & Plimmer, 1998).

3.2.2. Dissipation study of thiocyclam at high dose: detection of nereistoxin metabolites

To monitor metabolites of nereistoxin at detectable concentrations (>1 µg/kg), thiocyclam was applied at higher concentration (20 mg/kg).

The second experience consisted in a dissipation kinetics study of thiocyclam, monitoring nereistoxin and metabolites previously described in bibliography (Roberts et al., 1998) (Figure 2). Because there are not commercially available standards of these metabolites, they were calculated using the matrix matched calibration curve obtained for nereistoxin and expressed as nereistoxin content.

Results of SFO kinetics model was similar to low dose (Table 2 and Figure S1). DT_{50} of thiocyclam was slightly smaller than at low dose, 0.5 days (low dose) and 0.3 days (high dose) and k value was 1.5 days^{-1} at low dose and 2.4 days^{-1} at high dose. In relation to nereistoxin, its concentration was 1.8 mg/kg one day after application, similar to thiocyclam concentration (2 mg/kg). Its maximum concentration was at 7 days, 11 mg/kg, being stable until 30 days (10 mg/kg) and at the end of the monitoring period (60 days), the concentration was still 3 mg/kg, indicating the high persistence of nereistoxin in tomato.

In addition, due to thiocyclam degrades very quick (2 days or less), it can be possible that in routine control analysis this compound cannot detected. However residues of nereistoxin, originated from thocyclam, can remain in fruits and vegetables during longer periods as it was observed in this study. This could be a potential risk for consumers since toxicity of nereistoxin is really high causing respiratory problems (Park et al., 2015). According to these results, in addition to thiocyclam, nereistoxin should be monitored in routine control analysis to detect these type of compounds and this could be included in a possible MRL definition of thiocyclam (its precursor molecule) to ensure food safety.

Finally, four metabolites of nereistoxin were detected (Figure 3). They are metabolites 3, 5, 7 and 8 (Figure 2). Metabolite 3 was originated from the sulfonation (+SO) of one sulfur atom from the ring of nereistoxin and metabolite 7 by the sulfonation of both sulfur atoms. Metabolite 5 came from the rupture of sulfur-sulfur linkage followed by methylation and sulfonation of both sulfurs. Metabolite 8 occurred from the demethylation ($-CH_3$) of one methyl group of amine followed by the rupture of the two carbon-sulfur bonds from the nereistoxin ring (Figure 1).

Metabolites 5 and 7 were detected only one day, metabolite 5 at 22 days after application at concentration around 1.5 μg/kg and metabolite 7, one day after application at 10 μg/kg. The other two metabolites were detected throughout the study, as it can be observed in Figure 4. Metabolite 3 was detected 15 days after application (3 μg/kg) and increased its concentration until 45 days (75 μg/kg) and later decreased (60 days) at 60 μg/kg. Metabolite 8 was detected during the whole study. The highest concentration value was at 15 days (15 µg/kg) and later decreased until the end of the experience (3 µg/kg). The appearance of metabolites was confirmed at high dose application of thiocyclam, and one of them (metabolite 3) was detected at concentrations higher than 50 µg/kg, so it has to be considered in further studies in order to obtain information about potential risk to the human health. As at low dose studies, a mass balance was also performed observing that after one day of thiocyclam application, most of the 50 % of the initial content of thiocyclam was degraded into nereistoxin, which was stable till 30 days after application, and after that, this compound was degraded.

3.3. Risk assessment studies

Laboratory trials revealed that thiocyclam degraded very quickly, and approximately 90% of it was degraded in 1 day when both studies, at low and high dose, were performed, indicating that the dose does not influence in the dissipation rate. Thiocyclam degrades rapidly to nereistoxin and this remains in vegetables during a long time (more than 60 days). However there are few data about the nereistoxin toxicity and related compounds, and only acceptable daily intake (ADI) data for thiocyclam was provided, which is 0.0125 mg/kg bw/d (University of Hertfordshire, 2007). In addition, nereistoxin toxicity was studied in aquatic organism (bog frog) and in mammals (mouse), providing values for median lethal oral concentration (LC_{50}) of 0.122 mg/L for frog (Environmental Protection Agency, 2019a) and the median lethal oral dose (LD_{50}) of 92.6 mg/kg for mouse (Dai et al., 2020). In both cases, these values are smaller than thiocyclam toxicity (LC_{50} 1 mg/L and LD_{50} 156 mg/kg) (Environmental Protection Agency, 2019b). This reveals that nereistoxin was more toxic than its parent compound. Also, as it was revealed in this work, nereistoxin degrades into other metabolites, whose toxicity should also be evaluated. Therefore, more data related to nereistoxin toxicity

(ADI values or acute reference doses (ARD)) is needed, due to its persistence in vegetables, as well as risk assessment of thiocyclam is directly associated with nereistoxin, and additional data about its toxicity in humans is required.

4. CONCLUSIONS

To sum up, thiocyclam dissipation kinetics was first studied in tomato samples at two dose levels using LC-HRMS. Thiocyclam persistence was really low, being a non-persistent substance ($DT_{50} < 1$ day), degrading to nereistoxin, a natural toxin that persists during a long time in vegetables. Persistence of nereistoxin was higher than 60 days, since it was detected during the whole monitoring period. In addition, four metabolites of nereistoxin, were also detected during dissipation studies, detecting two of them (metabolite 3 and 8) during all monitored period. Because the use of thiocyclam and related substances has increased in the last few years these substances need to be studied deeply, in order to ensure food safety and nereistoxin should be included in routine laboratory analysis as a marker of the use of thiocyclam.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found in the online version

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no competing financial interest

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TABLES

Table 1. UHPLC-Orbitrap-MS parameters for thiocyclam and nereistoxin in solvent at 100 µg/L.

	Ionization mode	**Precursor ion**			**Fragments**			**Retention Time (min)**
		Exact mass	**Adduct**	**Mass error (ppm)**	**Exact mass**	**Molecular formula**	**Mass error (ppm)**	
Thiocyclam	Positive	182.01264	$[M+H]^+$	-0.42	136.95471	$C_3H_5S_3$	-2.6	3.89
					103.97530	$C_3H_3S_2$	-2.9	
					73.01122	C_3H_5S	-3.1	
Nereistoxin	Positive	150.04057	$[M+H]^+$	-0.02	104.98338	$C_3H_5S_2$	2.1	4.08
					72.00354	$C_2H_5S_2$	2.9	
					61.01140	C_3H_3S	-3.4	

Table 2. SFO kinetic model parameters and tomato dissipation (DT_{50}) of thiocyclam

	Dose	
Parameters	Low (1 mg/kg)	High (20 mg/kg)
C_0 (µg/kg)	720.38	19999.99
k (days^{-1})	1.50	2.38
DT_{50} (days)	0.46	0.30
R^2	0.920	0.904

Figure legends

Figure 1. Thiocyclam and nereistoxin behaviour at a) low dose (1 mg/kg) and b) high dose (20 mg/kg). (Error bars obtained for n = 3).

Figure 2. Nereistoxin metabolites previously described by Roberts et al.(Roberts et al., 1998)

Figure 3. Extracted ion chromatograms of nereistoxin metabolites detected in tomato sample at high dose.

Figure 4. Metabolite behaviour according to nereistoxin concentration in tomato at high dose.

Figure(s)

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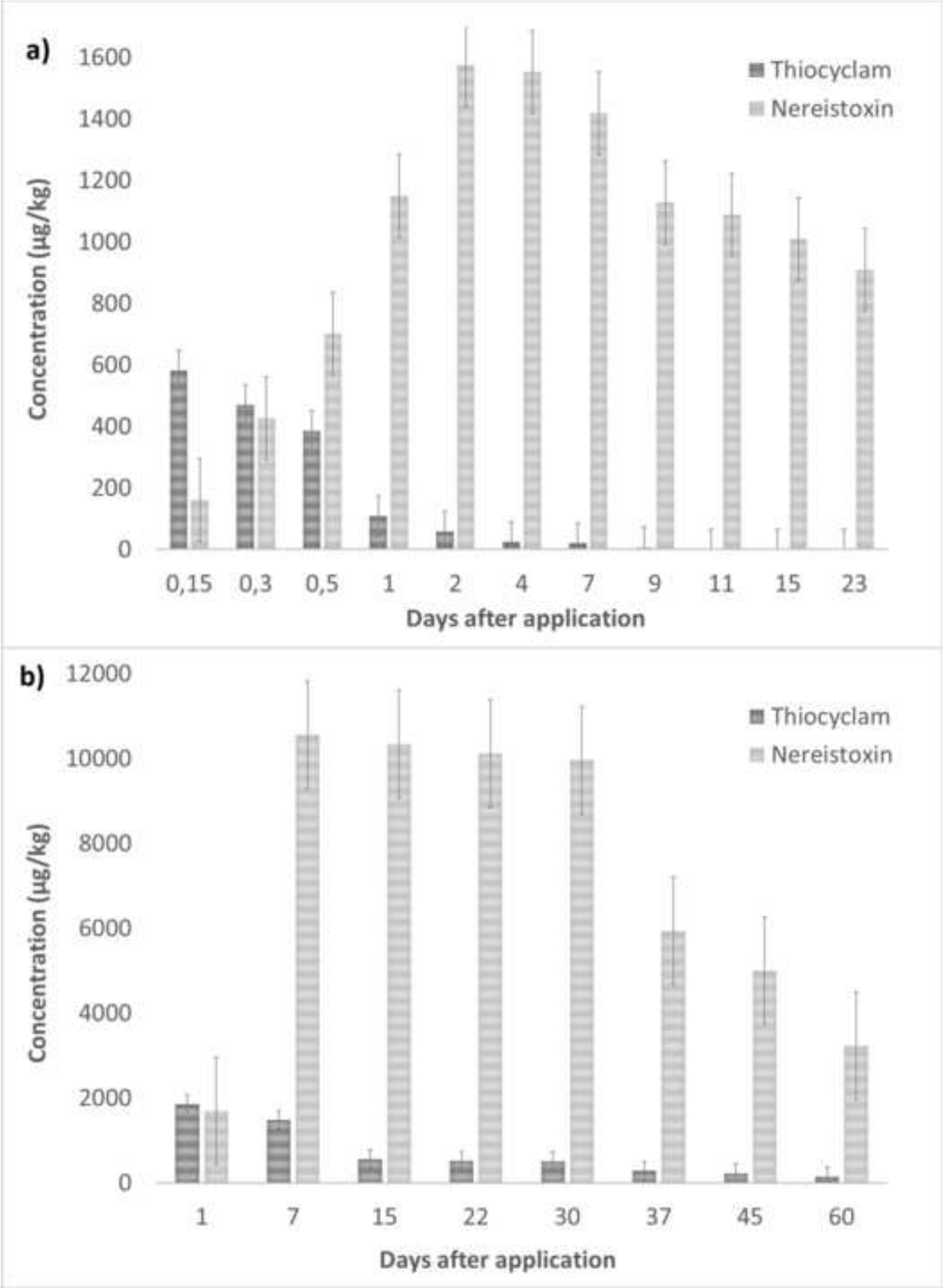

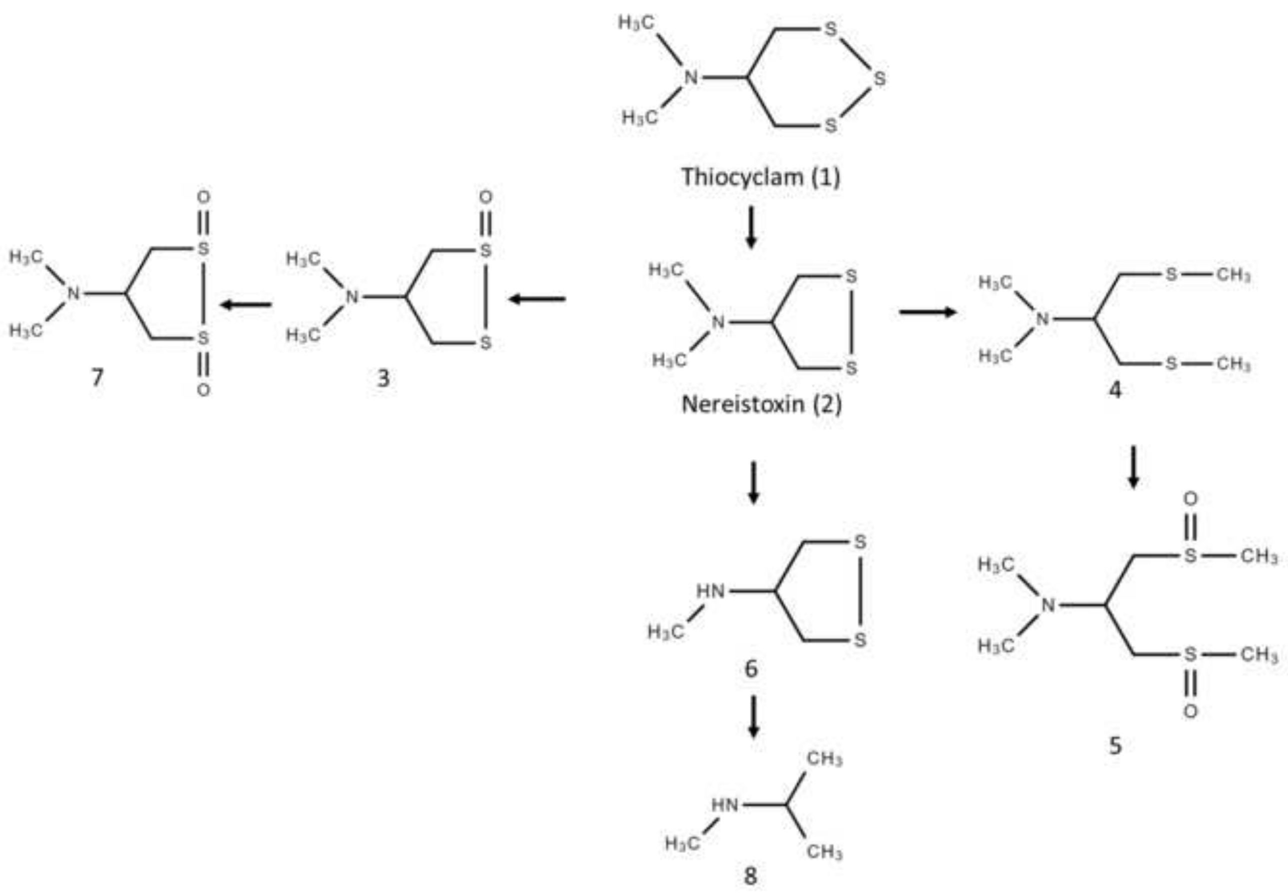
Thiocyclam (1)
Nereistoxin (2)
3
4
5
6
7
8

Figure(s)
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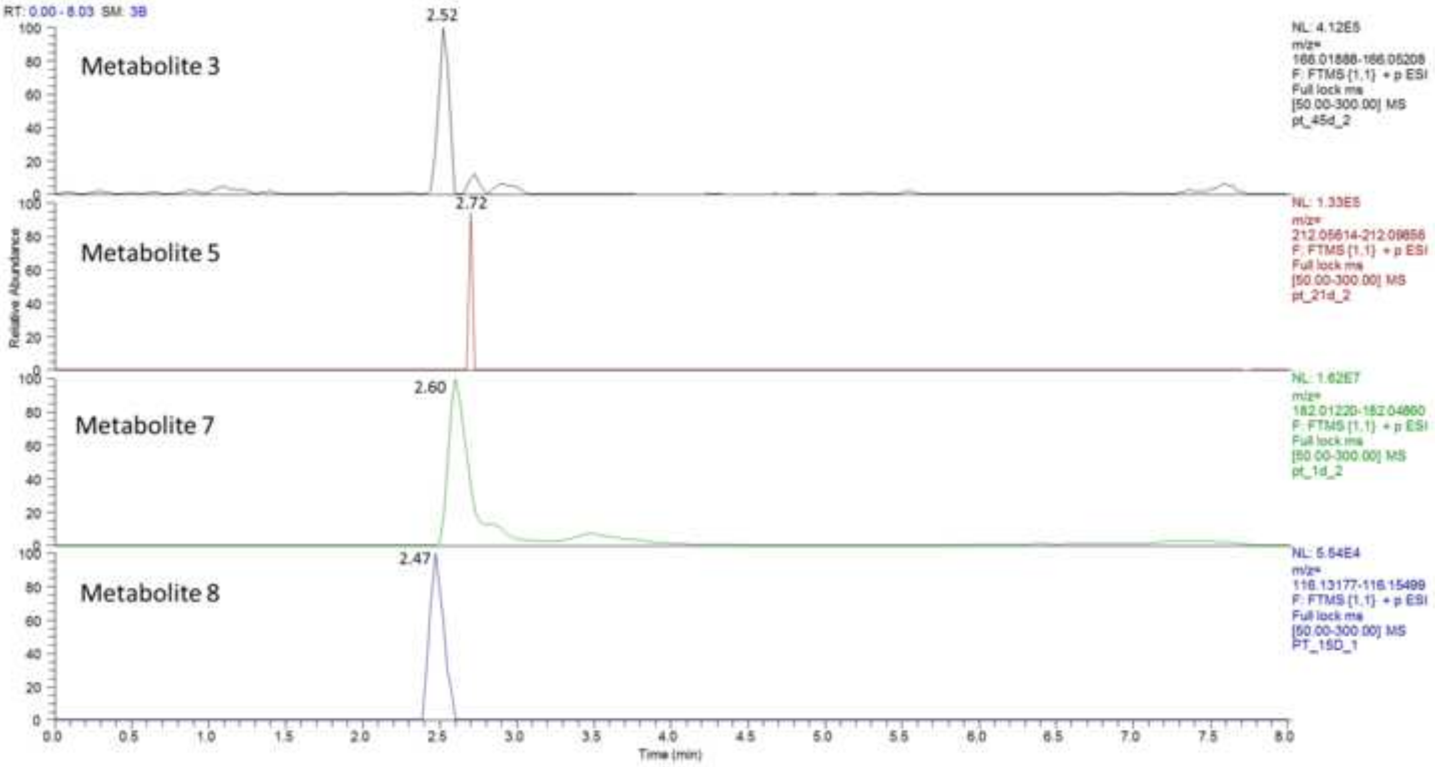

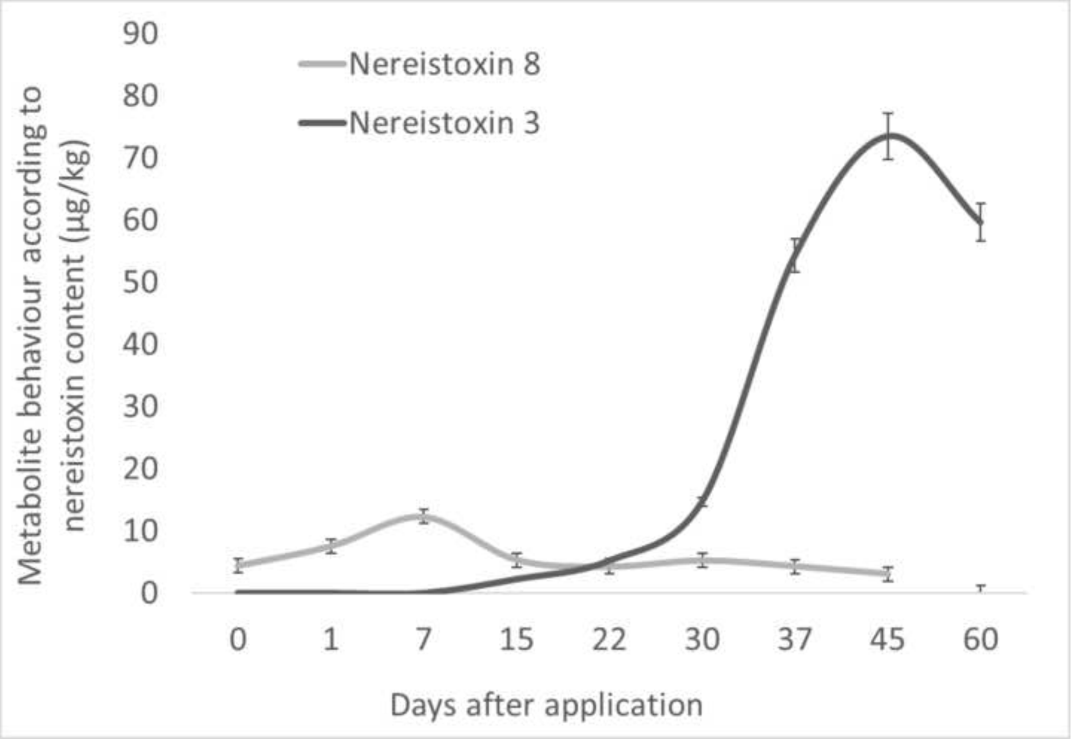
Metabolite behaviour according to nereistoxin content (µg/kg)
90
80
70
60
50
40
30
20
10
0
Nereistoxin 8
Nereistoxin 3
0
1
7
15
22
30
37
45
60
Days after application

Journal Name: Food Chemistry

SUPPLEMENTARY MATERIAL to the paper entitled: Dissipation study and risk assessment of the proinsecticide thiocyclam and nereistoxin in tomato using liquid chromatography high resolution mass spectrometry

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Table of contents

Section 1. Materials, reagents and apparatus

Individual standard solutions of each compound (1000 mg/L) were prepared in acetonitrile (ACN) and they were stable for six months. Intermediate solutions of the two compounds (10 mg/L) were prepared in ACN and were stable for 1 month. Both types of solutions were stored at -21ºC.

ACN and methanol (MeOH) (both LC-MS grade) were supplied by Fluka (St. Louis, MO, USA). Water (LC-MS grade) was purchased from J.T. Baker (Deventer, The Netherlands) and acetic and formic acid were acquired from Fisher Scientific (Erembodegem, Belgium). Magnesium sulfate, sodium chloride, sodium citrate dibasic sesquihydrate and sodium citrate tribasic dehydrate were provided by Sigma-Aldrich (St. Louis, MO, USA)

An analytical balance AB204-S (Mettler Toledo, Greifensee, Switzerland), a vortex mixer WX from Velp Scientifica (Usmate, Italy), an ultrasound Elmasonic S 80 H from Elma Schmidbauer (Germany) and a Centronic BL II centrifuge (J.P. Selecta, Barcelona, Spain) were used for sample preparation.

A Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend^{TM}, Thermo Fisher Scientific, San Jose, CA, USA) was used for chromatographic analysis. A Hilic column (Zorbax Hilic Plus (100 mm × 2.1 mm, 3.5 μm particle size) was coupled to a C_{18} column (Zorbax Eclipse Plus C18, 100 mm × 2.1 mm, 1.8 μm particle size), both purchased from Agilent Technologies (Santa Clara, CA, USA). They were used for separation of the selected compounds in the UHPLC system. Flow rate was set at 0.2 mL/min, and the injection volume was 10 μL. LC system operates in gradient mode, and it started at 85% of eluent B, kept constant for 1 min, and decreased to 65% B in 2 min and later to 0% B in 2 min. This composition was kept constant 1 min and returned to the initial composition in 0.5 min and held for 1.5 min. The total running time was 8 min and the mobile phase was composed by a water solution of 0.1% formic acid (eluent A) and ACN (eluent B).

A single mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive^{TM}, Thermo Fisher Scientific, Bremen, Germany), was coupled to LC system using an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA). Different ESI parameters were set: sheath gas (N_2, >95%), 35 (arbitrary units, a.u.); auxiliary gas (N_2, >95%), 10 (a.u.); spray voltage, 4 kV; capillary voltage, 35 V; skimmer voltage, 18 V; tube lens voltage, 95 V; capillary temperature, 300 °C; heater temperature, 305 °C. Four acquisition functions, with the following characteristics, were used to acquire the MS spectra: (1) full ESI+ MS, without fragmentation (higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 50,000 FWHM; scan time = 0.25 s; (2) all-ions fragmentation (AIF), ESI+, with fragmentation (collision energy 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s, (3) full MS, ESI- using the settings described in the first function, and (4) AIF, ESI- using the same settings that described for the second acquisition function. Mass range was set at *m/z* 50–300.

External calibration mode was used for the acquisition of the chromatograms. Xcalibur^{TM} version 3.0, with Quanbrowser and Qualbrowser were used for the sample processing.

Table S1. Recoveries (%) obtained using different extraction methods at 100 µg/kg.

Extraction method	**Thiocyclam**	**Nereistoxin**
QuEChERS citrate-buffered[a]	95	49
QuEChERS citrate-buffered with acidified ACN	65	71
QuEChERS citrate-buffered with acidified ACN and sonication step	77	70

[a] Lehotay, S. J., Son, K. A., Kwon, H., Koesukwiwat, U., Fu, W., Mastovska, K., … Leepipatpiboon, N. (2010). Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. *Journal of Chromatography A*, *1217*(16), 2548–2560. https://doi.org/10.1016/j.chroma.2010.01.044

Table S2. Validation parameters of the optimized method in tomato samples

Analites		**Thiocyclam**	**Nereistoxin**
R^2		0.9986	0.9995
Matrix effect[a]		21%	19%
LOQ (µg/kg)		10	10
Recovery (%)	***10 µg/kg***	77	72
	100 µg/kg	82	74
Intra-day Precision (% RSD)[b]	***10 µg/kg***	9 (11)[c]	11 (12)
	100 µg/kg	8 (11)	14 (15)

[a] Matrix effect = ((matrix calibration curve slope/ solvent calibration curve slope) – 1) x 100%

[b] n = 10

[c] Inter-day precision in parenthesis.

Table S3. Models tested to fit residual thiocyclam concentration in tomato at low dose.

Model	**Zero order**	**First order (SFO)**	**One-and-a-half-order**	**Second order**
Tomato low dose				
Residual concentration curve	$C\ (t) = 339.14 - 40.13 \text{ x } t$	$C(t) = 720.38\ e^{-1.5t}$	$C\ (t) = \left(\frac{1}{\sqrt{720.38}} + \frac{1.49 \text{ x } t}{2}\right)^{-2}$	$C\ (t) = \frac{910.66}{1 + (910.66 \text{x} 0.004t)}$
Square of sum of residuals	$2\text{x}10^{5}$	$6.5\text{x}10^{3}$	$3.2\text{x}10^{8}$	$1.9\text{x}10^{4}$

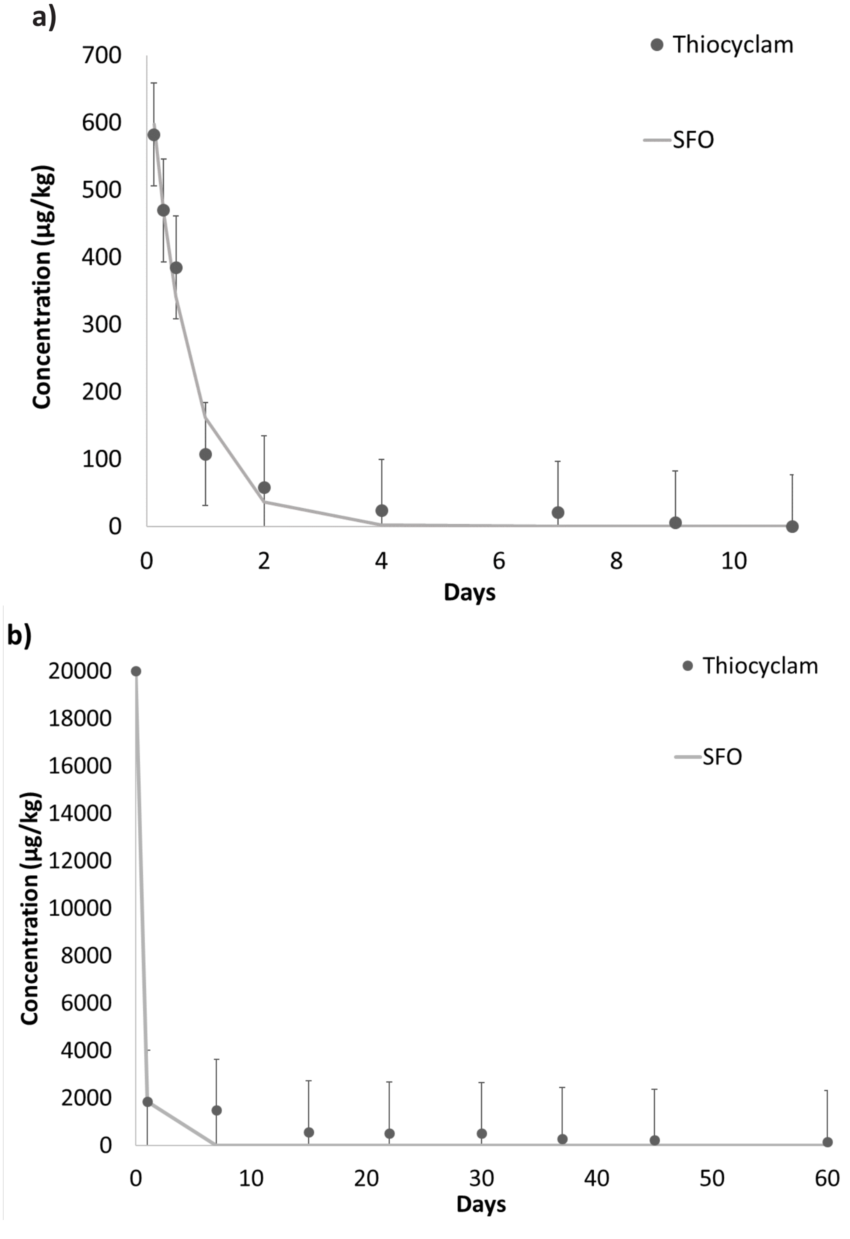

Figure S1. Concentration of thiocyclam (adjusting to kinetic model "Single First-Order Rate" (SFO)) in tomato a) at low dose and b) at high dose (Error bars obtained for n = 3)

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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CAPÍTULO
4

Estudios de disipación de herbicidas e identificación de sus metabolitos en matrices ambientales

1. INTRODUCCIÓN

Los herbicidas al igual que los insecticidas son ampliamente usados en la actualidad. Se aplican en el medio con el fin de eliminar malas hierbas, que pueden llegar a ser perjudiciales para los cultivos de interés. Suelen ser aplicados sobre suelos de cultivos agrícolas, como patata, cucurbitáceas etc…, que por su tamaño o modo de crecimiento (en forma de arbusto) están más afectados por estas malas hierbas. Al ser aplicados directamente al suelo, cobra gran relevancia el estudio del comportamiento de estos compuestos en este medio, así como en el agua o aire con el fin de asegurar la calidad ambiental. Además, los herbicidas pueden degradarse en metabolitos, permaneciendo en el medio y en algunos casos produciendo daños en la flora y la fauna [1]. Este es el caso de quizalofop-p y quizalofop descritos previamente por la EFSA, como metabolitos comunes de los herbicidas propaquizafop, quizalofop-p-etil y quizalofop-p-terfuril [2]. Sin embargo, muchos de ellos no han sido estudiados aún, por lo que es interesante emplear herramientas como la HRMS, que mediante el modo de análisis *full scan* puede monitorizar todos los componentes de la muestra para realizar estudios de sospechosos (*suspect screening)* y determinar todos los metabolitos descritos previamente, como por ejemplo los que EFSA declaró para la familia del quizalofop en sus estudios de evaluación de riesgos [2]. Para ello, se hizo uso de bases de datos creadas en nuestro laboratorio o adquiridas comercialmente. Además, se llevaron a cabo análisis de compuestos no descritos previamente, *unknown,* mediante el empleo de softwares especiales de análisis.

Los métodos de extracción más empleados en matrices ambientales son la SLE [3] o el método QuEChERS [4] para suelos, mientras que para aguas, la técnica de referencia es SPE [5]. Como técnicas de análisis destacan LC o GC acoplada a MS [6].

Por consiguiente, los trabajos incluidos en este capítulo se han focalizado en el desarrollo de métodos analíticos para el estudio del comportamiento de herbicidas específicos en matrices medioambientales, tales como suelos y aguas, (**Artículos científicos VI, VII y VIII**), incluyendo los metabolitos descritos previamente en bibliografía. También se ha realizado la búsqueda de metabolitos procedentes de cada uno de los compuestos objetivo mediante *suspect screening* o *unknown* haciendo uso de bases de datos y de software que permiten establecer rutas metabólicas, así como de búsqueda de desconocidos (**Artículos científicos VI, VII y VIII)**.

- **Artículo científico VI.** Degradation studies of quizalofop-p and related compounds in soils using liquid chromatography coupled to low and high resolution mass analyzers

- **Artículo científico VII.** Behavior of quizalofop-p and its commercial products in water by liquid chromatography coupled to high resolution mass spectrometry

- **Artículo científico VIII.** Degradation studies of dimethachlor in soils and water by UHPLC-HRMS: putative elucidation of unknown metabolites

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Publicación VI

DEGRADATION STUDIES OF QUIZALOFOP-P AND RELATED COMPOUNDS IN SOILS USING LIQUID CHROMATOGRAPHY COUPLED TO LOW AND HIGH RESOLUTION MASS ANALYZERS

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Degradation studies of quizalofop-p and related compounds in soils using liquid chromatography coupled to low and high resolution mass analyzers

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HIGHLIGHTS

- LC-Orbitrap was used for degradation studies of quizalofop and related compounds.
- Degradation studies of different quizalofop-related products were evaluated.
- Enantiomeric determination of quizalofop has been investigated using LC-QqQ.
- The dissipation of quizalofop in two types of soils has been studied.

GRAPHICAL ABSTRACT

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ABSTRACT

A comprehensive degradation study of quizalofop-p, quizalofop-p-ethyl, quizalofop-p-tefuryl and propaquizafop in soil samples have been firstly performed using ultra high performance liquid chromatography coupled to Orbitrap mass spectrometry (UHPLC-Orbitrap-MS). Thus, metabolites or degradation products, such as CHHQ (dihydroxychloroquinoxalin), CHQ (6-chloroquinoxalin-2-ol), PPA ((*R*)-2-(4-hydroxyphenoxy)propionic acid) and 2,3-dihydroxyquinoxaline were also monitored. An extraction procedure based on QuEChERS procedure was used. Acidified water (0.1 M hydrochloric acid) and acidified acetonitrile (1% acetic acid, (v/v)) were used as extraction solvents, and magnesium sulfate and sodium chloride were used as salts. Dispersive solid phase extraction with C_{18} as sorbent, was needed as a clean-up step. Several commercial products (Panarex®, Master-D® and Dixon®) were used to evaluate the degradation of the target compounds into their metabolites. The concentration of the main active substances (quizalofop-p-tefuryl, quizalofop-p-ethyl and propaquizafop) decreased during the degradation studies, whereas the concentration of quizalofop-p increased. Dissipation rates of half-live of quizalofop-p were also evaluated, and it was observed that this compound is easily degraded, obtaining values lower than 1 day. Taking into account that quizalofop-p is the *R* enantiomer of quizalofop, a chiral separation was performed by liquid chromatography coupled to tandem mass spectrometry, concluding that in samples containing quizalofop-p-tefuryl, there was a 15% contribution from the *S* enantiomer and a 85% contribution from the *R* enantiomer. Metabolites such as PPA, CHHQ and CHQ were detected in soil samples after 15 days of application commercial product at concentrations between the limits of detection (LOD) and the limits of quantification (LOQ). CHQ and CHHQ were detected at concentrations higher than the LOQ in samples after 50 and 80 days of application, with their concentration increasing during this time up to 500%.

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1. Introduction

Quizalofop-p, also known as (*R*)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionic acid, is an aryloxyphenoxypropionate compound. It is a systemic herbicide, absorbed by the leaves with translocation throughout the plant (Mantzos et al., 2016), and it is commonly used for post-emergence control of annual and perennial grass weeds in broad-leaved crops like soya beans (Roberts et al., 1998). This compound is the *R* enantiomer of quizalofop (2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionic acid), which is the parent compound, and its use as pesticide has not been approved. Nevertheless its metabolites can be used as phytosanitary products (European Union, 2002). Quizalofop-p is the main active substance of quizalofop, and other compounds such as quizalofop-p-ethyl (ethyl (2*R*)-2-{4-[(6-chloroquinoxalin-2-yl)oxy]phenoxy}-propanoate) and quizalofop-p-tefuryl ((*RS*)-tetrahydrofurfuryl (*R*)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionate) can be used as main substances of commercial products. Another metabolite related to quizalofop is propaquizafop (2-[(Isopropylideneamino)oxy]ethyl(2*R*)-2-{4-[(6-chloro-2-quinoxalinyl)oxy]phenoxy}propanoate). This compound is an isopropylideneamino ester of quizalofop that has been scarcely evaluated. In addition, there are a number of common metabolites to all of this compounds, which can be detected in soil, water, crops or air as CHHQ (dihydroxychloroquinoxalin), CHQ (6-chloroquinoxalin-2-ol), PPA ((*R*)-2-(4-hydroxyphenoxy)propionic acid) and 2,3-dihydroxyquinoxaline (EFSA (European Food and Safety Authority), 2008).

Up to now, the maximum residue limit (MRL) in food commodities for quizalofop-p has only included the parent compound, quizalofop, which has not been authorized as herbicide, whereas propaquizafop has an independent MRL ("Pesticide database,", n.d.).

Up to our knowledge, the few papers that have studied this herbicide, quizalofop-p, and its metabolites were mainly focused on the chiral study and the degradation of the parent compound (Li et al., 2012; Liang et al., 2014; Ma et al., 2016), whereas in multiresidue methods focused studies the parent compound is included but not the metabolites (Kaczyński et al., 2016; Karasali et al., 2016; Lazartigues et al., 2011; Mantzos et al., 2016; Marchese et al., 2001). Therefore, more studies focused on the dissipation of quizalofop and related products are needed for determining the metabolites that could appear during the degradation of the parent compounds.

The extraction of quizalofop and quizalofop-p-ethyl in soil has been usually performed by solid-liquid extraction followed by a dehydration step (Ma et al., 2016; Li et al., 2012). When multiresidue methods are used, the extraction method applied is the European version of QuEChERS (Mantzos et al., 2013), using acetone or acetonitrile as extraction solvent.

For the analysis of the target compounds, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the most used analytical technique (Kaczyński et al., 2016; Karasali et al., 2016; Lazartigues et al., 2011; Marchese et al., 2001; Saha et al., 2015), but LC-UV has also been utilized (Guillén-Casla et al., 2011; Ma et al., 2016; Li et al., 2012) especially for the enantioselective degradation of quizalofop-p-ethyl. Up to our knowledge, there are no studies using high-resolution mass spectrometry (HRMS) for the analysis of these compounds. This could be explained by the high cost of these analyzers and the fact that they have not been implemented in routine laboratories yet. Nevertheless HRMS instruments, such as Orbitrap, have several advantages because they operate in the full scan mode (theoretically, no limitations in the number of monitored compounds) and the Independent Data Acquisition (IDA) mode enables the detection of a wide range of compounds at low concentration levels in complex sample matrices with high mass accuracy (Gómez-Pérez et al., 2012). This powerful analytical tool allows for the development of analytical strategies that combine: (a) target analysis (determination of specific priority analytes for which standards are available); (b) post-run target or retrospective screening analysis based on an accurate customized mass database of known parent molecules and some diagnostic fragment ions or isotopic pattern, and (c) non-target analysis (Coscollà et al., 2014). For these reasons, in this study, HRMS is used for the detection of target compounds (parent compounds and known metabolites) as a result of the degradation of quizalofop-p and related compounds in soils under field conditions.

The aim of this study is to understand the dissipation behavior of quizalofop and related compounds in soils, monitoring the parent compound and the appearance of metabolites due to the scarce studies focused on this issue. For that purpose, a new analytical method has been developed and validated for the quantitative determination of quizalofop and metabolites applying UHPLC coupled to Orbitrap-MS. Moreover, HPLC-QqQ-MS/MS was used for the enantiomeric separation of quizalofop.

2. Materials and methods

2.1. Equipment, material and reagents

Quizalofop (CAS registry No. 76578-12-6, purity 97,1%), quizalofop-p-ethyl (CAS registry No. 100646-51-3, purity 98,4%), quizalofop-p-tefuryl (CAS registry No. 200509-41-7, purity >99%), propaquizafop (CAS registry No. 111479-05-1, purity >99%), quizalofop-p (CAS registry No. 94051-08-8, purity >99%), 2,3-dihydroxyquinoxaline (CAS registry No. 15804-19-0, purity >99%) and PPA (CAS registry No. 94050-90-5, purity >99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CHHQ (CAS Registry No. 6639-79-8, purity ≥99%) and CHQ (CAS Registry No. 2427-71-6, purity >99%) were purchased from Cymit (Barcelona, Spain).

Commercial products, like Dixon® (propaquizafop 10% (v/v)) and Master-D® (quizalofop-p-ethyl 5% (v/v)) were purchased in Planeta Huerto (Alicante, Spain), while Panarex® (quizalofop-p-tefuryl 4% (v/v)) was donated by Massó S.A. (Barcelona, Spain).

Stock standard solutions of 1000 mg/L were prepared by dissolving 10 mg of the pure compound in 10 mL of acetonitrile (ACN), except for 2,3-dihydroxyquinoxaline, CHQ and CHHQ, which were prepared in a mixture of ACN:water (50:50 (v/v)). Intermediate solution of the compounds (10 mg/L) was prepared by taking 100 μL of each stock solution and diluting up to 10 mL with acetonitrile in a volumetric flask. Stock solution was stored at −21 °C and intermediate solutions were stored at 4 °C. Stock solution were stable for a year and the intermediate solution for 2 months.

ACN (LC-MS grade) and formic acid were acquired from Fluka (St. Louis, MO, USA). Water (LC-MS grade) and hydrochloric acid were acquired from J.T. Baker (Deventer, The Netherlands) and acetic acid was obtained from Panreac (Barcelona, Spain). Isopropanol, ethanol and *n*-hexane (LC-MS grade) were obtained from Sigma-Aldrich. Ammonium acetate and magnesium sulfate (Sigma-Aldrich), sodium chloride (J.T. Baker) and C_{18} (Supelco, Bellefonte, PA, USA) were used during the extraction procedure and for the preparation of the mobile phase.

A mixture of acetic acid, caffeine, Met–Arg–Phe–Ala–acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrat and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative) from Thermo-Fisher (Waltham, MA, USA) were employed for the accurate mass calibration of the Orbitrap analyzer.

For the treatment and preparation of samples, an analytical balance AB204-S from Mettler Toledo (Greifensee, Switzerland), a vortex mixer WX from Velp Scientifica (Usmate, Italy), a Reax 2 rotary agitator from Heidolph (Schwabach, Germany) and a Centronic BL II centrifuge from J.P. Selecta (Barcelona, Spain) were used.

2.2. UHPLC-Orbitrap-MS analysis

For chromatographic analysis Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) was used.

A Zorbax Eclipse C_{18} column (100 mm × 2.1 mm, 1.8 μm particle size) and a Zorbax Hilic Plus column (100 mm × 2.1 mm, 3.5 μm particle size) supplied by Agilent Technologies (Santa Clara, CA, USA) were used for chromatographic separation, using a flow rate of 0.2 mL/min. The mobile phase consisted of eluent A, which was a water solution of 0.1% acetic acid adjusted to pH = 4 with ammonium acetate and eluent B, ACN.

The step gradient was as follows: 0–1 min 15% A, then it was linearly increased to 60% in 6 min, and to 100% in 1 min, and then it was kept constant for 3 min. Finally, it returned to the initial conditions in 30 s and remained constant during 90 s. The total run time was 13 min. The column temperature was set to 25 °C and the injection volume to 10 μL.

The chromatographic system was coupled to a single mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen, Germany) that used an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA) in positive and negative ion modes. ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N2, >95%), 35 (adimensional); auxiliary gas (N2, >95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 °C; and capillary temperature, 300 °C. The mass spectra were acquired employing two alternating acquisition functions: (1) full MS, ESI+, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 full width at half maximum (FWHM); scan time = 0.25 s, (2) full MS, ESI− using the aforementioned settings, (3) all-ions fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s, and (4) AIF, ESI− using the settings explained for (3). The mass range in the full scan experiments was set to *m/z* 50–500.

The chromatograms were acquired using the external calibration mode and they were processed using Xcalibur™ version 2.2, with Quanbrowser, Qualbrowser, and Mass Frontier™ 6.0 (Thermo Fisher Scientific, Les Ulis, France).

2.3. HPLC-QqQ-MS/MS analysis to enantiomeric separation

Detection of (*S*)-quizalofop and quizalofop-p ((*R*)-quizalofop) was performed with an Agilent series 1290 RRLC instrument (Agilent, Santa Clara, CA, USA) equipped with a binary pump (G4220A), an autosampler thermostat (G1330B) and a column compartment thermostat (G1316C). The RRLC system was coupled to an Agilent triple quadrupole mass spectrometer (6460A) with a Jet Stream electronic spray ionization (ESI) source (G1958-65138). A Chiralpak AY3 column (150 × 4.6 mm, 3 μm particle size) from Daiciel (Barcelona, Spain) was employed for chiral separation. MassHunter (Agilent) was used for chromatographic data treatment.

The chromatographic separation was carried out with ethanol containing 0.3% formic acid (v/v) as mobile phase, using isocratic mode at a flow rate of 0.5 mL/min for 11 min. The injection volume was 5 μL and the column temperature was kept at 25 °C. Quizalofop and quizalofop-p were ionized in positive ESI mode and detected using selected reaction monitoring (SRM) mode. The selected precursor ion was *m/z* 345.0 (fragmentor voltage 125 V), and three product ions were monitored: *m/z* 299 (20 eV) selected as quantifier; *m/z* 243.9 (30 eV); and *m/z* 162.8 (40 eV).

Source gas temperature and sheath gas temperature were 325 °C and 400 °C, respectively. Source gas flow and sheath gas flow were 5 L/min and 11 L/min, respectively. Nebulizer was 45 psi. Capillary and nozzle voltage were 3500 V and 500 V, respectively.

2.4. Samples

Soils samples were collected in different areas of El Ejido, Almería (Spain) and characterized by different physicochemical parameters (see Supplementary material Table S1). They were dried at room temperature for two days and sieved (particle size <2 mm) before analysis.

2.5. Laboratory studies

Two types of soils, clay and sandy loam, were weighed (15 g of dry weight sample). Different volumes of water were added: 12 mL for clay soils (80% water content) and 6 mL for sandy loam soils (40% water content) in order to simulate natural conditions. After that, the soils were spiked with three different commercial products, Dixon® (propaquizafop 10%), Master-D® (quizalofop-p-ethyl 5%) and Panarex® (quizalofop-p-tefuryl 4%) at two concentration levels, normal dose and a double dose. Thus, the normal rate for each commercial product was: Dixon®, 1.50 L/ha (10.3 μL/g of soil); Master-D®, 2.5 L/ha (17.3 μL/g of soil); and Panarex®, 2.5 L/ha (17.3 μL/g of soil). The spiked soil samples were homogenized for 2 min.

Spiked samples were placed in Erlenmeyer flasks and they were stored at room temperature under normal sunlight conditions inside the laboratory. Samples were treated with the commercial product. Later they were taken at 24 h, 2, 3, 4, 5, 7, 15, 30, 50 and 80 days and analyzed. Two replicates per condition were used and the flasks were weighed every two days and the required amount of water was added if necessary (Dionisio and Rath, 2016).

2.6. Sample extraction

During the optimization of the sample extraction, several procedures described by Mantzos (Mantzos et al., 2013) and Saha (Saha et al., 2015) were tested. Briefly, the Mantzos procedure was based on the European version of the QuEChERS method, adding 5 mL of water and 10 mL of ACN to the sample, and performing a clean-up with C_{18} and $MgSO_4$. On the other hand, the Saha method was based on the original version of QuEChERS, but without clean-up.

The optimized extraction procedure performed in this study was as follows: 5 g of soil sample was introduced in a 50 mL plastic centrifuge tube. After that, 10 mL of acidified water (0.1 M hydrochloric acid) and 10 mL of acidified ACN (1% acetic acid, (v/v)) were added and shaken for 1 min in a vortex. Then, the sample was stirred for 1 h in a rotary shaker and 1 g of NaCl and 4 g of $MgSO_4$ were added, and the mixture was shaken vigorously for 1 min in a vortex. After that, the samples were centrifuged for 6 min at 5000 rpm (4136 ×*g*) and 2 mL of the supernatant was introduced in a 15 mL Eppendorf tube. 50 mg of C_{18} was added and shaken for 1 min. Then, the samples were centrifuged for 6 min at 5000 rpm (4136 ×*g*).

One mL of the supernatant was collected and injected into the UHPLC-Orbitrap-MS, whereas 200 μL of supernatant were redissolved in 800 μL of ethanol and injected into the UHPLC-QqQ-MS to perform the chiral separation.

2.7. Method validation

The validation of the analytical method was carried out according to SANTE guidelines (Document No. SANTE/11945/2015) (SANTE/EU, 2015), calculating the following parameters: matrix effect, linearity, working range, trueness (% recovery), precision (intra and inter-day), and limits of quantification (LOQs) and detection (LODs).

Matrix effect was evaluated by analyzing standards at different concentrations in solvent (ACN) and standards prepared in extracted blank matrix. For quizalofop-p, quizalofop-p-ethyl, quizalofop-p-tefuryl, propaquizafop, CHHQ and PPA, the concentrations were from 10 to 100 μg/L and for 2,3-dihydroxyquinoxaline and CHQ from 20 to 100 μg/L. Linearity was carried out using matrix-matched calibration, spiking blank extracts at five concentration levels of quizalofop-p and its metabolites (from 10 to 100 μg/L, with the exception of CHQ and 2,3-dihydroxyquinoxaline which ranged from 20 to 100 μg/L). The linearity of the calibration curves was studied using determination coefficients (R^2).

Trueness was studied in terms of recovery by spiking blank matrix (previously checked) at two different concentration levels for each analyte, at LOQs value and five or ten times greater than LOQs value, analyzing five replicates at each level.

Precision, which was estimated by performing intra and inter-day studies, was expressed as relative standard deviation (RSD) at the same concentration levels selected for trueness. Intraday precision (repeatability) was evaluated within the same day. Interday precision (reproducibility) was estimated by fortifying samples at the same concentration levels of intraday precision, and they were analyzed in ten different days.

LODs and LOQs were estimated by extracting and injecting blank samples spiked at low concentrations (1 to 50 μg/kg). LODs were set as the minimum concentration at which the characteristic ion is monitored with a mass error lower than 5 ppm. LOQs were estimated according to the lower concentration providing acceptable trueness values (70–120%) and precision (≤20%).

3. Results and discussion

3.1. UHPLC-Orbitrap MS optimization

First, the spectrometric characterization of the compounds was carried out following the same procedure described in previous studies (Gómez-Pérez et al., 2014; López-Ruiz et al., 2016). Table 1 shows the UHPLC-MS-Orbitrap parameters for the analysis of quizalofop-p and related compounds. Five compounds were ionized in positive mode, and three were ionized in negative mode. As these compounds belong to the same family, four analytes, quizalofop-p, quizalofop-p-ethyl, quizalofop-p-tefuryl and propaquizafop, shared common fragments. Nevertheless, the precursor ions were different as well as the retention time, and therefore, they could be determined without interferences between each other.

Secondly, chromatographic conditions were studied using conventional C_{18} (Zorbax Eclipse Plus C_{18}) as stationary phase. Mobile phase formed by MEOH:acidified water (0.1% formic acid) was evaluated. Poor results were obtained for 2,3-dihydroxyquinoxaline, which eluted at an early retention time. Therefore ACN:acidified water (0.1% formic acid) was studied, as ACN has been used by other authors for the determination of quizalofop-p (Lazartigues et al., 2011; Zhang et al., 2016). However bad peak shapes were obtained for the metabolites. For this reason the aqueous phase used by Lazartigues et al. (2011) (water solution of 0.1% acetic acid/ammonium acetate pH = 4) was tested, using MEOH and ACN as organic phase. The best results were obtained with water solution of 0.1% acetic acid/ammonium acetate pH = 4:ACN, so it was used for further experiments. Gradient elution was optimized in order to improve the retention time of the metabolites particularly for 2,3-dihydroxyquinoxaline. Three different gradient profiles were evaluated, and the best results were obtained with the gradient profile described in Section 2.2, which is shorter (total run time 13 min) than those provided by other authors such as Lazartigues et al. (2011) (total running time 19 min).

Using these conditions, peak shape was not suitable, as well as some compounds such as PPA eluted very early. That is why, in addition to Zorbax Eclipse plus C_{18}, other stationary phases such as Zorbax Eclipse Plus phenyl-hexyl, Zorbax Eclipse Plus Hilic and ODS Hypersil were tested. The last one provided the worst results, due to the metabolites eluting at a retention time lower than 1 min. Zorbax Eclipse Plus phenyl-hexyl gave better results than Hypersil, but one metabolite (PPA) eluted at 0.6 min. In order to increase the retention time of polar compounds Zorbax Eclipse plus C_{18} and Zorbax Eclipse plus Hilic were coupled and this tandem was evaluated, obtaining the best results. Fig. 1 shows the extracted ion chromatograms of the analytes in this study at a concentration of 500 μg/L, which corresponds to the injection of 5 ng of each compound.

3.2. HPLC-QqQ-MS/MS optimization

For the enantiomeric separation of (*R*,*S*)-quizalofop, an HPLC-QqQ-MS/MS method was developed. First, spectrometric characterization was performed by direct infusion of standard solutions of the two analytes at 10 mg/L in ACN at a flow rate of 0.2 mL/min. Both compounds were analyzed using ESI+. Full scan and MS/MS spectra were performed to acquire the most sensitive transitions. Further optimization was carried out to evaluate the intensity of precursor ion obtained from different fragmentor voltages (from 80 to 130 V) and collision energies (collision energy, from 10 to 50 eV) for each product ion. The characteristic precursor and product ions were described in Section 2.3.

Chromatographic separation was based on a previous study performed in our research group (Marín-Sáez et al., 2016). Chiralpak AY3 column was tested as a stationary phase with ethanol/0.1% DEA as mobile phase in isocratic mode, but no separation was achieved and bad peak shapes were obtained. Standard solutions of the analytes were prepared in different mixtures of ethanol:ACN 50:50, 80:20, 90:20 (v/v) to improve the elution of the compounds. The best results were obtained using a mixture of ethanol:ACN (80:20, v/v). Ethanol/0.1% formic acid was evaluated as a mobile phase due to its popularity in other studies on enantiomeric

Table 1
UHPLC-Orbitrap-MS parameters for the selected compounds.

	Ionization mode	Precursor ion			Fragments			Retention time (min)
		Exact mass	Adduct	Mass error (ppm)	Exact mass	Molecular formula	Mass error (ppm)	
Quizalofop-p	Positive	345.06366	$[M+H]^+$	1.0	299.05818	$C_{16}H_{12}O_2N_2Cl$	1.9	3.73
					244.03979	$C_{13}H_9ON_2Cl$	−0.5	
					197.01124	$C_8H_6O_2N_2Cl$	1.1	
Quizalofop-p-tefuryl	Positive	429.12118	$[M+H]^+$	1.5	299.05818	$C_{16}H_{12}O_2N_2Cl$	0.9	5.41
					271.06327	$C_{15}H_{12}ON_2Cl$	−0.7	
					243.06835	$C_{14}H_{12}N_2Cl$	0.4	
Quizalofop-p-ethyl	Positive	373.09496	$[M+H]^+$	1.3	299.05818	$C_{16}H_{12}O_2N_2Cl$	0.9	5.60
					271.06327	$C_{15}H_{12}ON_2Cl$	0.9	
					243.06835	$C_{14}H_{12}N_2Cl$	0.5	
Propaquizafop	Positive	444.13207	$[M+H]^+$	1.8	327.05310	$C_{17}H_{12}O_3N_2Cl$	0.7	5.83
					299.05818	$C_{16}H_{12}O_2N_2Cl$	0.9	
					271.06327	$C_{15}H_{12}ON_2Cl$	1.8	
					243.06835	$C_{14}H_{12}N_2Cl$	1.2	
2,3-dihydroxyquinoxaline	Positive	163.05020	$[M+H]^+$	0.8	145.03964	$C_8H_5ON_2$	1.3	2.81
					117.04554	$C_7H_5N_2$	4.9	
CHHQ	Negative	194.99668	$[M\text{-}H]^-$	−2.8	167.00067	$C_7H_4ON_2Cl$	−2.2	2.79
					131.02399	$C_7H_3ON_2$	−3.7	
CHQ	Negative	179.00079	$[M\text{-}H]^-$	−4.0	151.00575	$C_7H_4N_2Cl$	−4.2	3.17
					143.02399	$C_8H_3ON_2$	−3.5	
PPA	Negative	181.05063	$[M\text{-}H]^-$	−3.5	108.02168	$C_6H_4O_2$	−5.0	2.74
					93.03454	C_6H_5O	−4.9	

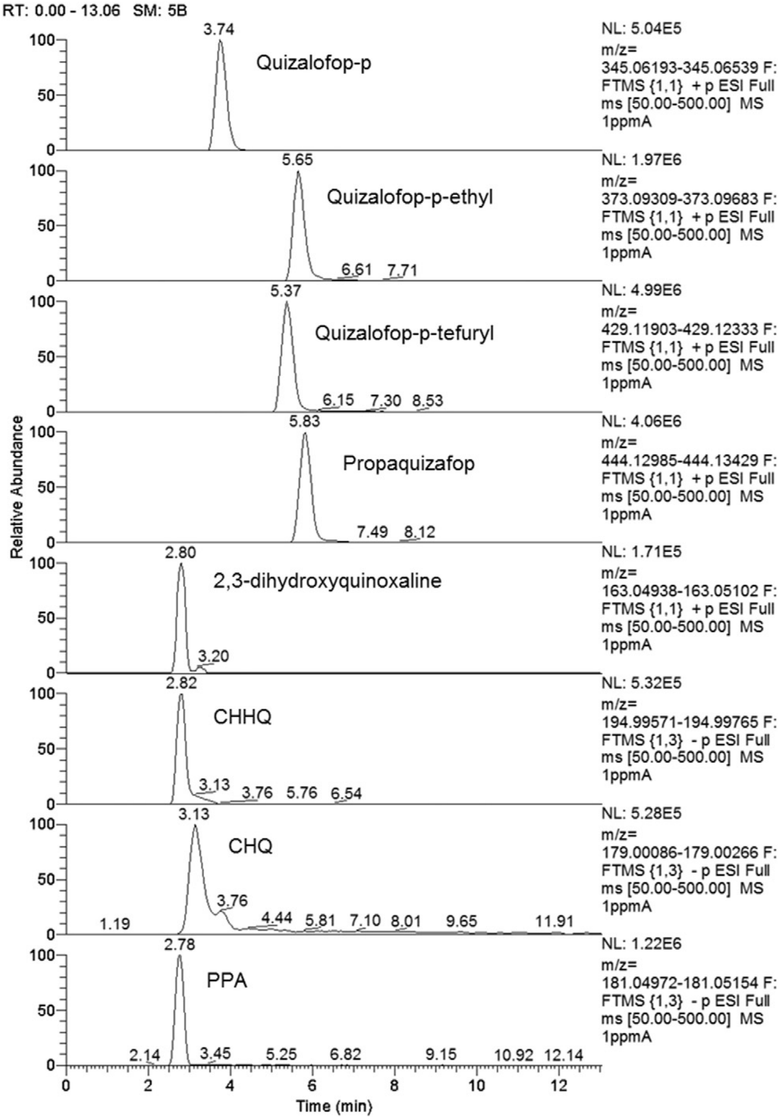

Fig. 1. Chromatograms of target analytes in solvent at 500 μg/L.

separation of quizalofop (Ma et al., 2016). Separation was good but resolution was still lower than 1, so it was decided to add more formic acid to the mobile phase. Ethanol/0.3% formic acid provided the best results for the chromatographic separation of the enantiomeric compound as it can be observed in Fig. 2. Retention time of the *S* enantiomer was 4.6 min and of the *R* enantiomer 5.3 min.

3.3. Optimization of the extraction procedure

Several extraction procedures were evaluated in this study. Initially, the extraction procedure developed by Mantzos et al. (2013) was evaluated, but poor recoveries were obtained for metabolites like CHQ, CHHQ, PPA and 2,3-dihydroxyquinoxaline (see Table 2). Then, the Saha et al. (2015) extraction procedure was evaluated and PPA, CHQ and 2,3-dihydroxyquinoxaline were not recovered (see Table 2). Next, other extraction solvents were tested, like acetone, methanol, ethyl acetate and ACN or 1% acetic acid ACN (see Table 2).

The best results were obtained with 10 mL of ACN acidified with 1% acetic acid, but the recoveries of more polar metabolites (CHHQ and 2,3-dihydroxyquinoxaline) were very low. Due to, 10 mL of water with 0.1 M of hydrochloric acid was evaluated before the addition of ACN (1% acetic acid). The results improved, and recoveries ranged from 19 to 90% (Table 2), so an aqueous solution of 0.1 M hydrochloric acid was added to the extraction procedure. Then, an agitation step was evaluated, testing 5, 15, 30, 45, 60 and 90 min. Recoveries between 72 and 120% (except for PPA) were obtained at 60 and 90 min (see Table S2). Thus 60 min was selected for further experiments in order to minimize extraction time. American QuEChERS (Anastassiades

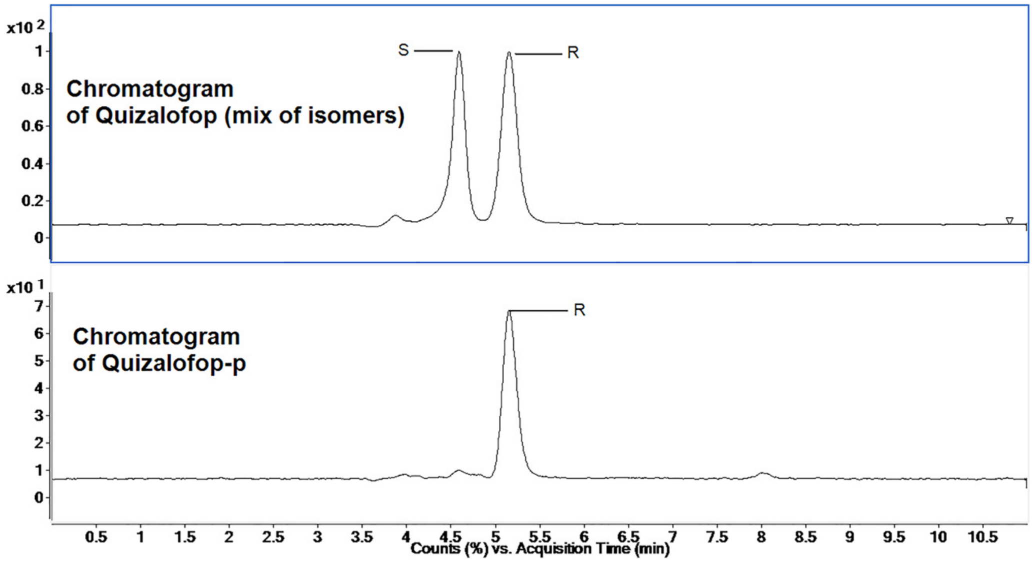

Fig. 2. Total ion Chromatograms of enantiomeric separation of quizalofop in UHPLC-QqQ-MS/MS (counts vs. Acquisition time (min)).

Table 2
Comparison of recovery values (at 200 μg/kg) when different extraction procedures were evaluated.

Recovery (%)								
Extraction method	Quizalofop-p	Propaquizafop	Quizalofop-p-ethyl	Quizalofop-p-tefuryl	2,3-dihydroxyquinoxaline	CHHQ	CHQ	PPA
Mantzos et al., 2013	<10	72	74	70	<10	<10	34	<10
Saha et al., 2015	<10	75	67	79	<10	<10	60	<10
Ethyl acetate extraction	79	90	92	96	25	42	95	<10
ACN extraction	<10	192	127	158	<10	<10	228	171
ACN 1% acetic acid extraction	63	87	79	86	<10	<10	59	<10
ACN 1% acetic acid and H_2O 0.1 M hydrochloric acid extraction	87	85	79	88	37	48	72	19
ACN 1% acetic acid and H_2O 0.1 M hydrochloric acid extraction + clean up (C_{18})	86	116	114	118	78	74	91	52

et al., 2003) and European QuEChERS (Lehotay et al., 2010) salt buffering step was tested and the former provided the best results (recoveries ranging from 69 to 100%).

Because of the high matrix effect observed for the metabolites, different clean-up steps were evaluated in order to test which sorbent provided the best results. 50 mg of PSA (primary secondary amine), GCB (black carbon graphitized), Florisil, C_{18}, aluminum oxide or *Z*-sep was added individually to a 1.5 mL supernatant sample. The matrix effect results are shown in Table S3. It can be observed that despite the type of sorbent used, strong matrix suppression effect was observed for CHHQ, CHQ and PA. In general, better results were obtained when 50 mg of C_{18} was used during this stage.

Table 3
Performance characteristics of the optimized method.

Method performance		Quizalofop-p	Propaquizafop	Quizalofop-p-ethyl	Quizalofop-p-tefuryl	2,3-dihydroxyquinoxaline	CHHQ	CHQ	PPA
R^2		0.9970	0.9952	0.9910	0.9955	0.9973	0.9943	0.9991	0.9916
Matrix effect[a]		0.33	0.75	0.60	0.70	0.06	0.40	0.05	0.11
LOD (μg/kg)		10	4	10	10	20	4	20	4
LOQ (μg/kg)		20	20	20	20	40	20	40	20
Recovery (%)	*20 μg/kg*[b]	110	98	71	117	112	92	111	50
	200 μg/kg	86	116	114	118	78	74	91	52
Intra-day precision (% RSD)[c]	*20 μg/kg*[b]	11	5	20	20	9	7	5	6
	200 μg/kg	16	12	8	17	17	4	18	13
Inter-day precision (% RSD)[d]	*20 μg/kg*[b]	2	17	11	19	3	13	9	15
	200 μg/kg	12	15	9	8	7	14	5	20

[a] Matrix effect = slope of calibration curve in matrix/slope of calibration curve in solvent.
[b] For 2,3-dyhydroxiquinoxaline and CHQ, the concentration was 40 μg/kg.
[c] n = 5.
[d] n = 5.

Finally, it can be observed in Table 2 that applying the proposed procedure, including clean-up step, suitable recoveries were obtained for most of the compounds included in this study, ranging from 74 to 118%, with the exception of PPA (recoveries slightly higher than 50%). In general the results are better than those provided in previous studies.

3.4. Method validation

The optimized method was validated in soils, studying the parameters previously mentioned, and the results are shown in Table 3. The matrix effect was evaluated comparing the slopes of the calibration curves of each

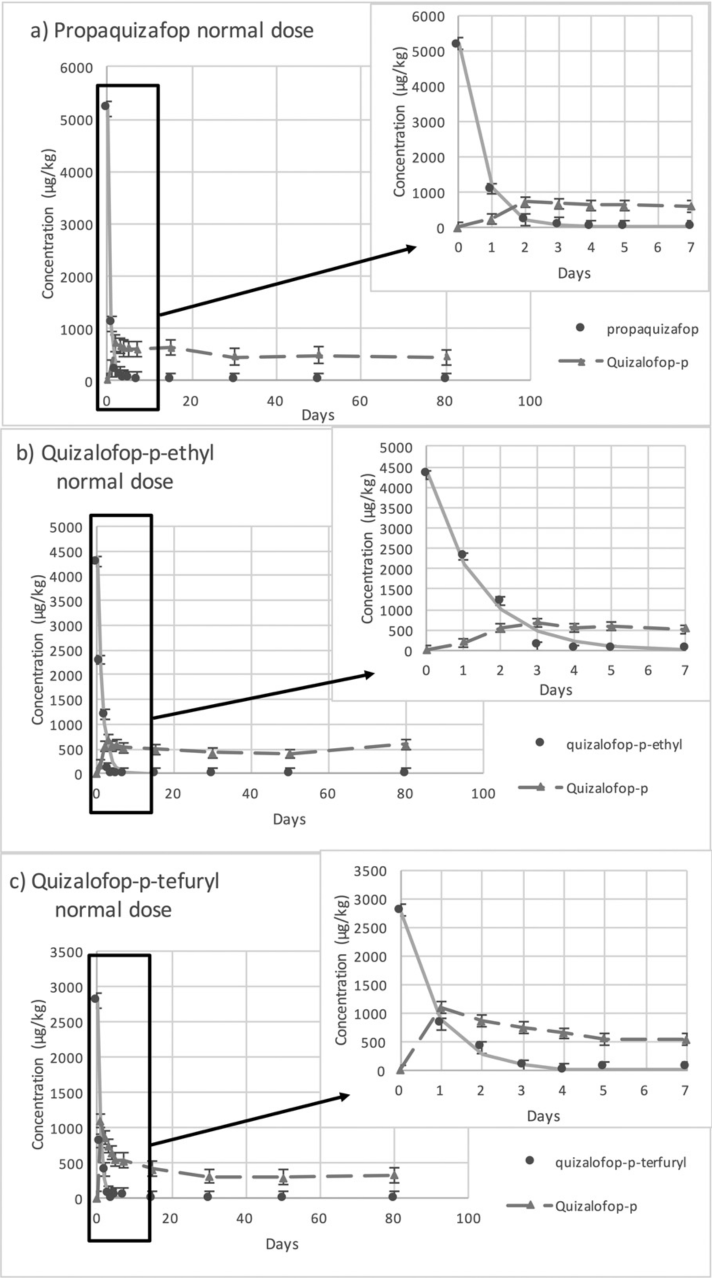

Fig. 3. Degradation of active substance propaquizafop (a), quizalofop-p-ethyl (b) and quizalofop-p-tefuryl (c) when normal dose was used (Error bars obtained for n = 2) as well as quizalofop-p appearance.

analyte prepared in blank matrix and in solvent (ACN), indicating matrix suppression if the ratio was lower than 0.8 and matrix enhancement if ratio was higher than 1.2. For all compounds, matrix suppression was observed, with matrix effect values lower than 0.8, and therefore matrix-matched calibration was used for quantification purposes.

The linearity and working range of the method was studied and the obtained determination coefficients (R^2) were higher than 0.99 for all target compounds. Linear range was from 10 to 100 μg/L, except for CHQ and 2,3-dihydroxyquinoxaline which ranged from 20 to 100 μg/L. Working range varied from 20 to 200 μg/kg for most of the compounds and from 40 to 200 μg/kg for CHQ and 2,3-dihydroxyquinoxaline.

Recoveries ranged from 71% (quizalofop-p-ethyl) to 118% (quizalofop-p-tefuryl) at the two concentration levels evaluated (see Table 3), except for PPA, so it can be concluded that recoveries are suitable. PPA had recoveries lower than 70%, but a correction factor could be used considering the low values of RSD obtained.

Intra-day precision ranged from 4 to 20% and inter-day precision (n = 5) ranged from 2 to 20%. Quizalofop-p and 2,3-dihydroxyquinoxaline had the lowest precision values (2 and 3% respectively) and PPA had the highest value (20%).

The lower limits of the method (LOD and LOQ) are also shown in Table 3. LODs ranged from 4 to 20 μg/kg. LOQs were 20 μg/kg for most of the analytes but for CHQ and 2,3-dihydroxyquinoxaline it was 40 μg/kg.

3.5. Laboratory trials

Two replicates of each commercial product were analyzed at various time intervals after its application, as it has been described in Section 2.5. The results of the degradation of the substance applying the kinetic model “Single First-Order Rate” (SFO) at normal dose are shown in Fig. 3, and the results for the double dose are described in Fig. S1 (see Supplementary material). Moreover, quizalofop-p was detected during the degradation of the main active substances, as shown in Fig. 3 and Fig. S1. In addition, the extracted ion chromatograms at 2 and 80 days after the fortification of commercial product with propaquizafop are shown in Figs. S2 and S3, revealing the detection of quizalofop-p, CHQ and CHHQ.

When propaquizafop (Dixon®) spiked samples were monitored, it was observed that the concentration of the main product decreased from 1098 μg/kg (1 day after application) to 43 μg/kg (4 days after application). Quizalofop-p was detected at 1 day after application at concentration of 243 μg/kg, and its concentration increased three times (720 μg/kg) the second day after application, and after that the concentration slowly decreased (Fig. 3a). Propaquizafop was not detected after 30 days, and the concentration of quizalofop-p was 461 μg/kg.

In relation to quizalofop-p-ethyl (Master-D®) applied samples, the concentration of quizalofop-p-ethyl decreased from 2300 μg/kg (after 1 day) to 22 μg/kg (after 4 days). Quizalofop-p was detected 1 day after the application of the commercial product at concentration of 193 μg/kg and its increased after 2 days up to 540 μg/kg (Fig. 3b).

Quizalofop-p-tefuryl (Panarex®) concentration decreased from 813 μg/kg (after 1 day) to 14 μg/kg (after 4 days). Quizalofop-p concentration considerably increased within the first two days (860 μg/kg at two days after application) (Fig. 3c). The low persistence of this compound is in accordance with previous studies (Mantzos et al., 2016).

The degradation observed in this study always followed the same pattern for the two different soils at the normal and at the double dose. The concentration of the main product decreased, especially from 1 to 7 days (see Fig. 3). From 7 to 30 days the concentration slowly decreased to concentrations below the LOD. The concentration of quizalofop-p increased, when the main product decreases, remained constant after 3 days and decreases after 30 days. The half-live values for quizalofop-p-ethyl, propaquizafop and quizalofop-p-tefuryl were low (see Table 4), approximately, from 0.5 to 1 days for the main compounds, so these compounds would have a low persistence.

In relation to other studies, the degradation of the main products is similar to the degradation of clothianidin (Zhang et al., 2016), chlorpyrifos and endosulfan (Dores et al., 2016) as these compounds degrade quickly in the first ten days. In relation to metabolites, there are scarce studies. In our previous study, flonicamid degradation has been studied in oranges (López-Ruiz et al., 2017), and one metabolite increased in concentration while the parent compound concentration decreased. This behavior is similar to that observed for quizalofop-p.

SFO kinetic model (Table 4) shows that the *k* value was different for the three active substances evaluated but the same at normal and double dose. For example, for propaquizafop, the *k* value was 1.55 days^{-1} for normal dose and 1.52 days^{-1} for double dose. For quizalofop-p-ethyl *k* value was 0.73 days^{-1} for normal dose and 0.72 days^{-1} for double dose, and DT_{50} ranged from 0.45 to 0.96 days (see Table 4). These results are in accordance with previous studies (Mantzos et al., 2016), which obtained DT_{50} values for quizalofop-*p*-ethyl ranging from 0.55 to 0.68 when SFO kinetic model was used, whereas *k* values are slightly higher (from 1.019 to 1.253 days^{-1}). Thus, it can be concluded that although the degradation is different for each active substance, it is independent of the dose of application. Moreover, *k* value is independent of the type of soils because the value is the same for the two soils studied.

Metabolites of the main compound were detected in samples after 15 days of application. Table 5 shows the concentration of the monitored metabolites. Metabolites of the main compound of quizalofop-p-ethyl (Master-D®) were detected in sandy loam soils. PPA and CHQ were detected after 15 days of application of the commercial product at concentration between the LOQ and LOD. After 50 and 80 days, CHQ and CHHQ were detected, at concentrations higher than the LOQ. For example for clay soils, when double dose of propaquizafop (Dixon®) was applied, the concentration of CHHQ was 30 μg/kg at 50 days and 182 μg/kg at 80 days. In quizalofop-p-ethyl (Master-D®) double dose samples, CHHQ concentration was 29 μg/kg at 50 days and 212 μg/kg at 80 days. CHQ concentration was 43 μg/kg at 50 days and 301 μg/kg at 80 days. In quizalofop-p-tefuryl (Panarex®) double dose samples, the concentration of CHHQ was 37 μg/kg at 50 days and 108 μg/kg at 80 days. For sandy-loam soils only CHQ was detected at concentration higher than its LOQ at 50 days, when propaquizafop (Dixon®) double dose was applied. It was detected at 42 μg/kg, whereas the concentration increased till 103 μg/kg 80 days after application. These results indicate that the detection of these metabolites are indicators of the application of parent compounds, and although the parent compound is degraded,

Table 4
SFO kinetic model parameters and soil dissipation (DT_{50} and DT_{90}) of propaquizafop, quizalofop-p-ethyl and quizalofop-p-tefuryl.

Parameters	Dixon (propaquizafop)		Master-D (quizalofop-p-ethyl)		Panarex (quizalofop-p-tefuryl)	
	Normal dose	Double dose	Normal dose	Double dose	Normal dose	Double dose
C_0 (μg/kg)	5199.33	10388.91	4382.82	8681.02	2788.45	5582.13
k (days)	1.55	1.52	0.74	0.72	1.15	1.68
DT_{50} (days)	0.45	0.46	0.94	0.96	0.60	0.41
DT_{90} (days)	1.48	1.51	3.14	3.18	2.01	1.37

Table 5
Detected metabolites in soil samples.

	Dose	Type of soil	15 days	30 days	50 days	80 days
PPA	Normal	Clay	<LOQ (Quizalofop-p-ethyl)	<LOD	<LOD	<LOD
		Sandy-loam	<LOD	<LOD	<LOD	<LOD
	Double	Clay	<LOD	<LOD	<LOD	<LOD
		Sandy-loam	<LOQ (Quizalofop-p-ethyl)	<LOD	<LOD	<LOD
CHHQ	Normal	Clay	<LOD	<LOD	<LOD	60 μg/kg (Propaquizafop) 56 μg/kg (Quizalofop-p-ethyl) 40 μg/kg (Quizalofop-p-tefuryl)
		Sandy-loam	<LOD	<LOD	<LOD	<LOQ (Propaquizafop) 13 μg/kg (Quizalofop-p-ethyl) <LOQ (Quizalofop-p-tefuryl)
	Double	Clay	<LOD	<LOD	30 μg/kg (Propaquizafop) 29 μg/kg (Quizalofop-p-ethyl) 37 μg/kg (Quizalofop-p-tefuryl)	182 μg/kg (Propaquizafop) 212 μg/kg (Quizalofop-p-ethyl) 108 μg/kg (Quizalofop-p-tefuryl)
		Sandy-loam	<LOD	<LOD	<LOD	13 μg/kg (Propaquizafop) 21 μg/kg (Quizalofop-p-ethyl) <LOQ (Quizalofop-p-tefuryl)
CHQ	Normal	Clay	<LOD	<LOD	<LOD	<LOD (Propaquizafop) 63 μg/kg (Quizalofop-p-ethyl) 21 μg/kg (Quizalofop-p-tefuryl)
		Sandy-loam	<LOQ	<LOD	<LOD	25 μg/kg (Propaquizafop) 37 μg/kg (Quizalofop-p-ethyl) <LOD (Quizalofop-p-tefuryl)
	Double	Clay	<LOD	<LOD	43 μg/kg (Quizalofop-p-ethyl)	301 μg/kg (Propaquizafop) 248 μg/kg (Quizalofop-p-ethyl) 106 μg/kg (Quizalofop-p-tefuryl)
		Sandy-loam	<LOQ	<LOD	42 μg/kg (Propaquizafop)	103 μg/kg (Propaquizafop) 102 μg/kg (Quizalofop-p-ethyl) 58 μg/kg (Quizalofop-p-tefuryl)

some metabolites can persist or appear in the environment 50 or 80 days after application.

3.5.1. Enantiomeric study

Due to the appearance of quizalofop-p in real samples, this compound was monitored by HPLC-QqQ-MS/MS using a chiral column, in order to know the percentage of quizalofop-p ((*R*)-quizalofop) and (*S*)-quizalofop in the studied samples. The results show that the composition of the isomers in samples was always the same (no enantiomeric transformation) and it does not depend on the number of days since application. Nevertheless, it was observed that each commercial product provided a different ratio of isomers. Thus, quizalofop-p-tefuryl (Panarex®) had the lowest percentage of isomer *R* (85%). The other products, propaquizafop (Dixon®) and quizalofop-p-ethyl (Master-D®) had 96% of *R* and 4% of *S* isomer.

Previous studies of quizalofop-p or related compounds have been mainly focused on the analysis of quizalofop-p in multiresidue methods (Lazartigues et al., 2011; Saha et al., 2015) or on the enantiomeric degradation (Li et al., 2012). Although not all commercial products and metabolites had previously been investigated, when the results are compared with published literature the same pattern for the degradation of quizalofop-p-ethyl was observed.

4. Conclusions

A new analytical method has been developed and validated for the determination of quizalofop-p and related compounds by UHPLC-Orbitrap-MS. Suitable LOQs, recovery and precision values were obtained for the reliable evaluation of the dissipation of quizalofop-p and related products. Due to the importance of the appearance of metabolites, UHPLC-Orbitrap-MS was used in target mode to monitor all the compounds generated in the process. Related degradation compounds followed the same pattern, degradation occurred quickly while quizalofop-p concentration increased and remained constant during the 80 days of experiment. Three metabolites, CHQ, CHHQ and PPA were detected in real samples after 15 days at concentrations lower than the LOQ and at 50 and 80 days at concentration higher than the LOQ. In conclusion, the metabolites were detected at higher concentration at 80 days, and they can be used as markers of the application of quizalofop or related products. In clay soils the concentration of the analytes was higher than in sandy-loam soils, except for CHQ at propaquizafop (Dixon®) normal dose. Enantiomeric determination of quizalofop-p has been studied with a chiral column and it was concluded that no enantiomeric transformation occurs during the monitored period.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.06.261.

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Publicación VII

BEHAVIOR OF QUIZALOFOP-P AND ITS COMMERCIAL PRODUCTS IN WATER BY LIQUID CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION MASS SPECTROMETRY

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Behavior of quizalofop-p and its commercial products in water by liquid chromatography coupled to high resolution mass spectrometry

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ABSTRACT

A degradation study of quizalofop-p and its commercial products (quizalofop-p-ethyl, quizalofop-p-tefuryl and propaquizafop) in water samples has been performed using ultra high-performance liquid chromatography coupled to Orbitrap mass spectrometry (UHPLC-Orbitrap-MS). CHHQ (dihydroxychloroquinoxalin), CHQ (6-chloroquinoxalin-2-ol) and PPA ((R)-2-(4-hydroxyphenoxy)propionicacid) were the main metabolites of this active substance (quizalofop-p) in water. The degradation of the parent compound has been monitored in distilled water. Several commercial products (Panarex®, Master-D® and Dixon®) were used to evaluate the degradation of the target compounds into their metabolites. The concentration of the main active substances (quizalofop-p-tefuryl, quizalofop-p-ethyl and propaquizafop) decreased during the degradation studies, whereas the concentration of quizalofop-p increased. DT_{50} of the main active substances ranged from 10 days to 70 days for most of the analytes, so it can be concluded that compounds are medium-high persistent in this matrix. Metabolites, such as PPA, CHHQ and CHQ, were detected in water samples after 7 days of the application of the commercial products at concentrations higher than their limits of quantification ($>$ 0.1 μg/L). CHQ was detected at 1400 μg/L after 75 days of the application of quizalofop-p-ethyl commercial product. CHHQ and CHQ were found at the highest concentrations at 7–45 days after the application of quizalofop-p-tefuryl, whereas PPA was detected at higher concentrations (up to 5.37 μg/L) in propaquizafop samples.

1. Introduction

Nowadays, one of the most relevant environmental issues is the occurrence of pesticide active substances and their metabolites in surface waters, wastewater effluents, and sediments (Ferrari et al., 2011). The increasing contamination of freshwater systems with industrial chemical compounds, which are released deliberately into the environment, is one of the key environmental problems society is facing today. More than one-third of the Earth's accessible renewable freshwater is used for agricultural, industrial and domestic purposes, and most of these activities lead to water contamination with numerous synthetic compounds. Chemical pollution of natural waters has already become a major public concern worldwide, since long-term effects on aquatic life and on human health are unknown (Loos et al., 2009).

Quizalofop-p, (*R*)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionic acid, is a systemic herbicide absorbed by the leaves with translocation throughout the plant. It is an aryloxyphenoxypropionate compound (Mantzos et al., 2016), used for post-emergence control of annual and perennial grass weeds in broad-leaved crops like beans, onions and potatoes (Roberts et al., 1998). Quizalofop-p has three different commercial products, quizalofop-p-ethyl (ethyl (*2R*)-2-{4-[(6-chloroquinoxalin-2-yl)oxy]phenoxy}-propanoate), quizalofop-p-tefuryl ((*RS*)-tetrahydrofurfuryl (R)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionate) and propaquizafop (2-[(Isopropylideneamino)oxy] ethyl(2R)-2-{4-[(6-chloro-2-quinoxalinyl)oxy]phenoxy}propanoate) (Fig. 1), and each compound is used for one specific application. All of these commercial products degrade to quizalofop-p under normal conditions in soils (López-Ruiz et al., 2017a) and a similar behavior could be expected in water. Moreover, this compound, quizalofop-p, can be degraded into different metabolites as CHHQ (dihydroxychloroquinoxalin), CHQ (6-chloroquinoxalin-2-ol) and PPA ((R)-2-(4-hydroxyphenoxy)propionic acid) (Fig. 2) that can be detected in soil, water, fruits, vegetables or air (EFSA European Food and Safety Authority, 2008).

The extraction of quizalofop-p and related compounds (quizalofop-p-ethyl, propaquizafop and quizalofop-p-tefuryl, metabolites not

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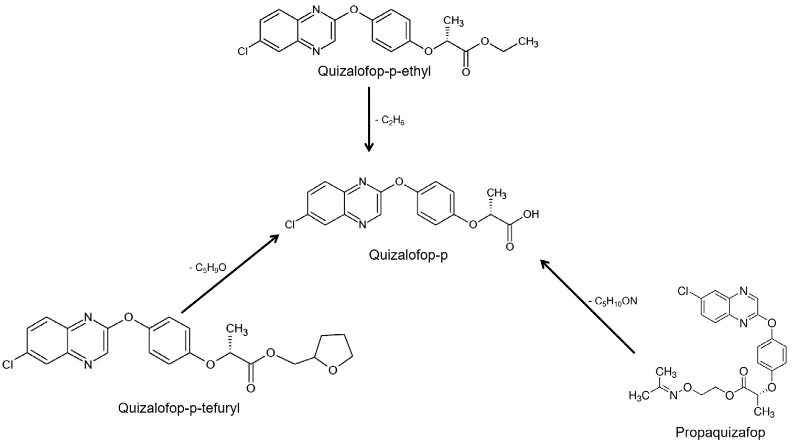

Fig. 1. Transformation scheme of the commercial products in the main active substance, quizalofop-p.

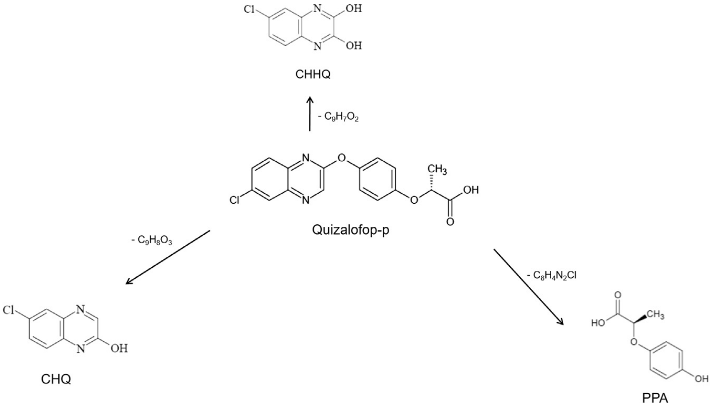

Fig. 2. Transformation scheme of the main active substance in its main metabolites.

included) from water samples has generally been performed by solid phase extraction (SPE), using Oasis HLB cartridges (Mantzos et al., 2016, 2013), strata X cartridges (Lazartigues et al., 2011) and multi-walled carbon nanotubes (MWCNTs) (Luo et al., 2014). For the elution of the compounds, several solvents as ethyl acetate (AcOEt) (Mantzos et al., 2016, 2013) and acetonitrile (ACN) (Lazartigues et al., 2011), or methanol (MEOH) containing 0.1 M chlorhydric acid (Luo et al., 2014) have been used.

Determination has been carried out by liquid chromatography coupled to mass spectrometry (LC-MS) (Lazartigues et al., 2011; Luo et al., 2014), but gas chromatography coupled to mass spectrometry was also applied in some studies (Mantzos et al., 2016, 2013). However, LC coupled to high resolution mass spectrometry (LC-HRMS) has only been used in a previous study (López-Ruiz et al., 2017a). For this reason, the development of a method based on HRMS for water sample analysis is important because the technique has a lot of possibilities. It allows analytical strategies that combine: (a) target analysis (determination of specific priority analytes included in a database, for which standards are available); (b) post-run target or retrospective screening analysis based on an accurate customized mass database of known parent molecules and some diagnostic fragment ions or isotopic pattern, and (c) non-target analysis, which is commonly performed applying several software tools (Coscollà et al., 2014; López-Ruiz et al., 2017b). Despite these benefits, LC-HRMS has several drawbacks as the high cost of the analyzer compared with a triple quadrupole analyzer, as well as well-trained staff and suitable software are needed.

This is the first time that degradation of quizalofop-p and its commercial products in water has been studied, considering that most of the previous studies have been focused on soils (López-Ruiz et al., 2017a) or only the main active substance had been studied in soils (Li et al., 2012; Ma et al., 2016) or rats (Liang et al., 2014). Thus, the main purpose of this study is the evaluation of the behavior of quizalofop-p and related compounds in water, monitoring the degradation of these compounds into metabolites. For that purpose, an analytical method has been developed and validated for the quantitative determination of quizalofop-p and metabolites applying UHPLC coupled to Orbitrap-MS for the identification of the compounds, applying target and non-target approaches for the detection of known and potential unknown metabolites of quizalofop-p.

2. Materials and methods

2.1. Material and reagents

Quizalofop (CAS registry No. 76578–12-6, purity 97,1%), quizalofop-p (CAS registry No. 94051–08-8, purity > 99%), quizalofop-p-ethyl (CAS registry No. 100646–51-3, purity 98,4%), quizalofop-p-tefuryl (CAS registry No. 200509–41-7, purity > 99%), propaquizafop (CAS registry No. 111479–05-1, purity > 99) and PPA (CAS registry No. 94050–90-5, purity > 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CHHQ (CAS Registry No. 6639–79-8, purity ≥ 99%) and CHQ (CAS Registry No. 2427–71-6, purity > 99%) were purchased from Cymit (Barcelona, Spain).

Commercial products, like Master-D® (quizalofop-p-ethyl 5% (v/v)) and Dixon® (propaquizafop 10% (v/v)) were purchased from Planeta Huerto (Alicante, Spain), while Panarex® (quizalofop-p-tefuryl 4% (v/v) was donated by Massó S.A. (Barcelona, Spain).

Stock solutions (1000 mg/L) were prepared weighing 10 mg of the pure compound, which was dissolved in 10 mL of a mixture of ACN: water (50:50 (v/v)). The multicompound working solution was prepared by mixing 100 µL of each stock solution in 10 mL of ACN to achieve a working mixture of 10 mg/L.

Stock and multicompound working solutions were stored at − 21 ºC. Stock solutions were stable for a year and multicompound working solution for 2 months.

ACN and MEOH (both LC-MS grade) were purchased from Fluka (St. Louis, MO, USA), water (LC-MS grade) was acquired from J.T. Baker (Deventer, Holland) and formic acid was obtained from Fisher Scientific (Erembodegem, Belgium). Oasis HLB flangeless SPE cartridges were purchased from Waters (Dublin, Ireland), Strata X cartridges from Phenomenex (Torrance, CA, USA) and Bond Elute Plexa cartridges from Agilent technologies (Santa Clara, CA, USA). Tetrabutylammonium acetate (TBA) was acquired from Sigma Aldrich.

A mixture of acetic acid, caffeine, Met–Arg–Phe–Ala–acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrat and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative) from Thermo-Fisher (Waltham, MA, USA) were used to accurate mass calibration of the Orbitrap analyzer.

2.2. Equipment

For the treatment and preparation of samples, an analytical balance AB204-S (Mettler Toledo, Greifensee, Switzerland) and a vortex mixer WX from Velp Scientifica (Usmate, Italy) were used.

For the UHPLC-Orbitrap-MS analysis, Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) was used, coupled to a mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen, Germany) using an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA).

The chromatographic separation was carried out with a Zorbax Eclipse C18 column (100 mm × 2.1 mm, 1.8 µm particle size) coupled to a Zorbax Hilic Plus (100 mm × 2.1 mm, 3.5 µm particle size) supplied by Agilent Technologies. The mobile phase consisted of eluent A, which was a water solution of 0.1% formic acid and eluent B, ACN, with a flow rate of 0.2 mL/min.

The elution gradient was as follows: 0–1 min 15% A; then it was linearly increased to 60% in 6 min, and to 100% in 1 min. This composition was kept constant during 3 min. Finally, it returns to the initial conditions in 30 s and remained constant during 90 s. The column temperature was set at 25 °C, the injection volume at 10 µL and total running time was 13 min.

The ESI parameters for the spectrometric detection were as follows: spray voltage, 4 kV; sheath gas (N_2, > 95%), 35 (adimensional); auxiliary gas (N2, > 95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 °C; capillary temperature, 300 °C. The mass spectra were acquired employing fouralternating acquisition functions: (1) full MS, ESI + , without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 full width at half maximum (FWHM); scan time = 0.25 s, (2) full MS, ESI- using the aforementioned settings, (3) all-ions fragmentation (AIF), ESI + , with fragmentation (HCD on, collision energy 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s, and (4) AIF, ESI- using the settings explained for (3). Mass range in the full scan experiments was set at *m/z* 50–600.

The chromatograms and spectra were acquired using the external calibration mode and they were processed using Xcalibur™ version 2.2, with Quanbrowser and Qualbrowser, and Mass Frontier™ 6.0 (Thermo Fisher Scientific, Les Ulis, France).

2.3. Laboratory studies

Dissipation studies have been carried out in darkness and sun light conditions at room temperature (average value 20ºC). Darkness conditions can simulate the conditions of groundwater and sun light conditions those of surface waters like rivers and swamps. 250 mL of distilled water (pH value 7.3 and conductivity value 2.2 µS/cm) were placed into amber (darkness conditions) and transparent bottles (sunlight conditions). After that, each commercial product, Dixon® (propaquizafop 10%), Master-D® (quizalofop-p-ethyl 5%) and Panarex® (quizalofop-p-tefuryl 4%), was individually analysed, spiking water samples at 1 mg/L and 5 mg/L for each compound. Then, each bottle (three replicates per condition were used) was agitated and hermetically closed. "Sunny" samples were stored at room temperature (maximum temperature: 23ºC, minimum temperature: 17ºC) under natural sunlight conditions, ensuring an average of 8 h of sunlight per day. They were placed on a shelf near the south window, inside the laboratory, and it was checked that the absorption of the containers is negligible between 310 and 800 nm and transmittance was higher than 90%. "Darkness" samples were stored in a cupboard under darkness conditions at the same temperatures of sunny samples inside the laboratory. Three replicates per condition were simultaneously taken from independent bottles at 24 h and 2, 3, 4, 6, 7, 15, 30, 45, 60, 75 and 100 days. Bearing in mind the commercial products evaluated (3), spiked levels (2), days monitored (12), light conditions (2) and number of replicates (3), a total of 432 samples were spiked and analysed. The final concentrations were fitted to kinetic model "Single First-Order Rate" (SFO), and characteristic parameters as initial concentration (C_0), rate constant (k) and half-lives (DT_{50}) have been estimated, applying Eq. (1):

$$C_t = C_0\, e^{-kt} \tag{1}$$

where C_t is the concentration at time t.

2.4. Sample extraction

Water sample extraction was carried out by a SPE procedure. In the first step, the cartridge was conditioned with 3 mL of ACN and equilibrated with 3 mL of water containing 5% of formic acid at atmospheric pressure. Then, 250 mL of samples, acidified with 0.1% of formic acid, were passed through the cartridge at one drop per second under vacuum. The cartridge was dried for 30 min under vacuum and then, the analytes were eluted with 3 mL of ACN at atmospheric pressure. MilliQ water blank samples were also submitted to the same SPE procedure. A preconcentration factor of 83.33 was obtained under these conditions.

2.5. Method validation

In order to perform reliable quantitative results, the UHPLC-MS-Orbitrap analytical method was validated according to SANTE guidelines (SANTE/EU, 2015), calculating matrix effect, linearity, trueness (% recovery), precision (intra and inter-day), and limits of quantification (LOQs) and detection (LODs).

The matrix effect was investigated by analyzing standards in solvent (ACN) and standards prepared in an extracted blank matrix. The calibration curves were prepared at different concentrations ranging from 8 to 100 μg/L of the target compounds except for CHQ (from 12 to 100 μg/L). The linearity of the method was studied using matrix-matched calibration at the same range, and it was evaluated by the determination coefficients (R^2).

Accuracy and precision (intra e inter-day precision) were established across the specified range of the analytical procedure by analyzing samples spiked with known amounts of the compounds (2 concentrations/5 replicates each). For all analytes, the tested concentrations were 0.1 and 1 μg/L of sample, except for CHQ (0.15 and 1 μg/L). Trueness is reported as percent of mean recovery ± relative standard deviation (RSD), expressed as %, and precision is reported as relative standard deviation (RSD) in %. Inter-day precision was studied in ten different days.

LODs and LOQs were estimated by injecting extracted blank samples spiked at low levels. LODs were determined as the minimum concentration at which the characteristic ion is monitored with a mass error lower than 5 ppm. For the estimation of LOQ, in addition to the characteristic ion, one fragment should be monitored at the same retention time and chromatographic peak shape than the characteristic one, being the mass error lower than 5 ppm. In addition, recovery and precision at LOQ value should be within 70–120% and lower than 20% respectively.

3. Results and discussion

3.1. Development and validation of the UHPLC-Orbitrap-MS method

Spectrometric and chromatographic conditions were optimized previously (López-Ruiz et al., 2017a) and LC-MS parameters of the monitored compounds are shown in Table S1. Extraction procedure was optimized due to the need of sample preconcentration, starting with the following conditions. Blank samples were fortified with the target compound at 1 μg/L and the extraction procedure was similar to that described in Section 2.4, except the water used for cartridge conditioning was not acidified. Oasis HLB, Strata X and Bond Elute Plexa cartridges were evaluated. The results are shown in Table S2. Strata X and Bond Elute Plexa provided poor recoveries for most of the compounds, whereas good recoveries, from 74% to 101%, were obtained when Oasis HLB was used.

In the second step, the effect of different parameters such as the conditioning and loading steps, as well as elution solvent were evaluated. First, the cartridge was conditioned with an organic solvent like ACN, and equilibrated with water. Acidified water was also tested during equilibration step, and different percentages of formic acid (0.1% and 5%) were tested. The best results were obtained when 5% of formic acid was used, achieving recoveries from 74% (PPA) to 101% (quizalofop-p-ethyl) (Table S2). Then, loading step was studied. The addition of water or aqueous solutions containing 0.1% formic acid, 1 mM of TBA and 0.1% formic acid/TBA to the sample was tested. Addition of formic acid was studied because the extraction performed with acidified solvents provided better results (López-Ruiz et al., 2017a). TBA was tested because it was expected that metabolites of quizalofop-p were not retained in the cartridge because of their polarity and TBA could form ionic pairs with those analytes. It can be seen in Table S3 that the best results were obtained when an aqueous solution containing 0.1% of formic acid was used. Finally, ACN was selected as elution solvent, because it was the best solvent for analytes.

Once the method had been optimized, it was validated in distilled water, studying the parameters previously mentioned in *Method Validation* section. The obtained results are shown in Table 1.

The linearity and working range of the method were studied with the calibration curves and determination coefficients (R^2) were higher than 0.99 for all target compounds. Working range was from 0.1 to 1.0 μg/L, except for CHQ that was from 0.15 to 1.00 μg/L.

The matrix effect was evaluated comparing the slopes of the calibration curves of each analyte prepared in blank matrix and in solvent (ACN), indicating matrix suppression if the ratio was lower than 0.8 and matrix enhancement if ratio was higher than 1.2. For all compounds, no matrix effect was observed. Therefore, solvent calibration was used for quantification purposes.

Recoveries ranged from 83% (quizalofop-p) to 120% (quizalofop-p-tefuryl) at the two concentration levels previously indicated (see Table 1), so it can be concluded that recoveries are suitable.

Intra-day precision ranged from 3 (CHQ/PPA) to 20% (propaquizafop). On the other hand, inter-day precision (n = 5) ranged from 4% to 20%. Quizalofop-p-ethyl had the lowest value (3%) and quizalofop-p-tefuryl the highest value (20%).

The lower limits of the method (LOD and LOQ) are also shown in Table 1. LODs were set at 0.06 μg/L for most of the compounds and 0.12 μg/L for CHQ metabolite, whereas LOQs were 0.1 μg/L for all the compounds except for CHQ (0.15 μg/L).

3.2. Laboratory trials

The results of the degradation of each substance applying the kinetic model "Single First-Order Rate" (SFO) at normal dose in sunny and darkness conditions are shown in Fig. 3, whereas the results for the fivefold dose were described in Fig. S1 (see Supplementary Material). Moreover, extracted ion chromatograms of one standard, control sample and spiked sample were included in Fig. S2.

Metabolites were detected during the degradation of the main active substances, showing the degradation results of quizalofop-p in Fig. 3 (normal dose) and Fig. S1 (fivefold dose).

3.2.1. Sunny conditions

When propaquizafop (Dixon®) applied samples were monitored, it was observed that the concentration of the main product decreased from 990 μg/L (1 day after application) to 475 μg/L (7 days after application), whereas the concentration of quizalofop-p was detected at 1 day after application (3 μg/L), and the concentration slowly increased until 59 μg/L, seven days after application. After that, the concentration quickly increased (632 μg/L) until forty-five days after application (Fig. 3a), and then, the concentration decreased (179 μg/L at seventy-five days). Propaquizafop was not detected after 75 days, whereas the concentration of quizalofop-p was 138 μg/L at 100 days after application.

In relation to quizalofop-p-ethyl (Master-D®) applied samples, the concentration of quizalofop-p-ethyl decreased gradually from 1400 μg/L (1 day) to 77 μg/L (100 days), whereas the presence of quizalofop-p was detected 4 days after the application of the commercial product (13 μg/L) and increased up to 307 μg/L after 60 days (Fig. 3b).

Quizalofop-p-terfuryl (Panarex®) concentration decreased from 1410 μg/L (1 day) to 12 μg/kg (100 days), while quizalofop-p concentration was detected 4 days after application (12 μg/L) and increased until 346 μg/L at 15 days (Fig. 3c). Thus it can be observed that the main compound of the commercial product is still detected 100 days after application, indicating its persistence in water samples, although quizalofop-p is detected few days after application.

When the behavior of quizalofop-p is evaluated at the two different concentrations tested it was observed that higher concentrations were observed at normal dose than at fivefold dose. For instance, at 45 days, quizalofop-p concentration was 632 μg/L at normal dose while at fivefold dose the concentration at 60 days was 250 μg/L. For the other commercial products, the behavior was similar, and higher concentrations were detected at normal dose than fivefold dose. Although further studies are needed to justify this behavior, this could be explained because the different degradation rate observed at normal and fivefold dose. Thus, k value as well as the concentration of quizalofop-p are higher at normal dose, which could indicate that at high degradation rates, the main compound is mainly degraded to quizalofop-p, whereas at lower values of k, the formation of metabolites is favored. It can be

Table 1
Validation results of the optimized method in water.

Method performance		Quizalofop-p	Propaquizafop	Quizalofop-p-ethyl	Quizalofop-p-terfuryl	CHHQ	CHQ	PPA
R^2		0.9902	0.9984	0.9922	0.9924	0.9940	0.9986	0.9900
Matrix effect[a]		0.82	0.97	1.06	1.03	1.18	0.84	1.14
LOD (μg/L)		0.06	0.06	0.06	0.06	0.06	0.12	0.06
LOQ (μg/L)		0.1	0.1	0.1	0.1	0.1	0.15	0.1
Recovery (%)	***0.1 μg/L***[b]	97	116	107	120	111	88	98
	1 μg/L	83	93	109	91	113	105	86
Intra-day precision (% RSD)[c]	***0.1 μg/L***[b]	16	18	4	10	4	15	5
	1 μg/L	18	20	6	10	14	3	3
Inter-day precision (% RSD)[d]	***0.1 μg/L***[b]	5	14	4	20	10	11	18
	1 μg/L	15	16	5	12	17	5	12

[a] Matrix effect = slope of calibration curve in matrix/slope of calibration curve in solvent.
[b] For CHQ, the concentration was 0.15 μg/L.
[c] n = 5.
[d] n = 5.

concluded that only a photodegradation process occurs in the samples because distilled water was used, avoiding biological processes that can also participate in the degradation of the parent compound if other type of matrix, as surface water, was used.

The concentration of the main product decreased according to SFO model during the study, and the concentration of quizalofop-p increased when the main product decreased, and after 60 days it also decreased. Quizalofop-p-ethyl, propaquizafop and quizalofop-p-terfuryl half-life is high (see Table 2), from 20 to 70 days at fivefold concentration for the main compounds, so these compounds would have a medium-high persistence (EFSA European Food and Safety Authority, 2008).

SFO kinetic model (Table 2) shows that *k* value is similar for the three active substances evaluated at normal and fivefold dose (from 0.01 to 0.08 days^{-1}). These results are in accordance with previous studies of other pesticides like pendimethalin (Chopra et al., 2015), with *k* values of 0.05 days^{-1} when SFO kinetic model was used.

Table 3 shows the concentration of the monitored metabolites. PPA

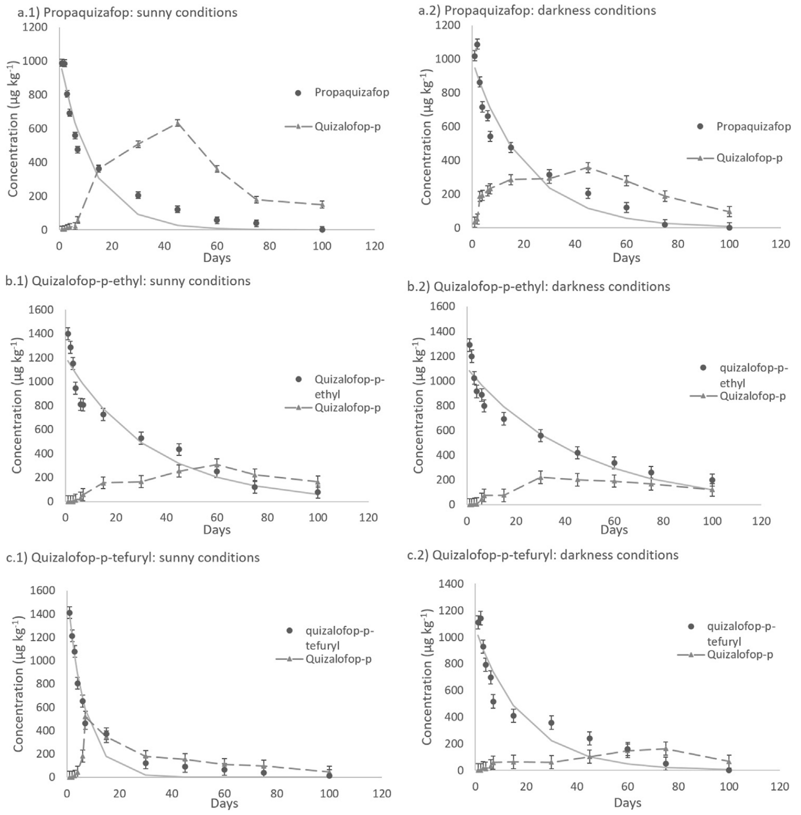

Fig. 3. Degradation of active substance propaquizafop (a), quizalofop-p-ethyl (b) and quizalofop-p-terfuryl (c), in sunny and darkness conditions, when normal dose was used, as well as quizalofop-p appearance. Error bars obtained for n = 3.

Table 2
Parameters from the fitting to a SFO kinetic model of propaquizafop, quizalofop-p-ethyl and quizalofop-p-terfuryl.

Parameters	Propaquizafop				Quizalofop-p-ethyl				Quizalofop-p-terfuryl			
	Normal dose		Fivefold dose		Normal dose		Fivefold dose		Normal dose		Fivefold dose	
	Sunny conditions	Darkness conditions	Sunny conditions	Darkness conditions	Sunny conditions	Darkness conditions	Sunny conditions	Darkness conditions	Sunny conditions	Darkness conditions	Sunny conditions	Darkness conditions
C_0 (µg/kg)	1033.90	994.43	4374.40	4783.59	1208.37	1104.72	6231.18	5388.53	1593.01	1067.43	5136.66	5022.36
k (days^{-1})	0.08	0.05	0.02	0.01	0.03	0.02	0.01	0.01	0.07	0.05	0.03	0.02
DT_{50} (days)	8.58	14.50	36.54	72.20	23.23	31.38	76.19	94.63	9.20	13.32	20.77	30.79
R^2	0.9534	0.9417	0.9336	0.9133	0.9214	0.9223	0.9656	0.9295	0.9060	0.9206	0.9872	0.9380

and CHHQ were detected at concentrations lower than 15 µg/L for all samples at the two application doses. However, CHQ was detected in most of samples at higher concentrations than 30 µg/L, so it could indicate that the compounds degraded mainly into CHQ. The maximum concentration for this compound was detected in quizalofop-p-ethyl samples at 1400 µg/L. Furthermore, it can indicate that quizalofop-p-tefuryl commercial product mainly degrades into CHHQ (from 5 to 13 µg/L) and CHQ (from 553 to 720 µg/L) up to 60 days. Then, the maximum concentration was for quizalofop-p-ethyl samples, detecting 5.94 µg/L for CHHQ and 1400 µg/L for CHQ. In relation to PPA, its maximum concentration was achieved in propaquizafop applied samples, whereas for the other commercial products, this compound was detected at the lowest concentrations throughout the monitoring period. There are not previous studies in water but the behavior of these compounds is similar to those observed in previous studies in soils (López-Ruiz et al., 2017a), where the parent compound disappears while the concentration of the metabolites increases, but the degradation rate of the parent compound is different. In soil studies the main product degrades in one week, while in water studies the main product degrades slowly during up to 100 days. This can be explained because microbiological degradation can occur in soil, whereas in water, only photodegradation was evaluated in sunny conditions, avoiding other degradation pathways. In relation to metabolites, there are also differences between soils and water samples. In soils samples, the metabolites were detected after 50 days of application and only CHHQ and CHQ were found at concentrations higher than LOQ (0.1 µg/L for CHHQ and 0.15 for CHQ). In water samples, metabolites have been detected 7 days after application of commercial products, and in addition to CHHQ and CHQ (also detected in soils), PPA was also found in water samples, indicating that different degradation pathways may occur in water and soil samples.

3.2.2. Darkness conditions

When the degradation of the parent compounds was monitored under darkness conditions, it can be observed that the behavior of the main active substances and quizalofop-p was similar than those in sunny conditions, but the degradation rate was lower. In addition, UV-spectra of the main compounds of the commercial products were measured to verify that the compounds absorb at wavelengths higher than 290 nm. As it has been shown in Fig. S3, it can be observed that all compounds absorbed at values higher than 290 nm, showing a characteristic band between 330 and 360 nm. Thus, the effect of the sun in the water is important for the degradation of these substances. For example, for quizalofop-p-ethyl samples in sunny conditions the main active substances ranged from 1400 to 19 µg/L, whereas in darkness conditions, they ranged from 1289 to 200 µg/L.

When the experimental data were fitted to SFO kinetic model (Table 2) the determination coefficient is higher than 0.90 for all the conditions evaluated, which means that more than 90% of the variability of the data can be explained by this model. It can be observed that *k* values are always higher in sunny than in darkness conditions, except for quizalofop-p-ethyl. In addition, rate constant is always higher at normal dose than at five dose (Chen et al., 2016; Chopra et al., 2015). Moreover, DT_{50} was higher at darkness (from 13.32 to 94.64 days) than at sunny (8.58–76.19 days) conditions in all commercial products, so the degradation speed was lower in darkness conditions in comparison with sunny conditions. It can be noted that in the darkness conditions, propaquizafop presents DT_{50} values 4.3–5.0 times longer than in sunny conditions, whereas quizalofop-p-ethyl ranged from 3.0 to 3.3 and quizalofop-p-terfuryl between 2.25 and 2.3 times. Therefore, the

Table 3
Detected metabolites under sunny conditions[a].

	Dose	7 days	15 days	30 days	45 days	60 days	75 days	100 days
PPA (µg/L)	Normal	0.12 (Q-p-E)	0.24 (Q-p-E)	1.52 (Q-p-E)	2.30 (Q-p-E)	1.39 (Q-p-E)	1.23 (Q-p-E)	1.13 (Q-p-E)
		1.05 (P)	2.27 (P)	4.64 (P)	3.41 (P)	2.94 (P)	2.20 (P)	5.37 (P)
		0.27 (Q-p-T)	0.53 (Q-p-T)	1.53 (Q-p-T)	1.82 (Q-p-T)	2.09 (Q-p-T)	1.68 (Q-p-T)	1.85 (Q-p-T)
	Fivefold	0.10 (Q-p-E)	0.14 (Q-p-E)				2.33 (Q-p-E)	
		0.55 (P)	0.68 (P)	0.54 (Q-p-E)	0.58 (Q-p-E)	3.07 (Q-p-E)	0.93 (P)	2.56 (Q-p-E)
		0.23 (Q-p-T)	0.54 (Q-p-T)	0.78 (P)	4.031 (P)	2.30 (P)	2.56 (Q-p-T)	0.32 (P)
CHHQ (µg/L)	Normal				0.11 (Q-p-E)			
		0.19 (P)	0.16 (P)	0.23 (P)	0.25 (P)	2.71 (Q-p-T)	3.54 (Q-p-T)	5.08 (Q-p-T)
		0.75 (Q-p-T)	0.96 (Q-p-T)	1.48 (Q-p-T)	1.54 (Q-p-T)			
	Fivefold	0.31 (Q-p-E)	0.61(Q-p-E)	2.95 (Q-p-E)	3.02 (Q-p-E)	3.29 (Q-p-E)	5.94 (Q-p-E)	9.37 (Q-p-E)
		1.62 (P)	2.16 (P)	2.39 (P)	3.22 (P)	1.36 (P)	1.25 (P)	2.25 (P)
		5.36 (Q-p-T)	5.66 (Q-p-T)	5.95 (Q-p-T)	12.48 (Q-p-T)	4.33 (Q-p-T)	4.03 (Q-p-T)	3.19 (Q-p-T)
CHQ (µg/L)	Normal	20.23 (Q-p-E)	23.56 (Q-p-E)	29.36 (Q-p-E)	39.75 (Q-p-E)	57.22 (Q-p-E)	63.14 (Q-p-E)	50.06 (Q-p-E)
		72.6 (P)	55.9 (P)	48.1 (P)	44.79 (P)	48.01 (P)	112.61 (P)	74.31 (P)
		60.71 (Q-p-T)	71.12 (Q-p-T)	86.61 (Q-p-T)	131.55 (Q-p-T)	180.93 (Q-p-T)	380.74 (Q-p-T)	140.12 (Q-p-T)
	Fivefold	243.63 (Q-p-E)	253.85 (Q-p-E)	289.28 (Q-p-E)	590.58 (Q-p-E)	592.55 (Q-p-E)	1399.49 (Q-p-E)	616.89 (Q-p-E)
		566.51 (P)	556.1 (P)	278.50 (P)	434.37 (P)	317.8 (P)	747.55 (P)	202.14 (P)
		553.33 (Q-p-T)	601.01 (Q-p-T)	362.53 (Q-p-T)	720.17 (Q-p-T)	512.57 (Q-p-T)	943.32 (Q-p-T)	753.32 (Q-p-T)

[a] In brackets the main compound used. If the main compound is not indicated, that means that this metabolite was not detected (< limit of detection). Abbreviations: P, propaquizafop; Q-p-E, quizalofop-p-ethyl; Q-p-T, quizalofop-p-tefuryl

Table 4
Detected metabolites under darkness conditions[a].

	Dose	7 days	15 days	30 days	45 days	60 days	75 days	100 days
PPA (μg/L)	Normal	1.05 (P) 0.13 (Q-p-T)	1.18 (P) 0.42 (Q-p-T)	0.73 (Q-p-E) 1.55 (P) 0.53 (Q-p-T)	0.38 (Q-p-E) 2.41 (P) 0.37 (Q-p-T)	0.15 (Q-p-E) 1.84 (P) 0.32 (Q-p-T)	0.16 (Q-p-E) 1.96 (P) 0.77 (Q-p-T)	0.31 (Q-p-E) 1.52 (P) 0.57 (Q-p-T)
	Fivefold	0.63 (P) 0.50 (Q-p-T)	0.72 (P) 2.04 (Q-p-T)	2.9 (Q-p-E) 5.07 (P) 2.64 (Q-p-T)	0.65 (Q-p-E) 14.03 (P) 3.74 (Q-p-T)	0.45 (Q-p-E) 8.55 (P) 1.62 (Q-p-T)	0.62 (Q-p-E) 7.14 (P) 0.76 (Q-p-T)	0.66 (Q-p-E) 7.13 (P) 0.67 (Q-p-T)
CHHQ (μg/L)	Normal	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
	Fivefold	< LOD	< LOD	0.43 (Q-p-T)	0.69 (Q-p-T)	0.57 (Q-p-T)	0.39 (Q-p-T)	0.50 (Q-p-T)
CHQ (μg/L)	Normal	7.50 (Q-p-E) 15.15 (P) 10.14 (Q-p-T)	6.36 (Q-p-E) 16.09 (P) 11.12 (Q-p-T)	8.29 (Q-p-E) 18.57 (P) 8.02 (Q-p-T)	6.30 (Q-p-E) 19.11 (P) 14.09 (Q-p-T)	5.15 (Q-p-E) 23.01 (P) 14.38 (Q-p-T)	3.12 (Q-p-E) 20.61 (P) 37.74 (Q-p-T)	3.12 (Q-p-E) 10.86 (P) 21.80 (Q-p-T)
	Fivefold	30.30 (Q-p-E) 130.6 (P) 37.4 (Q-p-T)	32.12 (Q-p-E) 51.1 (P) 35.23 (Q-p-T)	33.34 (Q-p-E) 41.50 (P) 32.96 (Q-p-T)	21.81 (Q-p-E) 60.12 (P) 57.15 (Q-p-T)	21.20 (Q-p-E) 67.44 (P) 62.57 (Q-p-T)	11.29 (Q-p-E) 79.55 (P) 157.46 (Q-p-T)	13.39 (Q-p-E) 31.35 (P) 61.75 (Q-p-T)

[a] In brackets the main compound used. Abbreviations: P, propaquizafop; Q-p-E, quizalofop-p-ethyl; Q-p-T, quizalofop-p-tefuryl

degradation rate depends on the commercial product applied, which has also been considered when the withdrawal period for these compounds have been set (Ministerio de Agricultura y Pesca Alimentacion y Medio Ambiente, 2009a, 2009b, 2006). That is why, when these commercial products are applied to vegetables or trees, the withdrawal period is higher for propaquizafop (40 days or more) than for of quizalofop-p-ethyl (21 days).

Metabolites appearance was lower than in sunny conditions (Table 4). For example, CHHQ was only detected in quizalofop-p-tefuryl samples at maximum concentration of 0.69 μg/L from 30 days. As it happens in sunny conditions, CHQ was the metabolite detected at the highest concentrations, but it did not exceed 150 μg/L, which is ten times lower than in sunny conditions. Therefore, it can be indicated that the sun effect was the key factor in the degradation of these compounds and some metabolites can persist or appear in the environment 100 days after application.

4. Conclusions

In the present study, dissipation of three herbicides of the same family, quizalofop-p-ethyl, propaquizafop and quizalofop-p-tefuryl in water was evaluated. Lab experimental experiences have been carried out in order to know the behavior of these compounds. Overall, the persistence of those compounds is medium to high because DT_{50} is higher than 15 days for most of the analytes. In relation to metabolites, they were detected after 7 days of the application of the commercial products and the metabolite detected at higher concentration was CHQ. In comparison to soil degradation, PPA, which was not found in soil, was detected in water. When sunny conditions were used, the metabolite concentrations are higher than in darkness conditions, so the sun was important in the dissipation of the main products. Moreover, it can be stated that this compound, CHQ, can be used as a marker for the technical products of quizalofop-p.

Finally, these results provide a comprehensive evaluation of the presence of quizalofop-p and its commercial products in water samples, monitoring their main transformation products, that can be used in environmental risk assessments studies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.03.094.

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Publicación VIII

DEGRADATION STUDIES OF DIMETHACHLOR IN SOILS AND WATER BY UHPLC-HRMS: PUTATIVE ELUCIDATION OF UNKNOWN METABOLITES

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Degradation studies of dimethachlor in soils and water by UHPLC-HRMS: putative elucidation of unknown metabolites

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Abstract

BACKGROUND: The analytical control of the presence of dimethachlor and its metabolites in environmental samples, such as water and soils, is a main concern. Degradation of this pesticide has been evaluated in two types of soils and two different water conditions at two concentration levels. For that purpose, a new liquid chromatography-mass spectrometry method has been developed and putative identification of new metabolites has been performed.

RESULTS: An analytical method based on ultra-high-performance liquid chromatography coupled to Orbitrap mass spectrometry (UHPLC-Orbitrap-MS) was developed to monitor the degradation of dimethachlor in environmental samples (water and soils). The degradation of dimethachlor in soils and groundwater samples has been monitored from 1 to 110 days after application of a plant protection product at two doses. Concentration of the parent compound slowly decreased in both matrices. DT_{50} values ranged from 40 to 70 days. Some metabolites were detected in the commercial product and in the samples one day after the application of the plant protection product. In addition, three new metabolites were putatively identified during dimethachlor degradation by untargeted analysis.

CONCLUSION: In this study, the degradation of dimethachlor into its metabolites has been studied in soils and water, using a UHPLC-Orbitrap-MS validated method. A putative elucidation of new metabolites of dimethachlor has been carried out applying HRMS and software tools. Degradation results allowed for understanding the behavior of dimethachlor in soils and water, and provided information regarding the possible risk of this pesticide and its metabolites to the ecosystem.

Supporting information may be found in the online version of this article.

Keywords: degradation; dimethachlor; environmental matrices; unknown analysis; metabolites

1 INTRODUCTION

In modern agriculture, the use of pesticides is a common practice to improve crop production. The presence of these compounds in agricultural run-off can be used as water quality indicators. Among the different classes of pesticides, herbicides are commonly used to control unwanted plants, but they have toxic side effects and negative environmental results[1]. However, general knowledge related to the consequences of the continuous use of herbicides is incomplete because previous studies have mostly been focused on the determination of the parent compound, whereas herbicide degradation products (referred to as metabolites in this study) have not been included in conventional studies. In fact, there have been scarce published methods focused on their analysis, due to the methodological difficulties and the requirements for advanced analytical instrumentation[2]. Nevertheless, degradation information is necessary to calculate the residual concentration and half-life (DT_{50}), that are indicators for environmental persistence, food safety, and potential health risks of herbicides on treated crops, especially those that are widely consumed and cultivated[3,4].

Chloroacetanilide herbicides are used to control broadleaved weeds and seasonal grasses[5]. For this reason, they are commonly detected in environmental samples as surface and groundwater, as well as some of their metabolites such as oxanilic acids (OA) and ethane sulfonic acids (ESA)[6,7], observing that the parent chloroacetamide herbicide undergoes environmental degradation[8].

Dimethachlor (2-chloro-N-(2-methoxyethyl)acet-2′,6′-xylidide) is a chloroacetanilide herbicide, like propachlor and metazachlor. It

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is a selective herbicide that is mainly absorbed by different parts of germinating plants, inhibiting the synthesis of very long chain fatty acids. The representative formulated product is 'Teridox 500 EC', an emulsifiable concentrate[9]. Several dimethachlor metabolites have been detected in soils, surface water or air[9], but they have not been included in the residue definition of dimethachlor yet. Both the parent compound and the following metabolites are commercially available as standard products: dimethachlor CGA 373464 ([(2,6-dimethylphenyl)-(2-sulfo-acetyl)amino]acetic acid sodium salt), dimethachlor SYN 530561 (2-[(2-hydroxyacetyl)-(2-methoxyethyl)amino]-3-methylbenzoic acid), dimethachlor SYN 528702 sodium salt (3-{2-[(2,6-dimethyl-phenyl)-(2-hydroxyacetyl)amino] ethylsulfanyl}-2-hydroxypropionic acid sodium salt) and dimethachlor oxalamic acid or dimethachor CGA 50266 (N-(2,6-dimethylphenyl)-N-(2-methoxyethyl)oxalamic acid), and they have been named as CGA, SYN, SYN Na and oxalamic acid respectively (Fig. S1, see in File S1). Additionally, European Food Safety Authority (EFSA) has reported other metabolites of dimethachlor that can also be detected in soils or water[9], although to our knowledge, there are no available standards. They are dimethachlor CGA 42443 (N-(2,6-dimethylphenyl)-N-(2-methoxyethyl)acetamide), dimethachlor CGA 72649 (2,6-dimethylaniline), dimethachlor CGA 369873 ((2,6-dimethylphenylcarbamoyl)-methanesulfonic acid sodium salt), dimethachlor CGA 102935 (N-carboxymethyl-N-(2,6-dimethylphenyl)oxalamic acid) and dimethachlor 39 981 (N-(2,6-dimethylphenyl)-2-hydroxy-N-(2-methoxyethyl)acetamide). They have been named by their numbers throughout this study (See File S1, Fig. S1). In relation to risk assessment reports, toxicity of dimethachlor was classified as moderate by oral ingestion ($LD_{50} = 1600$ mg kg^{-1} bw^{-1}) and oxalamic acid presented values of $LD_{50} > 2000$ mg kg^{-1} bw^{-1}. However, for the rest of metabolites there are no toxicity data yet. On the other hand, risk assessment studies[9] determined that oxalamic acid and CGA 42443 (only in anaerobic conditions) have to be monitored in soils with the parent compound, meanwhile in water, oxalamic acid, CGA, SYN, SYN Na, CGA 102935 and CGA 369873 have to be analyzed, as well as CGA 42443 in anaerobic conditions. Therefore, these reports show the need for monitoring the dimethachlor metabolites in water and soils.

To our knowledge, there are no analytical methods for the simultaneous determination of dimethachlor and related metabolites in soils and water, although this compound has been included in several multiresidue methods. Thus, the extraction of the parent compound in multiresidue methods from soils has been carried out by applying the QuEChERS (acronym of Quick, Easy, Cheap, Effective, Rugged and Safe) extraction method, using acetonitrile (ACN) and water as extraction solvent[10] or solid–liquid extraction with methanol (MeOH)[11]. For the extraction of this compound in multiresidue methods from water, solid phase extraction (SPE) with Strata X cartridges[10] and elution with ACN and ethyl acetate (AcOEt) or carbon cartridges, with MeOH containing 10 mmol L^{-1} of ammonium acetate as elution solvent, have been used[12].

Several studies focused on dimethachlor have been performed, and only a few of them are based on liquid chromatography-mass spectrometry (LC–MS)[10,12–14], but none of them had included dimethachlor metabolites. For this reason, a study focused on the degradation of dimethachlor and the determination of the metabolites in environmental samples using LC-high resolution mass spectrometry (HRMS) is needed, in order to get useful information for further risk assessment studies of this compound. HRMS has been selected due to it being the most suitable technique for the determination of analytes and their metabolites because of its capacity for both targeted (parent compounds and known metabolites) and untargeted analysis (unknown metabolites)[15–17].

The aim of this work was to monitor the behavior of dimethachlor in environmental samples and its degradation into its metabolites. For that purpose, the development and validation of an analytical method was carried out applying ultra high performance LC (UHPLC)-Orbitrap-MS, using targeted and non-targeted approaches for the identification of dimethachlor and metabolites.

2 MATERIALS AND METHODS

2.1 Material and reagents

Dimethachlor (CAS registry No. 50563–36-5, purity 99%), dimethachlor metabolite CGA 373464 (CAS registry No. 1196157–87-5, purity 99%), dimethachlor metabolite SYN 528702 sodium salt (CAS registry No. 1228182–52-2 purity 99%) and dimethachlor metabolite SYN 530561 (CAS registry No. 1138220–18-4, purity 99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethachlor oxalamic acid (CAS registry No. 1086384–49-7, purity 99%) was purchased from LGC standards (Augsburg, Germany).

Stock standard solutions of the individual compounds at 1000 mg L^{-1} were obtained by weighing 10 mg of commercial available standards in 10 mL of a mixture of ACN:water (50:50 *v v*$^{-1}$). Two working standard solutions, containing all the standards, were prepared at 10 and 1 mg L^{-1} by appropriate dilution of the stock standard solutions with ACN in a volumetric flask.

Stock solutions were stored at −21 °C and they were stable for 6 months, whereas intermediate solutions were stored at 4 °C and they were stable for 2 months. A fully description of materials, equipment and methods are described in File S1, *Section 1*.

2.2 Samples

Two agricultural soil samples (sandy loam and loam soils) were collected from two greenhouses located in Almería (Spain). At each greenhouse, six randomized subsamples (750–1000 g each one) were taken and they were mixed to get a composite sample (4.5–6.0 kg) for each type of soil. Both samples were homogenized, air-dried (2 days at room temperature) and sieved (particle size <2 mm) before the analysis and several physicochemical parameters for sandy loam and loam composite samples were monitored (see File S1, Table S1).

A composite groundwater sample (10 L) was collected from a water well located in Almería during 24 h. The pH of the sample was 7.1 and conductivity of 532 μS cm^{-1}.

2.3 Laboratory studies

2.3.1 Soils studies

Five grams of each soil (loam and sandy loam) of composite sample were weighed in Erlenmeyer flasks. Five milliliters of water were added to the loam soil (34% humidity) and 2 mL to the sandy loam (14% humidity) to mimic humidity conditions. Each sample was spiked with the commercial product (Teridox®) at both normal and double dose, and homogenized for 10 min. The normal dose was 2 L per ha (7 μL of Teridox® g^{-1} of soil) and the double dose was 4 L per ha (14 μL of Teridox® g^{-1} of soil). The Erlenmeyer flasks were hermetically closed and stored at room temperature (between 20–25 °C) under natural sunlight conditions (8 h of sunlight per day). The samples were analyzed at 0, 24 h and 7, 15, 30, 50, 70, 90 and 110 days (three replicates each time; a total of 54 samples/type of soil: two doses, three replicates, 9 monitoring points). The volume of water in each Erlenmeyer flask was kept constant throughout the study.

2.3.2 Water studies

Both sunlight and darkness conditions were tested in order to study the degradation of dimethachlor in water at room temperature. Sunlight conditions simulate the conditions of surface waters like swamps and rivers, whereas darkness conditions mimic groundwater habitat. For that, aliquots of 125 mL of composite water were placed into both transparent (sunlight conditions) and amber bottles (darkness conditions), and they were spiked with Teridox® at rate of 2 µL of Teridox® L^{-1} of water and 10 µL of Teridox® L^{-1} of water. Bottles were hermetically closed and the samples were homogenized by agitation during 15 min. Darkness samples were stored in a cupboard inside the laboratory (between 20–25 °C, darkness conditions). Sunny samples were stored at the same conditions as soils. They were placed on a shelf near the south window, and it was tested that the bottle absorption is not significant between 310 and 800 nm. Three replicates, at each sampling time, were analyzed at the same days that soil studies (a total of 54 samples/light condition: two doses, three replicates, nine monitoring points).

2.4 Sample extraction

2.4.1 Soil extraction

First, 5 g of soil sample was placed into a 50 mL plastic centrifuge tube. Then, 10 mL of water and 10 mL of ACN were added and the mixture was shaken for 1 min in a vortex. The samples were stirred for 1 h in a rotary shaker and centrifuged for 6 min at 5000 rpm (4136 *g*). Then, 2 mL of the supernatant was added in a 15 mL Eppendorf tube containing 50 mg of C18. The mixture was shaken for 1 min and centrifuged for 6 min at 5000 rpm (4136 *g*). Finally, 1 mL of the supernatant was collected and injected into the UHPLC-Orbitrap-MS.

2.4.2 Water extraction

Water sample extraction was carried out by a SPE procedure. First, the cartridge (Oasis HLB) was conditioned with 3 mL of ACN and equilibrated with 3 mL of water. Then, 125 mL of sample with 40 mg of tetrabutylammonium acetate (TBA) was passed through the cartridge at one drop per second. The cartridges were dried with air during 30 min under vacuum and the compounds were eluted with 1.5 mL of ACN.

2.5 Method validation

The analytical method was validated in both matrices (soil and water) following EPA validation guidance[18] and SANTE guidelines[19], testing several parameters: linearity, matrix effect, working range, trueness (% recovery), precision (repeatability and reproducibility), and lower limits (limits of detection, (LODs) and quantification (LOQs). More details are indicated in File S1, Section 2.

3 RESULTS AND DISCUSSION

3.1 Optimization of the UHPLC-Orbitrap MS method

First, the spectrometric characterization of dimethachlor and available metabolites was carried out. Through direct injection into the MS system of the individual standards at concentration of 500 µg L^{-1}, the monoisotopic ion of each compound was monitored according to its molecular formula. Fragments for each analyte were monitored in the spectra applying all ion fragmentation (AIF) mode. They were selected according to the criterion that mass error should be lower than 5 ppm, and the chromatographic peak, when these fragments are monitored, must be at the same retention time of the monoisotopic ion[17,20]. Because this analyzer does not allow selection of the precursor ion, Mass Frontier™ version 6.0 was used for the elucidation of the fragments. From the chemical structure of the compound, the fragmentation mechanisms and the corresponding fragment ions, with their corresponding exact masses, were determined. In addition, looking for common fragments of the target compounds was also possible. Another software used was Qualbrowser™, which provides an experimental formula from a selected ion, allowing for checking if that fragment comes from the parent structure. Table 1 shows the exact mass and molecular formula for the obtained fragments (two fragments per compound were monitored) when this approach is used and the total ion chromatogram, monoisotopic spectrum and fragment spectrum of each analyte have been shown in File S1, Fig. S2. The ionization mode was negative for CGA and SYN Na, while for the rest of analytes it was positive. For example, the loss of a methoxy group (-CH$_2$O) is common for dimethachlor and SYN, monitoring *m/z* 224.08367 and 236.09173 ions, respectively. Common fragments were also observed for some compounds as *m/z* 178.08078, which was detected for CGA and SYN Na, and it involves the loss of sulfonic (-SO$_3$) and ketone groups (C=O) for CGA, whereas for SYN Na, the loss of thiol (—S), hydroxyl (-OH) and acid groups (-COOH) was observed.

In the second step, the chromatographic conditions were studied. Firstly, organic mobile phase, using ACN and MeOH, was evaluated. It was observed that when ACN was used, the peak of oxalamic acid was split in two, whereas when MeOH was tested, SYN Na sensitivity was lower than that obtained using ACN. A mixture of MeOH and ACN (50:50, $v\,v^{-1}$) was also checked, providing good results for all analytes. Gradient elution was also tested to improve the elution time and the percentage of mobile phase. A generic gradient developed in previous studies was checked[17]: starting with a percentage of 95% of aqueous mobile phase lasting 1 min; linearly decreasing to 0% in 7 min and keeping constant during 4 min; returning to 100% in 30 s; and keeping constant 90 s. Although the peak shape of the compounds was suitable, the total running time was too long, so some steps of the gradient were shortened to reduce the total running time. Ramping step was reduced by 5 min (from 7 to 2 min) and holding step was 5 min. The overall running time was 10 min, obtaining a suitable elution for the analytes. Table 1 shows UHPLC-Orbitrap-MS parameters for the studied analytes, whereas Fig 1 shows the extracted ion chromatograms of the targeted compounds at 0.1 µg L^{-1}.

3.2 Extraction procedure optimization and validation of the analytical method

3.2.1 Optimization of soil procedure

For sample treatment, a previous extraction method, developed for quizalofop-p and metabolites in soils[16], was tested. However poor recoveries for CGA (< 10%) were obtained (see Table S2 in File S1). In consequence, the extraction procedure was modified in order to improve the recoveries, observing that they were better if the addition of acid to the extractant mixture (ACN-water) and salts (sodium chloride and magnesium sulphate) was avoided (see Table S2 in File S1). Results improved for all the compounds,

Table 1. Chromatographic and HRMS parameters of dimethachlor and metabolites

		Monoisotopic ion			Fragments				
Analytes name	Ionization mode	Exact mass	Adduct	Mass error (ppm)	Exact mass	Molecular formula	Mass error (ppm)	Neutral loss	Retention time (min)
Dimethachlor	Positive	256.10988	$[M+H]^+$	−1.9	224.08367	$C_{12}H_{15}ONCl$	−3.4	$[M\text{-}CH_2O]$	7.24
					148.11208	$C_{10}H_{14}N$	−3.0	$[M\text{-}C_3H_4O_2Cl]$	
Dimethachlor CGA	Negative	300.05473	$[M-H]^-$	0.7	256.06381	$C_{11}H_{16}O_4NS$	−4.1	$[M\text{-}CO_2]$	5.59
					178.08078	$C_{10}H_{15}O_2N$	−2.9	$[M\text{-}C_2HO_4S]$	
Dimethachlor SYN	Positive	268.11795	$[M+H]^+$	−2.0	236.09173	$C_{12}H_{14}O_4N$	−3.2	$[M\text{-}CH_2O]$	5.96
Dimethachlor SYN Na	Negative	326.10560	$[M-H]^-$	−0.3	178.08626	$C_{10}H_{11}O_2N$	−2.7	$[M\text{-}C_5H_{10}O_3S]$	6.19
					120.08078	$C_8H_{10}N$	−4.5	$[M\text{-}C_7H_{10}O_5S]$	
Dimethachlor oxalamic acid	Negative	250.10848	$[M-H]^-$	−3.7	178.12374	$C_{11}H_{16}ON$	−3.8	$[M\text{-}C_2O_3]$	6.12

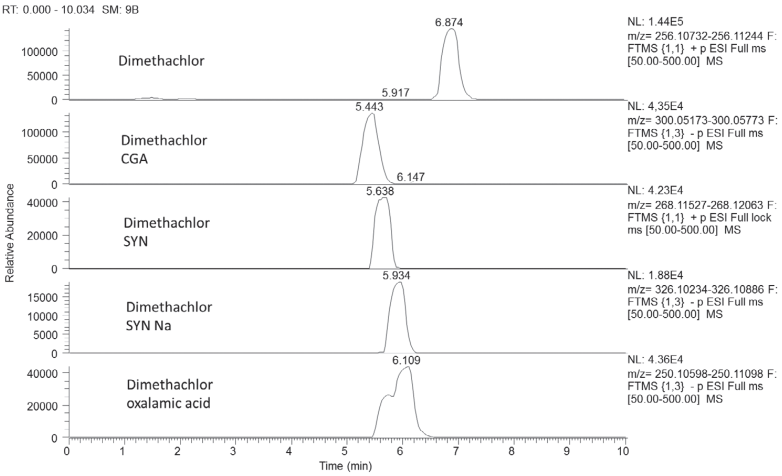

Figure 1. Extracted ion **c**hromatograms of the targeted analytes at 0.1 μg L^{-1}.

and recoveries ranged from 80 to 111% (Table S2 in File S1). Therefore it was decided to use the conditions described in '*Section 2.4.1. Soil extraction*'.

3.2.2 *Optimization of water procedure*

An SPE procedure was optimized for the extraction of the targeted compounds from water samples working with blank samples fortified at 1 μg L^{-1}. Using Oasis HLB cartridges conditioned with 3 mL of ACN and 3 mL of water, and 3 mL of ACN during the elution step, the recoveries were lower than 12% for most of the analytes except for dimethachlor (Table S3 in File S1), probably because of their higher polarity, so it was decided to test the addition of 40 mg of TBA in the sample to form ionic pairs[21]. Good recoveries were achieved for all analytes except for SYN Na. Strata X-A cartridges were tested, both alone and together to Oasis HLB cartridges, but recoveries did not improve in any case, especially for dimethachlor (Table S3 in File S1), so using Oasis HLB cartridges and adding TBA to the sample was selected for further studies, despite recoveries for SYN Na being slightly lower than 70%. Nevertheless, the recoveries were repetitive, and therefore, a correction factor of 1.47 was used for the quantitative determination of this compound.

3.2.3 *Validation study*

Table S4 in File S1 summarizes the characteristic parameters found in the validation of a new analytical method for determining dimethachlor and its metabolites in both matrices, using UHPLC-Orbitrap-MS. The working range was between 20 to 200 μg kg^{-1} for soils and between 0.1–1.0 μg L^{-1} for water. Good trueness and intra e inter-day precision values were achieved for all compounds. During sample analysis, an internal quality control was carried out, checking the linearity of the targeted compounds, as well as fortified samples (20 μg kg^{-1} for soils and 0.1 μg L^{-1} for waters) were also injected in order to check the efficiency of the extraction process.

3.3 Laboratory trials

The degradation results of dimethachlor, when the kinetic model 'Single First-Order Rate' (SFO) was applied in soils and water at normal dose, are shown in Figs. 2 and 3 (Figs S3 and S4 in File S1 show the results obtained for the double and fivefold doses for soils and water respectively). In order to identify other possible metabolites in the samples, metabolites described by EFSA in the risk assignment report[9], were included in the target list of the analyzed compounds, although standards were not available.

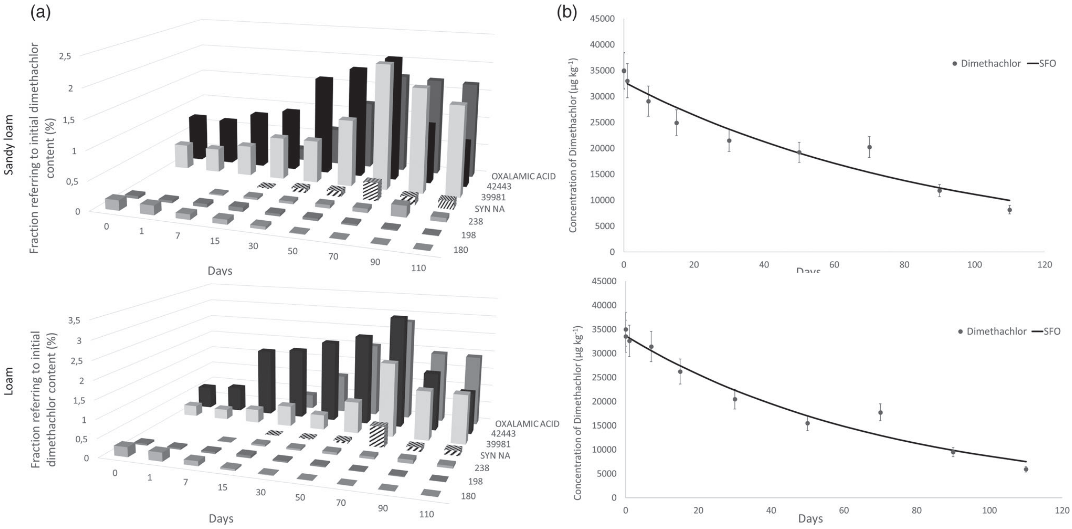

Figure 2. (a) Fraction referring to initial dimethachlor content (%) for all detected analytes and (b) concentration of the parent compound at normal dose (7 µL of commercial product kg^{-1} of soil) for soils (adjust to kinetic model 'Single First-Order Rate' (SFO. (Error bars obtained for n = 3) (Metabolites CGA 42443 and CGA 39981 are shown as 42 443 and 39 981 respectively and metabolites 238, 198 and 180 were metabolites unknown).

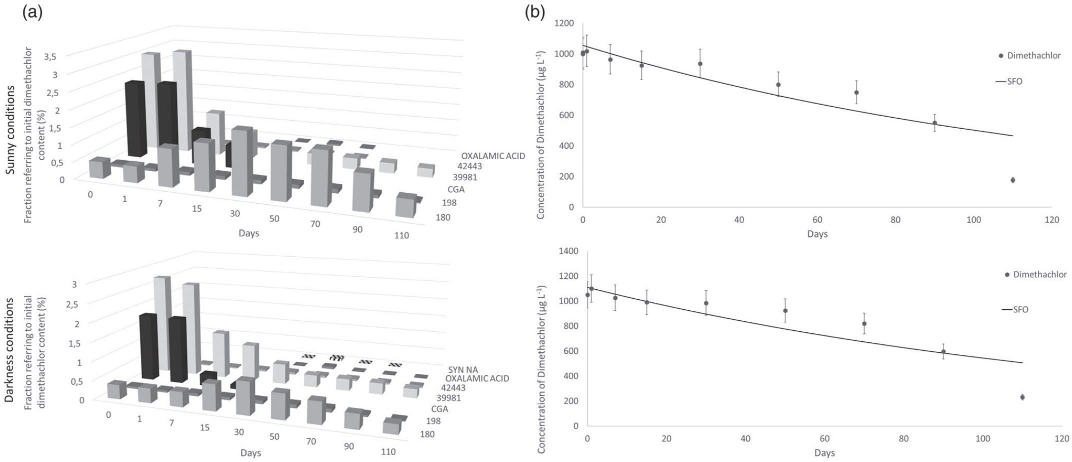

Figure 3. (a) Fraction referring to initial dimethachlor content (%) for all detected analytes and (b) concentration of the parent compound at normal dose (2 µL of commercial product L^{-1} of water) for water (adjust to kinetic model 'Single First-Order Rate' (SFO)). (Error bars obtained for n = 3) (Metabolites CGA 42443 and CGA 39981 are shown as 42 443 and 39 981, respectively, and metabolites 238, 198 and 180 were metabolites unknown).

3.3.1 *Soil trials*

The degradation results of dimethachlor in soils are shown in Table 2 and Fig. 2 and Fig. S3 in File S1, and they are fit to SFO model. It can be observed that the *k* value is similar for both doses (7 µL of commercial products g^{-1} of soil and 14 µL of commercial product g^{-1} of soil) and both types of soils (from 0.010 to 0.013 $days^{-1}$), and the determination coefficients are higher than 0.84. The DT_{50} of dimethachlor is high (see Table 2), from 50 to 70 days for all conditions, so this compound would have a medium-high persistence[22]. These results are similar to those obtained for TCP (transformation product of chlorpyriphos) in soils (DT_{50} 17–52 days)[23], and other pesticides like thiamethoxan (DT_{50} 34–60 days)[24] o fomesafen (DT_{50} 34–48 days)[25], which have similar DT_{50} values in soils. In addition, the results were compared with those obtained for two herbicides belonging to the same family, as metazachlor and prochloraz. It was observed that persistence (DT_{50}) in soils was 10.8 days and 223.6 days, respectively[26]. It can be highlighted that the DT_{50} of dimethachlor was between the values described for the two pesticides, due to metazachlor being non-persistent, meanwhile prochloraz was very persistent, observing the wide variability of the persistence of the compounds belonging to this family.

Table 2. SFO parameters of dimethachlor

Type of soil/ Conditions	Soil				Water			
	Sandy loam		Loam		Sunny		Darkness	
Parameters	Normal dose^{a}	Double dose^{a}	Normal dose^{a}	Double dose^{a}	Normal dose^{a}	Fivefold dose^{a}	Normal dose^{a}	Fivefold dose^{a}
C_0^{b}	32 812	56 160	33 663	53 256	1056	5138	1219	4743
k (days^{-1})	0.011	0.010	0.013	0.012	0.007	0.014	0.008	0.012
DT_{50} (days)	63.99	67.07	50.73	57.04	93.12	50.63	86.32	55.28
R^2	0.934	0.843	0.945	0.892	0.827	0.929	0.819	0.890

[a] Normal dose soil: 7 μL of commercial product kg^{-1} of soil; Double dose soil: 14 μL of commercial product kg^{-1} of soil; Normal dose water: 2 μL of commercial product L^{-1} of water; Fivefold dose water: 10 μgL of commercial product L^{-1} of water.
[b] Concentration units: 'μg kg^{-1}' for soil samples and 'μg L^{-1}' for water samples.

The SYN Na and oxalamic acid metabolites were detected 15 days after the application of the commercial product (at both doses as it can be observed in Fig. 2 and Fig. S3 in File S1), at concentrations of 5.8 μg kg^{-1} for SYN Na and 99.1 μg kg^{-1} for oxalamic acid in the case of sandy loam soils at double dose (14 μL of commercial product g^{-1} of soil) for instance. These concentrations increased until 70 days to decrease until the end of the monitoring period. For example, for oxalamic acid its concentration ranged from 1430 μg kg^{-1} (70 days) to 458 μg kg^{-1} (110 days). Similar behavior was observed for SYN Na. In relation to the other targeted metabolites, SYN and CGA, they were detected 50 days after application, but their concentrations were below the LOQ of the parent compound.

On the other hand, metabolites 39 981 and 42 443 were detected one day after the application of the commercial product, most of them can be present in the technical product or originated when it was mixed with water for application (Table S5 in File S1). It is shown in Fig. S5 in File S1, the extracted ion chromatogram of the commercial product Teridox®, where it can be observed that metabolites 39 981 and 42 443 are putatively detected in the commercial product.

Despite no standards were available for metabolites 39 981 and 42 443, their concentrations were estimated using the matrix matched calibration curve obtained for the parent compound (Table S5 in File S1). They approximately were at 189 and 437 μg kg^{-1} for sandy loam soils at double dose (14 μL of commercial product g^{-1} of soil) for metabolites 39 981 and 42 443, respectively, one day after application of the commercial product. The metabolite 39 981 and 42 443 concentrations increased until 70 days and slowly decreased until the end of the monitoring period. For example, for sandy loam soils applied at double dose (14 μL of commercial product kg^{-1} of soil), concentration of the metabolite 39 981 was 968 μg kg^{-1}, whereas for 42 443 it was estimated at 222 μg kg^{-1} at 110 days. In relation to the other putative metabolites 369 873 and 72 649, they were detected at concentrations lower than LOQ of dimethachlor 50 days after application.

3.3.2 Water trials

Behavior of dimethachlor in water was similar to soils, and the parent compound slowly degraded throughout the monitoring period, from 1000 μg L^{-1} to 850 μg L^{-1} after 70 days of application for sunny conditions at normal dose (2 μL of commercial product L^{-1} of water) (Fig. 3). In addition, the degradation does not depend on the environmental tested conditions. The results of the degradation of dimethachlor in water also fit the SFO model, as it can be observed in Fig. 3, Fig. S4 in File S1 and Table 2 (determination coefficient is higher than 0.82). It can be pointed out that depending on the dose, the *k* value is different. Thus, meanwhile at normal dose it was 0.007 days^{-1} for sunny conditions and 0.008 days^{-1} for darkness conditions, at fivefold dose (10 μL of commercial product L^{-1} of water), it was slightly higher, 0.014 days^{-1} and 0.012 days^{-1}, respectively. The same pattern was observed for DT_{50}, which was 90 days for normal dose and 50 days for fivefold dose. Thus, it can be concluded that at higher doses, persistence of dimethachlor decreases quicker than at normal dose, in contrast to other pesticides like quizalofop-p, that at higher concentrations, DT_{50} is higher than at normal concentration[21]. Comparing the results obtained for dimethachlor in water with prochloraz and metazachlor herbicides, it can be indicated that persistence in water was 78.9 days for prochloraz and 216 days for metazachlor[26]. It can be highlighted that dimethachlor has a similar persistence than prochloraz in waters, and this is shorter than the value described for metazachlor. Metabolites of dimethachlor were detected one day after the application of commercial product but their behavior is different than in soils (Fig. 3 and Fig. S4 and Table S6 in File S1). CGA was detected one day after the application and its concentration was 243 μg L^{-1} for sunny conditions at normal dose (2 μL of commercial product L^{-1} of water) and 178 μg L^{-1} for darkness conditions at normal dose. The concentration at fivefold dose (10 μL of commercial product L^{-1} of water) was lower than for normal dose for both conditions, 93 μg L^{-1} for sunny conditions and 43 μg L^{-1} for darkness conditions. It can be indicated that at higher concentrations, CGA metabolites degrade slowly in relation to normal concentrations. On the other hand, the concentration for the other metabolites was higher when fivefold dose was applied. CGA was not detected at 30 days after application of the commercial product. In contrast to soils, degradation of CGA in water was observed at higher concentrations and from the first day after application.

SYN Na and oxalamic acid metabolites were detected at 30 days after application in the case of double dose, and their concentration increased at concentrations closed to 0.8 μg L^{-1} at 50 days and later decreased their concentration. SYN Na concentration decreased until 70 days at concentrations closed to 0.2 μg L^{-1}, and oxalamic acid until 110 days at concentrations of 0.2 μg L^{-1}. In the case of normal dose (2 μL of commercial product L^{-1} of water), oxalamic acid followed the same pattern that at five dose, meanwhile metabolite SYN Na was detected at concentrations lower than 0.05 μg L^{-1} during all experiments.

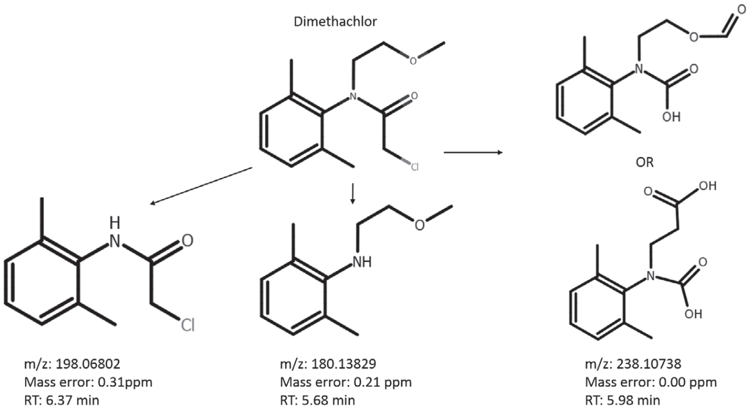

Figure 4. Structures of the new putative metabolites detected in the tested samples.

The metabolite 39 981 concentration decreased during monitoring period. Thus, it was detected one day after application at 128 µg L^{-1} for fivefold dose (10 µL of commercial product L^{-1} of water) and sunny conditions, and decreased until 12 µg L^{-1} at 110 days. Other EFSA metabolites, such as 102 935, 369 873 and 72 649, were detected at concentrations lower than 0.05 µg L^{-1} for all conditions.

The metabolite 42 443 was detected at lower concentrations than in soils, and its concentration increased slowly during monitoring until 15 days and later decreased. For example, for fivefold dose (10 µL of commercial product L^{-1} of water) and sunny conditions ranged from 1.4 (one day) to 2.5 (15 days) and 0.95 µg L^{-1} (110 days) (See Fig. 3 for normal dose and Fig. S4 in File S1 for fivefold dose).

3.3.3 *Untargeted analysis*

Raw files of each study were processed with MassChemSite®[27–29] and Compound Discoverer® (See selected settings in File S1, Section 3) in order to look for unknown compounds related to dimethachlor. At first, samples were classified according to the day after application of the commercial product, the dose and the type of sample. Thus four groups were processed independently: water at normal dose (2 µL of commercial product L^{-1} of water), water at fivefold dose (10 µL of commercial product L^{-1} of water), soils at normal dose (7 µL of commercial product kg^{-1} of soil) and soils at double dose (14 µL of commercial product kg^{-1} of soil).

Compound Discoverer® processing was carried out by a workflow of '*degradants*'. From the structure of the parent compound, common metabolic pathways[30] from different common transformations of pesticides in environmental samples like dehydration, reduction, reductive dechlorination, methylation, desaturation, oxidation and oxidative dichlorination were evaluated obtaining 1500 features. Features were filtered according to intensity of the signal (higher than 1e5) (900 features), retention time (between 1 to 9 min) (700 features) and background was subtracted (300 features): For that, a blank sample was processed and used to eliminate potential false positives in the samples. When the data were filtered, a further study was developed; all possible compounds (300) were studied in order to identify potential metabolites. To achieve a reliable elucidation of the potential compounds, it was considered how the signal changed throughout the monitoring period, if the mass error was lower than 10 ppm and if the transformation can be related to the parent compound according to the transformation pathways provided by Compound Discoverer® and MassChemSite®. Bearing this in mind, a total of 86 compounds were studied in detail.

When this strategy was used, two new metabolites where tentatively detected in water samples, meanwhile an additional one was also detected in soil samples. However, Compound Discoverer® did not show the final structure of the detected metabolites, and it only provided the molecular formula, so the elucidation of the structure is not a straightforward step. Therefore, another software tool, MassChemSite®, was used in order to confirm the appearance of these metabolites and a reasonable structure can be proposed. This software provided the reaction as well as the mass spectrum, the parent and the proposed metabolite with the matches and mismatches for the full scan spectra and the HCD mass spectra[28,31]. Using the last one, it compared the different ions and search for possible common ions, in order to corroborate if it came from the same family due to in most cases the parent compound having common fragments with its metabolites[20]. With this software tool, three new metabolites were tentatively elucidated[32] and the possible structures were identified for two of them but for the third one, putative structures are provided. Figure 4 shows the structure of the new detected metabolites, whereas Fig. S6 in File S1 shows extracted ion chromatograms, experimental and theoretical spectrum of the new metabolites a) metabolite 198 *m/z*, b) metabolite 180 *m/z* and c) metabolite 238 *m/z*. In addition in Table S7 and Fig. S7 in File S1 information about the fragments of each metabolite was provided. The metabolite 198 *m/z* is originated from the transformation of the parent compound, dimethachlor, by a cleavage of a carbon-nitrogen linkage and later the hydrogenation of the nitrogen. It was detected with a mass error of 0.31 ppm. The compound eluted at retention time of 6.37 min and because of the presence of chlorine, it has a characteristic isotopic pattern. The metabolite 180 *m/z* is detected at 5.68 min with 0.21 ppm of mass error and comes from the cleavage of a ketone carbon-nitrogen linkage followed by the hydrogenation of amino nitrogen. Finally, for the metabolite 238 *m/z* two possible structures were elucidated. The first involved the loss of chlorine group from the parent compound and the formation of an acid and aldehyde group. The second involved the formation of the same acid group elucidated for the first structure and the formation of the other acid group instead of the aldehyde group. It was detected with a mass error of 0.0 ppm and a retention time of 5.98 min.

In relation to the reliability of the identification process, the criteria indicated by Schymanski *et al.*[32] were applied, bearing in mind that different levels can be proposed during the identification of new compounds applying HRMS. Thus, the metabolites 198 *m/z* and 180 *m/z* correspond to level 2, whereas the metabolite 238 *m/z* can be set at level 3, because two possible structures were possible, achieving similar identification levels than in other studies[33–35] that used all the MS information obtained for the tentative elucidation.

The concentration of these metabolites was evaluated using the same procedure that was used for those metabolites for which commercial standards are not available. In soil samples, the metabolites 180 *m/z* and 198 *m/z* were detected in the commercial product (Fig. S5 in File S1) and one day after application in soil samples. For example, for loam soils at double dose (14 μL of commercial product g^{-1} of soil) the concentration of the metabolite 180 *m/z* decreased from 136 to 10 μg kg^{-1} at 50 days, meanwhile the metabolite 198 *m/z*, increased its concentration from 33 to 451 μg kg^{-1} at 15 days and later decreased the concentration slowly until 10 μg kg^{-1} (110 days after the application of the commercial product). On the other hand, the metabolite 238 *m/z* was detected 7 days after application at concentration of 10 μg kg^{-1} for sandy loam soils at double dose (14 μL of commercial product g^{-1} of soil). Then, its concentration increased until 70 days (108 μg kg^{-1}) and later decreased (Fig. 2 and Fig. S3 in File S1).

In water samples, the metabolites 180 *m/z* and 198 *m/z* were detected one day after the application and their concentration increased until 30 days and later decreased. For example, the concentration of the metabolite 180 *m/z* increased from 15 (one day) to 52 μg L^{-1} (30 days) for fivefold dose (10 μL of commercial product L^{-1} of water) sunny conditions. On the other hand, it increased from 12 to 20 μg L^{-1} at fivefold dose in darkness conditions. Later, its concentration decreased until 110 days, 18 μg L^{-1} for sunny conditions and 10 μg L^{-1} for darkness conditions. The concentration of the metabolite 198 *m/z* increased from 3 to 6 μg L^{-1} in both cases (2 μL of commercial product L^{-1} of water and 10 μL of commercial product L^{-1} of water) until 30 days and later decreased to 2 μg L^{-1} at 110 days (Fig. 3 and Fig. S4 in File S1).

To summarize, the behavior of those new metabolites is the same in both soils and at both doses. The metabolites 238 *m/z* and 198 *m/z* concentration increased and later decreased, while the metabolite 180 *m/z* decreased its concentration (Fig. 3 and Fig. S4 in File S1). In water samples, the concentration of metabolites increased until 30 days and later decreased until 110 days.

4 CONCLUSIONS

Degradation of dimethachlor was first studied in soils and water, and metabolites were detected in incurred samples using a new optimized and validated UHPLC-Orbitrap-MS analytical method. Lab trials have been developed to monitor the behavior of dimethachlor and its metabolites. The persistence of dimethachlor is medium to high in both matrices because half-life is higher than 50 days. Regarding metabolites, they were detected one day after the application of the commercial product. In addition, untargeted analysis was carried out and three new metabolites of dimethachlor were putatively elucidated in environmental samples. According to the concentrations of the metabolites, a limited part of the dimethachlor is degraded into them and further studies are needed to understand other mechanisms involved in the biodegradation of this pesticide. These data give us a first appraisal of the occurring of dimethachlor in environmental matrices, including the monitoring of its metabolites. This information may be utilized in further environmental risk assessment studies.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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CAPÍTULO

5

Estudios de disipación de fungicidas e identificación de sus metabolitos en matrices alimentarias y ambientales

1. INTRODUCCIÓN

Los fungicidas son aplicados, tanto en cultivos al aire libre como en cultivos de invernadero, con el fin de prevenir o eliminar hongos. Al igual que el resto de grupos de plaguicidas, éstos deben de ser estudiados con el fin de garantizar la calidad y la seguridad alimentaria además de unas condiciones ambientales óptimas. El comportamiento de los fungicidas es crucial para conocer todas las pautas que hay que seguir antes y después de su aplicación en los cultivos. El plazo de seguridad es uno de los conceptos más importantes y determina el tiempo de espera necesario antes de recolectar el fruto, y asegura que el fungicida aplicado se ha eliminado casi por completo o sus niveles son inferiores a los MRLs establecidos por el organismo competente. Se requieren métodos analíticos para garantizar que los frutos no exceden la concentración máxima permitida de sustancia activa con acción fungicida legislada por la UE [1]. Además, los fungicidas se transforman en metabolitos, que pueden permanecer inalterados en el fruto o en el medioambiente incluso durante un tiempo mayor al del compuesto progenitor [2]. En el caso de los fungicidas estudiados en esta Tesis, para propamocarb se conoce que existen sales derivadas de éste, indicadas por EFSA en sus ensayos de riesgos, donde determinan que éstas han de ser controladas e incluidas en el MRL del compuesto progenitor [3]. Para el resto de fungicidas estudiados no se incluye ningún metabolito en sus respectivos MRLs. De modo que es de gran interés en la presente Tesis Doctoral llevar a cabo un estudio de los posibles metabolitos de los fungicidas objeto de estudio mediante UHPLC-HRMS y el modo de trabajo *unknown*. Esta se basa en la búsqueda de compuestos desconocidos en las matrices estudiadas mediante dos softwares de análisis. Uno de ellos (MassChemSite®) está basado en la simulación de rutas metabólicas del compuesto progenitor mediante reacciones simples que se podrían dar en condiciones reales como la oxidación, reducción, metilación etc, de donde surgen los posibles metabolitos que son buscados a lo largo de todo el espectro de masas adquirido. El otro (Compound Discoverer®) se basa principalmente en el empleo de bases de datos espectrales y herramientas de análisis quimiométrico, cuyo fin es buscar a lo largo del espectro, una molécula desconocida en la muestra y asignarle una fórmula molecular [4].

Los trabajos incluidos en este capítulo se han focalizado en el desarrollo de métodos analíticos para el estudio del comportamiento de fungicidas específicos en matrices alimentarias, tales como tomate, pepino y calabacín, (**Artículos científicos IX y X**) y medioambientales tales como suelos y aguas (**Artículos científicos XI y XII**). Por otro lado, también se ha realizado la búsqueda de metabolitos procedentes de cada uno de los compuestos objetivo mediante análisis *unknown* (**Artículos científicos IX, X, XI y XII)**.

- **Artículo científico IX.** Dissipation studies of famoxadone in vegetables under greenhouse conditions using liquid chromatography coupled to high-resolution mass spectrometry: putative elucidation of a new metabolite

- **Artículo científico X.** Dissipation kinetic studies of fenamidone and propamocarb in vegetables under greenhouse conditions using liquid and gas chromatography coupled to high-resolution mass spectrometry

- **Artículo científico XI.** Residues and dissipation kinetics of famoxadone and its metabolites in environmental water and soil samples under different conditions

- **Artículo científico XII.** Dissipation kinetics of fenamidone, propamocarb and their metabolites in ambient soil and water samples and unknown screening of metabolites

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Publicación IX

DISSIPATION STUDIES OF FAMOXADONE IN VEGETABLES UNDER GREENHOUSE CONDITIONS USING LIQUID CHROMATOGRAPHY COUPLED TO HIGH-RESOLUTION MASS SPECTROMETRY: PUTATIVE ELUCIDATION OF A NEW METABOLITE

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Dissipation studies of famoxadone in vegetables under greenhouse conditions using liquid chromatography coupled to high-resolution mass spectrometry: putative elucidation of a new metabolite

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Abstract

BACKGROUND: Famoxadone is a pesticide that is used to control fungal diseases and its dissipation in vegetables should be monitored. For that purpose, liquid chromatography coupled to mass spectrometry has been used.

RESULTS: The dissipation of famoxadone has been monitored in cucumber, cherry tomato and courgette under greenhouse conditions at different doses (single and double), using ultra high-performance liquid chromatography coupled to Orbitrap mass spectrometry (Thermo Fisher Scientific, Bremen, Germany). The concentration of famoxadone increased slightly just after the application of the commercial product and then decreased. The half-lives (DT_{50}) of famoxadone are different for each matrix, ranging from 2 days (courgette single dose) to 10 days (cucumber double dose). The main metabolites, 4-phenoxybenzoic acid and 1-acetyl-2-phenylhydrazine, were not detected in vegetable samples. Other metabolites described by the European Food and Safety Authority, such as IN-JS940 [(2RS)-2-hydroxy-2-(4-phenoxyphenyl)propanoic acid], IN-KF015 [(5RS)-5-methyl-5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione] and IN-MN467 [(5RS)-5-methyl-3-[(2-nitrophenyl)amino]-5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione], were detected in the three matrices. Untargeted analysis allowed for the putative elucidation of a new metabolite of famoxadone in cucumber (up to 290 μg kg^{-1}) and cherry tomato (up to 900 μg kg^{-1}) samples.

CONCLUSION: The dissipation of famoxadone has been investigated in three vegetables: tomato, cucumber and courgette. The persistence of famoxadone was low in the three matrices (DT_{50} less than 10 days). Metabolites of famoxadone were monitored, detecting IN-JS940, IN-MN467 and IN-KF015, and the putative elucidation of a new metabolite of famoxadone was performed by applying software tools.

Supporting information may be found in the online version of this article.

Keywords: famoxadone; UHPLC-Orbitrap-MS; dissipation; metabolites; putative identification; greenhouse conditions

INTRODUCTION

Famoxadone [(RS)-3-anilino-5-methyl-5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione] is a fungicide that is used to protect agricultural products against various fungal diseases on fruiting vegetables such as tomatoes and cucurbits. When crops are sprayed with this pesticide, it reaches the inner parts of the plant and degrades into several metabolites. Many of them are biologically active and they can have high toxicity.[1]

Metabolites are generated by metabolic and simple reactions, which are common among several families of pesticides, such as pyrethrins or neonicotinoids. Famoxadone can be degraded into a variety of metabolites, such as 4-phenoxybenzoic acid and 1-acetyl-2-phenylhydrazine. In addition, the European Food and Safety Authority (EFSA) has proposed other metabolites,[2] such as IN-JL856 [(2RS)-2-hydroxy-2-(4-phenoxy phenyl)-N′-phenylpropanehydrazide], IN-JS940 [(2RS)-2-hydroxy-2

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-(4-phenoxyphenyl)propanoic acid], IN-KF015 [(5RS)-5-methyl-5-(4-phenoxyphenyl)-1,3- oxazolidine-2,4-dione], IN-KZ007 [(5RS)-3-anilino-5-[4-(4-hydroxyphenoxy)phenyl]-5-methyl-1,3-oxazolidine-2,4-dione], IN-MN467 [(5RS)-5-methyl-3-[(2-nitrophenyl)amino]-5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione], benzene and 1,5-diphenylcarbono hydrazide (see Supporting information, Fig. S1). These metabolites could be detected in fruits and vegetables and, in some cases, they can be more toxic than the parent compound,[3,4] or its toxicity can be similar, as is the case with one of these metabolites, as benzene (see Supporting information, Table S1). In relation to half-lives (DT_{50}), they can be shorter than 11 days in soils, whereas, in water, they have been estimated as being up to 1000 days (see Supporting information, Table S1). Therefore, knowing the behavior of these metabolites in vegetables is interesting, despite the attempts to estimate (DT_{50}) by applying several models.[5] Thus, the development of a method that allows for the determination of these metabolites in vegetables is required.

In the USA, the maximum residue limit (MRL) of famoxadone in tomatoes is 1 mg kg^{-1}, whereas, in cucurbits (cucumber and courgette), it has been set at 0.3 mg kg^{-1}.[6] On the other hand, in the European Union, the MRL of famoxadone in tomatoes is 2 mg kg^{-1} and 0.2 mg kg^{-1} in cucurbits (cucumber and courgette),[7] and the Acceptable Daily Intake (ADI) of famoxadone is 0.012 mg kg^{-1} body weight per day. However, neither are the metabolites included in the MRL definition, nor have AID values been set for these metabolites. For these reasons, it was necessary to determine how famoxadone degrades into its metabolites and their behavior, aiming to check whether they should be included in the MRL definition of the active substance and in the ADI. Therefore, in the present study, in addition to monitor 4-phenoxybenzoic acid and 1-acetyl-2-phenylhydrazine during the dissipation process of the parent compound, an untargeted screening was carried out to search for other possible metabolites.

During recent years, several studies describing multiresidue methods, where famoxadone has been determined in fruits and vegetables, have been published.[8–12] A scarce bibliography exists with respect to studies of the behavior of famoxadone in fruits and vegetables.[13–15] However, in those studies, only the parent compound famoxadone had been monitored, whereas the main metabolites (4-phenoxybenzoic acid and 1-acetyl-2-phenylhydrazine) and other potential metabolites proposed by the EFSA have not been included. Therefore, the evaluation of the behavior of famoxadone in greenhouse conditions as well as its dissipation into its metabolites is investigated for the first time in the present study. High-resolution mass spectrometry (HRMS) has become an essential tool for the identification of known and unknown compounds in several fields, such as pharmacokinetics, human health studies, metabolomics, natural products research, food and environmental analysis.[16,17] When the spectrum of an unknown compound is evaluated, a reasonable first step is the comparison of the experimental spectrum with those obtained from standards collected in databases and spectral libraries. However, when the study is focused on looking for unknown pesticide metabolites, the use of software tools is required to screen the samples and search for possible metabolic pathways, taking into account the structure of the parent compound. In such a case, Compound Discoverer® and MassChemsite®[18,19] comprise powerful tools that can be used in this field.

The aim of the present study is to investigate the dissipation kinetics of famoxadone in vegetables and monitor its metabolites under greenhouse conditions at single and double doses when the commercial product is used. Accoridngly, an analytical method has been developed and validated for the quantitative determination of famoxadone and its metabolites by applying ultra-high-performance liquid chromatography–Orbitrap mass spectrometry (UHPLC–Orbitrap-MS) (Thermo Fisher Scientific, Bremen, Germany). In addition, non-targeted approaches have been used.

MATERIALS AND METHODS

Equipment, materials and reagents

Famoxadone (CAS registry No. 131807–57-3, purity >99%) and metabolites, such as 1-acetyl-2-phenylhydrazine (CAS registry No. 114–83-0, purity >98%) and 4-phenoxybenzoic acid (CAS registry No. 2215-77-2, purity >97%), were purchased from Sigma-Aldrich (St Louis, MO, USA).

Stock standard solutions (1000 mg L^{-1}) were prepared by dissolving 10 mg of the pure compound in acetonitrile (ACN) (10 mL). An intermediate solution of the three compounds (10 mg L^{-1}) was prepared by taking 100 μL of each stock solution and diluting up to 10 mL with acetonitrile in a volumetric flask. Stock and intermediate solutions were stored at −21 °C. Stock solutions were stable for a year and intermediate solution was stable for 2 months.

ACN and methanol (MeOH) (both LC-MS grade) were acquired from Fluka (St Louis, MO, USA). Water (LC-MS grade) was acquired from J.T. Baker (Deventer, Holland) and formic acid was obtained from Fisher Scientific (Erembodegem, Belgium). Magnesium sulfate (Sigma-Aldrich) and sodium chloride (J.T. Baker) were used during the extraction procedure.

A mixture of acetic acid, caffeine, Met-Arg-Phe-Ala-acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), as well as a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrat and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative) (from Thermo Fisher Scientific), was employed to allow accurate mass calibration of the Orbitrap analyzer.

For the treatment and preparation of samples, an analytical balance AB204-S (Mettler Toledo, Greifensee, Switzerland), a mixer (Aicok, Beijing, China), a vortex mixer WX (Velp Scientifica, Usmate, Italy), a Reax 2 rotary agitator (Heidolph, Schwabach, Germany) and a Centronic BL II centrifuge (J.P. Selecta, Barcelona, Spain) were used.

A Transcend 600 LC (Thermo Fisher Scientific, San Jose, CA, USA) was used for chromatographic analysis. A Zorbax Eclipse Plus C_{18} column (100 mm × 2.1 mm, 1.8 μm particle size) supplied by Agilent Technologies (Santa Clara, CA, USA) was used. A flow rate of 0.2 mL min^{-1} was set for separation of the selected compounds in the UHPLC system. The mobile phase consisted of eluent A, which was an aqueous solution of 0.1% formic acid and 4 mmol L^{-1} ammonium formate, and eluent B, which was MeOH.

The step gradient was as follows: 0–1 min 95% A; then, it was linearly decreased to 0% in 1 min, and remained constant during 5 min. After returned to the initial conditions in 30 s, the composition was kept constant during 90 s. The total running time was 9 min. The column temperature was set at 25 °C and the injection volume was 10 μL.

The chromatographic system is coupled to a single mass spectrometer (Orbitrap Exactive; Thermo Fisher Scientific) using an electrospray interface (ESI) (HESI-II; Thermo Fisher Scientific, San Jose, CA, USA) in positive and negative mode. ESI parameters

Table 1. Parameters of greenhouse trials in line with existing reporting recommendations[a]

	Famoxadone	4-phenyxybenzoic acid	1-acetyl-2-phenylhydrazine
CAS-RN	131 807–57-3	2215-77-2	114–83-0
Study location	Vicar, Almeria, Spain		
Study characteristics	Hydroponic crop under greenhouse conditions		
Application dose (kg ha^{-1})	0.4 (single), 0.8 (double)		
Application dates	13, 20 and 27 March 2017		
Treated plant component	Leaf		
Formulation (%)	22.5 WG of famoxadone		
Air temp (greenhouse indoor) (°C)	16.5		
Binomial plant name	*Solanum lycopersicum* L. (tomato) *Cucumis sativus* L. (cucumber) *Cucurbita pepo* L. (courgette)		
Plant stage at pesticide application	Bloom		
BBCH scale[b]	60		
Sample plant component	Fruit with peel		
Sample mass (kg)	1		
Kinetic models used	SFO, IORE, DFOP		

[a] Fantke *et al.*[22]
[b] The BBCH scale (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie) is used to identify the phenological development stages of plants.
SFO, single first order; IORE, indeterminate order rate equation; DFOP, double first order parallel.

were as follows: spray voltage, 4 kV; sheath gas (N_2, > 95%), 35 (adimensional); auxiliary gas (N_2, >95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 °C; capillary temperature, 300 °C. The mass spectra were acquired employing two alternating acquisition functions: (i) full MS, ESI+, without fragmentation (higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 50 000 full width at half maximum (FWHM); scan time = 0.25 s; (ii) all-ions fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy 30 eV), mass resolving power = 10 000 FWHM; scan time = 0.10 s; (iii) full MS, ESI− using the aforementioned settings; and (4) AIF, ESI− using the settings oulined for (ii). Mass range in the full scan experiments was set at *m/z* 50–600.

The chromatograms were acquired using the external calibration mode and processed using Xcalibur, version 2.2, with Quanbrowser and Qualbrowser (Thermo Fisher Scientific, Les Ulis, France). For fragment identification, Mass Frontier, version 7.0 (Thermo Fisher Scientific) was used. This software used the chemical structure of the compound to determine the fragmentation mechanisms and the corresponding fragment ions with their exact masses.

Untargeted analysis was carried out with Compound Discoverer, version 2.1 (Thermo Fisher Scientific) and MassChemSite, version 2.0 (Molecular Discovery Ltd, London, UK).

Sample extraction

Extraction method was based on the well-known QuEChERS method.[20] Briefly, 1 kg of sample was crushed and homogenized as established by current regulation[21] and then stored in the freezer at −21 °C. Then, 10 g of homogenized sample was placed in a 50-mL plastic centrifuge tube. Next, 10 mL of ACN was added and shaken for 10 min in a rotary shaker. Subsequently, 1 g of NaCl and 4 g of $MgSO_4$ were added, and the mixture was shaken vigorously for 1 min in a vortex. Then, the sample was centrifuged for 10 min at 4136 × *g* and 1 mL of the supernatant was collected and injected into the UHPLC-Orbitrap-MS.

Greenhouse trials

Greenhouse trials were performed in winter and spring of 2018 (from January to May) in a greenhouse located in Vícar, Almería, Spain. A hydroponic greenhouse, using bags of coconut fiber, was selected for the present study, as indicated in Table 1. The suspension concentrate formulation of famoxadone (225 g L^{-1}) was dissolved in water and sprayed by using a manual sprayer (volume 1.5 L, maximum pressure 1.0 MPa), with one conical nozzle operated at 40 psi (275 kPa). To investigate the dissipation of the pesticide in tomato, cucumber and courgette samples, 0.75 g of commercial product (Equation Pro®; DuPont, Wilmington, DE, USA) was dissolved in 1.5 L of water and sprayed in each bag of crop (400 g of product per hectare). In the case of double dose, 1.5 g of commercial product in 1.5 L of water (800 g of product per hectare) was applied on the surface of the plant (Table 1). Three applications were carried out, as indicated in Table 1. After 0, 1, 2, 3, 5, 7, 9, 11, 15, 21, 23, 30 and 41 days of application, 1 kg of representative vegetable samples was collected randomly. Temperatures ranged from 20 to 30 °C during the day and from 10 to 20 °C during the night in the greenhouse. Plants were watered twice per day during monitoring period and several parameters,[22] such as BBCH or plant stage, have been included in Table 1.

The dissipation kinetics of famoxadone in vegetables was determined by plotting the residue concentration of famoxadone *versus* time. The residual concentration and DT_{50} of famoxadone was determined using the model 'Single First-Order Rate' (SFO),[23–26] and characteristic parameters such as initial concentration (C_0) and rate constant (k) have been estimated, applying Eqn (1), whereas overall dissipation half-lives (DT_{50} or $T_{1/2}$) have been calculated using Eqn (2), with C_t being the concentration at time t:

$$C_t = C_0\, e^{-kt} \tag{1}$$

$$DT_{50} = \ln 2/k \tag{2}$$

In addition to this, other models,[27] such as double first order parallel (DFOP), N^{th}-Order Rate Model and Indeterminate Order Rate Equation Model (IORE), were also tested.

Table 2. UHPLC-Orbitrap-MS parameters for famoxadone and its main metabolites

		Precursor ion			Fragments			
	Ionization mode	Exact mass	Adduct	Mass error (ppm)	Exact mass	Molecular formula	Mass error (ppm)	Retention Time (min)
Famoxadone	Positive	392.16048	$[M+NH_4]^+$	−0.4	195.08002	$C_{14}H_{11}O$	−2.2	6.75
					331.14334	$C_{21}H_{19}O_2N_2$	2.3	
1-Acetyl-2-phenylhydrazine	Positive	151.08659	$[M+H]^+$	−0.2	108.06869	$C_6H_8N_2$	4.1	5.10
					92.05020	C_6H_6N	4.9	
4-Phenoxybenzoic acid	Negative	213.05572	$[M-H]^-$	−2.1	169.06526	$C_{12}H_9O$	−2.7	6.44
					93.03345	C_6H_5O	−0.4	

Method validation

The developed method was validated to check its performance by a validation procedure[21] that involves the evaluation of several parameters, such as linearity, matrix effect, trueness (% recovery), inter- and intra-day precision, limits of detection (LODs) and limits of quantitation (LOQs). Further details are provided in the Supporting information, Section S1.

RESULTS AND DISCUSSION

Optimization of UHPLC-Orbitrap MS analysis

A mixture of 1 mg L^{-1} of the analytes was injected into the system and analyzed to characterize the compounds. The protonated ion of each compound was monitored according to its molecular formula. Exact masses were selected according to the criterion that mass error was lower than 5 ppm for the targeted compounds. Then, fragments of each analyte were studied, acquiring the spectra when a HCD fragmentation step was applied. Using the information provided by Mass Frontier, the comparison of MS and all ion fragmentation (AIF, pseudo MS/MS) spectra at the retention time of the parent compound was studied to determine the different ions. The most intense ions observed in the AIF spectra were selected in both traces (with and without HCD). Thus, if one ion was observed in both spectra but the intensity was higher in the AIF spectrum, this might indicate that this ion had been fragmented from the parent compound. In this case, the experimental formula, obtained using the software Qualbrowser, was evaluated to determine whether it originates from the parent structure. Table 2 shows the exact mass and molecular formula for the fragments when this approach is used. The ionization mode was negative for 4-phenoxybenzoic acid, whereas it was positive for the rest of analytes. For example, the loss of a methoxy group (-CH_2O) and the loss of an amide group (-C_2H_4NO) was observed for 1-acetyl-2-phenylhydrazine, providing the ions *m/z* 108.06869 and 92.05020, respectively. For 4-phenoxybenzoic acid, the most characteristic ion is a result of the loss of the acid group (-COOH), providing the fragment *m/z* 169.06526. For the parent compound, the fragments originate from the cleavage of cyclopentane that is present in its structure. For example, fragment *m/z* 331.06869 is obtained because of the fragmentation of the amide group of the cyclopentane and the loss of a carboxylate group (-COO). Finally, Table 2 shows a summary of the MS parameters for the targeted compounds.

Then, the chromatographic conditions were evaluated to obtain the best peak resolution. First, the mobile phases formed by MeOH and an aqueous solution of 0.1% formic acid (0.1%) and MeOH and water containing 0.1% formic acid and 4 mmol L^{-1} ammonium formate were tested. Poor results were obtained for the parent compound when water acidified with 0.1% formic acid was used, whereas better signals were obtained when ammonium formate was used because the ammonium adduct favored the ionization of the analyte. Thus, better results for this compound were obtained when ammonium formate was added to the mobile phase. ACN was also tested as organic mobile phase, although the peak shape of 4-phenoxybenzoic acid was better with MeOH. Gradient elution was evaluated aiming to reduce the total analysis time, taking into account the analytes eluted at 6 min approximately, and the total time analysis was 14 min using the initial gradient profile. This consisted of 0–1 min 95% A; then it was linearly decreased to 0% in 7 min, and remained constant during 4 min; finally returned to the initial conditions in 30 s and kept constant during 90 s. In this sense, the percentage of aqueous eluent was reduced from 95% to 0% in 1 min but then kept constant for 5 min. Using these conditions, a good peak shape was obtained with a total run time of 9 min (see Supporting information, Fig. S2).

The extraction procedure was based on the methodology of the QuEChERs method[20] using ACN and a mixture of salts as described above. Suitable recoveries for the three analytes were achieved (75–85%) at 50 μg kg^{-1} and so no further conditions were evaluated.

Method validation development

Cucumber was used as a reference matrix because the three studied matrices belong to high-water content commodities according to the SANTE classification.[21] The results of the validated parameters are provided in the Supporting information, Table S2. More information about the validation data is provided in the Supporting information (Section S2).

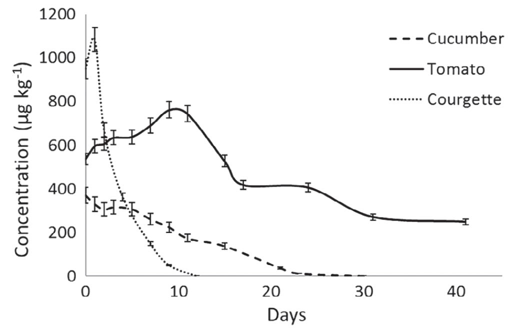
Greenhouse trials

Targeted analysis

Approximately 1 kg of samples was collected and crushed at the aforementioned time intervals after commercial product application, as described above. Famoxadone dissipation, when the kinetic model ‘Single First-Order Rate’ (SFO) was used at single and double dose for cucumber and courgette, is shown in the Supporting information (Figs S3 and S4). Figure 1 shows the dissipation of famoxadone in all matrices at single and double dose.

Cherry tomato trials showed that the concentration of famoxadone increased up until 11 days of application of commercial product for both doses, and later slowly decreased until 41 days. For single dose, the dissipation percentage at 11 days and 41 days was 67%, whereas, for double dose, it was 43% (Fig. 1).

(a) **Single dose**



(b) **Double dose**

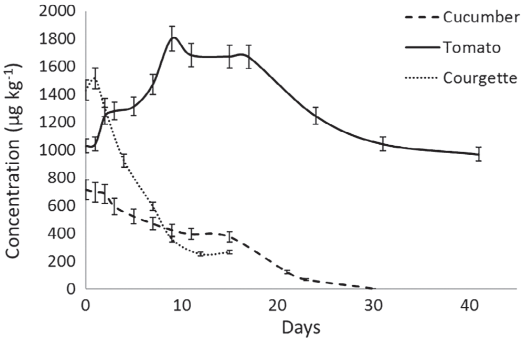


Figure 1. Dissipation curves of the parent compound, famoxadone, at (a) single dose and (b) double dose. (Error bars obtained for n = 3).

These results indicated that, at single dose, the parent compound degrades quicker than at double dose. Moreover, the data showed that, at double dose, the dissipation process was not carried out properly, possibly because, at higher concentrations, the plant can be phytostressed. In other studies,[28] the dissipation of famoxadone was evaluated in watermelon, peel and soil at one dosage, such that, after 35 days, more than 90% of the initial residue dissipated, which is faster than the degradation in the present study.

During the trials, plants leaves at double dose were falling and drying as the days progressed. The SFO kinetic model was applied with this matrix but the model did not fit well ($r^2 = 0.70$ for single dose and $r^2 = 0.045$ for double dose). Accordingly, other models,[27,29] such as DFOP and the N^{th}-Order Rate Model or (IORE), were applied but the fit was even worse than that obtained by SFO model, as shown in the Supporting information (Table S3).

These models were also applied to the experimental data of the other two matrices (cucumber and courgette), although the best results were obtained when the SFO model was used. The results were also compared with those published previously. Feng *et al.*[15] performed a comparison of different kinetics models to monitor the dissipation of famoxadone in vegetables and the best model was also SFO model, as in the present study. For this reason, it was decided that famoxadone dissipation can be explained by the SFO model but, in the case of tomato, further studies are needed because poorer determination coefficients were obtained for the models tested.

Table 3. SFO kinetic model parameters and vegetables dissipation (DT_{50}) of famoxadone

Matrix	Cucumber		Courgettes	
Parameters	Single dose	Double dose	Single dose	Double dose
C_0 (µg kg^{-1})	392	768	1097	1588
k (days^{-1})	0.073	0.065	0.249	0.138
DT_{50} (days)	9.42	10.66	2.78	5.01
r^2	0.856	0.905	0.920	0.951

C_0, initial concentration; k, rate constant; DT_{50}, half-life.

The concentration of famoxadone during cucumber trials decreased until 30 days after application, except at day 5 after application of commercial product, where the concentration increased slightly (see Supporting information, Fig. S3). For example, at single dose, the concentration increased up to 315 µg kg^{-1} (3 days) to 346 µg kg^{-1} (5 days). Day temperatures were slightly lower than the other days, and plants were only watered once, and so this can explain why the concentration of the parent compound increased slightly. Water content could be lower in cucumber and the concentration of the pesticide would be higher. The k value is the same for both doses (0.07 days^{-1}), as shown in Table 3, indicating that dissipation in cucumber was independent of the dose of commercial product applied, and this can be confirmed by DT_{50}, which is 10 days for both doses. Comparing the results at single dose for tomato and cucumber, it was observed that the dissipation of the parent compound in tomato was slower than in cucumber. For cucumber, famoxadone residues were not detected at 30 days after application of commercial product, whereas, for tomato, at 30 days, the concentration of famoxadone was 300 µg kg^{-1}. These results can be explained by cherry tomatoes being smaller than cucumber and its surface/weight ratio was higher than in cucumber, such that vegetables with the highest surface/weight ratio will show higher residue contents, as reported by Angioni *et al.*[11] who compared two types of tomatoes.

In relation to courgette trials, famoxadone concentrations increased up at 2 days after the application of commercial product and later decreased quickly until 12 days in the case of single dose (see Supporting information, Fig. S4). In comparison with the other matrices (cucumber and tomato), famoxadone degrades faster. After application, courgette plants were slowly dying and did not grow, possibly as a result of the low temperatures and a plague of white fly. The SFO kinetic model (Table 3) shows that the k value is different for both doses and this can affect the dissipation of the compound. For single dose, the k value is higher and so famoxadone degrades quicker when single dose was applied compared to double dose. DT_{50} for single dose was 2.78 days, whereas it was 5.01 days at double dose. This can be explained by other factors, such as the low temperature (i.e. kinetics depends on temperature) and the white fly, also affecting the behavior of the plants at double dose, modifying dissipation values.

In summary, famoxadone dissipation follows the same pattern in all matrices, being increased at first and later decreased. This behavior is similar to flonicamid in fruits and vegetables,[1,30] as a result of both pesticides being systemic. Thus, after the application of the compound, the pesticide was not totally absorbed and, after a few days, the whole amount of pesticide was absorbed.

For tomato, the concentration of famoxadone was not higher than its MRL (1 mg kg^{-1}) 3 days after application of commercial product at single dose, (waiting period), whereas, for cucumber and courgettes, at 7 days (waiting period), the concentration of famoxadone was 0.35 mg kg^{-1} in cucumber, which is higher than its MRL (0.3 mg kg^{-1}), and 0.15 mg kg^{-1} in courgettes (MRL 0.3 mg kg^{-1}).

The main metabolites of famoxadone (4-phenoxybenzoic acid and 1-acetyl-2-phenylhydrazine) were not detected in vegetable samples at concentrations higher than LOQ. Nevertheless, other metabolites of famoxadone described in the EFSA risk assessment report[2] were detected in the samples. However, there are no commercially available standards for those metabolites and so their detection and quantification were carried out using the parent compound as standard.

IN-JS940, IN-MN467 and IN-KF015 metabolites were detected in all matrices. The concentration of metabolites, calculated using the parent compound as standard, was between the LOD and LOQ of the parent compound. For this reason, the peak areas of metabolites *versus* time (Fig. 2) and the estimated concentration (μg kg^{-1}) *versus* time (see Supporting information, Fig. S5) were monitored. It can be seen that metabolite IN-KF015 was also found in the commercial product Equation Pro® (see Supporting information, Fig. S6) and, for this reason, metabolite IN-KF015 was detected in all matrices at a higher concentration compared to the other metabolites. Dissipation of metabolites was similar for both doses. The amount of metabolite IN-MN467 increases from 1 to 15 days after the application of commercial product and later it decreases for tomato and cucumber. On the other hand, it was only detected 2 days after application for both doses in courgette. Metabolite IN-KF015 behavior is different for each matrix. In cucumber and courgette samples, the concentration decreased during the monitoring period meanwhile in tomato increased up until 17 days and later decreased. Metabolite IN-JS940 was detected at 15 days for cucumber and disappeared after 21 days. In tomato, it was detected at 7 days, then slightly increased until 11 days and later decreased. In courgette, it was only detected at 15 days after application, as shown in Fig. 2.

In comparison with other studies, dissipation of famoxadone in cucumber is in accordance with the studies reported by Shabeer *et al.*[13] In that study, DT$_{50}$ for single and double dose in raisin samples was 12 days, whereas, in the present study, it was 10. In relation to the other matrices, Angioni *et al.*[11] evaluated the presence of famoxadone in two types of tomato. Famoxadone was detected in tomato until 24 days after application of the pesticide, whereas, in the present study, famoxadone residues were present 41 days after the commercial product was sprayed. This can be explained by the present study being performed in winter–spring, whereas the former was carried out in summer, affecting the temperature with respect to the dissipation of the compound.

In summary, famoxadone dissipation follows the same pattern for cucumber and courgette. For tomato, it was difficult to determine its behavior because the experimental data were not well-fitted at any tested model. Three metabolites, IN-JS940, IN-MN467 and IN-KF015 were first detected in samples after 1 day (IN-MN467 and IN-KF015) and 7 days (IN-JS940) of the application, although at low concentrations in relation to the concentration of the parent compound. For example, the concentration of metabolite IN-MN467 at 15 days in cucumber at double dose was 5 μg kg^{-1}, whereas, for famoxadone, it was 400 μg kg^{-1}. In addition, the main metabolites were not detected in samples.

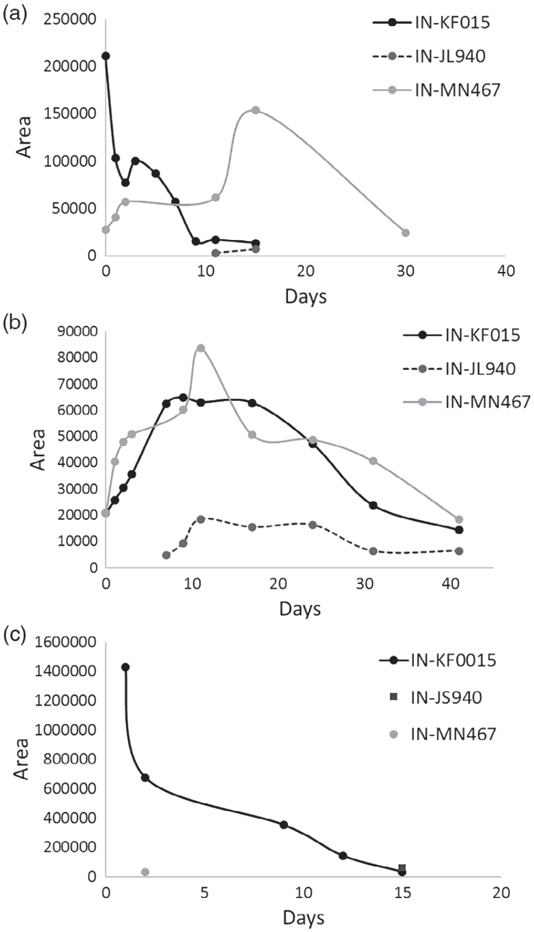


Figure 2. Metabolite areas according during monitoring period for double dose experiments for (a) cucumber, (b) tomato and (c) courgette.

The conclusion was that, at 7 days (waiting period), the concentration of the parent compound is higher than its MRL for cucurbits, and metabolites were detected in the samples at 30 days in cucumber, 40 days for tomato and 15 days in courgette. Nevertheless, no metabolites were detected at high concentrations, and their concentrations are low in relation to the parent compound, such that the inclusion of metabolites in the maximum residue limit of famoxadone was not necessary.

Unknown analysis

For the identification of unknown compounds, samples were classified according to the day after the application of the commercial product, as well as the dose and the type of matrix. Thus, six groups were processed independently: cucumber (single and double dose), courgette (single and double dose) and cherry tomato (single and double dose).

Raw files of each study were processed with MassChemSite and Compound Discoverer, aiming to identify unknown compounds related to famoxadone. Compound Discoverer processing

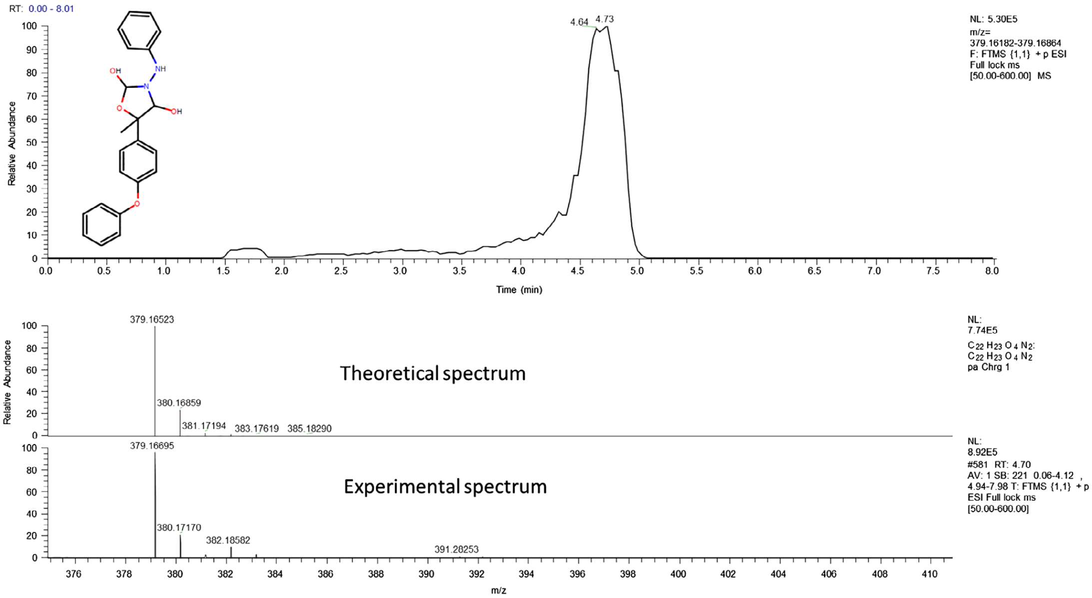


Figure 3. Total ion chromatogram, theoretical and experimental spectrum of the new metabolite.

was carried out using a workflow of 'degradants and unknown compounds', which means that, from the structure of the parent compound, possible metabolic pathways from different transformations such as dehydration, reduction, methylation, desaturation and oxidation were searched and, in addition, ChemSpider (http://www.chemspider.com) and *m/z* Cloud (https://www.mzcloud.org) databases were used to identify potential compounds. Raw data were filtered according to the intensity of the signal (higher than 1e4), retention time (between 2 and 7 min) and background was subtracted (a blank sample of tomato, cucumber and courgette were processed and used to eliminate possible false positives in the samples). When the data were filtered, a further study was developed; all possible compounds were studied to identify potential metabolites. To achieve a reliable identification of the potential compounds, consideration was made of how the signal varied over the days in the samples, as well as if the mass error was lower than 10 ppm and if the transformation can be related to the parent compound.

When this strategy was used, a new metabolite was detected in tomato and cucumber samples. Compound Discoverer, only provided the molecular formula ($C_{22}H_{22}N_2O_4$) of this new compound and did not show the final structure of the detected metabolite, and so determination of the structure is not straightforward.

Therefore, another software tool, MassChemSite, was used to confirm a reasonable structure of this new metabolite. This software provided the reaction, as well as the mass spectrum, the parent and the possible metabolite, with the matches and mismatches for the full scan spectra and the HCD mass spectra. Using the last one, the different ions were compared and possible common ions were searched, aiming to corroborate whether they originated from the same family, considering that, in most cases, the parent compound has common fragments with its metabolites. With this software tool, determination of this new metabolite was performed, and the possible structure was identified. Figure 3 shows the extracted ion chromatograms, as well as the experimental and theoretical spectrum of the new metabolite.

This new metabolite is generated by the reduction of the carbonyl groups present in the famoxadone structure to alcohol. Most likely, it was formed when famoxadone was combined with the matrix because it was not detected in the solution of commercial product with water. This new metabolite was detected with a mass error of 4.5 ppm and a retention time of 4.73 min. The structure of the new compound has been proposed using all of the MS information[31] and in accordance with the study by Schymanski *et al.*,[32] who proposed different levels to identify new compounds via HRMS.

The concentration of that metabolite was tested in relation to the concentration of the parent compound because there was no commercial standard available. The metabolite was detected 1 day after the application of the commercial product and the dissipation is similar for both doses but not for both matrices. In cucumber, the metabolite concentration increased after the application, whereas, in tomato, the concentration increased and later decreased, and this was the same for both doses. The behavior in cucumber indicates that the concentration may decrease after 30 days, as occurs happens in tomato (Fig. 4). Comparing the concentration of that metabolite with the others, it was found to be higher than those observed for the known metabolites. For these reasons, the MRL of famoxadone should include this new metabolite because the sum of the parent compound and the new metabolite would probably overcome the MRL of famoxadone for tomato and cucumber matrices.

CONCLUSIONS

The dissipation of famoxadone was studied in three vegetables using LC coupled to HRMS. Greenhouse trials have been carried

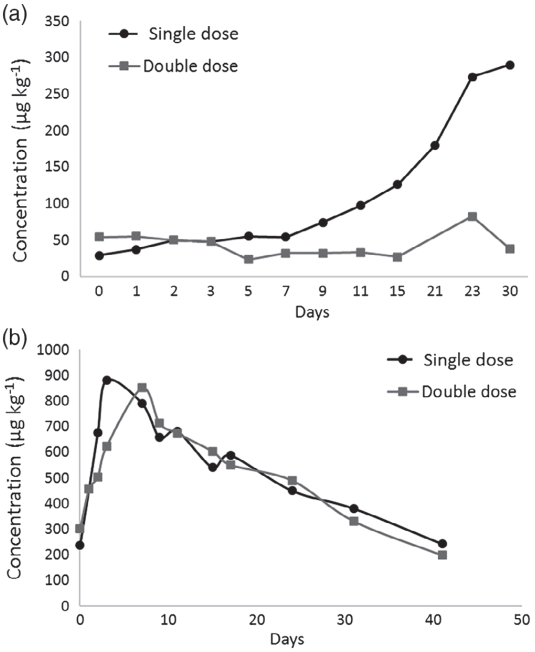


Figure 4. New metabolite concentration (µg kg^{-1}) during monitoring period for single and double dose experiments for (a) cucumber and (b) tomato.

out aiming to determine the behavior of the compound. The persistence of famoxadone was low in vegetables because DT$_{50}$ was less than 10 days. Metabolites of famoxadone were monitored during the study and two were detected 1 day after the application of the commercial product and one after 7 days. Also, a retrospective analysis was carried out and one new metabolite of famoxadone was putatively elucidated in vegetable samples. Finally, these results provide an evaluation of the dissipation of famoxadone in vegetables such as tomato, cucumber and courgette, monitoring their main transformation products and identifying one putative metabolite, which appeared at higher concentrations than the other metabolites.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Publicación X

DISSIPATION KINETIC STUDIES OF FENAMIDONE AND PROPAMOCARB IN VEGETABLES UNDER GREENHOUSE CONDITIONS USING LIQUID AND GAS CHROMATOGRAPHY COUPLED TO HIGH-RESOLUTION MASS SPECTROMETRY

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Dissipation kinetic studies of fenamidone and propamocarb in vegetables under greenhouse conditions using liquid and gas chromatography coupled to high-resolution mass spectrometry

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HIGHLIGHTS

- HRMS was used to study the degradation of fenamidone and propamocarb in vegetables.
- Degradation in tomato, cucumber and courgette under greenhouse conditions.
- Persistence of the compounds was low, with DT_{50} values lower than 35 days.
- Known and unknown metabolites were detected in the incurred samples.
- Five new metabolites were putative elucidated applying several software tools.

GRAPHICAL ABSTRACT

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ABSTRACT

In this study, fenamidone, propamocarb and their transformation products were monitored in cherry tomato, cucumber, and courgette samples. A mixture of both compounds, which have different physicochemical characteristics, are commercially available (Consento®). For analysis, ultra high-performance liquid chromatography coupled to Orbitrap mass spectrometry (UHPLC-Orbitrap-MS) and gas chromatography coupled to Q-Orbitrap mass spectrometry (GC-Q-Orbitrap-MS) were used. The dissipation of these active ingredients was monitored at two doses (normal and double dose) from 1 to 40 days after the application of the commercial product. Half-lives (DT_{50}) were lower than 30 days for both compounds, which indicates low persistence. Metabolites of both compounds were also monitored due to in some cases these can be more dangerous for human health than the parent compounds. The metabolites monitored were RPA 410193 ((5S)-3-anilino-5-methyl-5-phenylimidazolidine-2,4-dione), acetophenone, 2-phenylpropionic acid, 5-methyl-5-phenylhydantoin and 5-methylhydantoin for fenamidone, and propamocarb hydrochloride (propyl 3-(dimethylamino)propylcarbamate hydrochloride), *N*-oxide propamocarb (propyl [3-(dimethylnitroryl)propyl]carbamate), oxazoline-2-one propamocarb (3-[3-(dimethylamino)propyl]-4-hydroxy-4-methyl-1,3-oxazolidin-2-one), 2-hydroxypropamocarb and *n*-desmethyl propamocarb (propyl [3-(methylamino)propyl]carbamate) for propamocarb. In addition, they were detected one day after the application of commercial product, being RPA 410193, the metabolite detected

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at the highest concentration in samples. Retrospective analysis of incurred samples allowed putative identification of four possible new metabolites of propamocarb and one of fenamidone.

1. Introduction

Pesticides are used in order to reduce the production losses due to pest and maintain the food quality. Their use has increased worldwide because of their rapid action. However, the presence of pesticide residues in fruits and vegetables, among other foods, has become a major concern for consumer health, especially in developing countries (Fantke et al., 2012; Skovgaard et al., 2017).

Pesticides lead to the contamination of waters, soil and vegetables. In the European Union (EU) the approval of pesticides are strictly regulated (European Union, 2009), reducing the number of authorized substances due to most of them do not comply with regulatory requirements of the EU. However, their use is regulated at level of Member States and in one country a substance might be registered for use on a certain crop while in another it might not be registered for use on the same crop. The active ingredients of some pesticides are absorbed by plants and they are converted by biotransformation processes in other substances. These, known as metabolites, are produced by biochemical reactions that naturally occur in the cell metabolism, and they depend on the molecular structure of the pesticide parent compound and other factors as type of plant, crop conditions and temperature (Fenner et al., 2013). These degradation products, also named metabolites, are also detected during the dissipation of pesticides under field experimental (Corta et al., 2000; Picó et al., 2018).

Propamocarb (propyl [3-(dimethylamino)propyl]carbamate) is a systemic fungicide with protective action against phycomycetous diseases (Hu et al., 2007), whereas fenamidone ((S)-5-methyl-2-methylthio-5-phenyl-3-phenylamino-3,5-dihydroimidazol-4-one) is a systemic foliar fungicide used to control oomycete diseases such as early and late blights (Fig. S1, see supplementary data) (Mohapatra and Deepa, 2012). The mixture of both fungicides, propamocarb and fenamidone, commercially available as Consento$^{®}$, has good performance in controlling downy mildew and blight in tomato, cucumber and other crops.

On one hand, fenamidone is known to degrade into a variety of metabolites, such as RPA 410193 ((5S)-3-anilino-5-methyl-5-phenylimidazolidine-2,4-dione), acetophenone, 2-phenylpropionic acid, 5-methyl-5-phenylhydantoin and 5-methylhydantoin (Fig. S1) (EFSA (European Food and Safety Authority), 2016). Similarly, propamocarb degrades into propamocarb hydrochloride (propyl 3-(dimethylamino)propylcarbamate hydrochloride), *n*-oxide propamocarb (propyl [3-(dimethylnitroryl)propyl]carbamate), oxazoline-2-one propamocarb (3-[3-(dimethylamino)propyl]-4-hydroxy-4-methyl-1,3-oxazolidin-2-one), 2-hydroxypropamocarb and *n*-desmethyl propamocarb (propyl [3-(methylamino)propyl]carbamate) (Brancato et al., 2017). Table S1 shows physico-chemical properties of fenamidone, propamocarb and metabolites, including toxicity values and metabolite fractions for each one, which has been performed in soil studies (EFSA (European Food and Safety Authority), 2016, 2006; University of Hertfordshire, 2007). Up to our knowledge, there are not toxicity values for metabolites, so further studies evaluating the presence of these compounds in food is needed.

EU has set the maximum residue limit (MRL) of propamocarb in tomatoes at 4 mg/kg, and for cucurbits (cucumber and courgettes) has been set at 5 mg/kg (Commission, 2016). These MRLs include the sum of propamocarb and propamocarb salts but the metabolites described above have not been included. On the other hand, acceptable daily intake (ADI) was 0.29 mg of propamocarb/kg bw/day. The MRLs of fenamidone do not include its metabolites and they are 1 and 0.2 mg/kg in tomato and cucurbits, respectively. Regarding ADI value, it was set at 0.03 mg/kg bw/day for fenamidone, whereas for two of its metabolites, RPA-410193 and 5-methylhydantoin, they were set at 0.0094 mg/kg bw/day and 0.0064 mg/kg bw/day respectively, being the last one the main metabolite of fenamidone (fraction of 0.35, Table S1). In addition, the EU recently refused the approval of fenamidone as active substance (European Commision, 2018), due to toxicity studies are not provided as well as metabolites risk assessment was necessary to satisfy the requirements set by Regulation (EC) No 1107/2009 (European Union, 2009).

In this field, metabolites could be included in analytical methods, especially by retrospective methods, because they can be more toxic than their parent compounds. In consequence, it is important to include possible metabolites in pesticide residue analyses, as well as to assess if fenamidone or propamocarb have been illegally applied to food crops. For example, organic farming practices do not permit xenobiotic pesticide usage, but if only the metabolites are present, analytical methods that only monitor the active ingredients could not catch potential illegal pesticide usage in organic farming.

Despite there are multiresidue methods that include both compounds (fenamidone and propamocarb) (Angioni et al., 2012; de Melo Abreu et al., 2006; Dedola et al., 2014; González-Rodríguez et al., 2009), there are scarce bibliography monitoring the dissipation of these compounds. Two recent studies (Chen et al., 2017; Manikrao and Mohapatra, 2016) described their dissipation in soils and vegetables but metabolites were not monitored. So current analytical methods should be optimized or re-evaluated for the determination of these metabolites.

In addition to known metabolites, new metabolites can be detected in the incurred samples and, for that purpose, high-resolution mass spectrometry (HRMS) has become an essential tool for the identification of known and unknown compounds in food and environmental analysis (Coscollà et al., 2014; Gómez-Pérez et al., 2015; Munaretto et al., 2016). The first step in the study of the spectrum of an unknown compound is the comparison of the experimental spectrum with those obtained from reference standards collected in databases and spectral libraries. According to this, automated metabolite identification by using prediction tools may represent an important advance to detect new unknown chemicals, especially when the study is focused on the identification of unknown pesticide metabolites (Jaén-Gil et al., 2018). In that case, software as Compound Discoverer$^{®}$ and MassChemSite$^{®}$ (Brink et al., 2014) are powerful tools that can be used in this field.

The aim of this study is the development of new analytical methods to study the behaviour of fenamidone and propamocarb in different types of vegetables and their dissipation into their metabolites under greenhouse conditions at two different application doses. In addition, the information could be used to evaluate the exposure and risk of human health of fenamidone and propamocarb. For that purpose, analytical methods have been developed and validated for the quantitative determination of fenamidone, propamocarb and their metabolites, applying two powerful tools as GC-Q-Orbitrap-MS and UHPLC-Orbitrap-MS, respectively, due to the different physico-chemical properties of the compounds, applying targeted and unknown analysis.

2. Materials and methods

2.1. Materials and reagents

Fenamidone (CAS registry No. 161326-34-7, purity >99%), acetophenone (CAS registry No. 98-89-2, purity >99%), 2-phenylpropionic acid (CAS registry No. 492-37-5, purity >99%), 5-methyl-5-phenylhydantoin (CAS registry No. 6843-49-8, purity >99%), 5-methylhydantoin (CAS registry No. 616-03-5, purity >99%) and propamocarb (CAS registry No. 24579-73-5, purity >99%), were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPA 410193 (CAS registry No. 332855-88-6, purity >99%) was supplied by Santa Cruz Biotechnology (Heidelberg, Germany). Consento® was provided by Bayer Crop Science (Valencia, Spain).

Stock solutions of each compound (1000 mg/L) were prepared by dissolving 10 mg of pure compound in methanol (MeOH) (10 mL). Intermediate solutions of the seven compounds (10 mg/L) were prepared by taking 100 μL of each stock solution and diluting up to 10 mL with MeOH (in case of acetophenone and propamocarb) or in ethyl acetate (EtOAc) (the rest of analytes) in a volumetric flask. Both, stock and intermediate solutions were stored at −21 °C. Stock solutions were stable for a year and intermediate solution for 2 months.

Acetonitrile (ACN) and MeOH (both LC-MS grade) were acquired from Fluka (St. Louis, MO, USA). Water (LC-MS grade) was acquired from J.T. Baker (Deventer, Holland) and formic acid and EtOAc (both LC-MS grade) were obtained from Fisher Scientific (Erembodegem, Belgium). Magnesium sulfate (Sigma-Aldrich) and sodium chloride (J.T. Baker) were used during the extraction procedure.

A mixture of acetic acid, caffeine, Met–Arg–Phe–Ala–acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrat and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative), from Thermo-Fisher (Waltham, MA, USA), were used to accurate mass calibration of the Orbitrap analyzer.

2.2. Liquid chromatography-mass spectrometry

A Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) was used for chromatographic analysis of propamocarb and acetophenone. A Zorbax Eclipse Plus C_{18} column (100 mm × 2.1 mm, 1.8 μm particle size) supplied by Agilent Technologies (Santa Clara, CA, USA) was used for the chromatographic separation of the compounds. A flow rate of 0.2 mL/min was set. The mobile phase consisted of eluent A, which was an aqueous solution of 0.1% formic acid and eluent B, which was MeOH.

The step gradient was as follows: 0–1 min 95% of A; then it was linearly decreased to 75% in 2 min and remained constant during 1 min. Later it was decreased to 50% in 2 min, to 0% in 4 min and remained constant during 4 min. After returned to the initial conditions in 0.5 min, the composition was kept constant 1 min. The total running time was 16 min. The column temperature was set at 25 °C and the injection volume was 10 μL.

The LC chromatographic system is coupled to a single mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen, Germany) using an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA) in positive and negative ion mode. ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N2, >95%), 35 (adimensional); auxiliary gas (N2, >95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 °C; capillary temperature, 300 °C. The mass spectra were acquired employing two alternating acquisition functions: (1) full MS, ESI+, without fragmentation (higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 FWHM; scan time = 0.25 s; (2) all-ions fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s, (3) full MS, ESI- using the aforementioned settings, and (4) AIF, ESI- using the settings explained for (2). Mass range in the full scan experiments was set at *m/z* 50–400.

2.3. Gas chromatography-mass spectrometry

A GC system Thermo Fisher Scientific Trace 1310 with an autosampler Triplus RSH (Thermo Scientific™, Thermo Fisher Scientific, San Jose, CA, USA) was used for the analysis of fenamidone, RPA 410193, 2-phenylpropionic acid, 5-methyl-5-phenylhydantoin and 5-methylhydantoin. A Varian VF-5ms (30 m × 0.25 mm, 0.25 μm film thickness) supplied by Agilent Technologies (Santa Clara, CA, USA) with a precolumn (1.5 m × 0.25 mm) supplied by Supelco (Bellefonte, PA, USA) were used. Helium (99.9999%) was used as carrier gas at a constant flow rate of 1 mL/min. The GC system was operated at an injector temperature of 220 °C.

When the instrument was in standby mode, the injector split ratio was set at 20:1. When the syringe was placed into the injector, splitless mode was switched on for 2 min, and after that, the split valve was open again with a flow rate of 50 mL/min to clean the glass liner and avoid carry-over effects. It was finally reduced to 20 mL/min at 2 min. Septum purge was 5 mL/min during the analysis. Injection volume was 2 μL. Column temperature was initially set at 40 °C, and it was held for 1 min. Then it was increased at 20 °C/min to 300 °C, which was held for 10 min. The total running time was 24 min.

The chromatographic system was coupled to a mass spectrometer Q-Exactive Orbitrap Thermo Fisher Scientific (Q-Exactive™) operating in the electron ionization mode (EI, −70 eV). The Q-Exactive was operated in full scan mode between 40 and 350 *m/z*. The temperatures of the transfer line and ionization source were set at 250 °C. The analysis was performed with a filament delay of 5 min to prevent instrument damage.

Both LC and GC chromatograms were acquired using the external calibration mode and they were processed using Xcalibur™ version 3.0, with Quanbrowser and Qualbrowser, and Trace Finder 4.0 (Thermo Fisher Scientific, Les Ulis, France).

Unknown analysis was carried out with Compound Discoverer™ version 2.1 (Thermo Fisher Scientific) and MassChemSite 2.0 (Molecular Discovery Ltd, London, UK).

2.4. Sample extraction

The extraction procedure was the well-known QuEChERS method (Anastassiades et al., 2003). Briefly, 1 kg of sample fruits was crushed and homogenised as established by current regulations (SANTE/EU, 2017), storing it in the freezer at −21 °C. After that, 10 g of homogenised sample was placed in a 50 mL plastic centrifuge tube and 10 mL of ACN were added and shaken for 10 min in a rotatory shaker. After that, 1 g of NaCl and 4 g of $MgSO_4$ were added, and the mixture was shaken vigorously for 1 min in a vortex. Finally, the sample was centrifuged for 10 min at 5000 rpm (4480 rcf).

For UHPLC-Orbitrap analysis, 1 mL of supernatant was collected and injected into the system. For GC-Q-Orbitrap analysis, 1 mL of supernatant was dried under nitrogen current and redisolved in 500 μL of EtOAc prior injection.

2.5. Greenhouse trials

Parameters of greenhouse trials were summarized in Table 1. Cherry tomatoes, courgettes and cucumber were collected in winter and spring (from February to May), 2018, from hydroponic crops of a greenhouse located in Almería, Spain (Table 1). Maximum and minimum temperatures ranged from 15 °C to 40 °C. The greenhouse was a hydroponic crop, using two bags of three plants in each one for each dose. The plant protection product of fenamidone (75 g/L) and propamocarb (375 g/L) was dissolved in water and sprayed by using a manual sprayer (1.5 L volume, max. pressure 1.0 MPa) equipped with one conical nozzle operated at 40 psi (275 kPa). To investigate the dissipation of these pesticides in cherry tomato, cucumber and courgette plants, 6 mL of commercial product (Consento®) were dissolved in 1.5 L of water and sprayed on the surface of the plant three times with intervals of 10 days (which is the normal dose indicated by the manufacturer) at 2 L/ha. In the case of double dose, 12 mL of commercial product was dissolved in 1.5 L of water and the mixture was also applied three times on the surface of the plants (Table 1). One kg of vegetable samples was randomly collected at 0, 1, 2, 3, 5, 7, 9, 11, 15, 21, 23, 30 and 40 days after application.

The dissipation kinetics of fenamidone and propamocarb pesticides in vegetables were determined by plotting residue concentration versus time (Fantke and Juraske, 2013). The residual concentration and half-life of fenamidone and propamocarb was determined using the "Biphasic model" (Morrica et al., 2002; Ntow et al., 2007), and characteristic parameters as pesticide concentration (C_0, C_1), rate constant (k_1 and k_2) and half-lives (DT_{50}) have been estimated, applying Eq. (1), where C_t is the concentration at time t.:

$$C(t) = C_0 e^{-k_1 t} + C_1 e^{-k_2 t} \tag{1}$$

2.6. Strategy for unknown analysis

Unknown analysis was carried out using software tools as MassChemSite® and Compound Discoverer®. For the identification of unknown compounds, samples were classified according to the compound, the day after application of commercial product, the dose and the type of matrix. Thus, twelve groups were processed independently: cucumber (normal and double dose), courgette (normal and double dose) and cherry tomato (normal and double dose) for fenamidone and propamocarb.

Raw files of each study were processed in order to look for unknown compounds related to fenamidone and propamocarb. Compound Discoverer® processing was carried out by a workflow of "degradants and unknown compounds" that means that from the structure of the parent compound, possible metabolic pathways from different transformations like, dehydration, reduction, methylation, S-dealkylation, sulfonation, desaturation and oxidation were searched. In addition, Chemspider and m/zCloud databases were used to identify potential compounds. When the processing has been carried out, results were filtered according to intensity of the signal (higher than 1e4), retention time (between 2 and 14 min) and subtracting background (a blank sample of cherry tomato, cucumber and courgette were processed and used to eliminate possible false positives in the samples). When the data were filtered, a further study was developed, and all potential compounds were studied in order to identify potential metabolites. To achieve a reliable elucidation of potential compounds, it was considered how the signal varied over the days in the samples, if the mass error was lower than 10 ppm and if the transformation can be related to the parent compound.

2.7. Method validation

In order to perform reliable quantitative results, the UHPLC-MS-Orbitrap and GC-Q-Orbitrap-MS analytical methods were validated according to SANTE guidelines (SANTE/EU, 2017), calculating matrix effect, linearity, trueness (% recovery), precision (intra and inter-day), and limits of quantification (LOQs). More details are indicated in Electronic supplementary data Section 1 and 2.

3. Results and discussion

In this study seven compounds with different physico-chemical characteristics were analysed. For this reason LC and GC were used. Thus, acetophenone and propamocarb were analysed by LC-HRMS and fenamidone, RPA 410193, 2-phenylpropionic acid, 5-methyl-5-phenylhydantoin and 5-methylhydantoin were determined by GC-HRMS.

Table 1
Parameters of greenhouse trials in line with existing reporting recommendations[a].

Information	Fenamidone	Propamocarb	Acetophenone	RPA 410193	2-phenylpropionic acid	5-methyl-5-phenylhydantoin	5-methylhydantoin
CAS-RN	161326-34-7	24579-73-5	98-89-2	332855-88-6	492-37-5	6843-49-8	616-03-5
Study location	Vícar, Almería, Spain						
Study characteristics	Hydroponic crop under greenhouse conditions						
Application dose (kg ha^{-1})	0.4 (single), 0.8 (double)						
Application dates	27th February 2018 and 7th and 17th of March 2018						
Treated plant component	Leafs and stems						
Formulation (%)	7.5% of fenamidone and 37.5% of propamocarb						
Air temp (greenhouse indoor) (°C)	16.5						
Binomial plant name	*Solanum lycopersicum* L.(tomato) *Cucumis sativus* L. (cucumber) *Cucurbita pepo* L. (courgette)						
Plant stage at pesticide application	Bloom						
BBCH scale[b]	60						
Sample plant component	Fruit with peel						
Sample mass (kg)	1						
Kinetic models used	Zero Order, First Order, Second Order, Biphasic						

[a] Fantke P, Arnot JA, and Doucette WJ. Improving plant bioaccumulation science through consistent reporting of experimental data. *J Environ Manage* 181:374–384 (2016).

[b] The BBCH-scale (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie) is used to identify the phenological development stages of plants.

3.1. Optimization of the analytical methods

Regarding UHPLC-MS-Orbitrap method, the spectrometric characterization of propamocarb and acetophenone pesticides was carried out by injecting an intermediate solution of each compound into the system. Two characteristic fragments were monitored for propamocarb, one of them, *m/z* 102, is obtained due to the fact that the cleavage of carbon-amide nitrogen linkage, losing an amine group (-$C_5H_{12}N$), whereas the other fragment, *m/z* 74, corresponds to the loss of an ethyl group (-C_2H_5) from the previous fragment (Table 2). The acetophenone fragments were obtained by the loss of methyl group (−CH_3, *m/z* 82) and methoxy group (−CH_3CO, *m/z* 77) (Table 2).

Regarding the chromatographic conditions, the best chromatogram was obtained using MeOH as the organic solvent, bearing in mind that when ACN was used, a tailing peak was observed for propamocarb (Fig. S2). Fig. S3a shows the chromatograms of the target compounds in the solvent, using the optimized elution gradient.

For the GC-MS-Q-Orbitrap analysis, four steps were investigated during the optimization of the analytical method. Firstly, an intermediate solution of the GC-amenable compounds (100 μg/L) was injected into the system for the spectrometric characterization. The most intense and selective peak was used as quantifier peak, and the second and the third ions were used as qualifiers (Table 2). However, only one qualifier ion was obtained for 5-methylhydantoin, because low sensitivity was achieved for the other fragments. Nevertheless, it must be emphasized that 5-methylhydantoin fulfills the SANTE guidelines criteria regarding identification criteria (SANTE/EU, 2017). The spectral library NIST 2.0 was used as model to find out the ions for each analyte. The exact masses of each peak were selected with the criterion that mass errors were lower than 5 ppm for the target compounds.

The second step was the optimization of chromatographic parameters. The injector temperature was tested from 180 to 250 °C. The best results were obtained when 220 °C was used, due to the fact that several analytes, as 5-methylhydantoin, were not detected at higher temperatures, whereas at 180 °C, broad peaks were obtained for most of compounds. The splitless time was also evaluated, testing 0.5, 1.0, 2.0 and 3.0 min. The best results were obtained when splitless time was set at 2 min, and sensitivity and peak shape were suitable for all analytes.

Thirdly, oven temperature was evaluated. The initial temperature was set at 40 °C or 70 °C. The results provided for both temperatures were similar, but sensitivity was lower at 70 °C (5-methylhydantoin), so 40 °C was selected as starting temperature. The maximum temperature of the oven was tested, setting maximum temperature at 260 and 300 °C. The best results were obtained at 300 °C, bearing in mind that at 260 °C the retention time was higher and the sensitivity of fenamidone and RPA 410193 was lower than that obtained at 300 °C. Fig. S3b shows a chromatogram of analytes studied spiked at 100 μg/L.

Finally, the optimization of the extraction method has been evaluated, and for that purpose, different modifications of QuEChERS method, European, AOAC and unbuffered QuEChERS, were tested, observing that the clean-up step, based on dispersive solid phase extraction, was not necessary (Lehotay et al., 2010). The best results were obtained when the unbuffered QuEChERS method was used, obtaining recoveries that ranged from 70 to 96% (Table S2). For the determination of GC-amenable compounds (fenamidone, RPA 410193, 2-phenylpropionic acid, 5-methyl-5-phenylhydantoin and 5-methylhydantoin), an extra step was necessary in order to avoid the direct injection of ACN extract into the GC, and 1 mL of supernatant was dried under nitrogen current and recomposed in 500 μL of EtOAc before GC analysis.

The results obtained during the validation of these methodologies are included in Section 2 of Supplementary data as well as in Table S3.

3.2. Greenhouse trials

Results of the overall dissipation studies in vegetables were fitted to the “Biphasic” kinetic model for propamocarb and fenamidone (Table 3). Other models (Fantke and Juraske, 2013) as Zero Order, Single First Order and Double First Order were tested, but the best fitting was obtained for the Biphasic kinetic model (Table S4 and S5).

3.2.1. Propamocarb trials: target analysis

Approximately 1 kg of sample was collected and crushed at the time intervals, described in Section 2.5, after the application of the commercial product. The overall dissipation results, applying the Biphasic kinetic model at normal and double dose per each matrix, are shown in Figs. S3a and b.

Concentration of propamocarb during cucumber trials increased for the first 5 days after application, and then decreased till 30 days. The concentration, at normal dose, increased up to 2600 μg/kg (5 days) and decreased to 20 μg/kg (30 days). This fact can be explained because propamocarb is a systemic fungicide and after the application, the pesticide is firstly transferred to the fruit, and then decreased exponentially. This behaviour is similar to the famoxadone fungicide (Feng et al., 2018). k_1 *and* k_2 values show that the dissipation process (k_1) is slightly lower at the beginning (0.15 days^{-1} for double dose) than later, where $k_2 = 0.20$ days^{-1}. At normal dose, k_1 is 0.13 days^{-1} while k_2 is 0.19 days^{-1} (Table 3). This indicates that dissipation was independent on the dose of

Table 2
UHPLC-Orbitrap-MS and GC-Q-Orbitrap-MS parameters for amenable compounds.

Pesticide	Quantifier ion		Qualifier ions			Retention Time (min)
	Exact mass	Mass error (ppm)	Exact mass	Molecular formula	Mass error (ppm)	
Propamocarb (LC)	189.1597	1.4	102.0549	$C_4H_8O_2N$	−4.2	5.9
			74.0237	$C_2H_4O_2N$	5.3	
Acetophenone (LC)	121.0648	2.2	77.0386	C_6H_5	4.7	10.5
			82.0413	C_5H_5O	4.9	
Fenamidone (GC)	238.1101	0.5	268.0903	$C_{15}H_{14}N_3S$	0.3	14.2
			237.1022	$C_{15}H_{13}ON_2$	−0.8	
RPA 410193 (GC)	120.0808	0.7	237.1022	$C_{15}H_{13}ON_2$	−0.4	14.6
			281.1159	$C_{16}H_{15}O_2N_3$	0.1	
2-phenylpropionic acid (GC)	150.0675	−0.07	105.0704	C_8H_9	−0.3	7.7
			77.0391	C_6H_5	−0.5	
5-methylhydantoin (GC)	114.0429	0.3	105.0502	$C_4H_7O_2N_2$	0.9	8.6
5-methyl-5-phenylhydantoin (GC)	175.0502	−0.7	119.0730	C_8H_9N	0.7	11.3

Table 3
Biphasic kinetic model parameters and vegetables dissipation (DT_{50}) of fenamidone and propamocarb.[a]

Matrix	Tomatoes		Cucumber		Courgettes	
Parameters	Normal dose	Double dose	Normal dose	Double dose	Normal dose	Double dose
Fenamidone						
C_0 (μg/kg)	1521.8	2564.5	3011.9	8600.30	1330.9	2780.39
C_1 (μg/kg)	−0.49	−0.20	−19.90	−19.20	−0.98	−0.50
k_1 (days^{-1})	0.03	0.05	0.14	0.21	0.14	0.18
k_2 (days^{-1})	0.79	22.90	0.17	0.25	14.49	1.69
DT_{50} (k_1) (days)	20.55	15.02	4.80	3.27	4.87	3.89
DT_{50} (k_2) (days)	0.87	0.03	4.10	2.78	0.05	0.41
Propamocarb						
C_0 (μg/kg)	1040.46	1902.99	1418.10	2885.83	2850.00	6481.01
C_1 (μg/kg)	−0.99	−0.49	−9.25	−8.45	−0.61	−0.06
k_1 (days^{-1})	0.08	0.02	0.13	0.15	0.10	0.16
k_2 (days^{-1})	2.00	22.99	0.19	0.20	22.33	10.75
DT_{50} (k_1) (days)	8.55	29.56	5.28	4.51	7.15	4.20
DT_{50} (k_2) (days)	0.34	0.03	3.70	3.44	0.05	0.06

[a] Abbreviations: C_0, C_1: pesticide concentration; DT_{50}: half-lives; k_1 *and* k_2, rate constant.

commercial product applied in cucumber, with similar k values.

In courgette trials, propamocarb concentrations increased for the first 2 days after application of commercial product, and later quickly decreased till 15 days when normal dose was used (Fig. S4a) as in cucumber trials. In the case of double dose, concentration decreased throughout the study (Fig. S4b). When Biphasic kinetic model was used (Table 3) k_1 and k_2 values demonstrated different behaviour as in cucumber, showing that the dissipation rate was slowly at the beginning, and quickly at the final. DT_{50} (k_1) for normal dose was 7.4 days whereas DT_{50} (k_2) was 0.05 days, similar to DT_{50} for double dose (4.2 and 0.06 days respectively).

Concentrations of propamocarb in cherry tomato trials increased for the first 5 days after application of commercial product for both doses, and later decreased in a different way for the two doses evaluated. For the normal dose, the concentration decreased by 85% from days 5–41, while it was 60% for the double dose (Fig. S4a and b1). Biphasic kinetic model was applied and the results indicated that at normal dose k_2 value was really small compared to double dose (2 days^{-1} and 23 days^{-1} respectively). So, the parent compound degrades faster at normal dose than at double dose. On the other hand, at normal dose DT_{50} (k_1) was three times smaller than at double dose (8.6 days and 29.6 days). Therefore, the dissipation rate in cherry tomato trials was quicker for normal dose. Bearing in mind these results, it can be said that propamocarb *k value* was different in each type of matrix (cherry tomatoes, cucumber and courgettes). Propamocarb degrades slower in tomato and cucumber than in courgette. This can be explained because courgettes were affected by a plague of white fly and the low temperatures during February and March.

In conclusion, propamocarb dissipation follows the same pattern in all matrices except in courgettes normal dose, where concentration decreased all period monitored. The general behaviour was increased the concentration at the beginning and then, decreased, as it was observed in other studies as famoxadone and oxathiapiprolin in tomatoes (Feng et al., 2018), dicamba, carbaryl and cyromazine in wheat (Fantke et al., 2013) and florasulam, carfentrazone-ethyl, fluroxypyr-meptyl and fluroxypyr in wheat (Pang et al., 2016). For cucumber and courgette, concentration of propamocarb did not overcome its MRL (5 mg/kg), as well as for cherry tomato (4 mg/kg) after its waiting period. A study performed by Al-Rahman et al. (2012) determined dissipation kinetics of propamocarb in tomatoes under open field conditions using Single Fist Order model, obtaining half-lives of 1.29 days. In our study Biphasic model was applied, and half-lives of 8 days for the first step and 0.34 days for the second step were achieved. Comparing degradation curves, it can be observed that in the case of the Al-Rahman study, the concentration of propamocarb decreased during the whole study meanwhile in our case, firstly increased and later decreased. Comparing the half-lives obtained in this study, (0.34 days) with the value provided by Al-Rahman (1.29 days), it can be concluded that propamocarb degrades faster in greenhouse than in open field conditions.

Metabolites were also monitored during the study. Due to there not being commercially available reference standards for propamocarb metabolites, their detection and quantification were carried out using parent compound as standard. Metabolites of propamocarb (oxazoline-2-one propamocarb and N-desmethyl propamocarb) were detected in all matrices after the first day of application of Consento® at concentrations lower than 50 μg/kg. Metabolites' behaviour was the same for the different dosages and matrices evaluated, and their amount increased. For example in courgette samples, oxazoline-2-one propamocarb concentration increased to 45 μg/kg (from 1 day to 4 days). Later their concentrations decreased as it can be observed in Fig. 1 (normal dose) and Fig. S5 (double dose). Other metabolites of propamocarb described in the EFSA risk assessment report (Brancato et al., 2017) were monitored in the samples but were not detected. It can be observed in Fig. 1 that the highest molar mass ratio of metabolites in relation to the initial concentration of the parent compound was obtained in tomato, obtaining conversion factors higher than 50%, whereas in the other two matrices, this was lower than 10%.

3.2.2. *Fenamidone trials: targeted analysis*

The overall dissipation results applying the Biphasic model are shown in Figs. S6a and b. Fenamidone behaviour, was the same for all matrices, and its concentration increased after application of Consento® and later decreased as occurred for propamocarb trials and other pesticides (Fantke et al., 2013; Feng et al., 2018; Pang et al., 2016). For example, in cucumber trials, concentration of fenamidone increased up to 5500 μg/kg for normal dose for the first 7 days after application and later decreased to 10 μg/kg (30 days after application). In courgette trials the concentration increased for the first 2 days after application of commercial product (2600 μg/kg for normal dose) and later decreased quickly (15 days after application) as it can be observed in Fig. 2. Finally in cherry tomato trials, the concentration increased for the first 3 days after application of commercial product for both doses (for example for normal dose 2100 μg/kg), and later decreased quickly during the monitoring period. In relation to Biphasic results for cucumber, DT_{50} (k_1 and k_2) were 4.8 and 4.1 days for normal dose and 3.3 and 2.8 days for double dose (Table 3).

In cherry tomato, DT_{50} (k_1 and k_2) at normal dose were 20.6 and

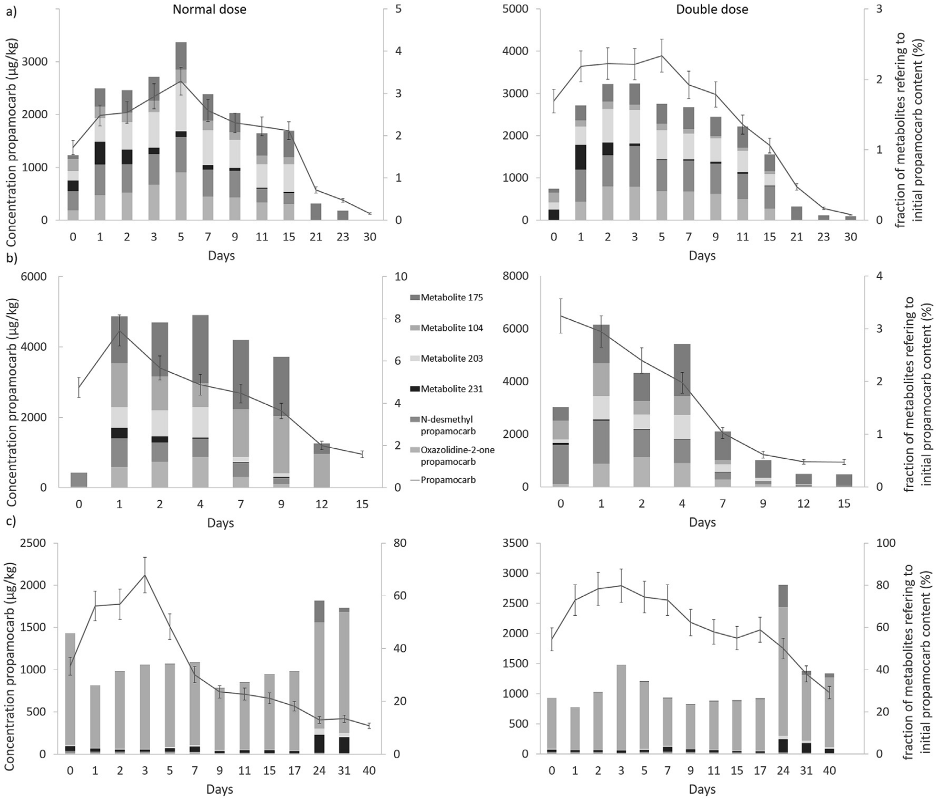

Fig. 1. Dissipation of propamocarb (µg/kg) and metabolites fraction during monitoring period for normal dose experiments for a) cucumber, b) courgette and c) tomato.

0.9 days respectively, and k_1 *value* was 0.03 days^{-1} and k_2 0.79 days^{-1}. In comparison with the other matrices, in courgette trials, fenamidone degraded quicker as occurred in the case of propamocarb. In addition fenamidone persistence was low, being DT_{50} lower than 30 days as propamocarb (Dores et al., 2016; EFSA (European Food and Safety Authority), 2016, 2006).

For all matrices, concentration of fenamidone overcome its MRL 0.2 mg/kg in the case of cucurbits and for cherry tomato (1 mg/kg) after its waiting period (3 days). Therefore it was necessary to review the MRLs of fenamidone in order to ensure food safety.

Comparing results obtained with the previous data provided by bibliography, it can be highlighted that in the case of cucumber, fenamidone half-live was 4 days for both doses, the kinetic model was not indicated (Mohapatra and Deepa, 2012). Our results, provided a half-life between 2.8 and 4.8 days for both doses, so these results are really similar to that obtained by Mohapatra et al. (Mohapatra and Deepa, 2012). On the other hand, Angioni et al. (2012) determined fenamidone residues in two types of tomatoes, detecting the parent compound 24 days after application of the compound, whereas in our case, residues were detected 40 days after application.

On the other hand, metabolites as acetophenone and RPA 410193 were detected in all matrices at concentrations ranging from 10 to 150 µg/kg in the case of normal dose. Moreover, 5-methyl-5-phenylhydantoin was detected in cucumber samples at double dose but at concentrations lower than 20 µg/kg. Thus, it can be indicated that 5-methyl-5-phenylhydantoin can be detected only when the parent compound was applied at higher concentrations. In relation to the behaviour of metabolites, the concentration of RPA 410193 slightly decreased at the beginning (1 day) and then it increased up (4 days) and finally decreased for both doses. In the case of cucumber trials at two doses, its concentration decreased at 12 days meanwhile in courgette it decreased at 4 days and in cherry tomato at 24 days. Acetophenone behaviour was similar in cherry tomato and cucumber, and this was similar to RPA 410193. However, in courgette at normal dose, its concentration increased up (from 15 µg/kg to 60 µg/kg) and later decreased to 12 µg/kg 5-methyl-5-phenylhydantoin concentration increased to 20 µg/kg (3 days) and decreased to values below LOQ (15 days) at double dose. Fig. 3 and S5 show the dissipation of fenamidone metabolites detected in all matrices. For this compound, the highest molar mass ratio of metabolites in relation to the initial concentration of the parent compound was obtained in courgette (Fig. 2), achieving conversion factors higher than 15%, although they are lower than those obtained for propamocarb.

3.2.3. *Unknown analysis*

Strategy defined in Section 2.6 was used and five new metabolites were detected by LC-HRMS in the three matrices evaluated, cucumber, cherry tomato and courgettes. However, Compound Discoverer® only provided the molecular formula, and it did not show the final structure of the detected metabolites, so the

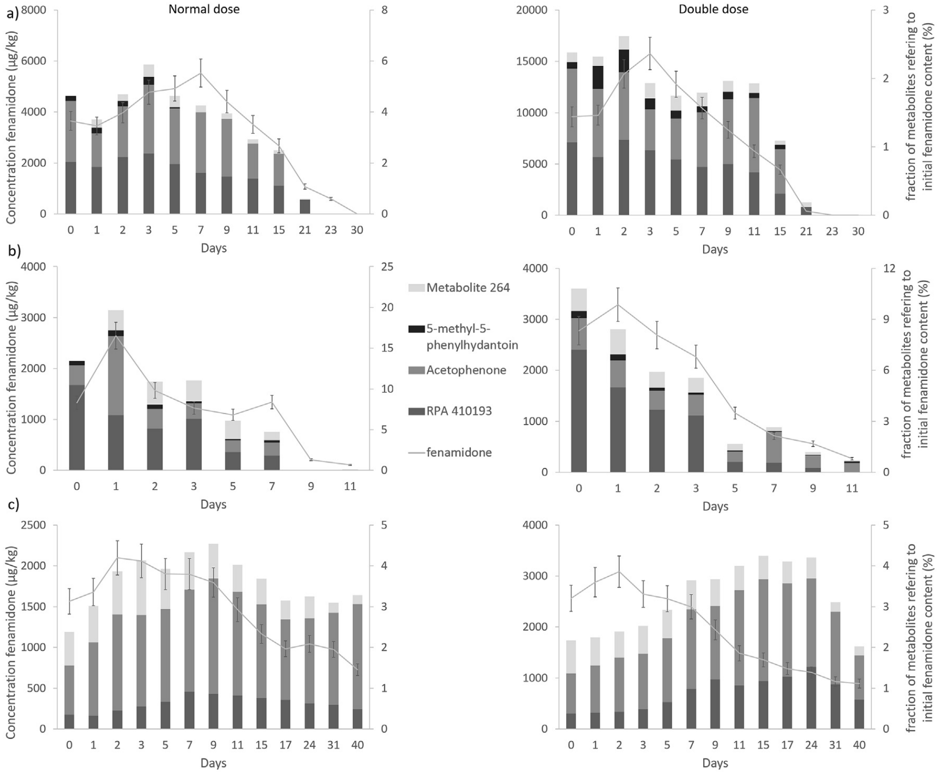

Fig. 2. Dissipation of fenamidone (µg/kg) and metabolites fraction during monitoring period for normal dose experiments for a) cucumber, b) courgette and c) tomato.

elucidation of the structure is not a straightforward step.

Therefore, another software tool, MassChemSite®, was used in order to confirm a reasonable structure of these new metabolites. This software provided the reaction as well as the mass spectrum of parent and the possible metabolite with the matches and mismatches for the full scan spectra and the HCD mass spectra. This software compared the different ions and searched for possible common ions, in order to corroborate if the ions came from the same family, due to the fact that in most of cases the parent compound has common fragments with its metabolites. Using this software tool, elucidated metabolites were confirmed and their possible structures were identified. Fig. 3(a–d) shows four putative metabolites of propamocarb, and Fig. 3e a new metabolite of fenamidone. Fig. 4, S7, S8, S9 and S10 show extracted ion chromatograms, experimental and theoretical spectrum of the new metabolites. According to Schymanski et al. (2014) different levels related to the identification of new compounds using HRMS can be set, and in this study, metabolites at levels 2 and 3 were detected. Thus, level 2 was related to the use of the information provided by fragments for the elucidation of exact mass, obtaining this information from MassChemSite®. This level corresponds with all metabolites elucidated except metabolite 203 *m/z*. Level 3 was related to the tentative structure candidates (for example isomers) for one exact mass. This is the case of metabolite 203 *m/z*. For this compound the software provided two structures corresponding to two possible isomers, but the elucidation of the final structure detected in the samples was not possible.

In the case of propamocarb, metabolite 104 *m/z* is originated from the successive cleavage of a carbon-carbon linkage and later the hydrogenation of nitrogen. It was detected with a mass error of 4.67 ppm and retention time of 1.40 min ($-C_5H_{11}N$). Metabolite 175 *m/z* was detected at 1.67 min (mass error −0.52 ppm) and it comes from the cleavage of a carbon-nitrogen linkage followed by the hydrogenation of nitrogen ($-CH_3$). For metabolite 203 *m/z* two possible structures were elucidated. They involved the aliphatic N-hydroxilation and the dehydrogenation of two carbons to form a cis or trans double bond. It was detected with a mass error of −0.62 ppm and retention time of 6.94 min. Metabolite 231 *m/z* comes from the dehydrogenation of nitrogen and formation of an amide group.

Metabolite 264 *m/z* was formed from the cleavage of carbon-sulfur group ($-CH_3S$). It was detected at 11.37 min with a mass error of −0.48 ppm.

The concentrations of these putative metabolites were evaluated using the same procedure as that used for propamocarb metabolites for which commercial standards are not available, and their detection and quantification were carried out using the parent compound as standard, plotting the molar ratio versus time (Figs. 2 and 3). In relation to propamocarb, metabolite 175 *m/z* was detected in the commercial product and in the sample collected 1 day after application. For the courgette samples, the concentration of metabolite increased from 60 to 90 µg/kg at 7 days and later decreased to 10 µg/kg at 15 days. For metabolite 231 *m/z*, it was detected 1 day after the application of commercial product and its

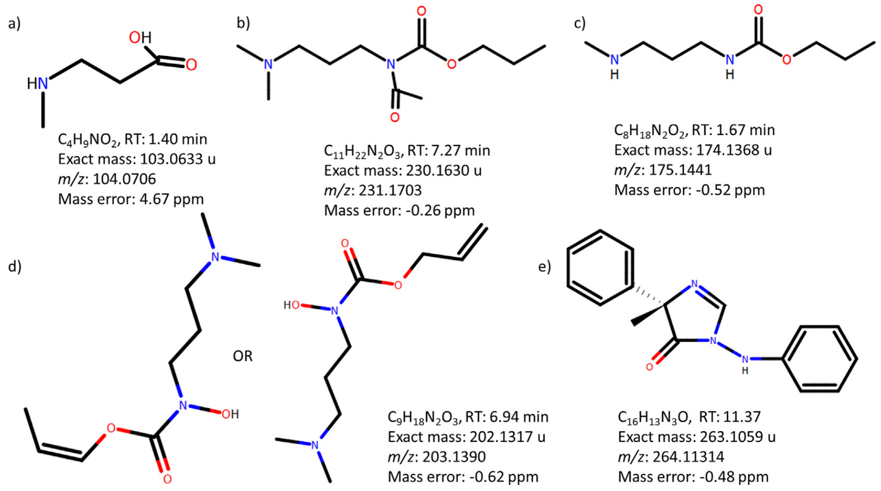

Fig. 3. Molecular formula, exact mass and retention time for the new metabolites of propamocarb, a) metabolite 104 *m/z* b) metabolite 231 *m/z* c) metabolite 175 *m/z*, d) metabolite 203 *m/z* and e) metabolite 264 *m/z*.

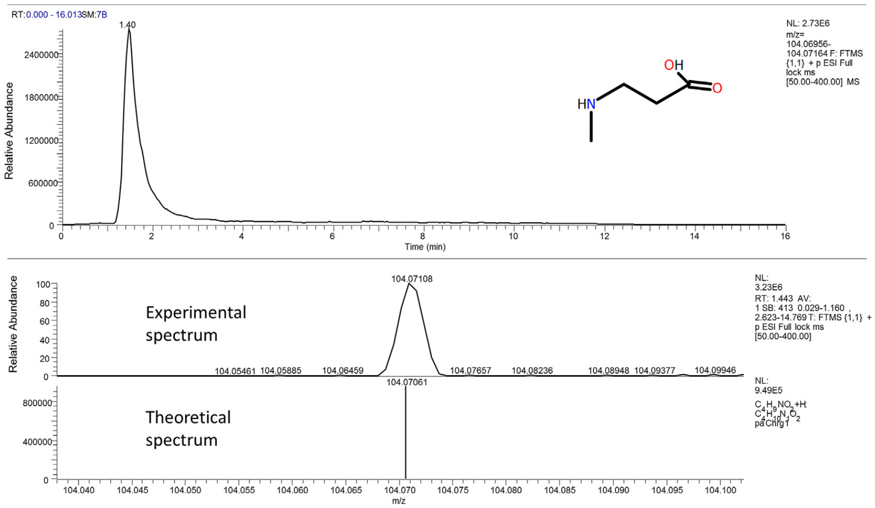

Fig. 4. Extracted ion chromatogram, theoretical and experimental spectrum of the new metabolite 104 *m/z*.

concentration slowly decreased to values lower than LOQ at double dose for cucumber samples. On the other hand, the metabolite 203 *m/z* was detected 1 day after application at concentration lower than LOQ. Its concentration increased to 17 μg/kg (7 days after application) and later decreased for cucumber samples at double dose. Finally metabolite 104 *m/z* had a similar concentration (approximately 250 μg/kg) from 1 day to 17 days in cherry tomato samples and later increased to 2000 μg/kg (24 days) and later decreased to 1200 μg/kg (40 days). It can be said that in cherry tomato samples metabolite 104 *m/z* was detected at the highest concentrations and its behaviour is slightly different than in cucumber samples, where its concentrations were constant or was hardly detectable (Fig. S10). Bearing in mind its higher concentrations, metabolite 104 *m/z* could be included in the MRL of propamocarb for tomatoes.

In relation to fenamidone, metabolite 264 *m/z* was detected 1 day after the application and its concentration increased up till 4 days and later decreased for all matrices. For example, the concentration of metabolite in cucumber samples at double dose increased from 11 (1 day) to 19 μg/kg (4 days) and later decreased. Its concentration was lower than LOQ 24 days after application. On the other hand, in case of cherry tomato samples at normal dose, the concentration increased from 10 to 18 μg/kg and later, its concentration decreased to values below LOQ 40 days after application. (Fig. S12).

4. Conclusions

In this study, dissipation of fenamidone and propamocarb was studied in vegetables, and metabolites were detected in incurred samples. LC and GC coupled to HRMS methods were developed and used for the comprehensive study of different analytes. Greenhouse trials were carried out in order to know the behaviour of these compounds in vegetables. The persistence of the parent compounds was low because DT_{50} values were lower than 30 days. In relation to metabolites, they were detected 1 day after the application of commercial product. In addition, retrospective analysis was carried out and four new metabolites of propamocarb and one for fenamidone were putative elucidated in cherry tomato, cucumber and courgette samples. Finally, these results provide a comprehensive overview of the presence of fenamidone and propamocarb in vegetables, monitoring their main transformation products, and some of them could be included in their MRL. Thus, for propamocarb, the new metabolite 104 *m/z* detected in this study, or RPA 410193 in the case of fenamidone, could be added to the MRL definition, because in most of the cases, the sum of both metabolites with their parent compound can be higher than the established MRL, although no toxicity data was provided.

Conflicts of interest

The authors declare no competing financial interest.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.03.118.

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Publicación XI

RESIDUES AND DISSIPATION KINETICS OF FAMOXADONE AND ITS METABOLITES IN ENVIRONMENTAL WATER AND SOIL SAMPLES UNDER DIFFERENT CONDITIONS

López-Ruiz, R.; Romero-González, R.; Garrido-Frenich, A.
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Residues and dissipation kinetics of famoxadone and its metabolites in environmental water and soil samples under different conditions★

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Dissipation kinetics

ABSTRACT

The dissipation of famoxadone as well as the behaviour of its metabolites in environmental samples such as water and soil is a major concern. In this study, the dissipation of the target compound in both matrices was carried out applying an analytical method based on ultra-high performance liquid chromatography coupled to Orbitrap mass spectrometry (UHPLC-Orbitrap-MS). The dissipation of famoxadone was monitored over a period of 100 days after the plant protection product, Equation Pro®, was administered to the target matrices. This study was performed at two doses, normal and double in the case of soils and fivefold instead of double dose in water. The concentration of famoxadone steadily decreased during the monitoring period in both matrices. Half-life (DT_{50}) values were lower than 30 days in most cases except for loam soils, for which it was 35 days. Therefore, persistence of this pesticide in both matrices was low. Famoxadone metabolites such as IN-KF015 ((5RS)-5-methyl-5-(4-phenoxyphenyl)-1,3- oxazolidine-2,4-dione) and IN-JS940 ((2RS)-2-hydroxy-2-(4- phenoxyphenyl) propanoic acid) were detected in both matrices and their concentration increased while the concentration of the parent compound decreased. Metabolite IN-JS940 was the compound detected at highest concentration for both matrices. In water the maximum concentration was 20% of the initial famoxadone content and in soils it was 50% of initial famoxadone content. In addition, another metabolite, IN-MN467 ((5RS)-5-methyl-3-[(2-nitrophenyl)amino]- 5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione), was detected in soils, following the same behaviour as the other metabolites. These results provided ample information about the behaviour of metabolites and the necessity of knowing their toxicity in both matrices in order to detect possible risks for living beings.

1. Introduction

Fungal diseases are controlled by fungicides in conventional agriculture. The use of pesticides has increased over the last decades, especially in Europe, with Spain and France being the countries using the highest amounts of pesticides (Eurostat and European Commission, 2007). Fungicides are bioactive toxic compounds that can influence soil productivity and agro ecosystem quality. They have different modes of action and can be used with a wide range of compounds or to target a specific group of fungi (Morton and Staub, 2008). The type of fungicide and application method depend on the crop (Lopez Santísima-Trinidad et al., 2018) and the amount of fungicide in direct contact with or "consumed" by target pests is a very small percentage of the applied amount. In most studies, the percentage of fungicide applied that reaches the target pest (0.3%) is far less than the quantity applied to the field and therefore a very high percentage (99.7%) of it is released into the environment, mainly in soils and water (Gámiz et al., 2016; van der Werf, 1996).

This study focused on the evaluation of the famoxadone, ((RS)-3-anilino-5-methyl-5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione), fungicide which is used to protect agricultural products against various fungal diseases.

Famoxadone can be degraded into a variety of metabolites such as 4-phenoxybenzoic acid and 1-acetyl-2-phenylhydrazine. The European Food Safety Agency (EFSA) has proposed other metabolites (EFSA (European Food and Safety Authority), 2015) such as IN-JL856 ((2RS)-2-hydroxy-2-(4-phenoxyphenyl)-N′-phenylpropanehydrazide), IN-JS940 ((2RS)-2-hydroxy-2-(4-

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phenoxyphenyl)propanoic acid), IN-KF015 ((5RS)-5-methyl-5-(4-phenoxyphenyl)-1,3- oxazolidine-2,4-dione), IN-KZ007 ((5RS)-3-anilino-5-[4-(4- hydroxyphenoxy)phenyl]-5-methyl-1,3- oxazolidine-2,4-dione), IN-MN467 ((5RS)-5-methyl-3-[(2-nitrophenyl) amino]- 5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione), benzene and 1,5-diphenylcarbono hydrazide. These metabolites could be detected in soils and water although in most cases, toxicity of metabolites has not been studied yet (EFSA (European Food and Safety Authority), 2015) as it can be observed in Table S1 (*Supplementary material*). Thus, the development of a new method that allows the simultaneous determination of these metabolites and their parent compound is required.

Some studies have developed multiresidue methods including famoxadone (Angioni et al., 2012; Dedola et al., 2014; Fenoll et al., 2010; Liu et al., 2011; Viñas et al., 2010). However, there is scarce bibliography evaluating famoxadone behaviour in soils (Feng et al., 2018; Ge et al., 2010; Liu et al., 2010; Shabeer et al., 2015) and to our knowledge, this compound has not been determined in water yet. In these studies, famoxadone was monitored but its metabolites were not evaluated, and only one study analyses the parent compound and its metabolites in vegetables (Lopez-Ruiz et al., 2019). Therefore, a comprehensive dissipation of famoxadone in environmental samples, including the main metabolites of famoxadone (4-phenoxybenzoic acid and 1-acetyl-2-phenylhydrazine) and other potential metabolites proposed by EFSA is required. The evaluation of the behaviour of famoxadone in soils and water as well as its dissipation into its metabolites is first studied in this paper. For this purpose, ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (HRMS) with electrospray ionization (ESI) was used. HRMS is widely used for the identification of known and unknown compounds in several fields such as metabolomics, pharmacokinetics, natural product research, human health studies, environmental analysis and food safety and quality (Schollée et al., 2017).

The objective of this study is the evaluation of the behaviour of famoxadone and its metabolites in soils and water as well as its kinetics dissipation into its metabolites under laboratory conditions at two doses. Determination of famoxadone and its metabolites was carried out by an analytical methodology based on UHPLC-Orbitrap-MS.

2. Materials and methods

2.1. Materials and reagents

Famoxadone (CAS registry No. 131807-57-3, purity >99%), 1-acetyl-2-phenylhydrazine (CAS registry No. 114-83-0, purity >98%) and 4-phenoxybenzoic acid (CAS registry No. 2215-77-2, purity >97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Stock standard solutions (1000 mg/L) were obtained by dissolving 10 mg of the commercial standard in acetonitrile (ACN) (10 mL). A working standard solution of the three compounds (10 mg/L) was prepared by pipetting 100 µL of each stock solution in a 10 mL volumetric flask, adding acetonitrile up to the mark. Both solutions were stored at −21 °C. The stability of the stock solutions has been evaluated, and working standard solution and stock standard solutions were stable for 2 months and one year respectively.

Plant protection product of famoxadone, Equation Pro®, was sold by Du Pont™ (Carreño, Asturias, Spain).

Water (LC-MS grade) was purchased from J.T. Baker (Deventer, Holland). Methanol (MeOH) and ACN (both LC-MS grade) were obtained from Fluka (St. Louis, MO, USA). Formic acid and acetic acid were purchased from Fisher Scientific (Erembodegem, Belgium).

Strata X-A cartridges were from Phenomenex (Torrance, CA, USA), and Oasis HLB flangeless SPE cartridges were acquired from Waters (Dublin, Ireland). Tetrabutylammonium acetate (TBA) was obtained from Sigma Aldrich.

To calibrate the Orbitrap analyzer, a mixture of Ultramark 1621, caffeine, acetic acid, and Met–Arg–Phe–Ala–acetate salt (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrat and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative) from Thermo-Fisher (Waltham, MA, USA) were used.

2.2. Equipment

An analytical balance AB204-S (Mettler Toledo, Greifensee, Switzerland), a Reax 2 rotatory agitator from Heidolph (Schwabach, Germany), a vortex mixer WX from Velp Scientifica (Usmate, Italy) and a Centronic BL II centrifuge (J.P. Selecta, Barcelona, Spain) were used for sample preparation.

A Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) was used for chromatographic analysis. A C_{18} analytical column, Zorbax Eclipse Plus C_{18} (100 mm × 2.1 mm, 1.8 µm particle size) purchased from Agilent Technologies (Santa Clara, CA, USA) was used (column temperature 25 °C). For separation of the selected compounds in the UHPLC system, flow rate was set at 0.2 mL/min, and the injection volume was 10 µL. The mobile phase was composed by a water solution of 0.1% formic acid and 4 mM ammonium formate, (eluent A) and MeOH (eluent B).

The gradient profile started at 90% of eluent A, and this composition was kept constant for 1 min; then it was linearly decreased to 0% in 1 min. This composition was held for 6 min (0% eluent A), before being returned to the initial conditions in 0.5 min (95% of eluent A), followed by a re-equilibration time of 1.5 min. The total running time was 9 min.

A single mass spectrometer, Orbitrap Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen, Germany), was used, and an electrospray ionization interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA) was selected. Positive and negative ionization modes were used. Different ESI parameters were set: sheath gas (N_2, >95%), 35 (arbitrary units, a.u.); auxiliary gas (N_2, >95%), 10 (a.u.); spray voltage, 4 kV; capillary voltage, 35 V; skimmer voltage, 18 V; tube lens voltage, 95 V; capillary temperature, 300 °C; heater temperature, 305 °C. Four acquisition functions, with the following characteristics, were used to acquire the MS spectra: (1) full ESI + MS, without fragmentation (higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 50,000 FWHM; scan time = 0.25 s; (2) all-ions fragmentation (AIF), ESI+, with fragmentation (collision energy 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s, (3) full MS, ESI- using the settings described in the first function, and (4) AIF, ESI- using the same settings that described for the second acquisition function. Mass range was set at *m/z* 50–600.

External calibration mode was used for the acquisition of the chromatograms. Xcalibur™ version 3.0, with Quanbrowser and Qualbrowser, and Mass Frontier™ 7.0 (Thermo Fisher Scientific, Les Ulis, France) were used for the sample processing.

2.3. Samples

Water samples were collected from a water well located in Almería (pH was 7.1 and conductivity was 532 µS/cm). Sandy loam and loam soil samples were collected in areas with different characteristics from Vícar, Almería (Spain). Samples were characterized according to different physicochemical parameters (pH, organic matter (%), carbon monoxide (%), % sand, % silt, % clay and % grit) (Table S2, see *Supplementary material*), observing that both soils (pH > 8) soils are alkaline. In addition, they were sieved (particle size <2 mm) and dried at room temperature for two days before analysis.

2.4. Laboratory trials

2.4.1. Water trials

Water samples were studied under darkness and sunlight conditions at room temperature (maximum temperature: 25 °C, minimum temperature: 20 °C). Darkness conditions simulated the underground water and sunlight conditions simulated surface waters like lakes and rivers. For that, 125 mL of water were deposited into transparent bottles (sunlight conditions) and amber (darkness conditions). Later, waters were spiked with Equation Pro® at 1 mg/L and 5 mg/L and each bottle was hermetically closed, and then, agitated. Sunlight samples were located inside the laboratory, and they were placed on a shelf near the south window. On the other hand, darkness samples were located in a cupboard. Three replicates per condition were taken at 24 h and 7, 15, 30, 50, 70 and 100 days, and analysed.

2.4.2. Soils trials

Aliquots of 15 g of sandy loam and loam soils were weighed, and different volumes of water were added in order to simulate humidity conditions: 2 mL for sandy loam (14% humidity) and 5 mL for loam soil (34% humidity). Then, the soils were spiked with the plant protection product (Equation Pro®) at two concentration levels, normal dose and double dose. The normal dose was 0.4 kg per ha (2.4 mg/g soil) and the double dose was 0.8 kg per ha (4.8 mg/g soil). Then the spiked samples were homogenised for 5 min and placed in Erlenmeyer flasks. They were placed at room temperature (minimum temperature: 20 °C, maximum temperature: 25 °C), with an average of 8 h of sunlight per day. Then, they were taken (three replicates per condition) at the same days of water trials and analysed. The flasks containing the soil were weighed every two days, and if necessary, the required amount of water was added.

2.5. Sample extraction

2.5.1. Water extraction

A solid phase extraction (SPE) procedure has been used for the extraction of famoxadone and metabolites from water. First, the cartridge (Oasis HLB) was conditioned with 3 mL of ACN and after that, 3 mL of water was added for equilibration of the cartridge. Then, 125 mL of sample with 40 mg of TBA were passed through the cartridge at one drop per second. Finally, the cartridge was dried (30 min) and the elution step was performed with 1.5 mL of ACN. Using these conditions, a preconcentration factor of 83.33 was achieved.

2.5.2. Soil extraction

First, place 5 g of soil sample into a 50 mL plastic centrifuge tube. Then, add 10 mL of water and 10 mL of acidified ACN (1% acetic acid). Shake the mixture for 1 min in a vortex and stirr for 1 h in a rotary shaker. After that, centrifuge the mixture at 5000 rpm (4136×g) for 10 min. Then, collect one mL of the supernatant and inject it into the UHPLC-Orbitrap-MS.

2.6. Method validation

Method performance has been evaluated by linearity, matrix effect, trueness (% recovery), precision (interday and intraday), limits of detection (LODs) and limits of quantitation (LOQs) (SANTE/EU, 2017).

The linearity has been performed by preparing matrix-matched calibration curves in blank matrix with five concentration levels, and the analytical range was set between LOQ and ten times the LOQ. For those samples with concentrations above the upper linear range, they were diluted to achieve concentrations within the linear range. Linearity was studied by plotting the peak area against the concentration of the standards, and it has been evaluated by the determination coefficient (R^2). Matrix effect was studied and for that purpose calibration curves in solvent (ACN) and in extracted blank matrix (soil and water) were built and compared. The matrix effect was calculated as follows: matrix effect (ME, %) = [(slope of calibration curves in matrix/slope of calibration curves in solvent)-1] x 100%. Values lower than −20% or greater than +20% correspond to matrix induced signal suppression and enhancement respectively.

LODs and LOQs were calculated performing the extraction of blank samples that were previously spiked with the targeted compounds at low concentrations (from 1 to 30 μg/kg). LODs were set as the lowest concentration at which the characteristic ion can be monitored with a mass error lower than 5 ppm. For the estimation of LOQ, one fragment has to be monitored at the same retention time and chromatographic shape than the characteristic ion, and the mass error should be lower than 5 ppm. In addition, acceptable trueness (70–120%) and precision (≤20%) values should be achieved.

Trueness was tested in terms of recovery. For that, blank samples were spiked at two different concentration levels for each analyte, at LOQ value (10 μg/kg for soils and 0.1 μg/L for water) and ten times the LOQ (100 μg/kg (soils) and 1 μg/L (water)), analysing five replicates at each level. Precision was estimated by performing intraday and interday studies at the same levels as trueness, expressed as RSD %. Intraday precision (repeatability) was tested at two different concentration levels for each analyte within the same day performing five replicates at each level. Interday precision was evaluated at the same levels and replicated in five different days.

3. Results and discussion

3.1. Optimization of extraction procedure and validation

Although UHPLC-Orbitrap-MS conditions were optimized in a previous study (Table 1) (Lopez-Ruiz et al., 2019), the extraction procedure was optimized for both matrices, water and soils. For more information see Supplementary Material Section 1. *Extraction procedure optimization*.

Both optimized methods for the extraction of famoxadone from soils and water, were validated, and results are summarized in Table 2. In addition, results were discussed in Supplementary Material Section 2. *Method Validation*.

3.2. Lab trials

Dissipation kinetics of famoxadone in water and soils was calculated by plotting residue concentration against time. The residual concentration and half-life of famoxadone (DT_{50}) was determined using the “Single First-Order Rate” (SFO) model applying Eq. (1).

$$C_T = C_0\, e^{-kt} \qquad (1)$$

where C_0 is the initial concentration, k is the rate constant (k) and C_T is the concentration at time t (Briones and Sarmah, 2019). Figs. 1

Table 1
UHPLC-Orbitrap-MS parameters for famoxadone and its main metabolites.

Analyte	Ionization mode	Precursor ion			Fragments			Retention Time (min)
		Exact mass	Adduct	Mass error (ppm)	Exact mass	Molecular formula	Mass error (ppm)	
Famoxadone	Positive	392.16048	$[M + NH_4]^+$	−0.4	195.08002	$C_{14}H_{11}O$	−2.2	6.8
					331.14334	$C_{21}H_{19}O_2N_2$	2.3	
1-Acetyl-2-phenylhydrazine	Positive	151.08659	$[M+H]^+$	−0.2	108.06869	$C_6H_8N_2$	4.1	5.1
					92.05020	C_6H_6N	4.9	
4-Phenoxybenzoic acid	Negative	213.05572	$[M-H]^-$	−2.1	169.06526	$C_{12}H_9O$	−2.7	6.4
					93.03345	C_6H_5O	−0.4	

Table 2
Validation parameters of the optimized method in soils and water.

Analytes		Famoxadone	1-Acetyl-2-phenylhydrazine	4-phenoxybenzoic acid
Soils				
R^2		0.988	0.995	0.991
Matrix effect[a]		181	26	96
LOD (µg/kg)		2	10	10
LOQ (µg/kg)		20	20	20
Recovery (%)[b]	20 µg/kg	94 (13)	75 (14)	72 (10)
	200 µg/kg	96 (3)	89 (10)	113 (6)
Inter-day precisión (% RSD)[c]	20 µg/kg	13	18	15
	200 µg/kg	9	15	7
Water				
R^2		0.995	0.998	0.998
Matrix effect[a]		198	−52	58
LOD (µg/L)		0.05	0.05	0.05
LOQ (µg/L)		0.10	0.10	0.10
Recovery (%)[b]	0.1 µg/L	75 (15)	72 (10)	75 (16)
	1 µg/L	106 (12)	113 (12)	85 (14)
Inter-day precisión (% RSD)[c]	0.1 µg/L	19	11	18
	1 µg/L	15	8	12

[a] Matrix effect = (ME, %) = [(slope of calibration curves in matrix/slope of calibration curves in solvent)-1] x 100%.
[b] Intra-day precision (% RSD) in parenthesis (n = 5).
[c] n = 5.

and 2 show the SFO model for famoxadone dissipation in water and soils respectively. Other models (Fantke and Juraske, 2013), such as zero order, one-and-a-half-order and second order were tested in water and soils but the best fit was obtained when the SFO model was applied (Table S5).

In addition, concentrations of metabolites described by EFSA (Figures S1, S2 and S3) (EFSA (European Food and Safety Authority), 2015) were estimated using the matrix matched calibration curve obtained for famoxadone as there are no commercially available standards for these compounds. They were expressed in relation to the initial concentration of the famoxadone. Also, a new metabolite of famoxadone detected in a previous study (Lopez-Ruiz et al., 2019) (Fig. S4) was monitored and its concentration was estimated using the same procedure as used for EFSA metabolites. Unknown analysis was also performed in order to look for unknown metabolites, but only the metabolites described above were detected in the samples.

3.2.1. *Water trials*

The results of the dissipation of famoxadone in water (Fig. 1 and Table 3) show that behaviour was the same for both conditions (sunlight and darkness), decreasing concentration throughout the monitored period. For example, in case of sunlight conditions at fivefold dose, the concentration of famoxadone decreased from 4500 µg/L at 1 day to 120 µg/L at 100 days whereas for darkness conditions at normal dose, the concentration went from 920 µg/L to 20 µg/L k value was higher in case of normal dose (for example 0.058 days^{-1} for normal dose and 0.049 days^{-1} for double dose in the case of darkness conditions). However, persistence of famoxadone was higher under sunlight conditions than under darkness conditions at both concentration levels (22 and 23 days for sunlight at normal and fivefold dose respectively and 12 and 14 days for darkness at normal and fivefold dose). This could suggest that the dissipation of famoxadone is not affected by sunlight and that other dissipation processes such as pH and the microorganisms that can be present in water, could be more important (Ruiz-Marrondo, 2001), although further studies are needed.

With regard to metabolites, IN-KF015 and IN-JS940 were the only two metabolites detected in water at concentrations higher than the famoxadone LOQ (Fig. 3). Behaviour of metabolite IN-JS940 was the same for both conditions/doses. This was the main metabolite detected (from 0.4 to 20.0% of initial famoxadone content) and when the concentration of the parent compound decreases, its concentration increased until the end of the monitoring period. For example, in darkness conditions at normal dose the concentration of the metabolite increased from 0.4% on the first day after application to 8% at 100 days in relation to initial famoxadone content. In sunlight conditions at normal dose, it increased from 2% to 20% of initial famoxadone content. Metabolite IN-KF015 was detected under sunlight at low concentration (<0.01%) in relation to the initial concentration of the parent compound. It was detected at double dose between 70 and 100 days when its concentration had the highest value whereas prior to that, its concentration was negligible compared with the initial concentration of the parent compound (Fig. 3). Therefore, the highest molar mass ratio of metabolites in relation to the initial concentration of the parent compound was obtained in sunlight conditions at normal dose (Fig. 3), obtaining conversion factors higher than 18% for only for one metabolite (IN-JS940) as metabolite IN-KF015 was not detected. In the case of fivefold dose concentration, the highest molar

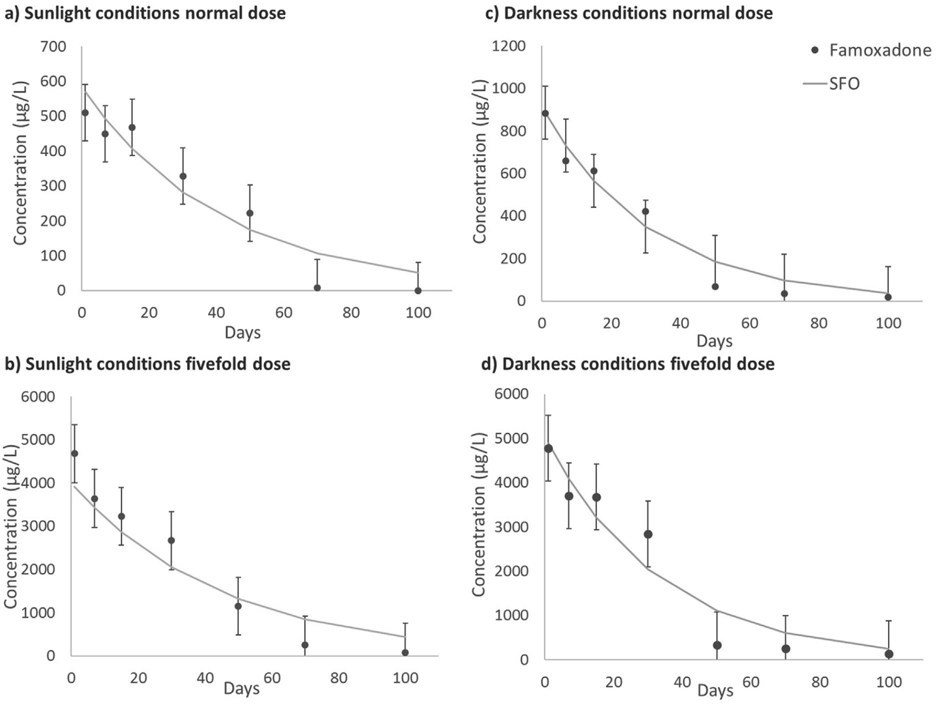


Fig. 1. Concentration of the parent compound (adjusting to kinetic model "Single First-Order Rate" (SFO)) at: a) sunlight conditions normal dose, b) sunlight conditions fivefold dose, c) darkness conditions normal dose and d) darkness conditions fivefold dose in water. (Error bars obtained for n = 3).

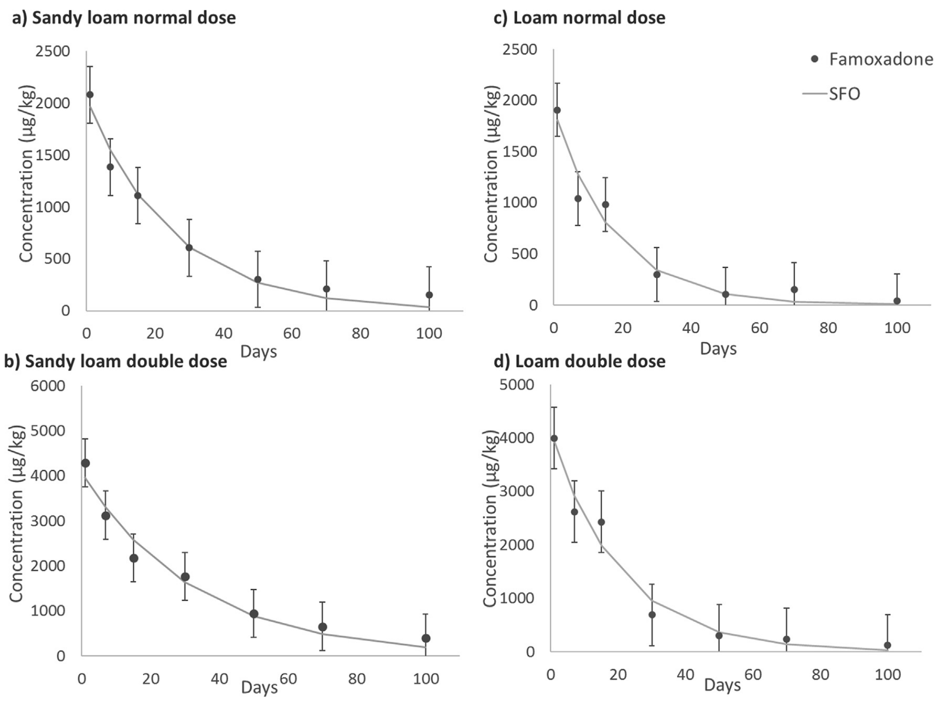
ratio was 5% and included both metabolites (IN-KF015 and IN-JS940). Despite dissipation of famoxadone being lower under sunlight conditions, metabolites were detected at higher concentrations than in darkness conditions. This can be explained considering that sunlight conditions favoured the degradation of famoxadone into IN-JS940, whereas other degradation routes are favoured under darkness conditions and provide other metabolites or degradation products that are not detectable by the proposed methodology. Furthermore, mass balance of famoxadone and metabolites was provided in Fig. S5. As can be observed, the total amount of famoxadone and metabolites decreased until 15 days after application of the commercial product after which it remained constant until 30 days. After that, the amount decreased and the final amount was almost zero after 60 days. Finally, metabolite IN-JS940 toxicity has to be evaluated since its concentration increased during all the parent compound dissipation (from 1 to 100 days). In consequence, it possibly persists in water for longer than 100 days and could be toxic to aquatic organisms.

3.2.2. Soil trials

Dissipation of famoxadone was different for each type of soil (Fig. 2 and Table 3). Whereas DT_{50} for sandy loam soils at normal dose was 17 days, in the case of loam soils it was 20 days, indicating that the persistence of famoxadone was similar in loam and in sandy soils. Regarding the dose, the behaviour is the same in both matrices as that observed in water. The *k* value was slightly higher in the case of normal dose, being 0.040 and 0.057 days $^{-1}$ for sandy loam and loam respectively and 0.030 and 0.018 days^{-1} for double dose. Concentration decreased during all experiments for both conditions and types of soil but at different velocity as can be observed from *k* values. For example, in the case of sandy loam at normal dose, the concentration of famoxadone decreased from 2100 µg/kg (the first day after application) to 600 µg/kg at 30 days whereas for loam soils it decreased from 1900 µg/kg to 250 µg/kg respectively. Comparing both matrices (water and soil), it can be concluded that persistence is low ($DT_{50} < 30$ days) in most cases except for loam soils, where DT_{50} was around 35 days so persistence can be considered low to medium (Ortiz, 2008).

IN-KF015, IN-MN467 and IN-JS940 were the metabolites detected in soils (Fig. 4). The other metabolites were not detected at concentrations above the LOQ of famoxadone. Metabolite IN-JS940, as in water, was the main metabolite detected. It was detected at a high percentage in relation to the parent compound (50%). Metabolites IN-MN467 and IN-KF015 were also detected in both types of soils but at a small percentage in relation to initial famoxadone content (<5%). Although they were detected in both soils, their behaviour was different. In the case of sandy loam soils, their concentration increased until 70 days after the application of the plant protection product and later quickly decreased until 100 days. For example, for metabolite IN-JS940 at normal dose, concentration increased from 1% at 1 day to 50% at 70 days and later decreased until 15% in relation to initial famoxadone content. In loam soils their concentration increased until 30 days and later slowly decreased until 100 days. Metabolite IN-KF015 increased its concentration from 0.1% at 1 day to 3% at 30 days and later decreased to percentages lower than 0.1%. This could be explained because leaching is expected to be higher in sandy soil than in loam soil and therefore concentration of these compounds increased for a longer period of time in sandy loam soils than in loam soils.

In comparison with water, the amount of metabolite IN-KF015


a) Sandy loam normal dose

b) Sandy loam double dose

c) Loam normal dose

d) Loam double dose

Fig. 2. Concentration of the parent compound (adjusting to kinetic model "Single First-Order Rate" (SFO)) at: a) sandy loam normal dose, b) sandy loam double dose, c) loam normal dose and d) loam double dose in soils. (Error bars obtained for n = 3).

Table 3
SFO kinetic model parameters and water and soil dissipation (DT_{50}) of famoxadone.

Parameters	Water Sunny		Water Darkness		Sandy loam		Loam	
	Normal dose	Fivefold dose	Normal dose	Fivefold dose	Normal dose	Double dose	Normal dose	Double dose
C_0 (µg/kg)	912	5041	1916	4124	2045	4093	1916	4050
k (days^{-1})	0.032	0.030	0.058	0.049	0.040	0.030	0.057	0.018
DT_{50} (days)	22	23	12	14	17	23	20	35
R^2	0.889	0.918	0.913	0.937	0.981	0.979	0.962	0.938

was higher and metabolite IN-MN467 was detected in soils. The highest molar mass ratio of metabolites in relation to the initial concentration of the parent compound was obtained in soils (Fig. 4), achieving conversion factors higher than 50% compared to water, which was 18%. In addition, as molar mass ratio values in soils were higher than in water and metabolites increased their concentration until 70 days in sandy loam soils, an evaluation of metabolites toxicity is also required in soil samples. Mass balance (Fig. S5) indicated the same information as in water trials as throughout the monitored period the balance indicated loss of analytes and from 80 to 100 days, the concentration loss was smaller compared to days prior to that.

Considering this was the first time that famoxadone metabolites were monitored in water and soils, a comparison of the data obtained with other studies is not possible. Nevertheless, dissipation of famoxadone in soils can be compared with other studies (Ge et al., 2010) that determined half-lives of famoxadone in soil at normal dose at 13.5 days. However, the current study provides half-lives of 17 days in sandy loam and 34 in loam soils so in the case of sandy loam soils the value is similar to those reported by Ge et al. On the other hand, comparing our results of half-lives with the data obtained from the Pesticide database (University of Hertfordshire, 2007) in their laboratory trials, our results for loam soils (34.4 days) are similar to the results from the Pesticide database of 41 days.

4. Conclusions

The dissipation of famoxadone in soils and water has been evaluated applying a new and validated UHPLC-Orbitrap-MS method. Metabolites of famoxadone were detected in incurred samples. The behaviour of famoxadone and its metabolites was tested by lab trials. The persistence of famoxadone is low in both matrices (DT_{50} was lower than 30 days) except in loam soils where it was low to medium (DT_{50} was 35 days). Behaviour of metabolites was similar and they were detected 1 day after the application of the plant protection product and their concentration increased while famoxadone decreased. In the case of water studies, metabolites concentration increased during the whole study and never decreased. Therefore, it can be concluded that they are present in

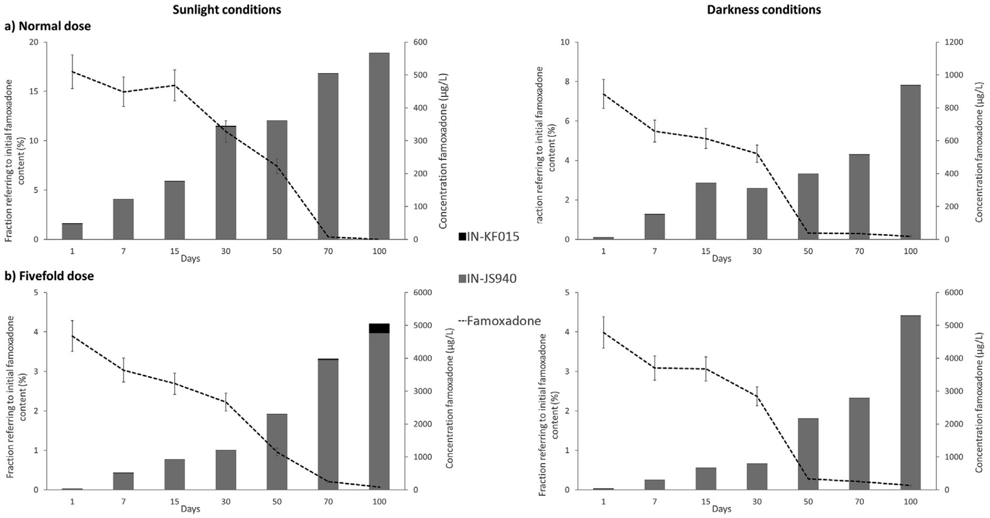


Fig. 3. Metabolite behaviour according to the concentration of famoxadone during monitoring period for water experiments at: a) normal dose and b) fivefold dose.

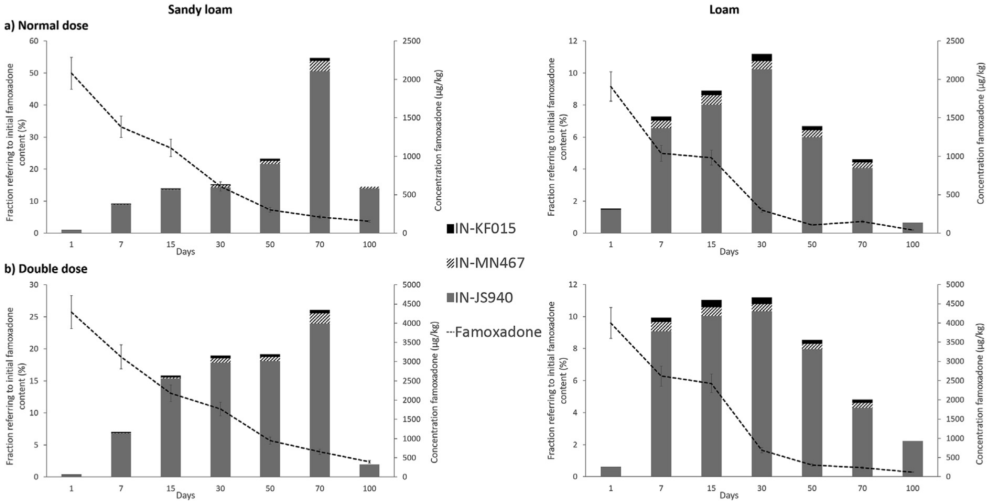


Fig. 4. Metabolite behaviour according to the concentration of famoxadone during monitoring period for soils experiments at: a) normal dose and b) double dose.

water for a longer period although the persistence of the parent compound was low. It should be noted that traditional metabolites such as 1-acetyl-2-phenylhydrazine and 4-phenoxybenzoic acid were not detected in incurred samples but metabolites proposed by EFSA were detected. Therefore, toxicity evaluation of metabolites proposed by EFSA is required in order to discover the possible risks that flora, fauna and humans may suffer.

Conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.05.123.

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Publicación XII

DISSIPATION KINETICS OF FENAMIDONE, PROPAMOCARB AND THEIR METABOLITES IN AMBIENT SOIL AND WATER SAMPLES AND UNKNOWN SCREENING OF METABOLITES

López-Ruiz, R.; Romero-González, R.; Garrido-Frenich, A.
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Research article

Dissipation kinetics of fenamidone, propamocarb and their metabolites in ambient soil and water samples and unknown screening of metabolites

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ABSTRACT

A fenamidone and propamocarb dissipation study was carried out applying ultra high-performance liquid chromatography coupled to Orbitrap mass spectrometry (UHPLC-Orbitrap-MS). Dissipation kinetics were evaluated in different types of soils and in water under different conditions (sunlight or darkness). In addition, a plant protection product containing both compounds was applied at two doses: (i) single and (ii) double dose in soils, and (i) single and (ii) fivefold dose in water. The fenamidone and propamocarb concentration decreased during the monitored period (100 days), obtaining high persistence in the case of water studies ($DT_{50} > 50$ days) and low to medium persistence in soils ($DT_{50} < 50$ days). No Observed Effect Concentration (NOEC) and concentration causing 50% lethality (EC50) were calculated and showed that fenamidone could cause toxic effects in soil and water organisms due to very high NOEC values (0.013 mg/L for aquatic invertebrates) while propamocarb did not cause any lethality. Fenamidone and propamocarb metabolites were also monitored with acetophenone and RPA-411639 ((5)-5-methyl-2-(methylthio)-3-(4-S nitrophenyl)amino-5-phenyl-3,5-dihydro-4H-imidazole-4-one) being the main metabolites for fenamidone. These metabolites obtained concentration values of up to 25% initial fenamidone content which can be a risk for the environment and fauna but, despite the toxicity of these compounds, they have not been studied yet. Metabolite 175 m/z and propamocarb n-desmethyl were the main propamocarb metabolites with values of 3% of initial propamocarb content. Three new propamocarb metabolites were detected in water samples and one in soil, highlighting the capabilities of the proposed methodology for monitoring known metabolites and identifying new ones in environmental studies.

1. Introduction

Ensuring good quality and sufficient food supply worldwide is currently a primary concern among the global population and therefore pesticides are used to fight against pests and so minimize production losses (Marín-Benito et al., 2019). In this field, herbicides, insecticides and fungicides used to control soil insects, fungi, weeds and pathogens are generally applied to plants, hereby making soils their principal drain although they can also undergo degradation processes (Fantke et al., 2013; Ramakrishnan et al., 2019). Pesticides do not exclusively contaminate soils as water, crops or air can also be affected (Peña et al., 2019). For this reason, the European Union (EU) strictly regulated the approval of pesticides and their use (European Union, 2009) and authorities are reducing the number of permitted products.

In this paper, two systemic fungicides, fenamidone and propamocarb, were studied in soil and water. These compounds are used to prevent early and late blights and to protect plants against phycomycetous diseases. Both compounds are found in the plant protection product called Consento® (Chen et al., 2017; Manikrao and Mohapatra, 2016; Mohapatra and Deepa, 2012), which is commonly used in intensive agriculture in Almeria. Fenamidone penetrates the young leaves as it has translaminar and acropetic movement, has an effect on the cellular respiratory system and fixes on the waxy layers of the plant surface. Propamocarb is a systemic pesticide acting on the lipid formation necessary for the maintenance and formation of the cell membrane. The European Food Safety Authority (EFSA) concluded that the renewal of fenamidone could not be approved as information about its risk assessment and metabolites was not sufficient to comply with current Regulation (EC) No 1107/2009 (European Commision, 2018; European Union, 2009). This fact reinforces that the behaviour of this compound and its metabolites should be studied in order to provide more information. Propamocarb and fenamidone are absorbed by soils and water

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and they can be converted into metabolites by transformation processes. In addition to pesticide properties, multiple factors are responsible for the appearance of metabolites in environmental samples like soil or water. The physicochemical properties of water and soil (pH, texture or organic material content), biological properties (variety, density and microbial population activity) or weather conditions (temperature, rains, droughts) can all affect the transformation processes of these compounds (Canadian Centre for Occupational Health and safety, n.d.; Marín-Benito et al., 2019; Puerto Rodriguez et al., 2014). In addition, pesticide dissipation rates are an excellent indicator of how they are converted into their metabolites so metabolite behaviour should also be evaluated (Corta et al., 2000; López-Ruiz et al., 2018; Picó et al., 2018). Fenamidone and propamocarb degrade into several metabolites that have already been described (EFSA (European Food and Safety Authority), 2006, 2016; Lopez-Ruiz et al., 2019c) (Table S1). However, to our knowledge, these pesticides and their metabolites have never been monitored simultaneously in environmental samples, making doing so highly desirable, especially considering that in some cases the metabolites persist longer than the parent compound. Toxicity values and physicochemical properties of parent compounds and metabolites are very useful but in the case of metabolites, toxicity has not been studied yet (Lopez-Ruiz et al., 2019c).

In relation to maximum residue limit (MRL) in fruit and vegetables, propamocarb has values of 5 mg/kg in the case of cucurbits or watermelon and 0.3 mg/kg in potatoes (European Commission, 2016) but these values are merely expressed as the sum of propamocarb and its salts and do not include the metabolites described above. The values for fenamidone are 0.2 mg/kg in the case of cucurbits and watermelon and 0.02 mg/kg for potatoes (European Commission, 2016). López-Ruiz et al. (Lopez-Ruiz et al., 2019c) evaluated the acceptable daily intake (ADI) of both compounds and established limits of 0.29 mg of propamocarb/kg bw/day and 0.03 mg/kg bw/day for fenamidone. They also mention ADI values for two fenamidone metabolites (RPA-410193 and 5- methylhydantoin) which were set at 0.0094 mg/kg bw/day and 0.0064 mg/kg bw/day respectively.

Although some multiresidue methods have been developed for fenamidone and propamocarb determination in fruits and vegetables, there is scarce bibliography focused on the dissipation of these compounds (Angioni et al., 2012; de Melo Abreu et al., 2006; Dedola et al., 2014; Golge et al., 2018; González-Rodríguez et al., 2009; Ribeiro Begnini Konatu and Sales Fontes Jardim, 2018). For instance, three recent studies (only one for both compounds (Chen et al., 2017)) described their dissipation kinetics in vegetables and soils (Abd-Alrahman and Almaz, 2016; Chen et al., 2017; Manikrao and Mohapatra, 2016). Manikrao et al. (Manikrao and Mohapatra, 2016) studied fluopicolide and propamocarb dissipation kinetics in cabbage and soils and Abd-Alrahman et al. (Abd-Alrahman and Almaz, 2016) studied different commercial propamocarb products in potato. Finally, only one study has evaluated the parent compound dissipation kinetics and metabolite behaviour in vegetables (Lopez-Ruiz et al., 2019c).

In addition, previously unstudied metabolites could be identified in the incurred samples when using appropriate techniques. Liquid chromatography (LC) or gas chromatography (GC) coupled to high-resolution mass spectrometry (HRMS) are the main techniques used for identifying targeted and non-targeted compounds in fields such as environmental or food analysis (Coscollà et al., 2014; Gómez-Pérez et al., 2015; Jaén-Gil et al., 2018). New metabolite identification in this field requires the use of software tools such as MassChemsite® or Compound Discoverer® (Brink et al., 2014).

The purpose of this paper is to study fenamidone and propamocarb dissipation kinetics in soil and water and monitor their metabolites. Metabolite behaviour and toxicity had not been studied yet so it was necessary to evaluate their presence in environmental samples. No Observed Effect Concentration (NOEC) and concentration causing 50% lethality (EC_{50}) were calculated to determine toxicity in soils and water samples. In order to do so, analytical methods based on UHPLC-Orbitrap-MS were developed and validated for the simultaneous determination of parent compounds and metabolites. To our knowledge, this is the first study where known and unknown metabolites of fenamidone and propamocarb were monitored in environmental samples.

2. Materials and methods

2.1. Materials, apparatus and reagents

Analytical standards (>99% purity) of propamocarb, fenamidone, acetophenone and 5-methyl-5-phenylhydantoin were acquired from Sigma-Aldrich (St. Louis, MO, USA) while RPA-410193 was sold by Santa Cruz Biotechnology (Heidelberg, Germany). The plant protection product (Consento®) was supplied by Bayer Crop Science (Valencia, Spain).

Individual standard solutions of each compound (1000 mg/L) were prepared in methanol (MeOH) and they were stable for a year. Intermediate solutions of the five compounds (10 mg/L) were prepared in acetonitrile (ACN) and were stable for 2 months. Both types of solutions were stored at −21 °C.

Oasis HLB flangeless SPE cartridges were acquired from Waters (Dublin, Ireland), and Strata X-A cartridges from Phenomenex (Torrance, CA, USA). Tetrabutylammonium acetate (TBA) was purchased from Sigma Aldrich.

ACN and MeOH (both LC-MS grade) were supplied by Fluka (St. Louis, MO, USA). Water (LC-MS grade) was purchased from J.T. Baker (Deventer, The Netherlands) and formic acid was acquired from Fisher Scientific (Erembodegem, Belgium).

A Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) coupled to a single mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen, Germany) was used for detecting the compounds using the previously described parameters from a recent study (Lopez-Ruiz et al., 2019c).

2.2. Laboratory trials and sampling

2.2.1. Soil trials

Soils were sampled from different areas in Almería (Spain) and collected and identified conforming to several parameters, as can be observed in Table S2. They were then kept waterless at ambient temperature for two days and sieved (particle size <2 mm) before analysis.

Soil trials were performed as follows: first the samples were weighed (20 g) in Erlenmeyer flasks and aliquots of water were added depending on the soil type to imitate humidity conditions: 5 mL for loam (34% humidity) and 2 mL for sandy loam (14% humidity). In order to maintain humidity constant during the monitoring study, the flasks were weighed every two days and the demanded volume of water was supplemented when necessary. The commercial product was applied to the soils at two concentration levels, single dose (2 L per ha (7 μL/g soil) and double dose (4 L per ha (14 μL/g soil). The spiked samples were then agitated for 2 min so as to homogenize the sample and kept at ambient temperature (max temp: 25 °C, min temp: 20 °C) under natural sunlight conditions ensuring an average of 8 h of sunlight per day. Three replicates per condition were collected and analysed at 24 h and 7, 15, 30, 50, 70 and 100 days.

2.2.2. Water trials

Water samples were collected from a water well in Almeria. The conductivity was 532 μS/cm and pH was 7.1.

Trials in water were conducted under two different conditions (darkness and sunlight conditions) and at two different doses. Darkness conditions simulated buried water conditions and sunlight conditions those related to surface waters like rivers. One hundred and twenty five mL of water were placed into amber (darkness conditions) and transparent bottles (sunlight conditions) and spiked with Consento® at rates of 1 mg/L and 5 mg/L referring to fenamidone content (75 g of fenamidone/L Consento® and 375 g propamocarb/L Consento®). Each bottle was agitated, hermetically covered and kept at ambient temperature (max temp: 25 °C, min temp: 20 °C). Then, sunlight samples were stored on a shelf under a south facing window inside the laboratory and darkness samples were placed in a cupboard under darkness conditions at the same temperatures as sunlight samples. Samples were collected between 1 and 100 days and three replicates per condition were analysed.

2.3. Sample extraction

2.3.1. Soil extraction

Soil sample extraction was based on solid-liquid extraction. First, 5 g of soil sample were weighed and placed into a 50 mL Falcon® tube. After that, 10 mL of water and 10 mL of MeOH were added and the sample was stirred in a rotary shaker for 1 h. Finally, the samples were centrifuged for 10 min at 5000 rpm (4136 *g*) and one mL of the supernatant was collected and injected into the UHPLC-Orbitrap-MS.

2.3.2. Water extraction

SPE procedure was carried out following manufacturer advice using SPE Oasis HLB cartridges. First, the cartridge was reconditioned with 3 mL of ACN and equilibrated with 3 mL of water. Then, 125 mL of samples were passed through the cartridge at one drop per second. After that, the cartridge was dried for 30 min and then the sample was eluted with 1.5 mL of ACN.

2.4. Method validation

UHPLC-MS-Orbitrap analytical method was validated in order to give reliable results according to SANTE guidelines (European Commission, 2017), calculating matrix effect, linearity, precision (intra and inter-day), limits of quantification (LOQs) and trueness (% recovery).

Linearity was studied calculating the determination coefficients (R^2) of the calibration curves, which ranged from 10 to 100 μg/kg for soil matrices and 0.1–1.0 μg/L for water. The matrix effect was tested by analysing standards in ACN and standards prepared in an extracted blank matrix (soil and water). Trueness was studied analysing samples spiked at the first and last concentration of the calibration curves (10 and 100 μg/kg for soils and 0.1 and 1.0 μg/L for water) using 5 replicates per level. Precision (intra and inter-day) studies were evaluated at the same levels selected for trueness, and inter-day precision was evaluated for ten days.

Limits of quantification (LOQs) were estimated by injecting extracted blank samples spiked at low levels. LOQ, was defined as the lower concentration that provides recovery between 70 and 120% and precision lower than 20% respectively.

2.5. Data analysis

Fenamidone and propamocarb dissipation kinetics in soils and water were determined by plotting residue concentration of the parent compound against time. The residual concentration and half-life of propamocarb and fenamidone were calculated using the "Single First-Order Rate" (SFO) model (Eq (1)), where concentration was expressed as 'C_0', rate constant as '*k*' and half-lives as 'DT_{50}' (Eq (2)). They were estimated applying Eq (1), where C_t is the concentration at time t.:

$$C_t = C_0 e^{-kt} \tag{1}$$

$$DT_{50} = \frac{\ln 2}{k} \tag{2}$$

3. Results and discussion

3.1. Optimization of the UHPLC-MS-Orbitrap method

The spectrometric characterization and chromatographic elution of propamocarb and the metabolite acetophenone were carried out in a previous study (Lopez-Ruiz et al., 2019c) (Table 1). Meanwhile, UHPLC-MS parameters for fenamidone and its metabolites (RPA-410193 and 5-methyl-5-phenylhydantoin) were studied and are defined in Table 1 and Fig. S1.

Extraction methods were developed for soils and water. The soil extraction method was optimized by testing different extraction procedures based on solid-liquid extraction (Table S3), which were previously optimized (Lopez-Ruiz et al., 2019b; López-Ruiz et al., 2017). Low recoveries were obtained for both methods. Acidification of acetonitrile with 1% of acetic acid was tested in order to obtain better recoveries for acetophenone and propamocarb. Recoveries improved for propamocarb (80%) but were lower than 10% for acetophenone and higher than 120% (150%) for 5-methyl-5-phenylhydantoin. Finally, MeOH was tested instead of ACN and good recoveries were obtained for all analytes. Propamocarb recoveries were 60% but due to suitable relative standard deviation being obtained (<20%), a correction factor can be applied for quantification purposes.

For compound extraction from water, different conditions were tested (Table S4). TBA was added to the sample as it provides suitable yields for similar compounds during the extraction process (Lopez-Ruiz et al., 2019b) and the results were compared to those obtained when no addition of TBA was used. The best results were obtained in the latter case, obtaining recoveries between 70 and 105%. Therefore, the final procedure described in Section 2.4.2 was used for further experiments.

3.2. Validation of the proposed methods

Validation results obtained for the targeted analytes and both matrices were between the ranges established by SANTE guidelines (European Commission, 2017). Performance characteristics are shown in Table 2 for soils and Table 3 for water. Recoveries were between 60% (propamocarb) and 120% (acetophenone) in soil samples, whereas for water samples they ranged between 61% (propamocarb) and 107% (5-methyl-5-phenylhydantoin). Intra and inter-day precision values were lower than 11% and 19% in soil and water samples respectively.

3.3. Laboratory trials

Data obtained from dissipation studies in soils and water were fitted to the SFO kinetic model for fenamidone (Table 4, Figs. S2 and S3) and propamocarb (Table 4, Figs. S4 and S5). Other models (Environmental Protection Agency, 2016; Fantke and Juraske, 2013) such as the double first order parallel (DFOP) and Nth-Order Rate Model or Indeterminate Order Rate Equation Model (IORE) were tested but the best fitting was obtained for SFO kinetic model ($R^2 > 0.80$). In addition, metabolite concentrations described by EFSA (EFSA (European Food and Safety Authority), 2016), which have no commercially available standards, were calculated using the matrix matched calibration curve obtained for fenamidone or propamocarb. They were expressed considering the initial concentration of each parent compound. New metabolites for both compounds previously detected in vegetables (metabolite 104 *m/z*, metabolite 231 *m/z*, metabolite 175 *m/z*, metabolite 203 *m/z* and metabolite 264 *m/z*) were also monitored (Fig. S6) (Lopez-Ruiz et al., 2019c).

3.3.1. Fenamidone study

We observed that fenamidone dissipation behaviour was different for both types of soils (sandy loam and loam soils) and the concentration decreased during the monitored period (Fig. S2). For sandy loam at single dose, the fenamidone concentration reduced from 3000 μg/kg at 1

Table 1
UHPLC-Orbitrap-MS parameters.

Pesticide	Ionization mode	Precursor ion			Fragments			Retention Time (min)
		Exact mass	Adduct	Mass error (ppm)	Exact mass	Molecular formula	Mass error (ppm)	
Fenamidone	Positive	312.11651	$[M+H]^+$	0.6	236.11822	$C_{15}H_{14}N_3$	3.2	12.2
					134.07127	$C_7H_8N_3$	2.9	
RPA-410193	Positive	282.12370	$[M+H]^+$	−1.3	120.08078	$C_8H_{10}N$	2.8	11.1
					92.04948	C_6H_6N	4.0	
5-methyl-5-phenylhydantoin	Positive	191.08150	$[M+H]^+$	3.4	120.08078	$C_8H_{10}N$	2.2	9.3
					65.03858	C_5H_5	4.9	
Propamocarb	Positive	189.15975	$[M+H]^+$	1.4	102.05496	$C_4H_8O_2N$	−4.2	5.8
					74.02365	$C_2H_4O_2N$	5.3	
Acetophenone	Positive	121.06479	$[M+H]^+$	2.2	77.03858	C_6H_5	4.7	10.5
					82.04132	C_5H_5O	5.2	

Table 2
Validation parameters of the optimized method in soil samples.

Analites		Fenamidone	RPA-410193	5-methyl-5-phenylhydantoin	Propamocarb	Acetophenone
R^2		0.9916	0.9976	0.9907	0.9947	0.9916
Matrix effect[a]		0.82	0.76	0.90	2.04	0.88
LOQ (μg/kg)		10	10	10	10	10
Recovery (%)	10 μg/kg	115	97	86	60	120
	100 μg/kg	108	98	87	56	95
Precision[b] *(% RSD)*[c]	10 μg/kg	4 (5)	2 (6)	7 (9)	14 (11)	4 (8)
	100 μg/kg	2 (6)	2 (8)	6 (8)	2 (6)	3 (4)

[a] Matrix effect = ((slope of calibration curves in matrix/slope of calibration curves in solvent) – 1) x 100%.
[b] Intra-day precision and inter-day precision (between parenthesis).
[c] n = 10.

Table 3
Validation parameters of the optimized method for water samples.

Analites		Fenamidone	RPA-410193	5-methyl-5-phenylhydantoin	Propamocarb	Acetophenone
R^2		0.9996	0.9956	0.9900	0.9926	0.9932
Matrix effect[a]		1.01	0.75	2.54	0.90	2.97
LOQ (μg/L)		0.1	0.1	0.1	0.1	0.1
Recovery (%)	*0*.1 μg/L	89	89	61	61	93
	1 μg/L	105	73	107	63	102
Precision[b] *(% RSD)*[c]	*0*.1 μg/L	10 (15)	5 (9)	17 (19)	2 (5)	10 (11)
	1 μg/L	8 (19)	3 (5)	14 (17)	4 (4)	13 (9)

[a] Matrix effect = (slope of calibration curves in matrix/slope of calibration curves in solvent) – 1) x 100%.
[b] Intra-day precision and inter-day precision (between parenthesis).
[c] n = 10.

Table 4
SFO kinetic model parameters and water and soils dissipation (DT_{50}) of fenamidone and propamocarb.

Matrix	Soil				Water			
Conditions	Sandy loam		Loam		Sunlight		Darkness	
Parameters	Single dose	Double dose	Single dose	Double dose	Single dose	Fivefold dose	Single dose	Fivefold dose
				Fenamidone				
C_0 (μg/kg)	3148.79	4947.65	2881.55	5232.10	1129.00	5506.14	1183.61	5218.21
k (days)	0.014	0.018	0.037	0.052	0.011	0.013	0.009	0.013
DT_{50} (days)	48.69	39.44	18.78	13.30	62.66	51.46	73.32	57.02
R^2	0.850	0.904	0.902	0.958	0.803	0.834	0.805	0.801
				Propamocarb				
C_0 (μg/kg)	8461.01	14,676.96	5562.10	11,724.01	5848.76	14,274.40	6214.73	12,892.30
k (days)	0.060	0.113	0.075	0.109	0.013	0.010	0.013	0.008
DT_{50} (days)	11.57	6.13	9.20	6.36	52.73	66.21	52.49	86.58
$\mathbf{R^2}$	0.912	0.850	0.986	0.992	0.923	0.816	0.914	0.877

day to 600 μg/kg at 100 days. In the case of loam conditions at single dose, the concentration decreased from 2700 μg/kg to 100 μg/kg in the same period of time. k values were different in both cases (Table 4). For sandy loam at single dose, k value was 0.014 $days^{-1}$ whereas in loam soils it was 0.037 $days^{-1}$. Consequently, persistence in sandy loam soils was higher than in loam soils as can be observed by evaluating DT_{50} values (Table 4), which were 49 days and 19 days in sandy loam and loam soils respectively. This could have occurred due to leaching, which is expected to be higher in sandy soil compared to loam soil, making persistence of this compound higher in sandy loam soils than in loam soils. When comparing the doses, we observed that DT_{50} was higher at single dose in both soils and so concluded that with a higher fenamidone concentration, the dissipation rate is also higher.

Metabolites (Fig. 1) RPA-410193, RPA-412708, RPA-411639 and metabolite 264 *m/z* were detected in soils at concentrations higher than fenamidone LOQ. Metabolite behaviour was comparable as their concentration increased up until 70 days, while parent compound concentration decreased, and then decreased at 100 days. RPA-411639 was the metabolite detected at the highest concentration (25% of fenamidone initial content), and its concentration ranged from 0.5% of initial fenamidone content at 1 day to 25% at 70 days and later decreased until 17%. Acetophenone was detected from 1 day to 15 days of commercial product application at concentrations no higher than 2% of initial fenamidone content during the monitoring study. For loam soils at double dose for example, its concentration increased from 0.1% (1 day) to 1.9% (15 days).

In water, fenamidone dissipation was similar for both conditions, darkness and sunlight (Fig. S3). The fitting of SFO kinetic model (R^2) was approximately around 0.8, which can be explained as the last point of concentration was slightly deviated (Fig. S3). Similar values were obtained when comparing k values for both conditions at single dose, 0.011 $days^{-1}$ for sunlight conditions and 0.009 $days^{-1}$ for darkness conditions (Table 4). When DT_{50} values (Table 4) were compared (63 days for sunlight single dose and 73 days for darkness single dose), persistence in water was the same under sunlight and darkness conditions so fenamidone dissipation was not affected by the sun in this study and therefore, neither photodegradation nor photolysis affect the degradation of this pesticide. Fenamidone behaviour when two different doses were tested showed k values were slightly higher at double dose, 0.011 $days^{-1}$ in case of sunlight conditions at single dose and 0.013 $days^{-1}$ in case of sunlight conditions at double dose (Table 4). Comparing soils and water trials, fenamidone persistence in water was higher ($DT_{50} > 50$) in comparison with soil trials, where DT_{50} values were lower than 50 days.

Metabolite behaviour is completely different in water trials compared with soil studies. In soils, RPA-411639 and RPA-412708 were the main metabolites (Fig. 1) but in water, acetophenone was the principal metabolite detected at maximum percentage of the initial fenamidone content of 20% (Fig. 2). Other metabolites such as RPA-410193, RPA-412708, RPA-411639 and metabolite 264 *m/z* were detected at percentages lower than 0.5%. Acetophenone concentration increased after applying the commercial product (from 4% to 20% of initial fenamidone content in the case of sunlight conditions at single dose) but after 15–30 days, the concentration decreased until the end of the monitoring period (0.2% of initial fenamidone content). For example, for sunlight conditions at double dose, the acetophenone percentage, considering the initial fenamidone content, evolved from 0.8% (1 day) to 4% (30 days) and 0.3% (100 days). The behaviour of the other metabolites, RPA-410193, RPA-412708, RPA-411639 and 264 *m/z*, showed an increase from 1 day to 100 days. For instance, the percentage of RPA-412708 rose from 0.1% (1 day) to 1% (100 days) for sunlight conditions at double dose.

Comparing these results with previous studies focused on fenamidone, Chen et al. (2017) obtained fenamidone half-lives in soils between 35 and 43 days, similar to our results for sandy loam soils (DT_{50} ranged from 40 to 49 days). The half-life data provided by the University of Hertfordshire (University of Hertfordshire, 2007) ($DT_{50} = 6.9$ days) differs from these results but it is close to the data obtained for loam soils in this study (DT_{50} ranged from 13 to 18 days). In the case of fenamidone dissipation in water, results can only be compared with the data provided by the University of Hertfordshire which provided two fenamidone half-life values at pH 7 and 20 °C, one related to photolysis ($DT_{50} = 6$ days) and the other to hydrolysis ($DT_{50} = 411$ days). These results are different to those obtained in this study (DT_{50} ranged to 50–70 days) because, as indicated above, photolysis does not affect compound degradation in water samples and more studies are needed to compare these results.

In order to put our results into perspective, we can compare them with the potential risks for soil and water organisms, which have to be evaluated in terms of No Observed Effect Concentration (NOEC) and concentration causing 50% lethality (EC_{50}) (Aurisano et al., 2019). NOEC values at 21 days were 0.31 mg/L (fish) and 0.013 mg/L (aquatic invertebrates) in the case of aquatic organisms and 0.63 mg/kg at 14 days in earthworms (soil organism) (University of Hertfordshire, 2007).

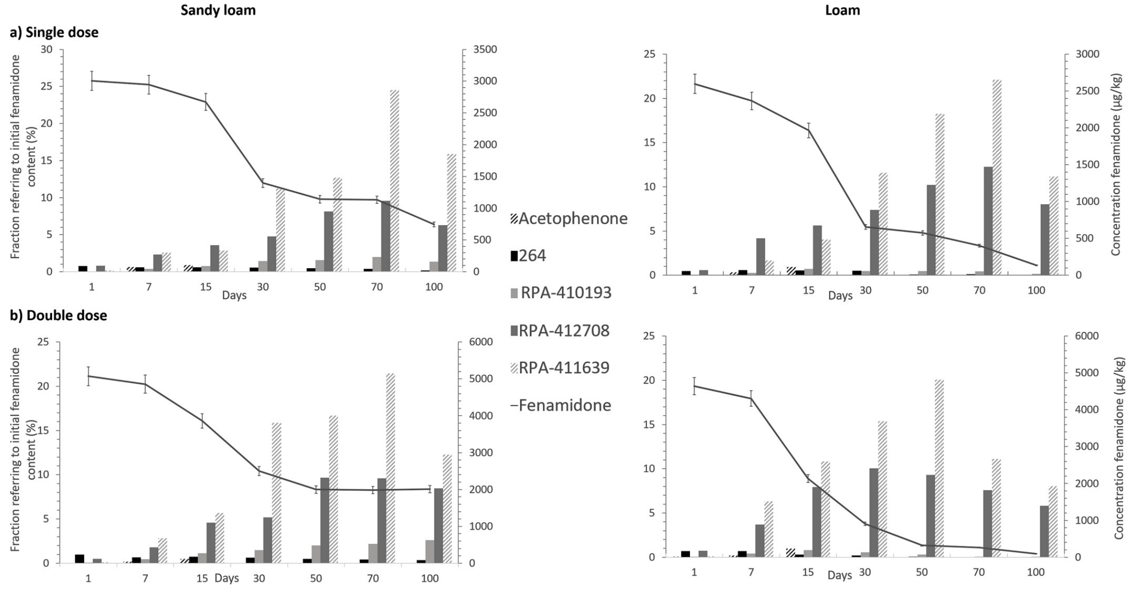


Fig. 1. Metabolite behaviour according to initial concentration of fenamidone for soils experiments at a) single dose and b) double dose. (Error bars obtained for n = 3).

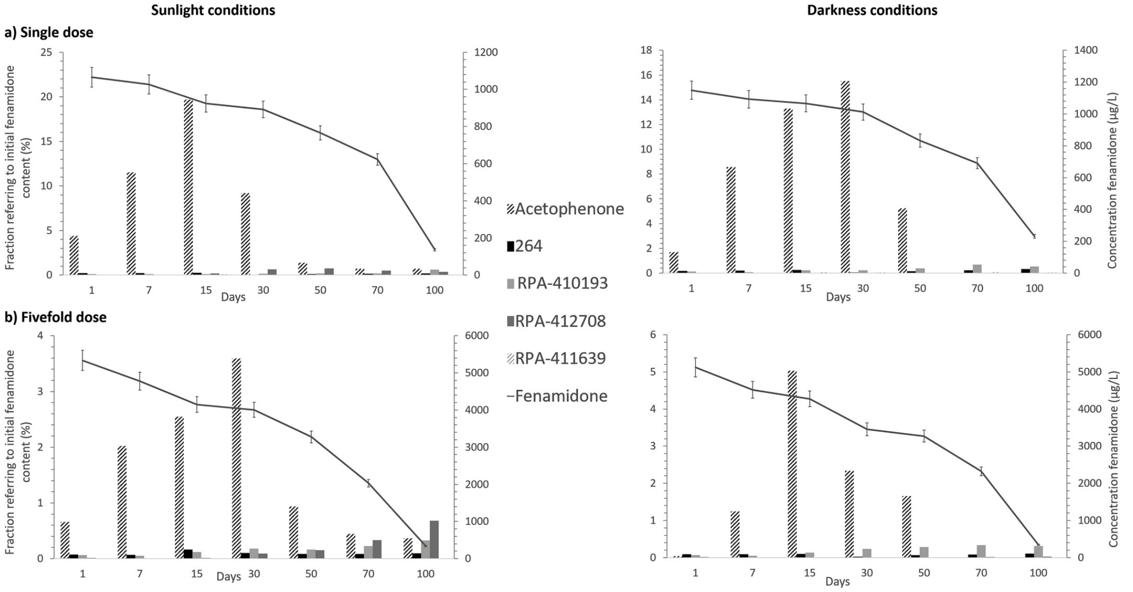


Fig. 2. Metabolite behaviour according to initial concentration of fenamidone for water experiments at a) single dose and b) fivefold dose. (Error bars obtained for n = 3).

These values are lower compared with the concentration values obtained in this study, 0.8 mg/L and 1.1 mg/L for sunlight and darkness conditions respectively (21 days), and 2.6 mg/kg and 2 mg/kg for sandy loam and loam soils (15 days). Considering these results, fenamidone could cause toxic effects in soil and water organisms due to its NOEC value and the concentrations found in environmental samples.

In the case of $EC_{50,}$ this parameter was set at 0.74 mg/L (fish) at 96 h and 0.19 mg/L (aquatic invertebrates) at 48 h and >25 mg/kg at 14 days in earthworms (University of Hertfordshire, 2007). For aquatic organisms, fenamidone can cause death whereas in the soil organism, the EC_{50} dose was considerably higher than the value obtained in each soil so it may not have any effect.

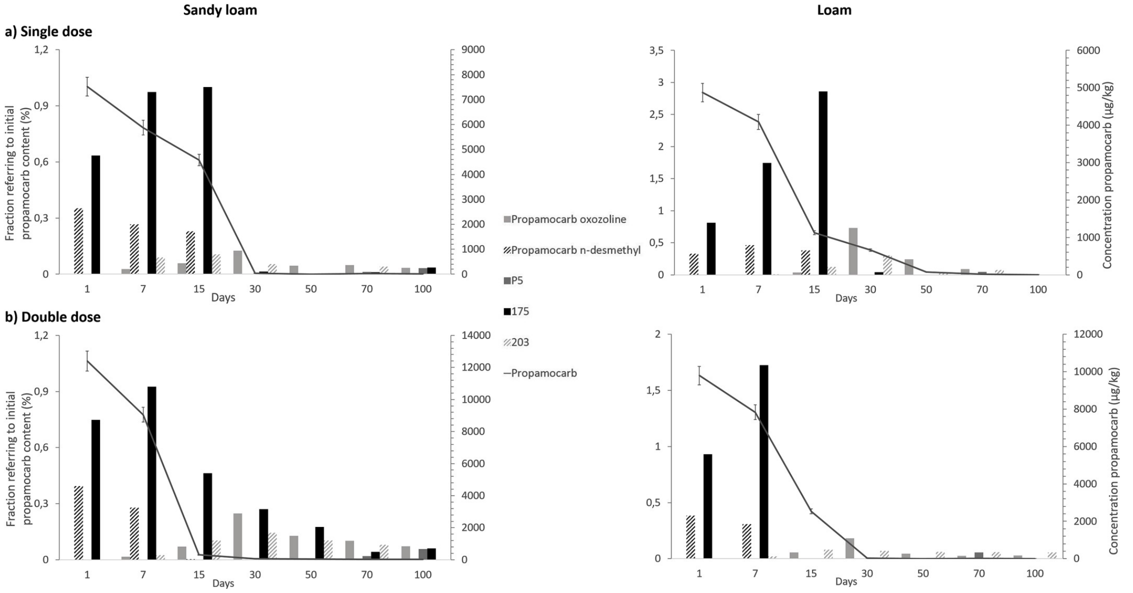


Fig. 3. Metabolite behaviour according to initial concentration of propamocarb for soils experiments at a) single dose and b) double dose. (Error bars obtained for n = 3).

3.3.2. Propamocarb study

Propamocarb dissipation in sandy loam and loam soils was similar (Fig. S4). The propamocarb concentration decreased during the monitored period as occurred with fenamidone. For example, in sandy loam soils double dose concentration dropped from 12,000 μg/kg (1 day) to 50 μg/kg (100 days). Degradation was not affected by the type of soil but it was by the application dose. In the case of double dose experiments, k value was twofold in relation to single dose (Table 4), so in double dose experiments degradation rate ($k = 0.11$ days^{-1}) was faster than in single dose ($k = 0.060$ days^{-1}) for sandy loam soils. Persistence (DT_{50}) was the same for both soils (Table 4) except when different doses were applied (Table 4). When the quantity of propamocarb was higher, dissipation was also higher (DT_{50} at single dose, 11.6 days, and 6.1 days at double dose). This behaviour was similar to results obtained from fenamidone in loam soils where the degradation rate was higher in the case of double dose experiments.

Propamocarb oxozoline, propamocarb n-desmethyl, P5, 175 *m/z* and 203 *m/z* metabolites were monitored in soils at values higher than propamocarb LOQ (Fig. 3). Their behaviour was the same in both soils/ doses except for metabolite 175 *m/z,* which showed different behaviour when different doses were evaluated. In sandy loam soils at single dose, its concentration rose until 15 days after commercial product application and then decreased. However, at double dose the increase was only observed until 7 days, after which it also dropped. The presence of propamocarb n-desmethyl decreased during the monitoring period. For sandy loam soils at double dose, its concentration decreased from 0.4% of initial propamocarb content to 0% at 30 days. It was also detected in the commercial product Consento® (Fig. S7). The other metabolites, P5, propamocarb oxozoline and 203 *m/z* increased in concentration while that of propamocarb lessened (from 0 to 30 days), after which they started decreasing until 100 days. For propamocarb oxoziline, the concentration went from 0% of initial propamocarb content at 1 day to 1% at 30 days and then decreased to 0.05% at 70 days in loam soils with single dose. The fraction of metabolites in relation to initial propamocarb content was not higher than 3% so the quantity of metabolites originated was not relevant when compared with the parent compound.

Propamocarb dissipation in water was similar in sunlight and darkness conditions (Fig. S5) but it was different when both doses were evaluated. When comparing the k value for both doses at sunlight conditions (Table 4), k value was 0.013 days^{-1} for single dose and 0.010 days^{-1} in the case of fivefold dose. Under darkness conditions, k value was 0.013 days^{-1} for single dose and 0.008 days^{-1} for double dose. It can be shown that persistence in water was slightly higher in the case of double dose. For example, for darkness conditions at single dose, DT_{50} was 52.5 days^{-1} and 86.6 days^{-1} at fivefold dose (Table 4). The same dissipation rate was obtained for sunlight and darkness conditions so the sun did not affect propamocarb degradation. Comparing these results with soil trials, propamocarb persistence in soil was lower ($DT_{50} < 12$) in comparison with water trials, where DT_{50} values were higher than 50.

Metabolite behaviour for propamocarb oxozoline, propamocarb n-desmethyl, P5, 175 *m/z* and 203 *m/z* were detected in both soil and water trials (Figs. 3 and 4) but it was different than in soils. In soils, metabolite 175 *m/z* was monitored at a higher percentage of the initial propamocarb content for all conditions. Propamocarb n-desmethyl was the main metabolite detected in water (0.7%). Propamocarb n-desmethyl behaviour content increased at the beginning and later decreased. The concentration of metabolite P5 increased during the monitored period in darkness conditions at single dose from 0% to 0.12% of initial propamocarb content. The other detected metabolites (propamocarb oxozoline, 175 *m/z* and 203 *m/z*) had similar behaviour to propamocarb n-desmethyl but their concentrations were very low compared with propamocarb n-desmethyl. The total percentage of metabolites referring to the initial propamocarb content was lower than 1% during the whole monitored period so metabolite behaviour was not very significant.

Data obtained for propamocarb dissipation can be compared with other studies such as Chen et al. (2017) where persistence obtained for soil trials are similar to our results (DT_{50} lower than 30 days). In addition, the University of Hertfordshire (University of Hertfordshire, 2007) gives similar values of DT_{50} for propamocarb in soils ($DT_{50} = 14$ days), whereas results in water have not been studied previously.

NOEC values at 21 days was >6.3 mg/L (fish) and 12.3 mg/L

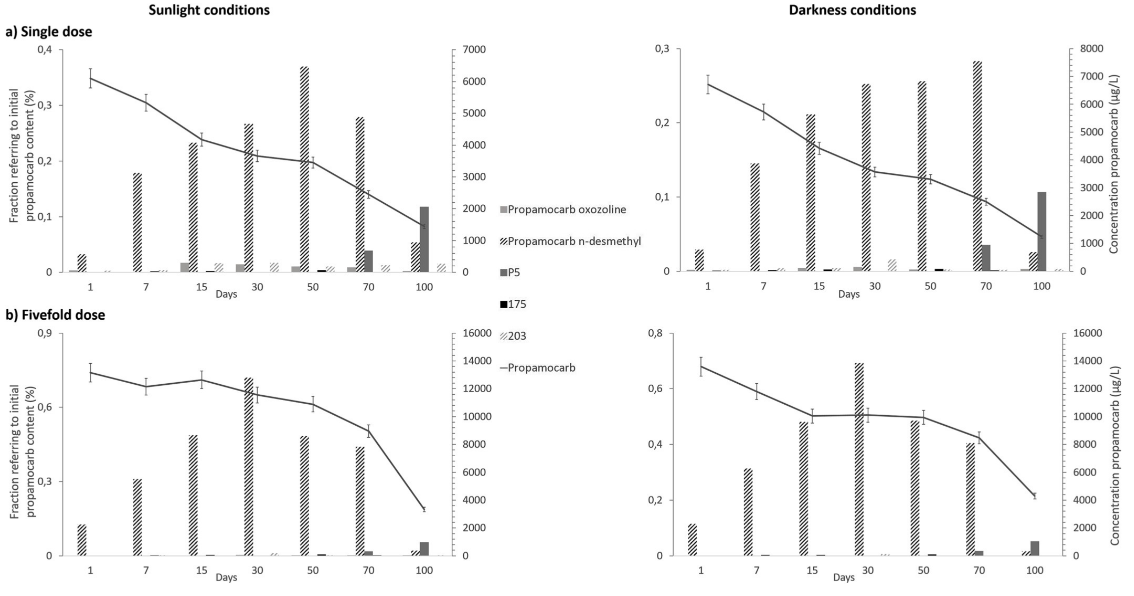


Fig. 4. Metabolite behaviour according to initial concentration of propamocarb for water experiments at a) single dose and b) fivefold dose. (Error bars obtained for n = 3).

(aquatic invertebrates) in the case of aquatic organisms whereas it was set at 362 mg/kg at 14 days in earthworms (soil organism). EC_{50} values were >99 mg/L (fish) at 96 h and >100 mg/L (aquatic invertebrates) at 48 h and >660 mg/kg at 14 days in earthworms (University of Hertfordshire, 2007). These values were compared with data obtained from dissipation studies. After 21 days of treatment the parent compound concentration was 3.8 mg/L and 4.0 mg/L for sunlight and darkness conditions respectively and 4.5 mg/kg and 1.2 mg/kg for sandy loam and loam soils (15 days). We can therefore conclude that propamocarb may not cause any danger to soil and aquatic organism because NOEC and EC_{50} values were higher than residues obtained in dissipation studies.

3.4. *Unknown analysis*

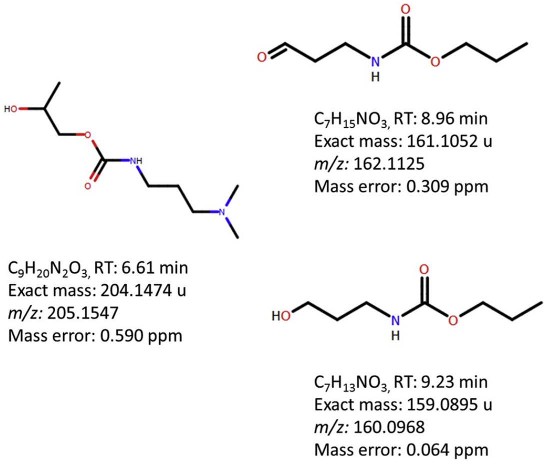
For the tentative identification of unknown metabolites, samples were sorted according to a number of previously studied parameters (Lopez-Ruiz et al., 2019a) such as days following the application of the plant protection product, parent compound, dosage and matrix. Sixteen groups were studied, eight for each analyte (propamocarb and fenamidone): sandy loam (single and double dose), loam (single and double dose), sunlight conditions (single and fivefold dose) and darkness conditions (single and fivefold dose). Workflow of two software (Compound Discoverer® and MassChemSite®) tools was set up and the parent compound structures were provided. Then, the software tools searched metabolic pathways from several transformation pathways like, oxidation, sulfonation, desaturation, methylation, S-dealkylation, dehydration and reduction.

Raw data was studied according to previously optimized values such as retention time, mass error, subtracting background and signal intensity and variation over the days (Lopez-Ruiz et al., 2019c). Compound Discoverer® was first used to obtain all possible compounds and then MassChemSite® was used to check the structure of new metabolites. The software compares the ions from the spectrum and searches for common ions with the parent molecule in order to corroborate if it comes from the same origin, bearing in mind that the parent molecule usually has common ions (fragments) with related molecules such as metabolites.

Using this strategy, three new propamocarb metabolites were elucidated with these tools in water and one of them was also detected in soils (Fig. 5). Tentative metabolites, using Schymanski classification (Schymanski et al., 2014), achieved level 2 according to this classification, which was associated with the data provided by the fragmentation in the elucidation of compound and exact mass. Figures S8, S9 and S10 display extracted ion chromatograms as well as experimental and theoretical spectrum of the putatively detected metabolites.

Propamocarb metabolites were generated by simple reactions. Metabolite 160 *m/z* ($C_7H_{13}NO_3$) originated from the fracture of a carbon-nitrogen union (-C_2H_6N) followed by the oxygenation of the carbon (+OH). This compound eluted at 9.23 min and the mass error was 0.064 ppm. Metabolite 162 *m/z* ($C_7H_{15}NO_3$) was monitored at 8.96 min (mass error 0.309 ppm) which can be explained because of the fracture of a carbon-nitrogen union (-C_2H_6N) succeeded by reduction of the carbon (C=O). Metabolite 205 *m/z* ($C_9H_{20}N_2O_3$) was formed by the oxygenation of a secondary carbon (+OH). It was monitored with a mass error of 0.590 ppm at 6.61 min.

These metabolites were studied in all the samples, monitoring their behaviour during the monitored period. The concentration was tested showing the fraction referring to initial propamocarb content in Fig. S11 for soils and S12 for water. Metabolite 160 *m/z* was only monitored in water samples and its concentration increased up until 50 days and later declined for both conditions/doses. In the case of sunlight conditions at single dose, its fraction evolved from 0.01% (1 day) to 0.04% (50 days) and to 0.012% (100 days). Metabolite 162 *m/z* was only monitored in water and it was detected 1 day after applying the plant protection product and then the amount decreased until 30 days, when the fraction in relation to the initial propamocarb content became negligible. Finally, metabolite 205 *m/z* was monitored in soil and water trials and it was the putative metabolite detected at the highest concentration. In soils, its concentration increased up until 7 days and later decreased quickly. For sandy loam single dose, its fraction referring to initial propamocarb content increased from 1% (0 days) to 7% (7 days) and became negligible at 70 days. On the other hand, in water its concentration increased up until 30 days (0.08% for sunlight single dose) and later decreased (0.02% for sunlight single dose).


$C_7H_{15}NO_3$, RT: 8.96 min
Exact mass: 161.1052 u
m/z: 162.1125
Mass error: 0.309 ppm

$C_9H_{20}N_2O_3$, RT: 6.61 min
Exact mass: 204.1474 u
m/z: 205.1547
Mass error: 0.590 ppm

$C_7H_{13}NO_3$, RT: 9.23 min
Exact mass: 159.0895 u
m/z: 160.0968
Mass error: 0.064 ppm

Fig. 5. New metabolites putatively elucidated using software tools.

4. Conclusions

Fenamidone and propamocarb dissipation was first studied in environmental samples using liquid chromatography coupled to high resolution mass spectrometry. Propamocarb persistence was low in soils (DT_{50} values lower than 12 days) whereas for fenamidone it was low to medium (DT_{50} values lower than 50 days). In water, persistence was high for both compounds because DT_{50} values were higher than 50 days and dissipation was the same under sunlight and darkness conditions. Fenamidone and propamocarb metabolites were monitored during this study. Fenamidone metabolites were detected at a maximum fraction to initial fenamidone content of 25% and propamocarb at a maximum percentage of 3% of initial propamocarb content. In the case of fenamidone, concentrations of the main metabolites were considerably higher compared to the parent compound so toxicity studies have to be performed in order to evaluate the possible risk to flora and fauna. In addition, unknown analysis was performed and three new propamocarb metabolites were putatively detected in soil and water samples. To conclude, a comprehensive evaluation of fenamidone and propamocarb dissipation in environmental samples, including their metabolites, has been provided.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2019.109818.

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CHAPTER 6

Comprehensive discussion

1. INTRODUCTION

Nowadays, despite of the development of organic farming, phytosanitary products or pesticides are widely used in fruits and vegetables growing. Its application represents one of the most important types of protection against pest. After the application of pesticides, they can remain in fruits and vegetables as residues and they can be incorporated into the food chain. Thus, pesticide residues must be at low concentrations to avoid harm in humans and in environment.

Knowing the behaviour of a pesticide after its application is a task of great interest. All the information generated in pesticide degradation studies is crucial to know the residual amounts of the active ingredients and to evaluate the environmental fate of them. For this reason, in this Thesis, the study of the behaviour of pesticides and their transformation into metabolites was the main concern. Analytical methodology based on HRMS, working in *full scan* mode was used because it is able to identify an "unlimited" number of compounds in the samples. It is a very useful tool for the detection of non-targeted compounds, allowing the development of suspect screening and elucidation of the structure of new metabolites by unknown studies.

2. MASS SPECTROMETRY TRENDS IN FOOD AND ENVIRONMENTAL SAFETY

In order to achieve the objectives of the current Thesis, a review of the recent analytical advances of the last decade in the field of UHPLC-MS was carried out (**Publication I**). Advantages and disadvantages between HPLC and UHPLC were discussed, being the main advantages of UHPLC the resolution capacity and the reduction of the analysis time. In addition, LRMS and HRMS applications were described. In the case of UHPLC-LRMS, the main applications were food safety, environment, metabolomics and lipodomics, food quality and biomedical studies. In UHPLC-HRMS, several working modes as screening analysis, target analysis and unknown analysis can be performed (**Figure** 6.**1**).

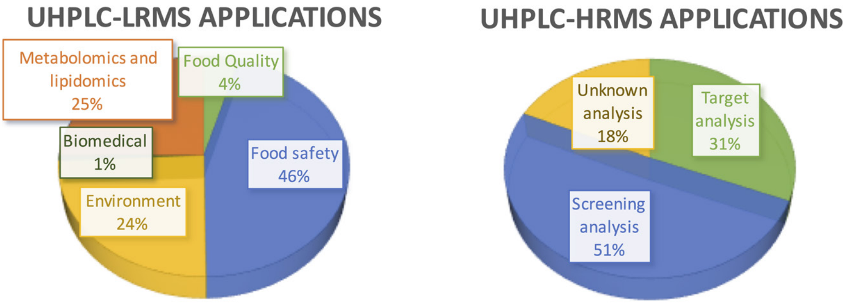


Figure 6.1. Main applications in UHPLC-LRMS and UHPLC-HRMS classified by fields of application. Copyrights with Permission of Elsevier.

Triple quadrupole (QqQ) or QTrap are the most widely used analysers in UHPLC-LRMS, being the main applications food safety, where pesticide residue analysis is the most important issue, especially in multiresidue methods, when a wide range of compounds are simultaneously analysed. The introduction of UHPLC was really important. Using these columns (particle size lower to 2 µm), analysis time was reduced by half for the same number of compounds, and peak shape and resolution between peaks were improved. Despite the fact that reversed phase was commonly used as stationary phase in UHPLC, other phases as chiral stationary phases, size-exclusion chromatography (SEC), ion-exchange chromatography (IEX) and hydrophilic interaction chromatography (HILIC) have been used and UHPLC improved the efficiency of the elution of the compounds in comparison with HPLC.

UHPLC-HRMS is a perfect combination between the ultra-high capacity of LC and the high mass resolution of MS, and the types of applications are different compared with LRMS. This can be explained because in HRMS, targeted analysis was in the background and other types of analysis as screening or unknown analyses are commonly performed. In that, the term metabolomics was introduced and the possibility to prepare another review that describes the last trends in metabolomics approaches for contaminant analysis in food came up (**Publication II**). Sample treatment, separation and detection modes, data acquisition and analysis were discussed. In this field, the description of a workflow was essential to follow a metabolomics scheme (**Figure 6.2.**).

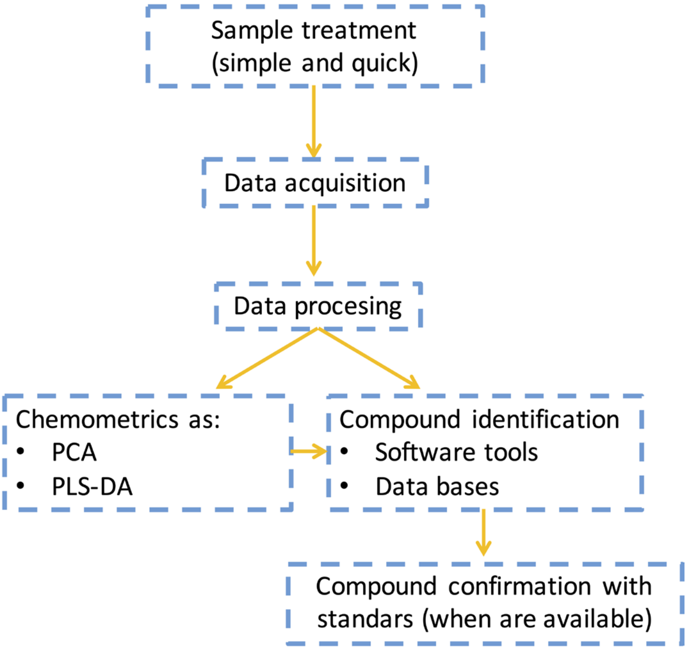


Figure 6.2. Typical workflow for food metabolomics analysis.

Main applications in this field were based on the use of non-targeted analysis mode, using databases (suspect screening) or different software tools (unknown analysis), but many of them employed chemometrics for a better compound identification. **Table 6.1** shows the main applications in metabolomics analysis. Pesticides were the main analytes, although in some cases metabolites have also been included. As sample treatment, the quality of the extraction is quick, simple and easy, where the majority of compounds were extracted from the sample. In this term, QuEChERS method for solid samples and "dilute and shoot" or SPE for liquid samples were widely used.

In relation to the separation and detection techniques, LC was the separation one most widely used, specially UHPLC. GC was also used but only in targeted mode. HRMS detection technique was used in metabolomics, utilizing Orbitrap or Q-TOF analysers but in addition, LRMS, using the QqQ analyser was also applied in targeted mode.

In data analysis step, chemometrics followed by compound identification were used. Chemometrics involves statistical methods to distinguish groups of samples using PCA or PLS-DA. The next step in data analysis was compound identification, an important issue in metabolomics analysis. In some cases, it has been performed after chemometrics stage, but when chemometric methods do not provided good results, compound identification was carried out using raw data. This stage involved the use of software tools as Compound Discoverer®, MassChemSite® or Metaboscape®. They can provide possible formulas from the parent molecule structure or compare the experimental full-MS and MS/MS spectra with the theoretical ones from different spectral databases, as *m/z* Cloud, Metlin, Human Metabolome Database (HMDB) or *m/z* vault.

Table 6.1. Main applications in metabolomics for the analysis of organic compounds in food.[a]

Analytes	***Matrices***	***Sample treatment***	***Separation and detection***	***Analysis mode***	***Data treatment***
Dimethenamid, saflufenacil and metabolites	Maize	QuEChERS	UHPLC-QqQ-MS	Targeted	Compound detection
Sulfonamides and metabolites	Baby food	QuEChERS	UHPLC-Orbitrap-MS	Targeted	Compound detection
Pesticides, antibiotics and mycotoxins	Bakery products	QuEChERS	UHPLC-Orbitrap-MS	Non-targeted	Chemometrics (PCA)
Pesticides and mycotoxins	Orange juice	Dilute and shoot	UHPLC-TOF-MS	Non-targeted	Chemometrics (PCA)
Pesticides	Honey	QuEChERS	LC-QTRAP-MS GC-QqQ-MS	Targeted	Compound detection
Fenamidone and propamocarb and metabolites	Cucumber, tomato and courgettes	QuEChERS	UHPLC-Orbitrap-MS	Targeted and non-targeted	Compound identification
Deoxyvalenol and metabolites	Crackers, biscuits and bread	Solid-liquid	UHPLC-Q-Orbitrap-MS	Targeted and non-targeted	^{13}C labelling study

Analytes	***Matrices***	***Sample treatment***	***Separation and detection***	***Analysis mode***	***Data treatment***
Carboxin and metabolites	Penauts	QuEChERS	UHPLC-QqQ-MS	Targeted	Compound detection
Pesticides	Baby food	QuEChERS Dilute and shoot	UHPLC-Q-Orbitrap-MS	Non-targeted	Compound identification
Pesticides, antibiotics and mycotoxins	Honey	Dilute and shoot	UHPLC-Orbitrap-MS	Non-targeted	Chemometrics (PCA and PLS-DA)
Contaminants	Infant formula	SPE	UHPLC-TOF-MS	Non-targeted	Chemometrics (PCA and PLS-DA)
Contaminants	Tea	QuEChERS Dilute and shoot	UHPLC-Q-TOF-MS	Non-targeted	Chemometrics (PCA and ICA)
Contaminants	Fish	No data	NMR	Non-targeted	Chemometrics (PCA and PLS-DA)

[a] Abbreviations: GC, gas chromatography; HRMS, high resolution mass spectrometry; ICA: independent component analysis; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; PCA, principal component analysis; PLS-DA, partial least square discriminant analysis; QqQ, triple quadrupole; SPE, solid phase extraction; TOF, time of flight; UHPLC, ultrahigh-performance liquid chromatography.

3. INSECTICIDE DISSIPATION STUDIES AND IDENTIFICATION OF THEIR METABOLITES IN FOOD MATRICES

Dissipation studies of insecticides were developed to evaluate their presence in fruits and vegetables. Their metabolites were also studied. To achieve these goals analytical methods were developed to evaluate two pesticides and their metabolites: flonicamid and its metabolites (TFNA, TFNG and TFNA-AM), in bell pepper (**Publication III**) and in orange (**Publication IV**), and thiocyclam and its metabolite (nereistoxin) in tomato (**Publication V**).

3.1. EXTRACTION TECHNIQUES

The extraction procedure is one of the most important step to determine as many compounds as possible with the best sensitivity. In the case of pesticides and their metabolites, the selected method has to extract compounds with different polarities, due to the fact that metabolites are sometimes small molecules with high polarity in comparison with the precursor compound. In the current Thesis, QuEChERS was the extraction method applied, but some modifications were performed in order to improve the extraction of metabolites. In flonicamid and metabolite studies (**Publication III** and **IV)**, original QuEChERS was employed, using acidified extraction solvent (1% formic acid), in order to improve the analytes extraction. In addition, and due to precision values were higher (>25%), a homogenization step with polytron was added, improving the precision values considerably.

Cleaning sorbents were also evaluated by d-SPE step in **Publication III**, but finally they were not necessary due to matrix (bell peper) effect was not significant. Moreover, in **Publication IV**, flonicamid and TFNA have matrix (orange) suppression, and the addition of PSA reduced significatively this effect. **Figure 6.3** summarizes the extraction methods used.

In the thiocyclam and its metabolite study (**Publication V)**, QuEChERS-based extraction was also used, but in this case, the citrate buffered version was the best option. However, recovery obtained for nereistoxin was not acceptable (49%) and therefore, the addition of acid in the extraction solvent was necessary. The acidification of the extraction solvent with 1% acetic acid was tested improving the recoveries of nereistoxin to 71% but reducing recoveries of thiocyclam till 65%. To solve that, another technique as sonication step was tested, improving the migration of the compounds to the extraction solvent. Finally, this was added to the final extraction method developed for thiocyclam and nereistoxin (**Figure 6.3**).

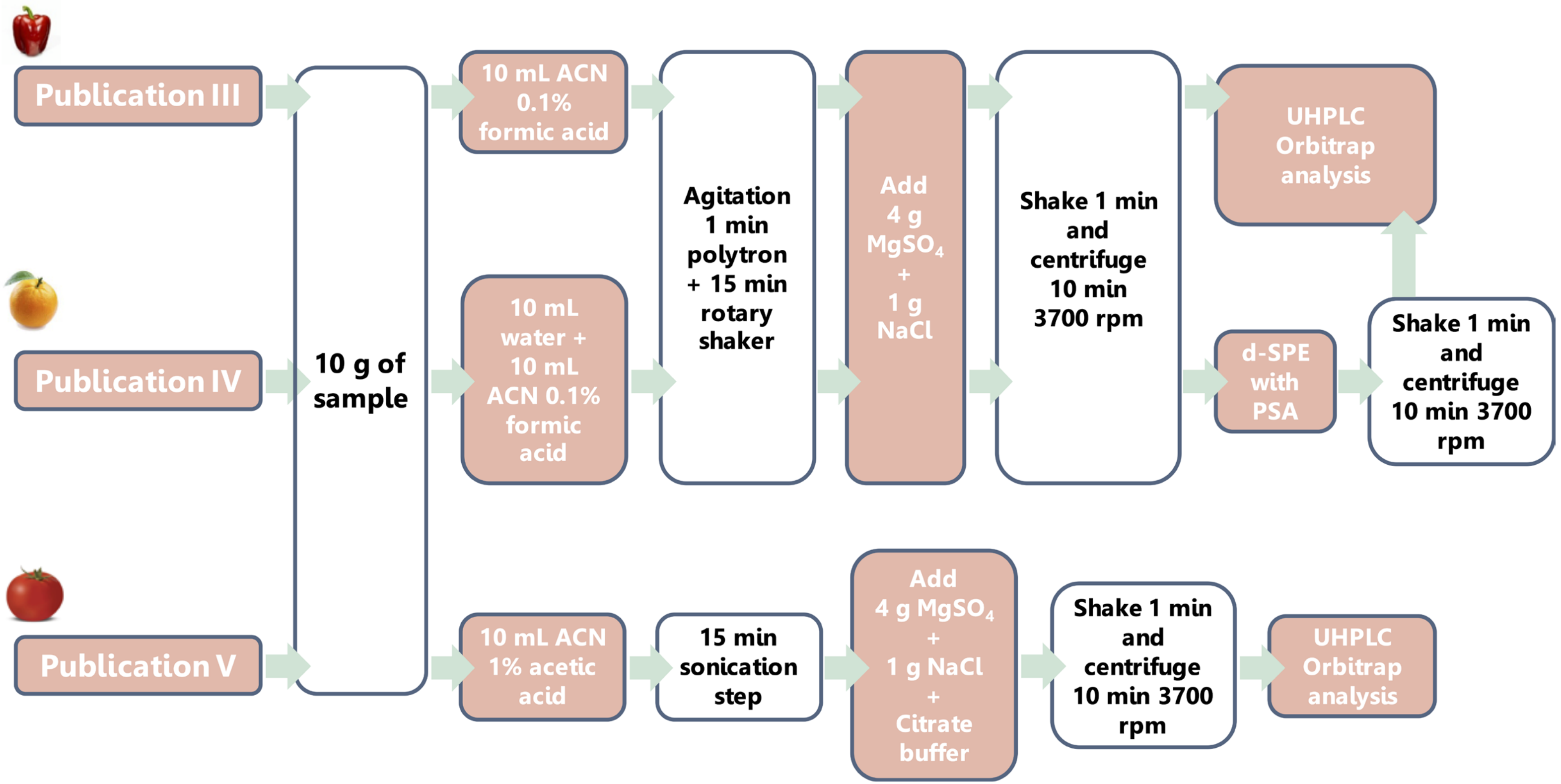


Figure 6.3. Scheme of the different extraction methods developed in Publication III, IV and V.

3.2. LIQUID CHROMATOGRAPHY - HIGH RESOLUTION MASS SPECTROMETRY

For insecticides and their specific metabolites separation, LC was the technique employed. In the case of flonicamid and its metabolites (**Publications III and IV**), stationary phase used was the described in bibliography (C_{18}), but the composition of the mobile phase was tested in order to improve the peak shape of one metabolite (TFNA). In this case, organic phase was evaluated, testing different solvents as methanol, acetonitrile and different mixtures of both solvents. The best results were obtained when a mixture of methanol:acetonitrile 80:20 (*v/v*) containing formic acid (0.1% *v/v*) was used. In thiocyclam study (**Publication V)**, several stationary phases were tested, as C_{18} (Zorbax Eclipse plus C_{18} and Hypersil gold aQ) and HILIC (Zorbax Hilic Plus). Thiocyclam and nereistoxin eluted quickly when C_{18} columns were tested, and when HILIC stationary phase was evaluated, peak shape and separation were not suitable. The coupling of both columns was tested, taking advantage of C_{18} columns (separation and peak shape) and HILIC column (retention time of polar compounds) properties, obtaining good results for thiocyclam and nereistoxin (**Figure 6.4**).

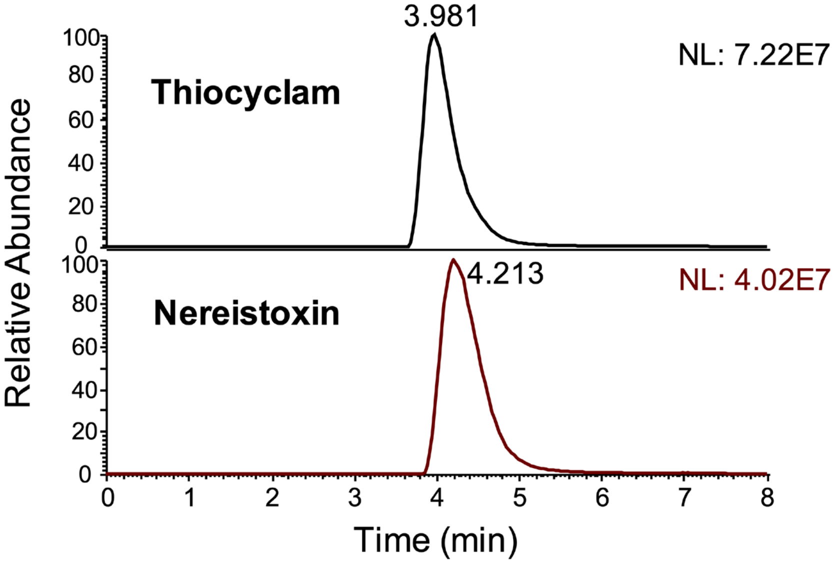


Figure 6.4. Extracted ion chromatogram of thiocyclam and nereistoxin in solvent (0.5 mg/L).

Finally, gradient elution was optimized. In **Publication III and IV** three different gradients were evaluated, modifying the percentage of water phase/organic phase used at the beginning. The best results were obtained when it started at 95% of aqueous phase (**Figure 6.5**).

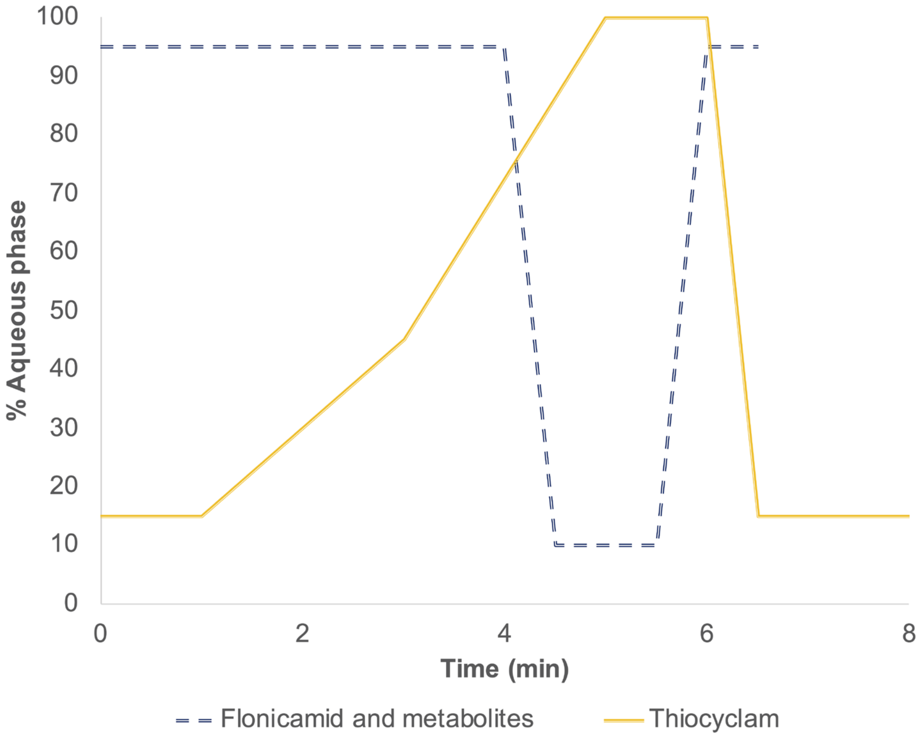


Figure 6.5. Gradient elution used in different publications (Publication III, IV and V)

On the other hand, for **Publication V,** in order to minimize potential matrix effect, the gradient profile was modified despite longer retention times could be achieved. The increase of the percentage of organic phase from 40% to 65% in the second step managed to delay the elution time of analytes and minimized the matrix effect (**Figure 6.5**). **Table 6.2** shows a summary of the chromatographic conditions for insecticides.

Table 6.2. Summary of LC conditions for the separation of insecticides and its metabolites.

LC Conditions	*Publication III and IV*	*Publication V*
Aqueous phase	Water 0.1% formic acid	Water 0.1% formic acid
Organic phase	Methanol:acetonitrile (80:20 *v/v*) containing 0.1% formic acid	Acetonitrile
Columns	Zorbax Eclipse Plus C_{18} (100 mm × 2.1 mm, 1.8 µm)	Zorbax Eclipse Plus C_{18} (100 mm × 2.1 mm, 1.8 µm) + Zorbax Hilic Plus (100 mm × 2.1 mm, 3.5 µm)
Column temperature	25°C	25°C
Injection volume	10 µL	10 µL
Flow rate	0.2 mL/min	0.2 mL/min
Runing time	6.5 min	8.0 min

In relation to the detection, HRMS was the technique used for the identification and determination of insecticides and their specific metabolites, and Orbitrap (Exactive) was the selected analyser.

Characterization of the pesticides and their metabolites was carried out following the same procedure in all cases. Thus, it was explained for the first time in this section, and it was the same for all pesticides studied in this Thesis. First, an intermediate solution of analytes was injected into the UHPLC-Orbitrap-MS system. The protonated or deprotonated ion of each compound was monitored according to its molecular formula, and the exact mass was selected with the criterion that mass error was lower than 5 ppm for the targeted analytes (**Figure 6.6**). Fragments of each analyte were assigned studying the spectra acquired after applying a fragmentation step by higher-energy collisional dissociation (HCD) in All-Ion-Fragmentation (AIF) mode. Software tools, as Mass Frontier™, were also used. This software used the chemical structure of the compound and determined the fragmentation mechanisms and the corresponding fragment ions, with their corresponding exact masses (**Figure 6.6**). In addition, another criterion was the comparison of MS and HCD (AIF) spectra at the retention time of the targeted compound, in order to determine ions (in the HCD spectra) at the same retention time of the target analyte and they were detected with mass error lower than 10 ppm.

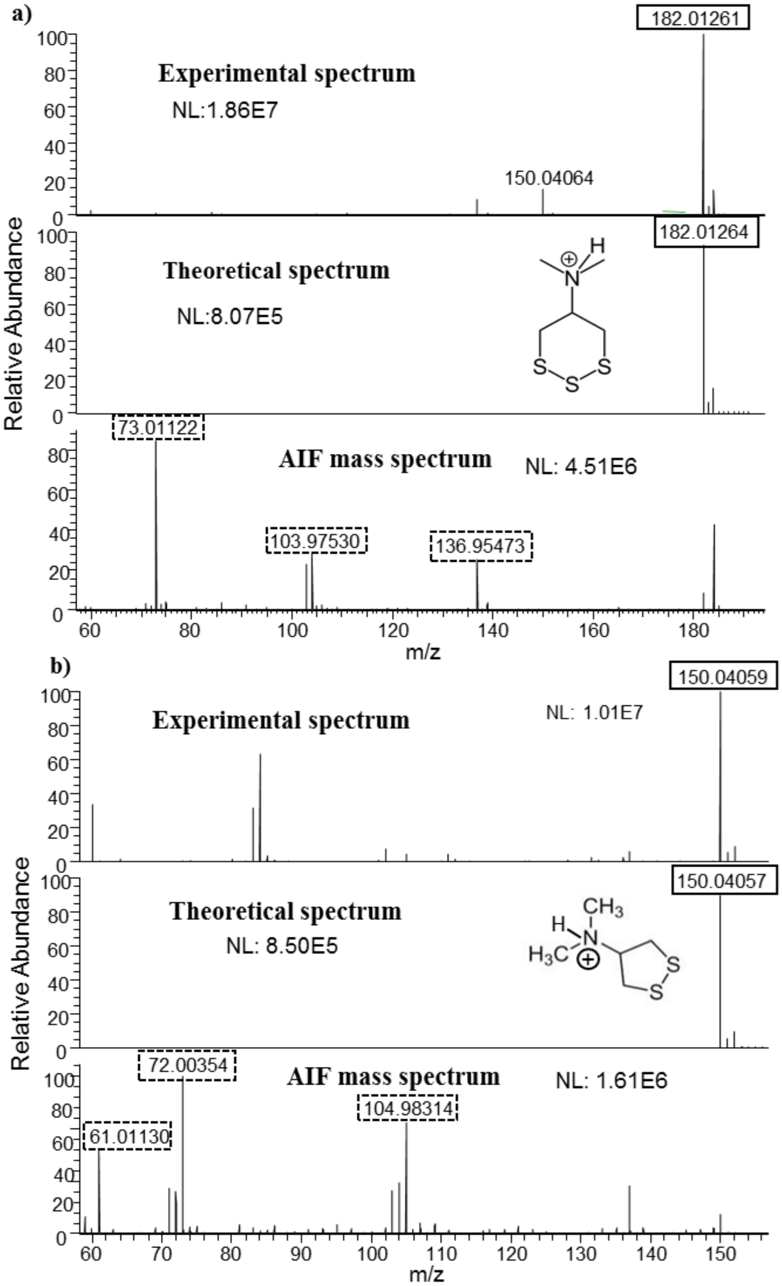


Figure 6.6. Experimental, theoretical and AIF mass spectra of (a) thiocyclam and (b) nereistoxin (at 1 mg/L).

Table 6.3 shows the HRMS conditions developed for flonicamid and its metabolites and thiocyclam and its metabolites respectively, observing that flonicamid and its metabolites have common fragments, $[C_7H_5ONF_3]^+$ and $[C_6H_5NF_3]^+$.

a)	***Precursor ion***		***Fragments***			
Compound	***Exact mass (m/z)***	***Mass error (ppm)***	***Exact mass (m/z)***	***Molecular formula***	***Mass error (ppm)***	***RT (min)***
Flonicamid	230.05357	-0.01	203.04267	$[C_8H_6ON_2F_3]^+$	-2.6	4.61
			174.01612	$[C_7H_3ONF_3]^+$	-0.1	
			148.03686	$[C_6H_5NF_3]^+$	-1.0	
TFNA	192.02669	0.01	174.01612	$[C_7H_3ONF_3]^+$	-1.8	4.58
			148.03686	$[C_6H_5NF_3]^+$	-0.7	
TFNG	249.04815	-0.01	203.04267	$[C_8H_6ON_2F_3]^+$	-0.5	4.48
			174.01612	$[C_7H_5ONF_3]^+$	-0.9	
			148.03686	$[C_6H_5NF_3]^+$	-1.5	
TFNA-AM	191.04267	-0.02	174.01612	$[C_7H_5ONF_3]^+$	3.9	4.09
			148.03686	$[C_6H_5NF_3]^+$	-0.3	

Table 6.3. HRMS parameters used for identification of a) flonicamid and its metabolites, and b) thiocyclam and nereistoxin.

b)	***Precursor ion***		***Fragments***			
Compound	***Exact mass (m/z)***	***Mass error (ppm)***	***Exact mass (m/z)***	***Molecular formula***	***Mass error (ppm)***	***RT (min)***
Thiocyclam	182.01264	-0.42	136.95471	$[C_3H_5S_3]^+$	-2.6	3.98
			103.97530	$[C_3H_3S_2]^+$	-2.9	
			73.01122	$[C_3H_5S]^+$	-3.1	
Nereistoxin	150.04057	-0.02	104.98338	$[C_3H_5S_2]^+$	2.1	4.21
			72.00354	$[C_2H_5S_2]^+$	2.9	
			61.01140	$[C_3H_3S]^+$	-3.4	

3.3. VALIDATION PROCEDURE

The validation of the analytical methods for insecticides in fruits and vegetables was carried out according to SANTE guidelines calculating the following parameters: matrix effect, linearity and working range, trueness (% recovery), precision (intra- and inter-day), LOQs and LODs. **Table 6.4** shows a summary of the validation results.

Table 6.4. Summary of the validation results in Publications III, IV and V.

Validation parameters	*Flonicamid and metabolites*	*Thiocyclam and metabolite*
Matrix effect[a]	-38% to 47%	19 to 27%
Linearity (R^2)	> 0.9930	> 0.9986
Trueness[b] *(%)*	74-102	72-82
Interday precision[b] *(%)*	2-17	11-15
Intraday precision[b] *(%)*	4-19	8-14
LODs//LOQs (µg/kg)	1-6//10-30	-//10

[a] Equation used: $Matrix\ effect = \left(\frac{slope\ in\ matrix}{slope\ in\ solvent} - 1 \right) x100\%$

[b] Concentrations tested: LOQs and 100 µg/kg; n=5

The matrix effect was investigated by analysing standards in solvent and standards prepared in an extracted blank matrix. For **Publication III and IV**, the calibration curves were obtained for different concentrations from 10 to 300 µg/kg; in the case of flonicamid and TFNG the calibration curve started at 10 µg/kg, meanwhile for TFNA it started at 20 µg/kg, and for TFNA-AM at 30 µg/kg. Values ranged from -38% to 47% (**Table 6.4**), so both suppression and enhancement effects were observed. In **Publication V**, matrix effect revealed matrix enhancement (values were from 19 to 27%).

Linearity was studied using matrix-matched calibration, testing the same levels as those utilized previously to evaluate the matrix effect. Linearity was checked by the determination coefficients (R^2). Flonicamid and its metabolites had linearity values higher than 0.9930 and thiocyclam and its metabolite higher than 0.9986.

Trueness (% recovery) and precision (intra and interday, % RSD) were established across the specified range of the analytical procedure by analysing spiked samples with known amounts of the compounds (2 concentrations/5 replicates each); intraday precision was studied in the same day and interday precision in ten different days. In the case of trueness, it ranged from 74-102% for flonicamid and its metabolites, and for thiocyclam and its metabolite, it was from 72-82%. Interday and intraday precision, in **Publication III and IV** ranged from 2-17% and 4-19% respectively, while in **Publication V**, between 11-15% and 8-14%, respectively.

LODs and LOQs were estimated by injecting spiked blank samples at low levels. LODs were assigned as the minimum concentration at which the characteristic ion is monitored with a mass error lower than 5 ppm. For the estimation of LOQ, in addition to the characteristic ion, one fragment should be monitored at the same retention time and chromatographic shape than the characteristic one, being the mass error lower than 10 ppm. In addition, the recovery and precision at LOQ should be within 70–120% and lower than 20% respectively. The LOD and LOQ values for flonicamid and its metabolites were 1-6 µg/kg (LODs) and 10-30 µg/kg (LOQs), meanwhile from thiocyclam and its metabolite, LOQs were set at 10 µg/kg.

3.4. DISSIPATION STUDIES

3.4.1. Flonicamid studies

In the current Thesis, the main purpose was to know the behaviour of insecticides in fruits and vegetables. For that, studies were carried out in order to monitor insecticide residues as well as their metabolites. Similar procedure was developed for all pesticides of the current Thesis. Firstly, a plant protection product of the selected insecticide was applied to fruits or vegetables, directly to the plant by foliar application (field studies) or through injection in the fruit with a syringe (laboratory studies). Two doses were applied, one at normal (recommended dose established by the manufacturer) and the other at a higher concentration, in order to observe the metabolites which not appeared when the pesticides were applied at a lower dose. Thus, vegetables and fruits were randomly collected, in a minimum period of 30 days. Then, the samples were analysed and pesticide residues and their metabolites were monitored. Flonicamid was studied in bell pepper (**Publication III**), and a commercial product of flonicamid (Teppeki®) was applied in bell pepper at dose of 0.1 g per hectare (two applications). The samples were randomly collected at least 10 days after the second application. For these studies, samples were applied and collected by the manufacturer, and later they were analysed by our research group. For this reason, data about the days sample was collected, was not provided.

Results of **Publication III** are shown in **Table 6.5**. Flonicamid was detected in seven samples at concentrations ranging from 11 to 98 µg/kg. In relation to the metabolites, TFNA was detected in only one sample at 20 µg/kg and TFNG was found in all the positive flonicamid samples at concentrations from 20 to 62 µg/kg. Moreover, TFNG was also detected in the other three samples where flonicamid was not detected at concentrations from 13 to 34 µg/kg. These results indicated the necessity of including all flonicamid metabolites in routine analysis, as some laboratories done, in order to fulfil the MRL definition of this compound, that includes only two metabolites of flonicamid (TFNA and TFNG).

Table 6.5. Concentration in µg/kg of flonicamid and its metabolites in bell pepper samples

Sample	*Flonicamid*	*TFNA*	*TFNG*	*Flonicamid sum*[a]	*TFNA-AM*
1	98.2	20.1	58.0	175.9	<LOQ
2	12.6	<LOQ	19.8	30.9	<LOD
3	<LOD	<LOQ	<LOD	<LOQ	<LOD
4	<LOD	<LOD	22.2	20.5	<LOD
5	<LOD	<LOD	34.0	31.4	<LOD
6	97.7	<LOD	42.2	136.7	<LOQ
7	<LOD	<LOD	<LOQ	<LOQ	<LOD
8	<LOD	<LOQ	<LOQ	<LOQ	<LOD
9	11.5	<LOD	61.9	68.7	<LOQ
10	<LOD	<LOD	12.7	11.8	<LOD

[a] $\mathit{Flonicamid\ sum} = 1C_F + \frac{Mm_F}{Mm_{TFNA}} C_{TFNA} + \frac{Mm_F}{Mm_{TFNG}} C_{TFNG}$

C_F, C_{TFNA} and C_{TFNG} is the concentration of flonicamid, TFNA and TFNG, and Mm_F, Mm_{TFNA} and Mm_{TFNG} show molecular mass of flonicamid, TFNA and TFNG.

In **Publication IV**, flonicamid and its metabolites were studied in orange under field and laboratory conditions. Commercial product was applied in orange at 0.15 g per L (field studies), and at 1 mg/kg (laboratory studies). Samples were collected at 24 h and 7, 15, 21, 30, 40 and 50 days, and also 60 and 70 days for laboratory samples. Results are shown in **Figure 6.7.** Behaviour of flonicamid was similar in both studies, and concentration of flonicamid increased (until the middle of the monitoring period) and later decreased. The only difference was that in laboratory studies the concentration of flonicamid increased till 30 days after the application, meanwhile in field studies until 15 days. This phenomenon can be explained considering the water loss over the monitoring period, observing the maximum concentration of flonicamid was 135 μg/kg (field studies) and 1325 μg/kg (laboratory studies).

Flonicamid metabolites were also monitored. In the case of TFNG, it can be highlighted that its concentration increased when flonicamid concentration decreased (**Figure 6.7**). It indicates that flonicamid was transformed into TFNG. In field studies, it appeared at 15 days after application (32 μg/kg), when flonicamid concentration started decreasing and TFNG increased its concentration until the end of monitoring period (68 μg/kg). Concentration of TFNG could have increased after monitoring period, but unfortunately no more oranges could be collected after that time. In order to confirm the latter statement, laboratory trials were planned and eventually performed. In laboratory studies, TFNG concentration was 25 μg/kg at 21 days and it was increasing until 70 days at 140 μg/kg. These results are in accordance with those obtained in the field study.

In the case of TFNA and TFNA-AM, TFNA was only detected at 31 μg/kg in field studies at 15 days after application of the commercial product, meanwhile TFNA-AM was not detected at a concentration higher than its LOQ (30 μg/kg)

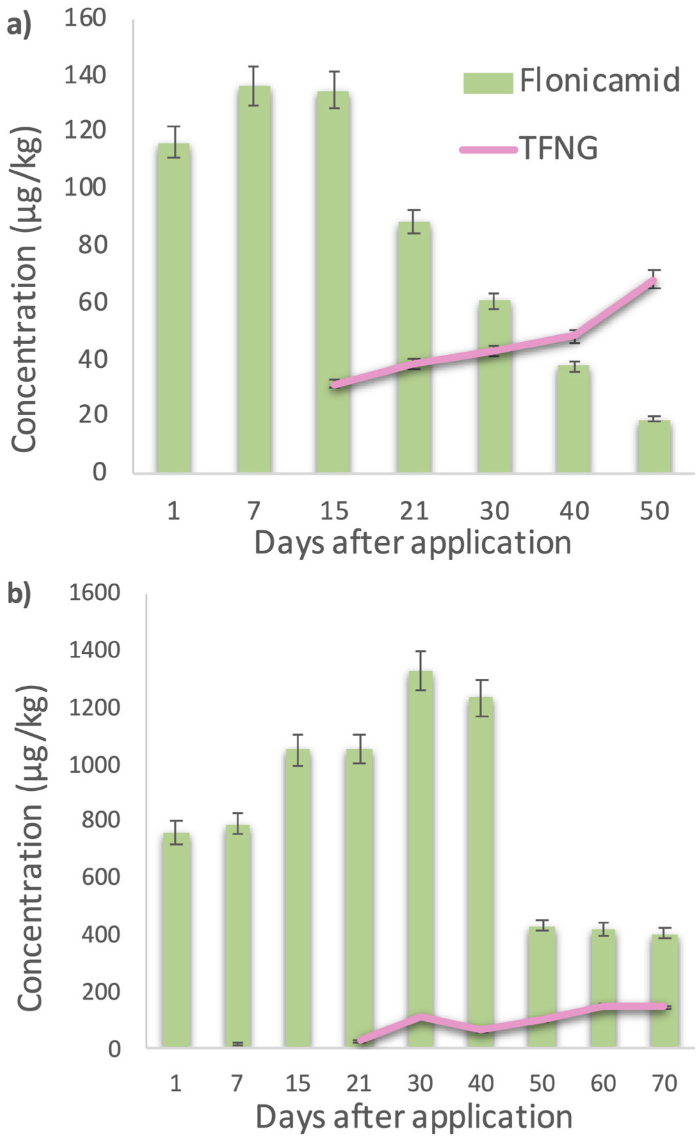


Figure 6.7. Evaluation of flonicamid and TFNG concentrations in incurred oranges during a) field studies and b) laboratory studies. Error bars obtained for n=3.

3.4.2. Thiocyclam studies

Thiocyclam was the next studied compound (**Publication V**). In addition to monitor thiocyclam residues and their metabolites, a dissipation kinetics study of thiocyclam was studied in tomato samples under laboratory conditions using a "Single First-Order Rate" (SFO) model (**Eq. 1**), and half-lives (DT_{50}) were also estimated (**Eq. 2.**)

$$C_t = C_0 e^{-kt} \quad \textbf{Eq. 1.}$$

$$DT_{50} = \frac{\ln 2}{k} \quad \textbf{Eq. 2.}$$

where C_0 is the initial concentration, *k* is the rate constant (k) and C_t is the concentration at time t.

Two studies were carried out in tomato under laboratory conditions. The first was developed at a theoretical concentration of 1 mg/kg of thiocyclam, and the second one at a concentration of 20 mg/kg. Results of SFO kinetic model were similar at both doses (**Table 6.6**).

Table 6.6. SFO kinetic model parameters and tomato dissipation (DT_{50}) of thiocyclam

	Studies	
Parameters	***1 mg/kg***	***20 mg/kg***
C_0 (µg/kg)	720.38	19999.99
k (days^{-1})	1.50	2.38
DT_{50} (days)	0.46	0.30
R^2	0.920	0.904

DT_{50} of thiocyclam was slightly smaller at 20 mg/kg studies (0.30 days) than at 1 mg/kg (0.46 days), and the *k* value was 1.50 days^{-1} at 1 mg/kg and 2.38 days^{-1} at 20 mg/kg. It indicated that thiocyclam persistence was low, being a non-persistent pesticide (DT_{50}<15 days) and dissipation into metabolites (nereistoxin) was really fast. After 2 days of the commercial product application, concentration of thiocyclam was approximately 10 times lower than the applied dose.

Nereistoxin, as the main metabolite of thiocyclam, was monitored over the study. Nereistoxin appearance was significant, and its concentration increased quickly. In **Figure 6.8**, it can be observed that few hours after the application of thiocyclam, nereistoxin concentration was increasing, and when 1 mg/kg of thiocyclam was added, nereistoxin maximum concentration was 1.6 mg/kg two days after application.

In addition to that, nereistoxin was really persistent, being stable in tomato after 60 days of the commercial product application. In addition, a mass balance was carried out and it was noted that thiocyclam was mainly degraded into nereistoxin, observing that 7 hours after the application, there was an equimolar mixture of both components, and after that, the amount of nereistoxin was higher, due to the dissipation of thiocyclam. According to these results, in addition to thiocyclam, nereistoxin should be monitored in routine control analysis to detect these types of compounds and this could be included in a new possible MRL definition of thiocyclam (its precursor molecule) to ensure food safety.

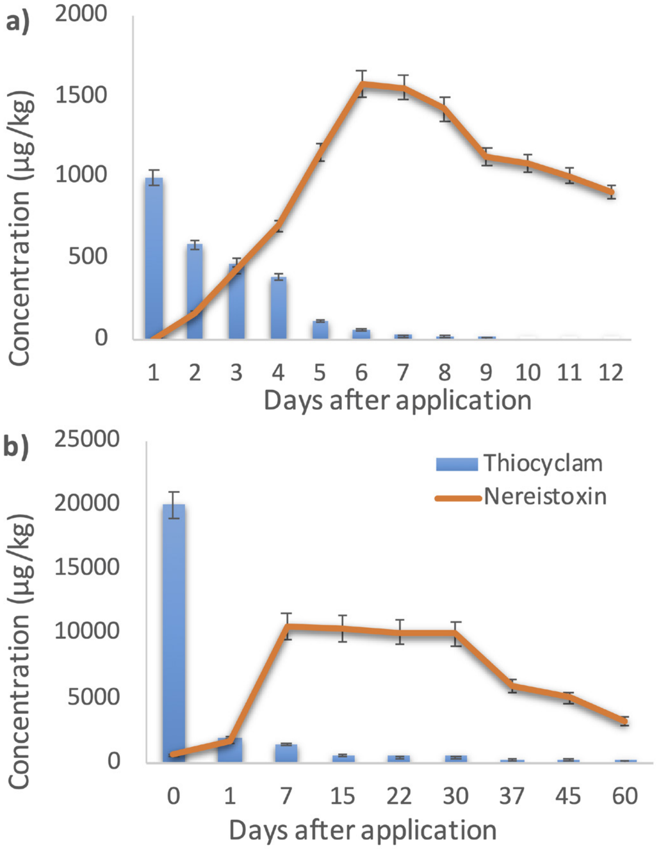


Figure 6.8. Evaluation of thiocyclam and nereistoxin concentrations in incurred tomatoes at a) 1 mg/kg studies and b) 20 mg/kg studies. Error bars obtained for n=3.

3.5. METABOLITE IDENTIFICATION STUDIES

Additionally, retrospective studies were carried out to determine new metabolites of the targeted pesticides. For thiocyclam metabolites, this study is summarized in **Publication V**. Thus, a bibliographic review was carried out to determine potential metabolites of this pesticide. Scarce bibliography was found, and up to our knowledge, only one book published in 1998 described a scheme of possible metabolites generated when thiocyclam degrades into nereistoxin, and they were defined as nereistoxin metabolites instead of thiocyclam metabolites (**Figure 6.9**). A database with molecular formula and exact mass of the six metabolites described was built, and a suspect screening of all samples during the all monitoring period at 20 mg/kg was performed.

When a suspect screening was performed, four nereistoxin metabolites were detected, metabolites 3, 5, 7 and 8 (**Figure 6.9**). Metabolites 5 and 7 were detected only one day. Metabolite 5 at 22 days after commercial product application, at 1.5 μg/kg. Metabolite 7 was detected 1 day after commercial product application at 10 μg/kg. The other two metabolites (3 and 8) were detected throughout the study (**Figure 6.10**).

Metabolite 3 was detected in the samples 15 days after application, and its concentration increased quickly to a maximum value of 75 μg/kg (45 days) and then decreased. Metabolite 8 was detected one day after application, and its concentration increased up to 15 μg/kg (15 days) and later decreased until 60 days where its concentration was 3 μg/kg. To conclude, the appearance of metabolites was confirmed at 20 mg/kg studies, and one of them (metabolite 3) was detected at concentrations higher than 50 μg/kg, so it should be considered in further studies in order to obtain information about potential risk to the human health.

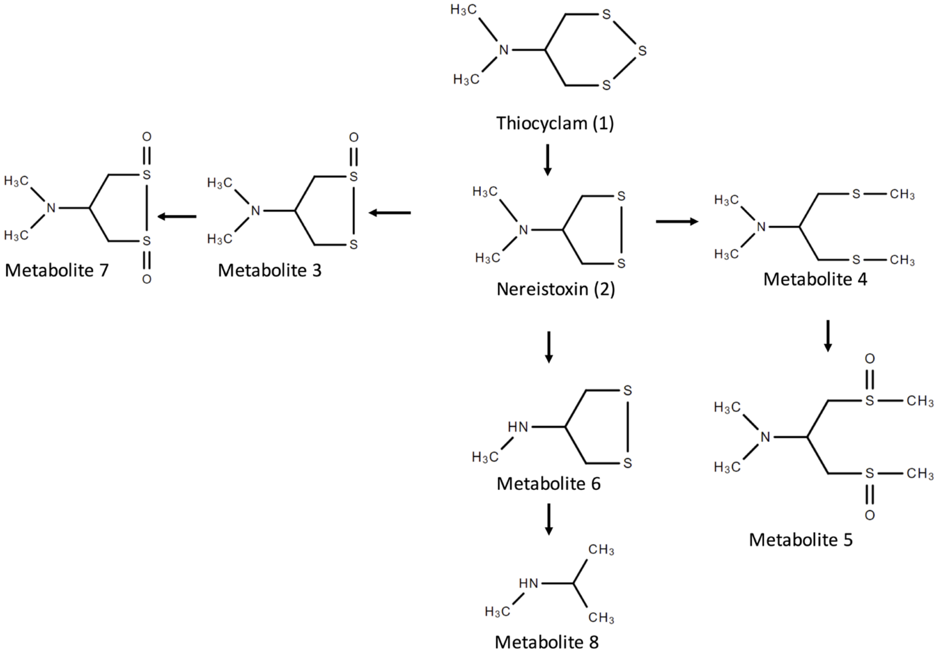


Figure 6.9. Nereistoxin metabolites from thiocyclam degradation.

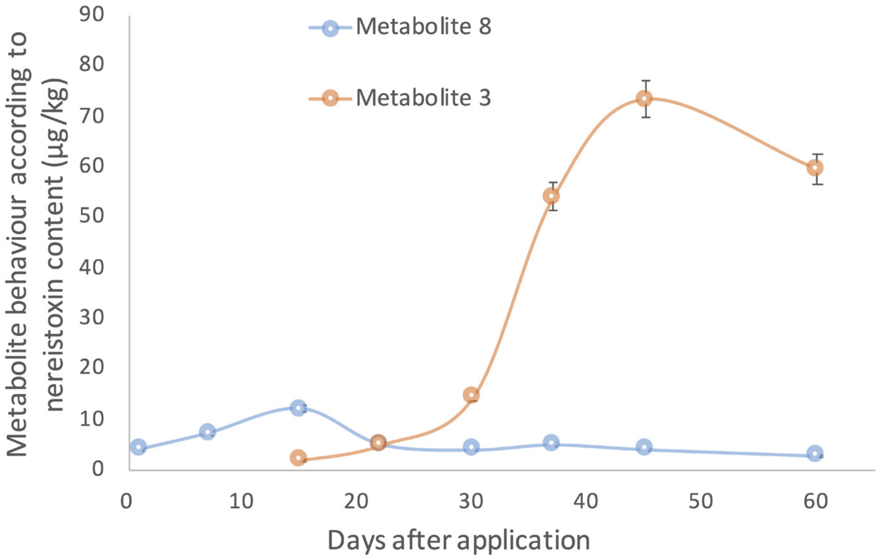


Figure 6.10. Behaviour of metabolites 3 and 8 according to nereistoxin concentration in tomato. Error bars obtained for n=3.

4. HERBICIDE DISSIPATION STUDIES AND IDENTIFICATION OF THEIR METABOLITES IN ENVIRONMENTAL MATRICES

To guarantee the environmental safety, the study of the behaviour of herbicides in the environment, as soils and water, is necessary. In this Thesis, three herbicides derivate from quizalofop-p (**Publication VI and VII**) and another one, dimethachlor, (**Publication VIII**) were studied in soils and water. In addition, their metabolites were monitored, and tentative identification of new metabolites was performed.

4.1. EXTRACTION TECHNIQUES

For environmental matrices, three different types of extraction procedures were applied: QuEChERS and SLE for soils, and SPE for water.

In **Publication VI**, the extraction of quizalofop-p and related compounds from soils were optimized (**Table 6.7)**. However, bad recoveries were obtained in the case of polar metabolites, so addition of acidified water was evaluated in order to hydrate the matrix and improve the extraction (**Table 6.7**). Also salt buffering step or additional clean-up step was tested, indicating the best results in **Table 6.7**.

Table 6.7. Different extraction procedures evaluated in soils for **Publication VI** (final method in bold and recoveries in %).[a,b]

Extraction method	***Quizalofop-p***	***Propaquizafop***	***Quizalofop-p-ethyl***	***Quizalofop-p-terfuryl***	***2,3-dihydroxyquinoxaline***	***CHHQ***	***CHQ***	***PPA***
Mantzos et al. [8]	< 10	72	74	70	< 10	< 10	34	< 10
Saha et al. [9]	< 10	75	67	79	< 10	< 10	60	< 10
Ethyl acetate	79	90	92	96	25	42	95	< 10
ACN	< 10	192	127	158	< 10	< 10	228	171
ACN 1% acetic acid	63	87	79	86	< 10	< 10	59	< 10
ACN 1% acetic acid and H_2O 0.1M HCl	87	85	79	88	37	48	72	19
ACN 1% acetic acid and H_2O 0.1M HCl + C_{18}	**86**	**116**	**114**	**118**	**78**	**74**	**91**	**52**

[a] Abreviations: ACN, acetonitrile; HCl, Hydrochloric acid; CHHQ, dihydroxychloroquinoxalin; CHQ, 6-chloroquinoxalin-2-ol; PPA, (R)-2-(4-hydroxyphenoxy)propionic acid. [b] Tested concentration 100 µg/kg, n=5.

The method developed for quizalofop-p and related compounds in soils was also tested for dimethachlor extraction in **Publication VIII,** as well as other modifications (**Table 6.8**). The best results were obtained when a mixture of acetonitrile and water was carried out with a C_{18} d-SPE clean-up step.

Table 6.8. Extraction methods tested in soils for **Publication VIII** (recoveries in %).[a]

Extraction method	***Dimethachlor***	***Dimethachlor oxalamic acid***	***Dimethachlor CGA***	***Dimethachlor SYN***	***Dimethachlor SYN Na***
Publication VI extraction	82	57	<10	63	20
Publication VI without salts	85	92	96	96	68
ACN and H_2O extraction + clean up (C_{18})	106	111	110	111	80

[a] Tested concentration 100 µg/kg, n=5.

In the case of water (**Publication VII**), a method based on SPE procedure for the extraction of quizalofop-p and related compounds was developed. Several SPE cartridges were tested as Oasis HLB, Strata X and Bond Elute Plexa cartridges. The best results were obtained when Oasis HLB cartridges were used. The second step was the evaluation of the different parameters such as the conditioning and loading steps, as well as elution solvent. Conditioning step was optimized evaluating the presence of acid as it was previously demonstrated in **Publication VI**, where the presence of acidic pH improved the analytes extraction. So different percentages of formic acid (0.1% to 5%) were evaluated, obtaining the best results when an aqueous solution containing 0.1% of formic acid was used (**Table 6.9**). Next, loading step was studied (**Table 6.9**) and acetonitrile was selected as elution solvent.

Table 6.9. Effect of the loading step on the recovery of the target compounds in water for **Publication VII** (tested concentration: 1 µg/L, n=5).

Loading step	*Quizalofop-p-ethyl*	*Quizalofop-p-tefuryl*	*Propaquizafop*	*Quizalofop-p*	*CHQ*	*CHHQ*	*PPA*
Sample	55%	60%	45%	22%	88%	26%	<10%
Sample with 0.1% formic acid	80%	95%	85%	72%	125%	95%	95%
Sample with 1 mM TBA[a]	96%	96%	88%	110%	32%	87%	93%
Sample with 1 mM TBA and 0.1% formic acid	96%	95%	85%	89%	149%	55%	95%

[a] TBA: Tetrabutylammonium acetate

For the extraction of dimethachlor and its metabolites in water, (**Publication VIII**), two types of cartridges (Oasis HLB and Strata SAX) were evaluated, as well as two different SPE procedures; the first one using acetonitrile and water to condition the cartridge and acetonitrile to elute the analytes, whereas the second one was similar but adding tetrabutylammonium acetate (TBA) to the sample. The best results were obtained when Oasis HLB cartridges were used and TBA was added (1 mM) to the sample (recovery range for most of the compounds 60-105%), even though recoveries for the metabolite dimethachlor SYN Na was slightly lower than 70%. Nevertheless, the recoveries were repetitive, and therefore, a correction factor of 1.47 was used for the quantitative determination of this compound (**Figure 6.11**).

To sum up, **Figure 6.11** shows a summary of the different extraction methods used in **Publications VI, VII and VIII** in environmental matrices.

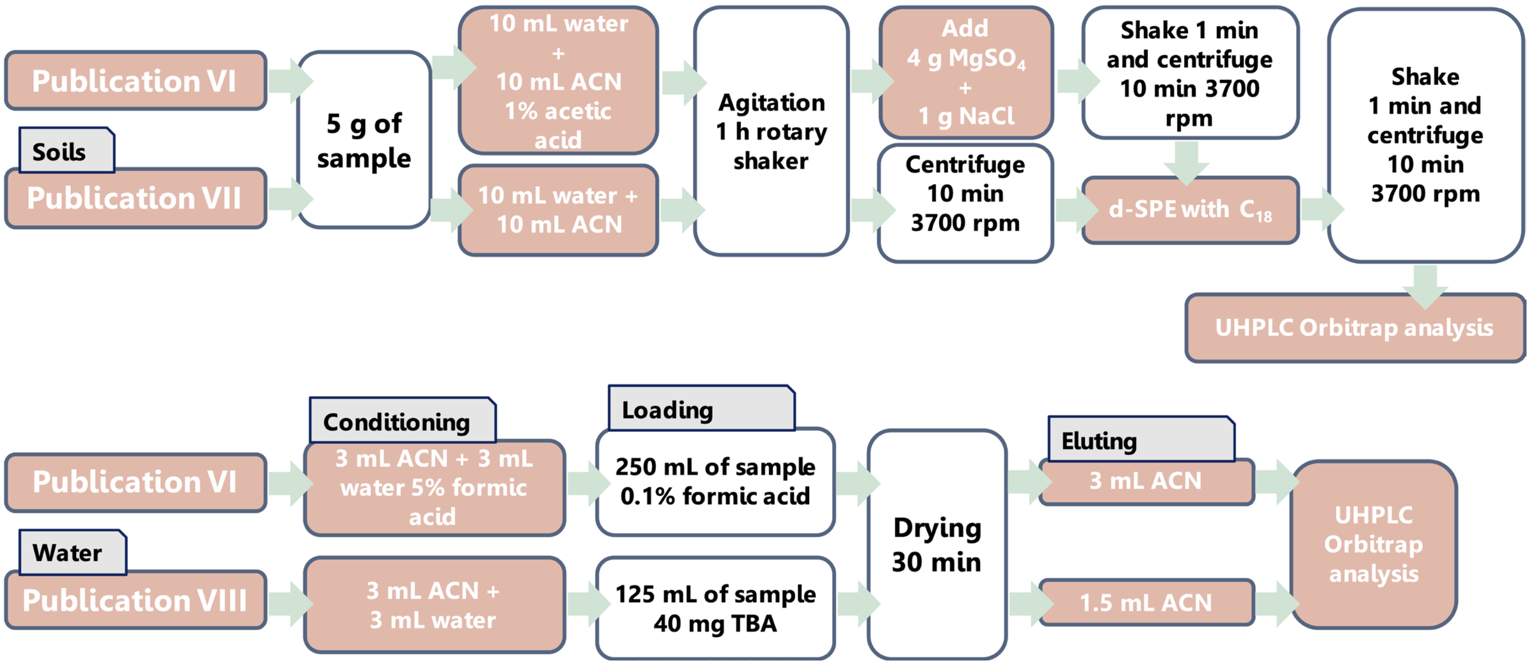


Figure 6.11. Scheme of the different extraction methods developed in Publication VI, VII and VIII.

4.2. LIQUID CHROMATOGRAPHY - HIGH RESOLUTION MASS SPECTROMETRY

Herbicides and their specific metabolites were determined using UHPLC-Orbitrap-MS. Chromatographic separation was optimized, evaluating the mobile phase composition using a conventional C_{18} column (Zorbax Eclipse Plus C_{18}). Mobile phases were tested, using as organic phase: acetonitrile or methanol and as aqueous phase: water containing 0.1% formic acid or water with 0.1% acetic acid/4mM ammonium acetate. For **Publication VI and VII**, the best results were obtained when acetonitrile:water 0.1% acetic acid/4mM ammonium acetate were used, meanwhile for **Publication VIII** a mixture of acetonitrile:methanol 50:50 (*v/v*) and water 0.1% formic acid was used. The next step was testing elution gradients, and three types of gradients were checked, obtaining the best results using the gradients shown in **Figure 6.12**.

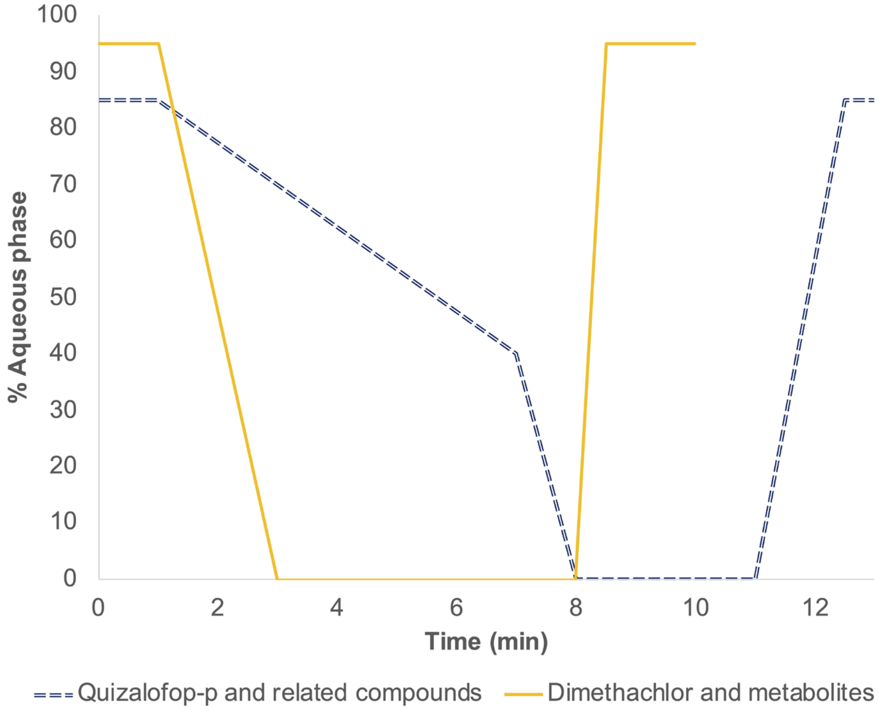


Figure 6.12. Gradient elution used in Publication VI, VII and VIII.

However, using these conditions, peak shape was not suitable in **Publication VI and VII**, and some compounds such as PPA eluted very early. That is why, in addition to Zorbax Eclipse Plus C_{18}, other stationary phases such as Zorbax Eclipse Plus phenyl-hexyl, Zorbax Eclipse Plus HILIC, Zorbax Eclipse Plus HILIC + Zorbax Eclipse plus C_{18} and ODS Hypersil were tested. The best results were obtained when Zorbax Eclipse Plus HILIC + Zorbax Eclipse Plus C_{18} were coupled as it was shown in **Figure 6.13.**

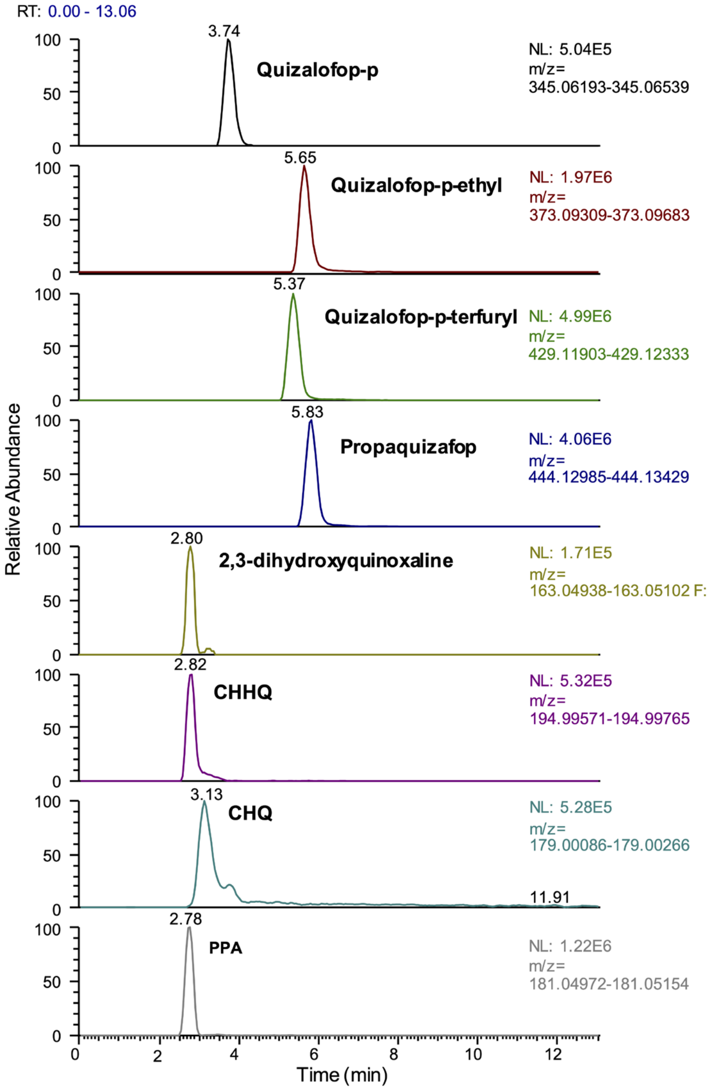


Figure 6.13. Extracted ion chromatograms of target analytes of Publication VI and VII in solvent at 500 μg/L.

Orbitrap-MS characterization was carried out as indicated in the previous section. **Table 6.10** shows the HRMS conditions developed for **Publication VI, VII** and **VIII**. For quizalofop-p and related compounds, five compounds were ionized in positive mode (quizalofop-p, quizalofop-p-ethyl, quizalofop-p-tefuryl, propaquizafop and 2,3-dihydroxyquinoxaline), and three (CHHQ, CHQ and PPA) were ionized in negative mode. These compounds belong to the same family, and therefore four analytes, quizalofop-p, quizalofop-p-ethyl, quizalofop-p-tefuryl and propaquizafop, shared common fragments. Nevertheless, the precursor ions as well as their retention time were different, and therefore, they could be determined without interferences between each other.

For dimethachlor and its metabolites, the ionization mode was negative for metabolites CGA and SYN Na, while for the rest of analytes it was positive. In relation to common fragments, for example, the loss of a methoxy group ($-CH_2O$) is common for dimethachlor and SYN, monitoring *m/z* 224.08367 and 236.09173 ions, respectively. Common fragments were also observed for some compounds as *m/z* 178.08078, which was detected for CGA and SYN Na, and it involved the loss of sulfonic ($-SO_3$) and ketone groups (C=O) for CGA, whereas for SYN Na, the loss of thiol (-S), hydroxyl (-OH) and acid groups (-COOH) was observed.

Table 6.10. HRMS conditions developed for: a) quizalofop-p-and related compounds and b) dimethachlor and its metabolites.

a)	***Precursor ion***		***Fragments***			
Compounds	***Exact mass (m/z)***	***Mass error (ppm)***	***Exact mass (m/z)***	***Molecular formula***	***Mass error (ppm)***	***RT (min)***
Quizalofop-p	345.06366	1.02	299.05818	$[C_{16}H_{12}O_2N_2Cl]^+$	1.9	3.73
			244.03979	$[C_{13}H_9ON_2Cl]^+$	-0.5	
			197.01124	$[C_8H_6O_2N_2Cl]^+$	1.1	
Quizalofop-p-terfuryl	429.12118	1.55	299.05818	$[C_{16}H_{12}O_2N_2Cl]^+$	0.9	5.41
			271.06327	$[C_{15}H_{12}ON_2Cl]^+$	-0.7	
			243.06835	$[C_{14}H_{12}N_2Cl]^+$	0.4	
Quizalofop-p-ethyl	373.09496	1.39	299.05818	$[C_{16}H_{12}O_2N_2Cl]^+$	0.9	5.60
			271.06327	$[C_{15}H_{12}ON_2Cl]^+$	0.9	
			243.06835	$[C_{14}H_{12}N_2Cl]^+$	0.5	
Propaquizafop	444.13207	1.88	299.05818	$[C_{16}H_{12}O_2N_2Cl]^+$	0.9	5.83
			271.06327	$[C_{15}H_{12}ON_2Cl]^+$	1.8	
			243.06835	$[C_{14}H_{12}N_2Cl]^+$	1.2	
2,3-dihydroxyquinoxaline	163.05020	0.82	145.03964	$[C_8H_5ON_2]^+$	1.3	2.81
			117.04554	$[C_7H_5N_2]^+$	4.9	
CHHQ	194.99668	-2.82	167.00067	$[C_7H_4ON_2Cl]^-$	-2.2	2.79
			131.02399	$[C_7H_3ON_2]^-$	-3.7	
CHQ	179.00079	-4.06	151.00575	$[C_7H_4N_2Cl]^-$	-4.2	3.17
			143.02399	$[C_8H_3ON_2]^-$	-3.5	
PPA	181.05063	-3.54	108.02168	$[C_6H_4O_2]^-$	-5.0	2.74
			93.03454	$[C_6H_5O]^-$	-4.9	

b)

	Precursor ion		**Fragments**			
Compounds	**Exact mass (m/z)**	**Mass error (ppm)**	**Exact mass (m/z)**	**Molecular formula**	**Mass error (ppm)**	**RT (min)**
Dimethachlor	256.10988	-1.91	224.08367	$[C_{12}H_{15}ONCl]^+$	-3.4	7.24
			148.11208	$[C_{10}H_{14}N]^+$	-3.0	
Dimethachlor CGA	300.05473	0.72	256.06381	$[C_{11}H_{16}O_4NS]^-$	-4.1	5.59
			178.08078	$[C_{10}H_{15}O_2N]^-$	-2.9	
Dimethachlor SYN	268.11795	-2.09	236.09173	$[C_{12}H_{14}O_4N]^+$	-3.2	5.96
Dimethachlor SYN Na	326.10560	-0.38	178.08626	$[C_{10}H_{11}O_2N]^-$	-2.7	6.19
			120.08078	$[C_8H_{10}N]^-$	-4.5	
Dimethachlor oxalamic acid	250.10848	-3.75	178.12374	$[C_{11}H_{16}ON]^-$	-3.8	6.12

In addition, in **Publication VI**, enantiomeric separation of *(R,S)*-quizalofop was developed. For that, two columns were evaluated, Chirobiotic column and ChiralpakAY3 column, which were used in a previous study performed in our research group. The best results were obtained when the ChiralpakAY3 column was employed but optimization of the mobile phase was needed, obtaining the best results when the mobile phase, ethanol, was acidified (0.3% formic acid) (**Figure 6.14**).

The detection of both enantiomers was performed using a QqQ analyser, instead of Orbitrap mass analyser. Both compounds were analysed using ESI+. Full scan and MS/MS spectra were performed to acquire the most sensitive transitions. Further optimization was carried out to evaluate the intensity of precursor ion obtained from different fragmentor voltages (from 80 to 130 V), and collision energies (collision energy, from 10 to 50 eV) for each product ion. The selected precursor ion was *m/z* 345.0 (fragmentor voltage 125 V), and three product ions were monitored: *m/z* 299 (20 eV) selected as quantifier; *m/z* 243.9 (30 eV); and *m/z* 162.8 (40 eV).

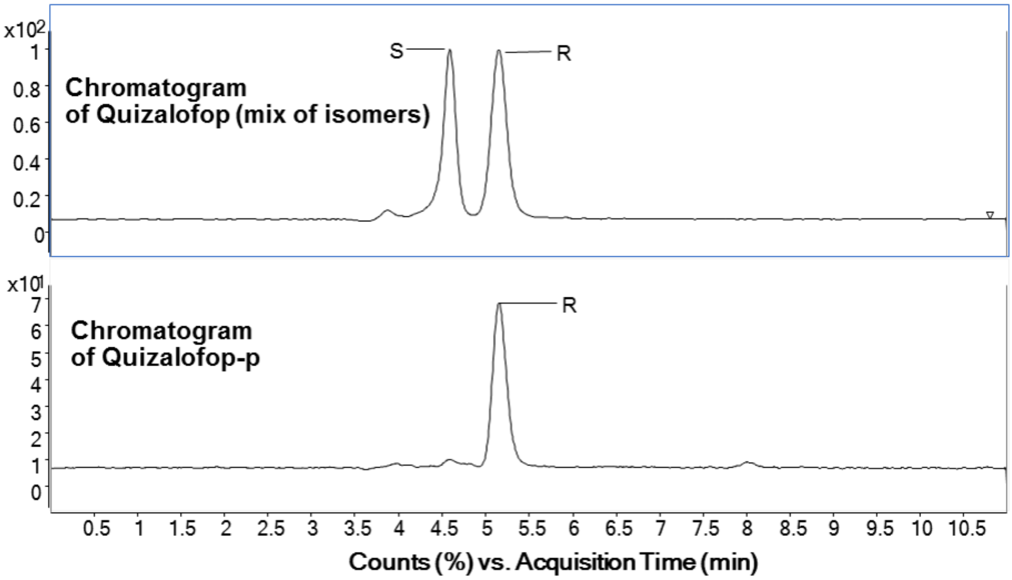


Figure 6.14. Total ion chromatograms of enantiomeric separation of quizalofop in UHPLC-QqQ-MS/MS (Counts vs. Acquisition time (min)).

4.3. VALIDATION PROCEDURE

Validation procedure for herbicides in soils and water were carried out according to SANTE guidelines calculating the same parameters as in previous section. **Table 6.11** shows a summary of the validation results for the targeted herbicides.

Table 6.11. Summary of the validation results in Publications VI, VII and VIII for soils and water matrices.

Validation parameters	*Quizalofop-p and related compounds*	*Dimethachlor and metabolites*
Matrix effect[a]	-95% to 18%	-95 to 445%
Linearity (R^2)	> 0.9900	> 0.9900
Trueness[b] *(%)*	50-120	68-118
Interday precision[b] *(%)*	2-20	3-19
Intraday precision[b] *(%)*	3-20	1-17
LODs	20 µg/kg (soil) and 0.1 µg/L (water)	
LOQs	200 µg/kg (soil) and 1 µg/L (water)	

[a] Equation used: $Matrix\,effect = \left(\frac{slope\,in\,matrix}{slope\,in\,solvent} - 1 \right) x100\%$

[b] Concentrations tested: LOQs and 200 µg/kg (soils) or 1µg/L (water): n=5

The matrix effect, in the case of quizalofop-p and related compounds (**Publication VI and VII**), ranged from -95% to 18%, so suppression effect was observed for these analytes. It ranged from -95% to 445% for dimethachlor and its metabolites (**Publication VIII**), having both matrix suppression and enhancement (**Table 6.11**). Linearity was studied from 20 to 200 µg/kg in the case of soils, and from 0.1 to 1 µg/L in water samples, obtaining R^2 values higher than 0.9900 (**Table 6.11**).

In the case of trueness (% recovery), for **Publication VI and VII**, it ranged from 50-120% and for **Publication VIII** from 68-118%. Precision (intra and interday, % RSD), in **Publication VI and VII,** ranged from 3-20% and 2-20% respectively, while in **Publication VIII** between 1-17% and 3-19%, respectively.

Finally, values of LODs and LOQs for soils were 20 µg/kg (LODs) and 200 µg/kg (LOQs) respectively, meanwhile for water, 0.1 (LOD) and 1 µg/L (LOQ) were set for both compounds and their metabolites.

4.4. DISSIPATION STUDIES

Dissipation of herbicides was studied in environmental matrices as soils and water in laboratory studies. Several conditions as the soil type (sandy loam and loam/clay), sunny and dark conditions (for water) and two different doses, normal dose (both matrices) and double dose (soils) and fivefold dose (water) were evaluated. Soils and water samples were treated with the commercial product of each pesticide (propaquizafop, quizalofop-p-ethyl, quizalofop-p-tefuryl) and samples were randomly collected until 100 days (quizalofop-p) or 110 days (dimethachlor). Samples were analysed and fitted to the SFO kinetic model.

4.4.1. Soils studies

Results of the SFO kinetic model for quizalofop-p related compounds (**Publication VI**) were shown in **Table 6.12**. The dissipation observed in this study always followed the same pattern for the two different soils (sandy loam and clay) at the normal and at the double dose. The concentration of the main products decreased (propaquizafop, quizalofop-p-ethyl and quizalofop-p-tefuryl), especially from 1 to 7 days **(Figure 6.15**), but they were detected until 30 days, when their concentration was lower than LOD.

Table 6.12. SFO kinetic model parameters and soil dissipation (DT_{50}) of quizalofop-p related compounds

Compounds	***Propaquizafop***		***Quizalofop-p-ethyl***		***Quizalofop-p-tefuryl***	
Dose	*Normal dose*	*Double dose*	*Normal dose*	*Double dose*	*Normal dose*	*Double dose*
C_0 *(µg/kg)*	5199	10389	4383	8681	2788	5582
k (days^{-1})	1.55	1.52	0.74	0.72	1.15	1.68
DT_{50} *(days)*	0.45	0.46	0.94	0.96	0.60	0.41
R^2	0.915	0.925	0.935	0.952	0.965	0.910

SFO kinetic model shows that the *k* value was different for the three products evaluated, but they were very similar at normal and double dose. Thus, it can be concluded that although the dissipation is different for each product (**Table 6.12**), it is independent of the application dose. Finally, persistence of the three products was low, with DT_{50} values < 1 day.

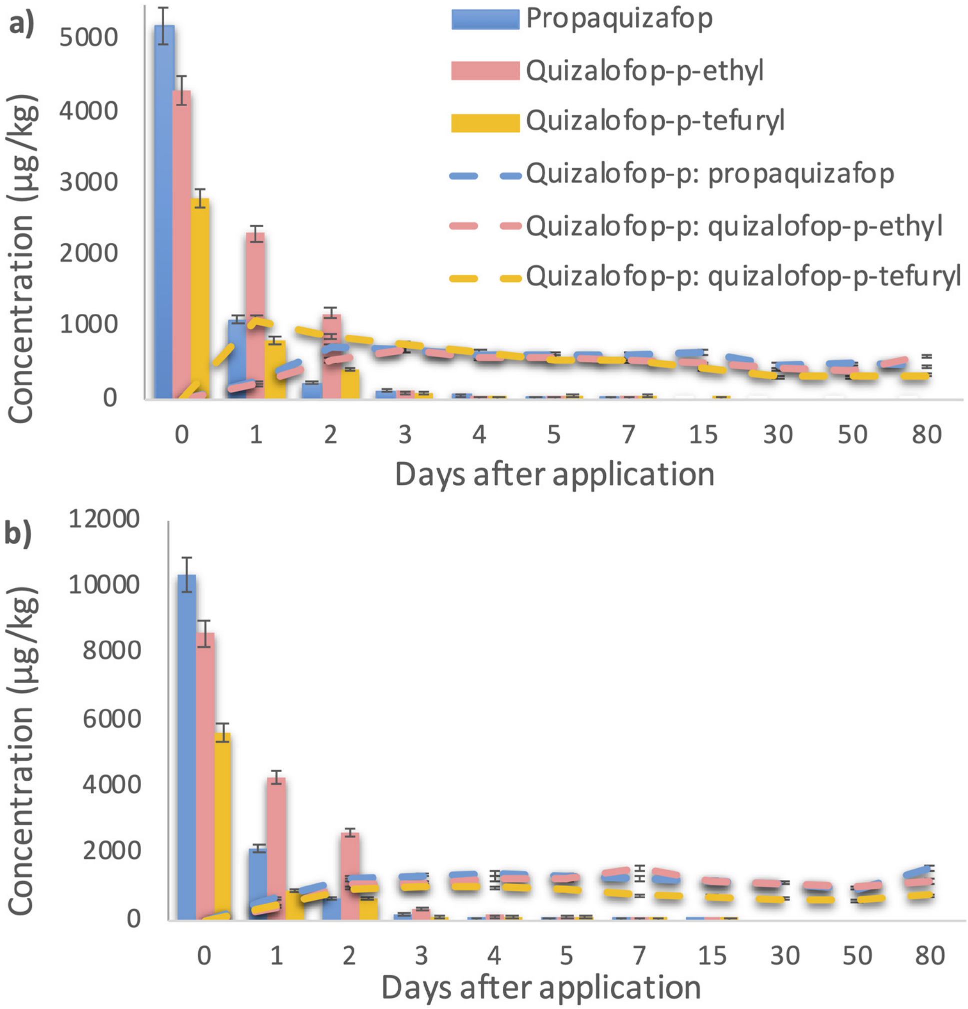


Figure 6.15. Evaluation of quizalofop-p (lines) and related compounds (bars) concentrations in soil samples during a) normal dose studies and b) double dose studies. Error bars obtained for n=3.

In relation to the main metabolite of the three products (quizalofop-p), concentration increased, when concentration of the three products decreased, and decreased after 30 days (**Figure 6.15**).

Dimethachlor dissipation was also discussed (**Publication VIII**) and SFO kinetic model revealed that it was similar at the two tested doses and in the two soils (**Table 6.14**). DT_{50} of dimethachlor is high, from 50 to 68 days for all conditions, so this compound would have a medium-high persistence.

Table 6.13. SFO kinetic model parameters and soil dissipation (DT_{50}) of dimethachlor

Parameters	***Sandy loam***		***Loam***	
	Normal dose	*Double dose*	*Normal dose*	*Double dose*
C_0 *(µg/kg)*	32812	56160	33663	53256
k $(days^{-1})$	0.011	0.010	0.013	0.012
DT_{50} *(days)*	63.99	67.07	50.73	57.04
R^2	0.934	0.843	0.945	0.892

Concentrations of dimethachlor and its known metabolites are shown in **Figure 6.16**. Dimethachlor concentration slowly decreased during all period monitored until 110 days, and the concentration of metabolites (SYN Na and Oxalic acid) increased until a maximum at 70 days and finally decreased until the end of the monitored period. As example, SYN Na and oxalamic acid metabolites were detected 15 days after the application of the commercial product at concentrations of 6 µg/kg for SYN Na and 99 µg/kg for oxalamic acid in the case of sandy loam soils at normal dose, and the concentration was 107 µg/kg and 603 µg/kg at 70 days respectively.

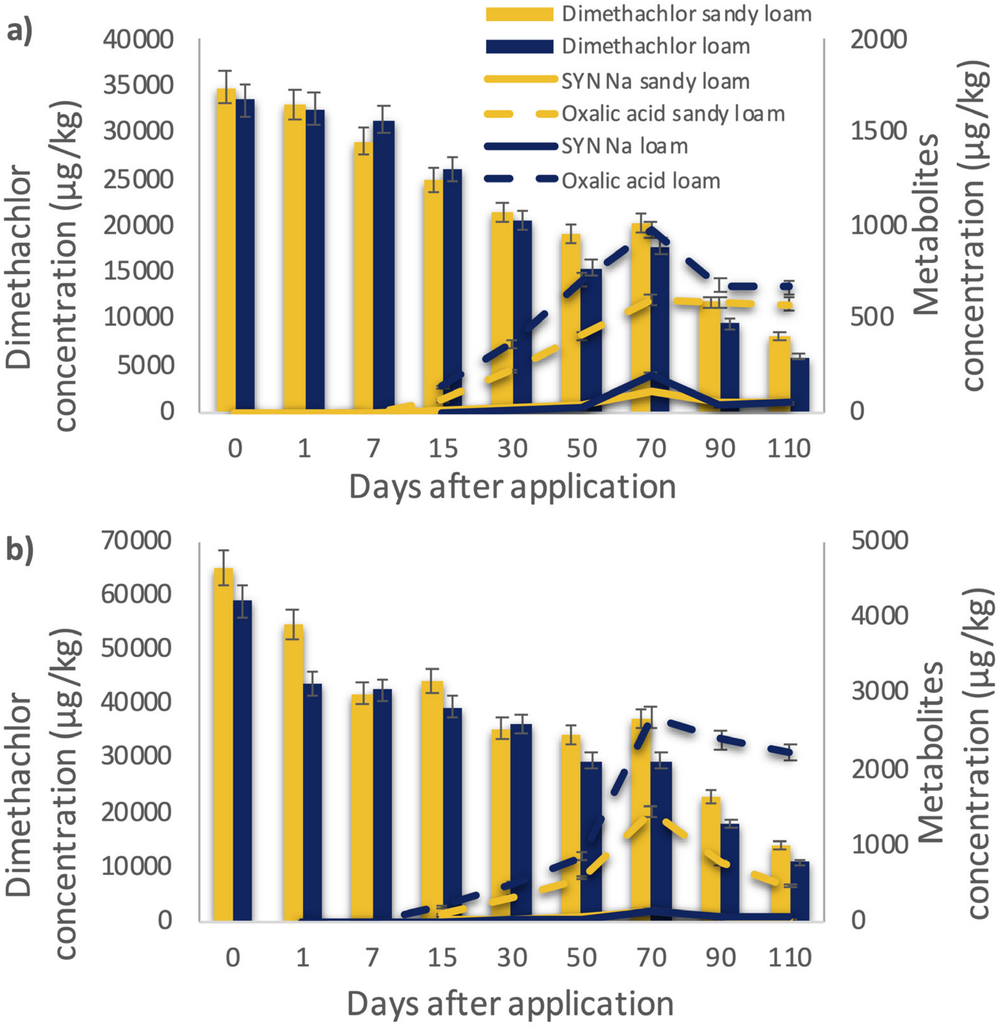


Figure 6.16. Evaluation of dimethachlor (bars) and metabolites (lines) concentrations in two types of soils (sandy loam and loam) at a) normal dose studies and b) double dose studies. Error bars obtained for n=3.

4.4.2. Water studies

In the case of water studies, **Publication VII** describes the behaviour of quizalofop-p and related compounds in that matrix. As indicated above, dark and sunny conditions at room temperature (average value 20°C) were evaluated. Dark conditions can simulate the conditions of groundwater, and sunny conditions those of surface waters like rivers and swamps. SFO kinetic model data was shown in **Table 6.14**. It can be observed that the main compound of the commercial product slowly decreased during the monitored period, and it was still detected 100 days after application, indicating its persistence in water samples (**Figure 6.17**). This was observed in DT_{50} values, and different degradation rates were observed at normal and fivefold dose. Thus, k value was slightly higher at normal dose, meanwhile DT_{50} was smaller at normal dose, which could indicate that persistence was higher at high doses. It can be noted that at dark conditions, propaquizafop presents DT_{50} values 4.3–5.0 times longer than in sunny conditions, whereas quizalofop-p-ethyl ranged from 3.0 to 3.3 times and quizalofop-p-terfuryl between 2.25 and 2.3 times. Therefore, the degradation rate depends on the commercial product applied, which had also been considered when the withdrawal period for these compounds was set. Thus, the withdrawal period is higher for propaquizafop (40 days or more) than for quizalofop-p-ethyl (21 days).

Quizalofop-p dissipation was monitored at the two different concentration levels, and it was observed that at normal dose, higher concentrations were obtained than at fivefold dose. This could be explained because the different degradation rate observed at normal and fivefold dose. Thus, k value as well as the concentration of quizalofop-p were higher at normal dose, which could indicate that at high degradation rates, the main compound was mainly degraded to quizalofop-p, whereas at lower k values, the formation of metabolites was favoured.

Table 6.14. SFO kinetic model parameters and water dissipation (DT_{50}) of quizalofop-p related compounds

Compounds	***Propaquizafop***				***Quizalofop-p-ethyl***				***Quizalofop-p-terfuryl***			
Dose	***Normal***		***Fivefold***		***Normal***		***Fivefold***		***Normal***		***Fivefold***	
Conditions	***Sunny***	***Dark***	***Sunny***	***Dark***	***Sunny***	***Dark***	***Sunny***	***Dark***	***Sunny***	***Dark***	***Sunny***	***Dark***
C_0 *(μg/kg)*	1033	994	4374	4784	1208	1105	6231	5389	1593	1067	5137	5022
k *(days^{-1})*	0.08	0.05	0.02	0.01	0.03	0.02	0.01	0.01	0.07	0.05	0.03	0.02
DT_{50} *(days)*	8.58	14.50	36.54	72.20	23.23	31.38	76.19	94.63	9.20	13.32	20.77	30.79
R^2	0.953	0.942	0.935	0.913	0.921	0.922	0.966	0.929	0.906	0.921	0.987	0.938

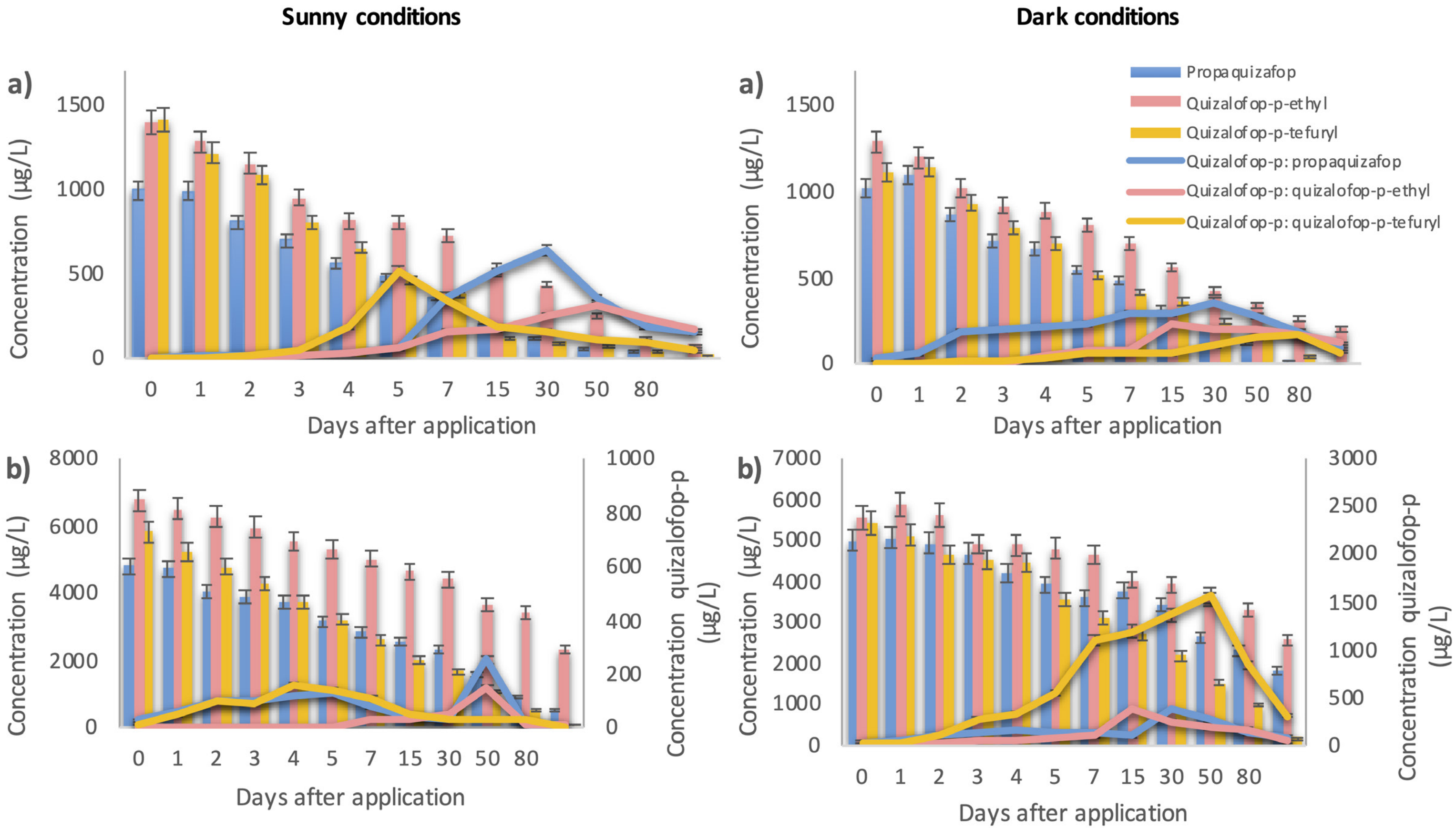


Figure 6.17. Evaluation of quizalofop-p (lines) and related compounds (bars) concentrations in water samples at a) normal dose studies and b) fivefold dose studies. Quizalofop-p comes from each compound indicated in the legend. Error bars obtained for n=3.

The behaviour of dimethachlor in water (**Publication VIII**) was similar than in soils, and the parent compound slowly degraded throughout the monitoring period. SFO parameters pointed that depending on the dose, *k* value was different (**Table 6.15**).

Table 6.15. SFO kinetic model parameters and water dissipation (DT_{50}) of dimethachlor

	Sunny		***Dark***	
Parameters	*Normal dose*	*Fivefold dose*	*Normal dose*	*Fivefold dose*
C_0 (μg/L)	1056	5138	1219	4743
k (days^{-1})	0.007	0.014	0.008	0.012
DT_{50} (days)	93.12	50.63	86.32	55.28
R^2	0.827	0.929	0.819	0.890

The same pattern was observed for DT_{50}. Thus, it can be concluded that at higher doses, persistence of dimethachlor decreased quicker than at normal dose, in contrast to other pesticides like quizalofop-p related compounds.

In relation to dimethachlor metabolites, in contrast to soil studies, where SYN Na and oxalic acid were detected, only CGA was detected in water samples (**Figure 6.18**). It was found one day after the application and its concentration at normal dose was 243 μg/L for sunny conditions and 180 μg/L for dark conditions. The concentration was smaller when fivefold dose was applied (93 and 43 μg/L, respectively). CGA was not detected at 30 days after application of the commercial product. In contrast to soils, degradation of CGA in water was observed at higher concentrations and from the first day after application.

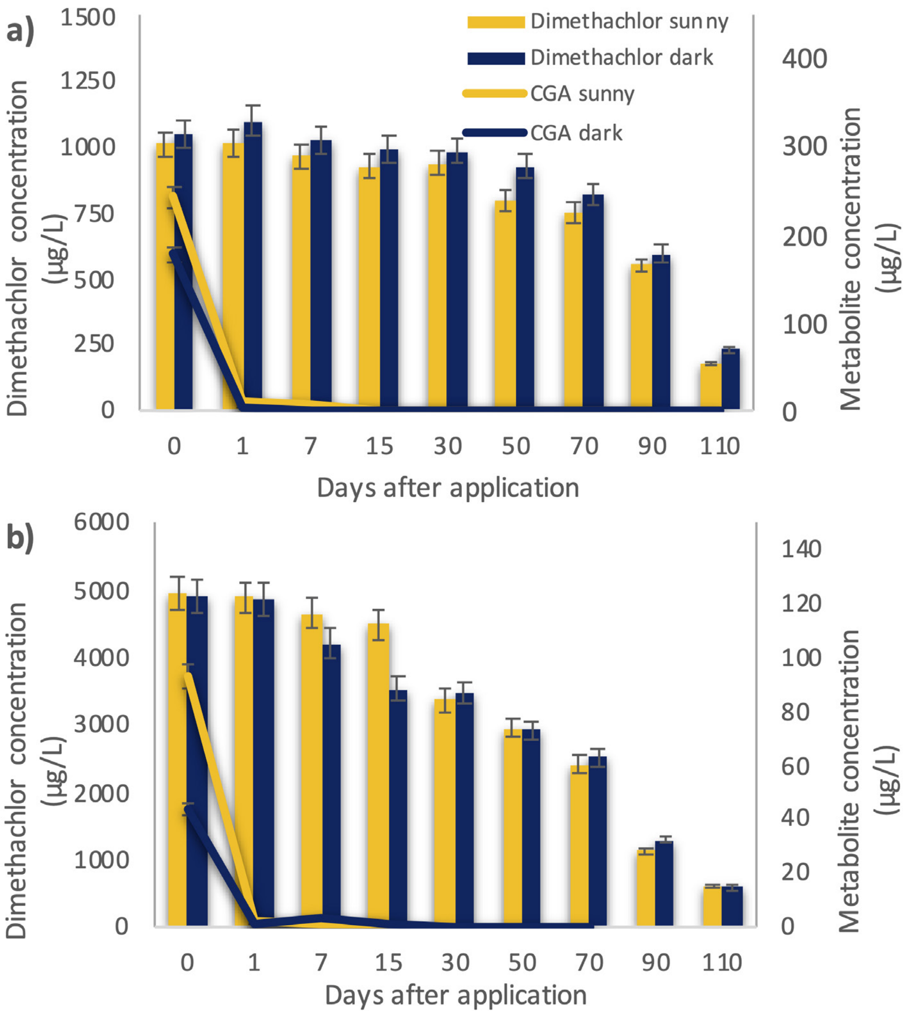


Figure 6.18. Evaluation of dimethachlor (bars) and metabolite (lines) concentrations under two conditions (sunny and dark) in water samples at a) normal dose studies and b) fivefold dose studies. Error bars obtained for n=3.

4.5. METABOLITE IDENTIFICATION STUDIES

First, a bibliographic review was done, obtaining a list of known metabolites of quizalofop-p and dimethachlor, which were mainly provided by EFSA in their risk assessment studies. **Figures 6.19 and 6.20** show the metabolites provided by EFSA. A home-made database was built, adding their formula and exact mass and they were searched in samples by suspect screening.

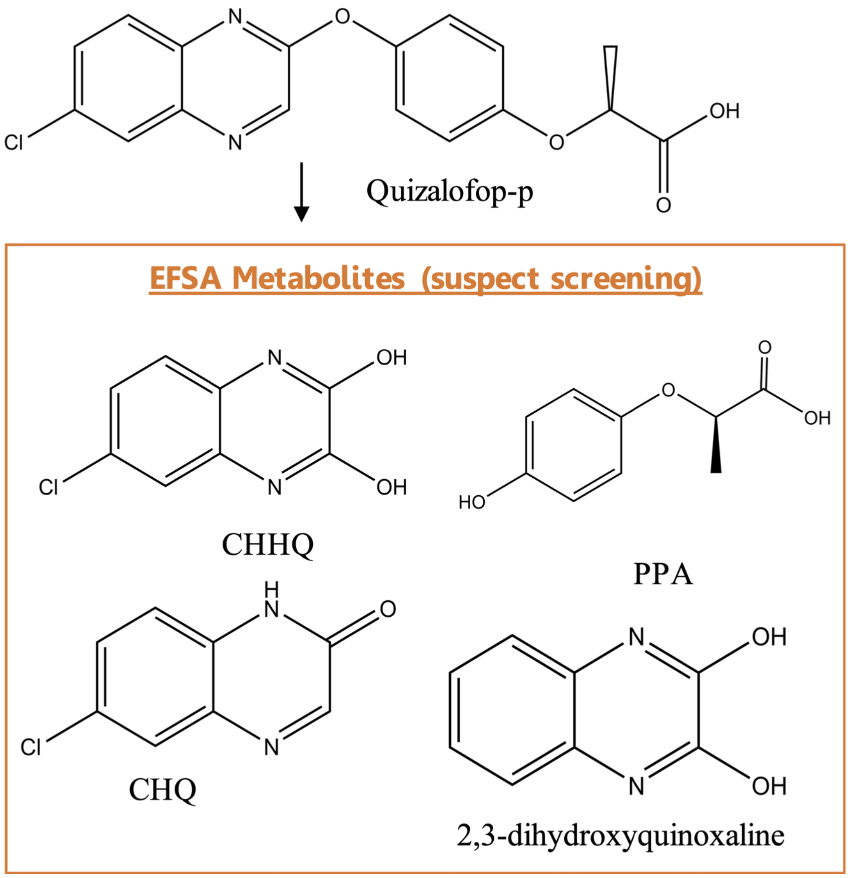


Figure 6.19. Quizalofop-p metabolites previously described by EFSA.

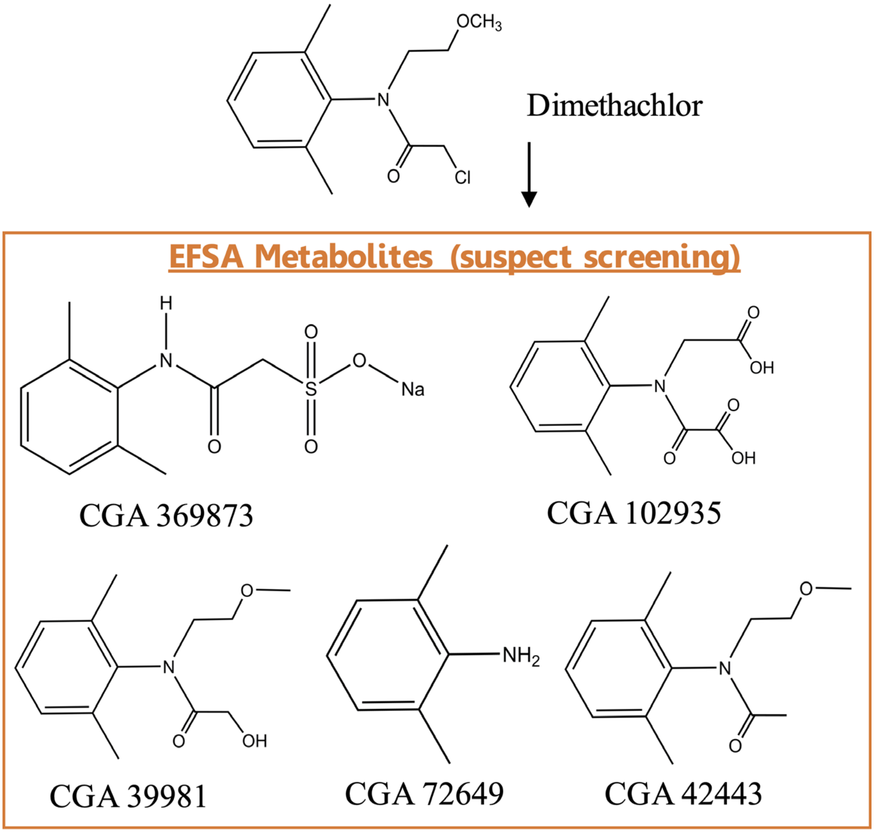


Figure 6.20. Dimethachlor metabolites previously described by EFSA.

In the case of quizalofop-p related compounds, metabolites were detected in soils (**Publication VI**), as well as in water (**Publication VII**) samples. The results in water are shown in **Figure 6.21**. In the case of soils samples, CHHQ and CHQ were detected at 50 and 80 days after application of commercial product at concentrations ranging from 13 µg/kg (CHHQ at 80 days) to 240 µg/kg (CHQ at 80 days), both when quizalofop-p-ethyl commercial product was applied. Also, for sandy loam soils, only CHQ was detected at concentration higher than its LOQ at 50 days, when propaquizafop double dose was applied. PPA was not detected in soil samples.

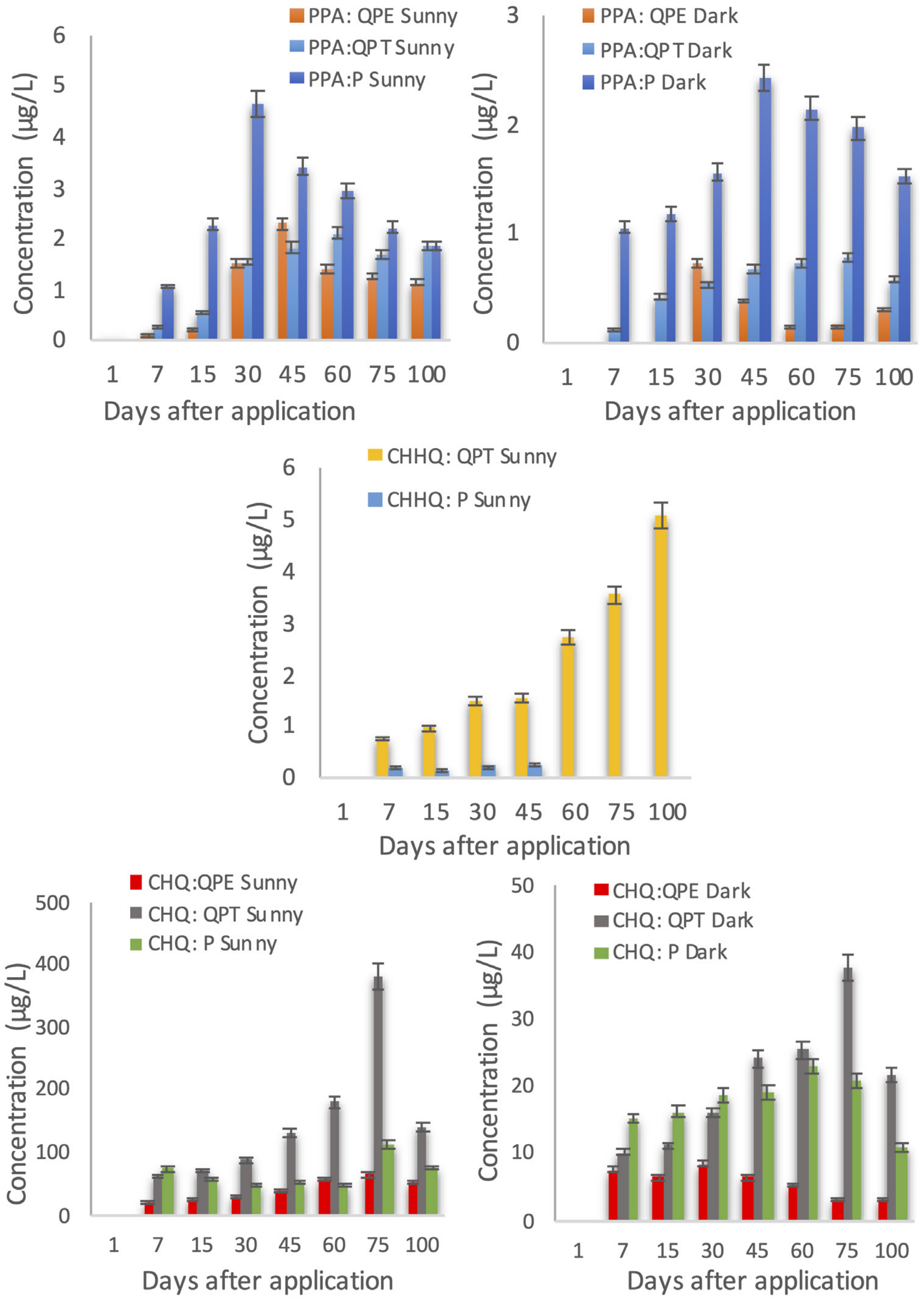


Figure 6.21. Evaluation of quizalofop-p metabolites concentrations under two conditions (sunny and dark) in water samples for the three commercial products (P: propaquizafop; QPE: quizalofop-p-ethyl; QPT, quizalofop-p-tefuryl). Error bars obtained for n=3.

Metabolites PPA and CHHQ were detected at concentrations lower than 15 µg/L for both conditions (sunny and dark), meanwhile CHQ was detected at concentrations higher than 10 µg/L up to 400 µg/L. In addition, it is important to highlight that metabolites appearance in dark conditions was lower than in sunny conditions. For instance, in sunny conditions, CHQ was the metabolite detected at the highest concentration, at 380 µg/L, which is ten times higher than that detected in dark conditions. Therefore, it can be indicated that the sun effect was the key factor in the degradation of these compounds, and some metabolites can persist or appear in the environment 100 days after the application of the parent compound.

For the dimethachlor metabolites study (**Publication VIII**), since no standards were available, their concentrations were estimated using the matrix matched calibration curve obtained for the parent compound (dimethachlor). For soil studies, the behaviour was the same in the two types of soils and at two doses. As example, **Figure 6.22** shows the behaviour for metabolites in sandy loam soils at normal dose. Metabolites 39981 and 42443 were detected one day after the application of the commercial product, and their concentration increased till 70 days, and later decreased. Finally, in water studies, metabolite 39981 concentration decreased during monitoring period and metabolite 42443 was detected at lower concentrations than in soils, and its concentration slowly increased during monitoring until 15 days, and later decreased.

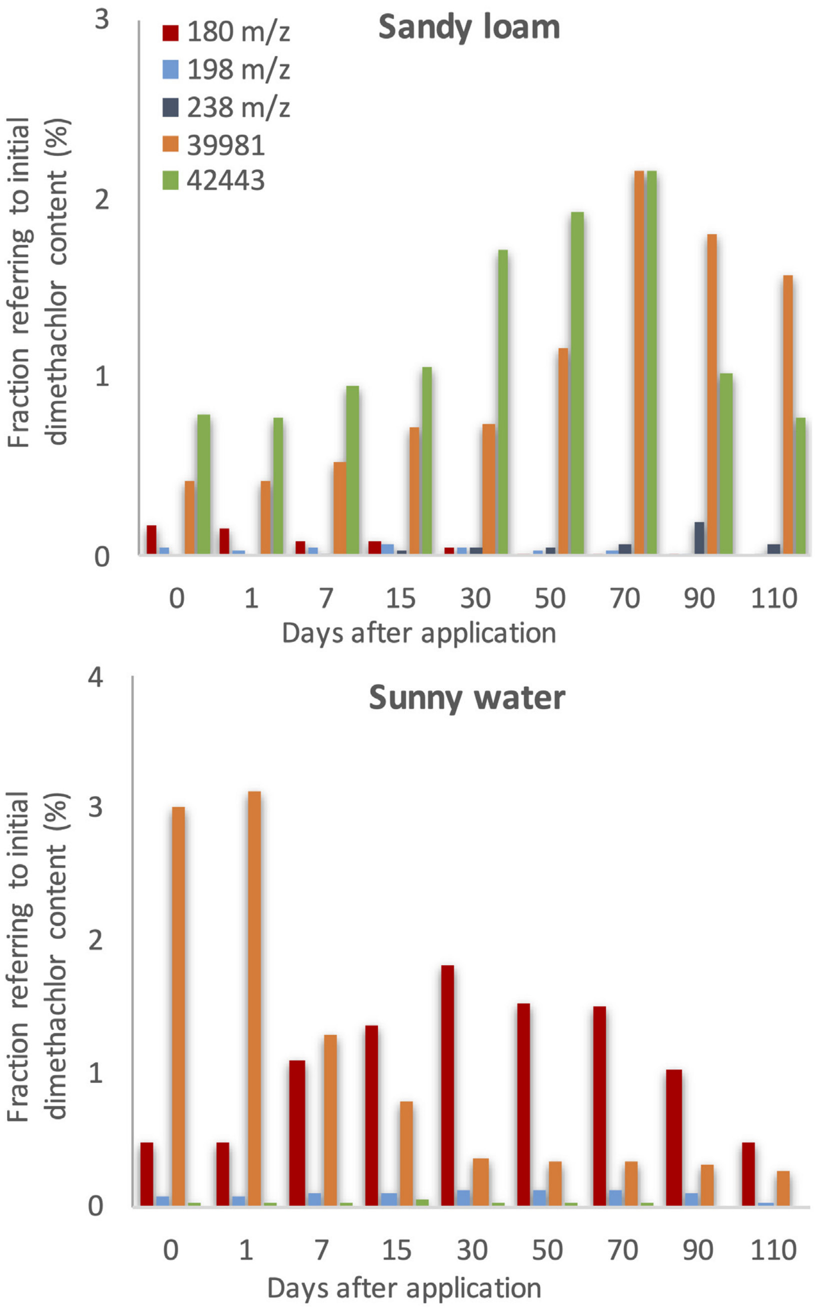


Figure 6.22. Evaluation of dimethachlor metabolites concentrations under two conditions (sandy loam soils and sunny water) at normal dose. Error bars obtained for n=3.

In addition to suspect screening, unknown analysis was carried out for the tentative elucidation of new metabolites of dimethachlor. For that purpose, several software tools were used. First, Compound Discoverer®

processing was carried out using a ‘degradants’ workflow. From the structure of the parent compound, common metabolic pathways from different common transformations of pesticides in environmental samples like dehydration, reduction, reductive dichlorination, methylation, desaturation, oxidation and oxidative dichlorination were evaluated. Lot of features were obtained that were filtered according to intensity of the signal (higher than 1e5), retention time and background subtraction. However, Compound Discoverer® did not show the final structure of the detected metabolites, but it only provided the molecular formula, so the elucidation of the structure is not a straightforward step.

Therefore, another software tool, MassChemSite®, was used in order to tentatively confirm the appearance of these metabolites and a reasonable structure can be proposed. This software provides the reaction as well as the mass spectrum, the parent and the proposed metabolite with the matches and mismatches for the full scan spectra and the HCD mass spectra. With this software tool, three new metabolites were tentatively elucidated, and the potential structures were identified for two of them (180 and 198 *m/z*) but for the third one, two tentative structures are provided (238 *m/z*) (**Figure 6.23**). These metabolites were quantified using the same procedure that was used for those metabolites for which commercial standards are not available. Metabolites behaviour in soils and water was shown in **Figure 6.22.**

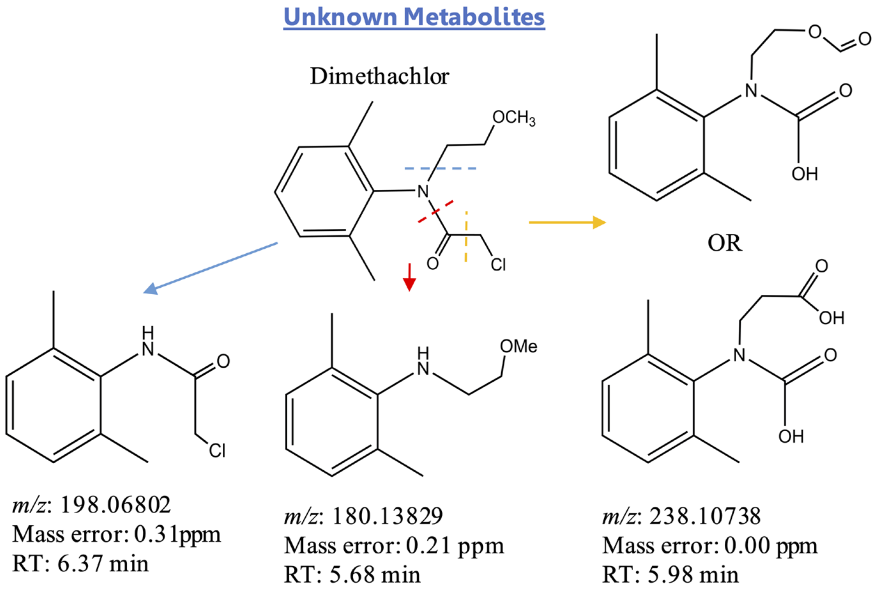


Figure 6.23. Dimethachlor metabolites tentatively identified

5. FUNGICIDE DISSIPATION STUDIES AND IDENTIFICATION OF THEIR METABOLITES IN FOOD AND ENVIRONMENTAL MATRICES

In this section, three fungicides, famoxadone, fenamidone and propamocarb and their metabolites were studied in both food and environmental matrices. Dissipation of famoxadone was developed under greenhouse (**Publication IX**) and laboratory conditions (**Publication XI**), and dissipation of fenamidone and propamocarb was also carried out under greenhouse (**Publication X**) and laboratory conditions (**Publication XII**).

5.1. EXTRACTION TECHNIQUES

To obtain the suitable extraction conditions for the determination of the target fungicides in vegetables as cucumber, tomato and courgette, original QuEChERS method and its two well-known versions (acetate and citrate) were tested, observing that the clean-up step, based on d-SPE, was not necessary. A scheme of the proposal methods was shown in **Figure 6.24**, obtaining recoveries from 75 to 85% (**Publication IX**) and 70% to 96% (**Publication X**) using the non-buffered version.

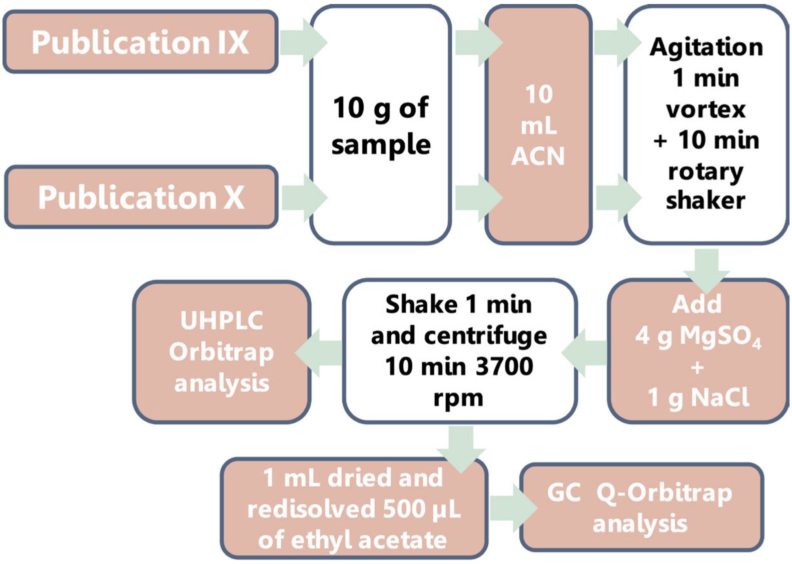


Figure 6.24. Extraction methods developed for vegetables in Publication IX and X.

In soil matrices, SLE method was the best option. In **Publication XI**, the best results were obtained when a mixture of acetonitrile (1% acetic acid) and water (50:50, *v/v*) was used, providing recoveries from 89-105% (**Table 6.16 and Figure 6.25**).

Table 6.16. Different extraction methods tested for famoxadone and metabolites in soils (final method in bold and recoveries %).[a]

Extraction method	*Famoxadone*	*1-acetyl-2-phenylhydrazine*	*4-phenoxybenzoic acid*
Acetonitrile and water (50:50, v/v)	55	98	<10
Acetonitrile, water and salts	112	142	<10
Methanol and water (50:50, v/v)	112	91	<10
Acetonitrile 1% acetic	87	61	<10
Acetonitrile 1% acetic acid and water	**89**	**101**	**105**

[a] Tested concentration 100 µg/kg, n=5.

In **Publication XII**, several extraction methods were tested for the extraction of fenamidone and propamocarb from soils, and the best results were obtained when methanol was used as solvent extraction. Propamocarb recoveries were 60%, but due to suitable relative standard deviation was obtained (<20%), a correction factor can be applied for quantification purposes (**Table 6.17** and **Figure 6.25)**.

Table 6.17. Different extraction methods tested for fenamidone, propamocarb and metabolites in soils (final method in bold and recoveries %).[a]

Extraction method	***Fenamidone***	***RPA-410193***	***5-methyl-5-phenylhydantoin***	***Propamocarb***	***Acetophenone***
Acetonitrile and water (50:50, v/v)	79	74	<10	86	<10
Acetonitrile, water and salts	114	94	103	162	139
Acidified Acetonitrile and water (50:50, v/v)	95	110	150	80	<10
Methanol and water (50:50, v/v)	**107**	**90**	**72**	**59**	**112**

[a] Tested concentration 100 µg/kg, n=5.

Finally, the extraction procedures used for fungicides in water were based on the methods developed for herbicides, quizalofop-p and dimethachlor. Only one condition was tested: sample addition with or without TBA. Oasis HLB cartridges and a simple conditioning with acetonitrile and water, and further elution with acetonitrile was employed. For famoxadone and its metabolites (**Publication XI**) the best results were obtained adding TBA (40 mg) to the sample (72-113%), meanwhile for fenamidone, propamocarb and their metabolites (**Publication XII**), good results were performed without the addition of TBA (**Figure 6.25**).

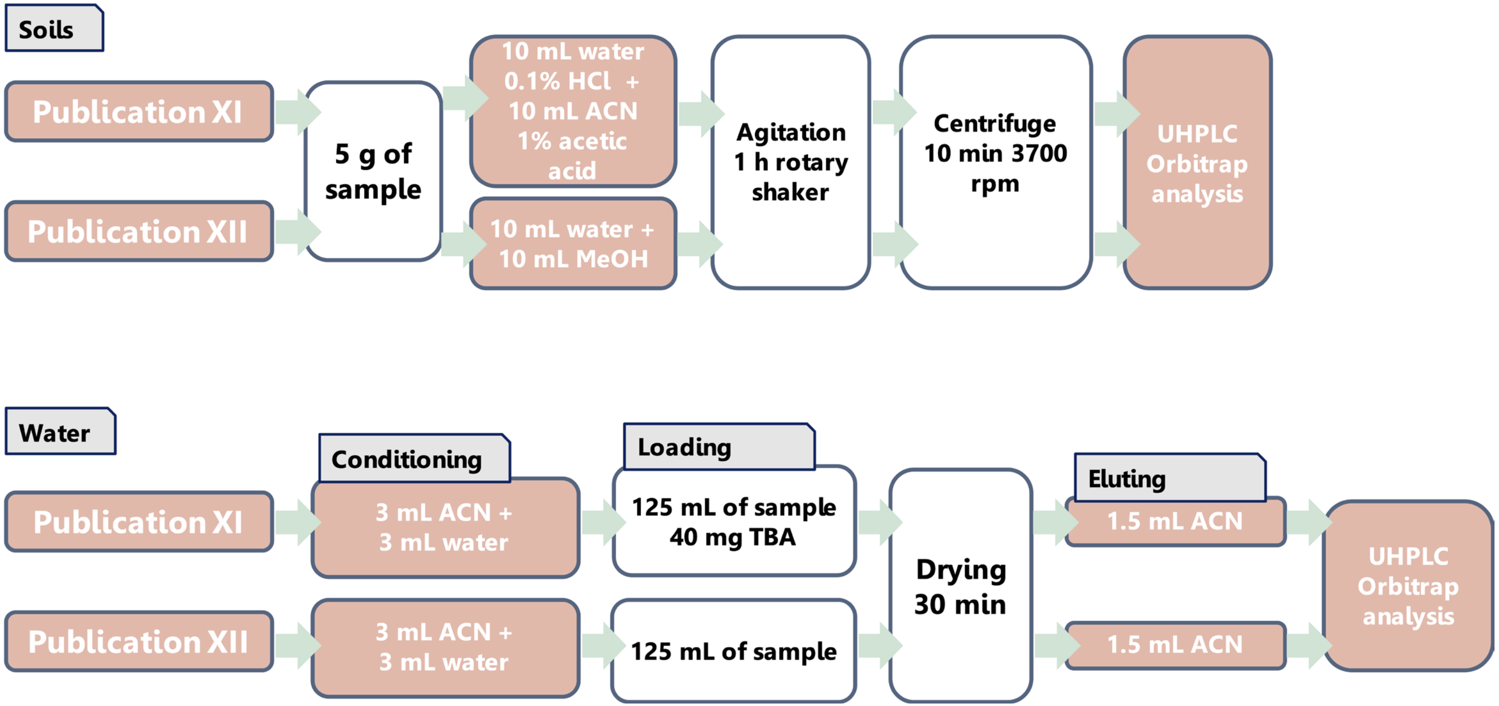


Figure 6.25. Extraction methods developed for soils and water in Publication XI and XII.

5.2. GAS AND LIQUID CHROMATOGRAPHY - HIGH RESOLUTION MASS SPECTROMETRY

Fungicides were determined using two chromatographic techniques, GC and LC. In **Publication IX, XI and XII**, UHPLC-Orbitrap-MS was the technique employed, meanwhile in **Publication X**, UHPLC-Orbitrap-MS was used for the determination of propamocarb and its metabolites, and GC-Q-Orbitrap-MS for fenamidone and its metabolites. LC conditions were optimized as in previous studies, using a C_{18} column, and mobile phase and the gradient profile were optimized. Acetonitrile and methanol were studied as organic phase, and water 0.1% formic acid and water containing 0.1% formic acid and 4 mM ammonium formate were evaluated as the aqueous phase. For famoxadone and its metabolites (**Publication IX and XI**), the best results were obtained when methanol and water containing 0.1% formic acid 4 mM ammonium formate were used, due to the fact that bad peaks for these compounds were obtained when acetonitrile was used, and ammonium adduct favoured the ionization of the analyte. For fenamidone, propamocarb and their metabolites (**Publication X and XII**), methanol and acidified water (0.1% formic acid) were employed, because a tailing peak for propamocarb was observed when acetonitrile was used (**Figure 6.26**).

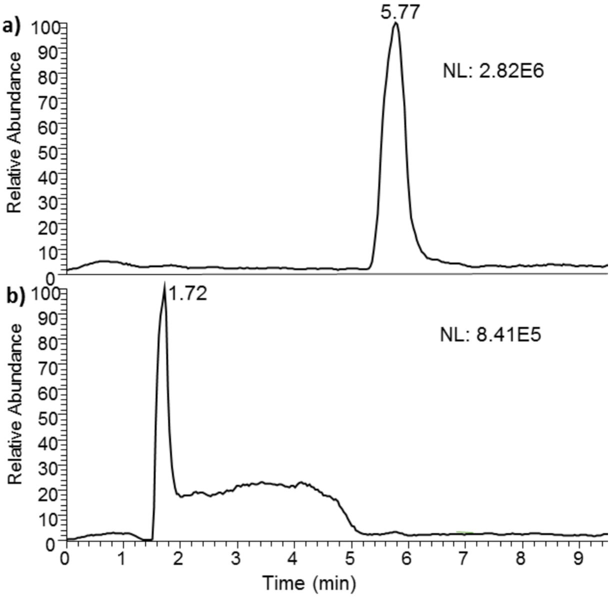


Figure 6.26. Total ion chromatogram of propamocarb (at 100 μg/L) using a) MeOH and b) ACN as organic solvent in the mobile phase.

Gradient elution was optimized using as starting point a similar gradient employed for quizalofop-p and related compounds (**Publications VI and VII)**. For **Publications IX** and **XI**, the elution of the compounds was suitable using this gradient, but the total analysis time was reduced, bearing in mind the retention time of the analytes where shorter than 7 min approximately, and the total analysis time was 14 min. Therefore, it was reduced in 5 min and the new gradient is shown in **Figure 6.27**.

For **Publications X** and **XII**, gradient profile was modified to improve the chromatographic separation of the compounds because some of the metabolites eluted very fast, showing in **Figure 6.27**, the optimized gradient profile.

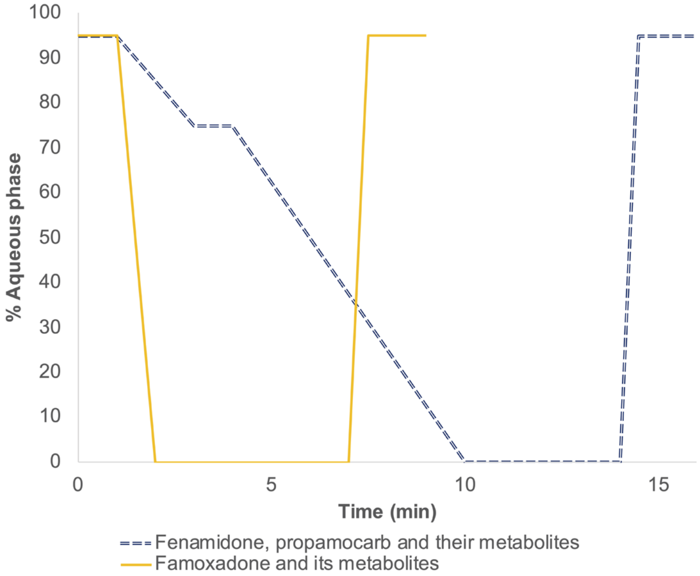


Figure 6.27. Elution gradient employed for chromatographic separation of fungicides.

For GC conditions three factors were investigated during the optimization of the chromatographic conditions. Injector temperature, split/splitless time and oven temperature. The injector temperature was tested from 180 to 250 °C. The best results were obtained when 220 °C was used, due to the fact that several analytes, as 5-methylhydantoin, were not detected at higher temperatures, whereas at 180 °C, broad peaks were obtained for most of compounds. The splitless time was also evaluated, testing 0.5,1.0, 2.0 and 3.0 min. The best results were obtained when splitless time was set at 2 min, and sensitivity and peak shape were suitable for all the analytes. Thirdly, oven temperature was evaluated.

The initial temperature was set at 40°C or 70 °C. The results provided for both temperatures were similar, but sensitivity was lower at 70 °C (5-methylhydantoin), so 40 °C was selected as starting temperature. The maximum temperature of the oven was tested, setting maximum temperature at 260 °C or 300 °C. The best results were obtained at 300 °C, bearing in mind that at 260 °C the retention time was higher and the sensitivity of fenamidone and RPA 410193 was lower than that obtained at 300 °C. In **Figure 6.28**, oven temperature gradient was shown.

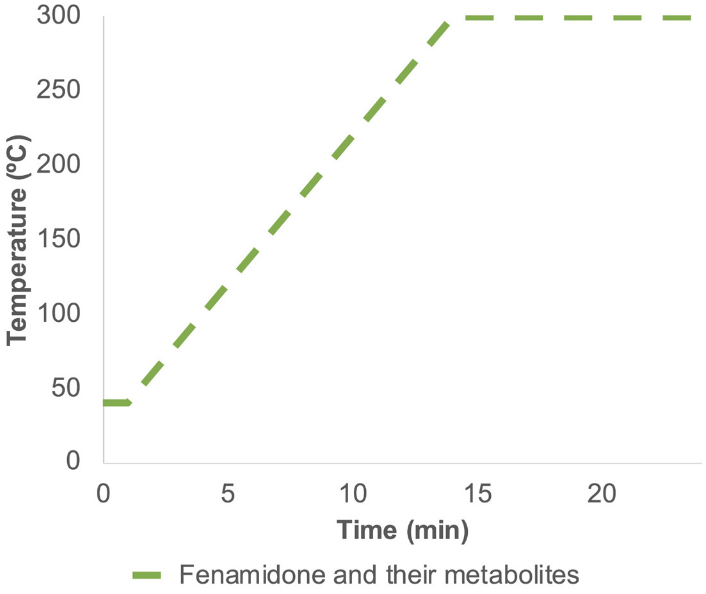


Figure 6.28. Oven temperature for Publication X.

In relation to spectrometric conditions, characterization of the compounds was developed as in previous studies for LC-MS amenable compounds. In **Publications IX and XI**, the ionization mode was negative for 4-phenoxybenzoic acid, whereas it was positive for the rest of the analytes. In relation to the fragments, the loss of a methoxy group ($-CH_2O$) and the loss of an amide group ($-C_2H_4NO$) was observed for 1-acetyl-2-phenylhydrazine, providing the *m/z* 108.06869 and 92.05020 fragment ions, respectively. For 4-phenoxybenzoic acid, the most characteristic fragment is a result of the loss of the acid group (-COOH), providing the fragment *m/z* 169.06526. For the parent compound, famoxadone, the fragments were obtained from the cleavage of cyclopentane that is present in its structure. For example, fragment *m/z* 331.06869 is obtained because of the fragmentation of the amide group of the cyclopentane and the loss of a carboxylate group (-COO). **Table 6.18** shows the HRMS conditions described above.

Table 6.18. HRMS conditions developed for famoxadone and its metabolites.

	Precursor ion		***Fragments***			
Compounds	***Exact mass (m/z)***	***Mass error (ppm)***	***Exact mass (m/z)***	***Molecular formula***	***Mass error (ppm)***	***RT (min)***
Famoxadone	392.16048	-0.45	195.08002	$[C_{14}H_{11}O]^+$	-2.2	6.75
			331.14334	$[C_{21}H_{19}O_2N_2]^+$	2.3	
*1-Acetyl-2-phenylhydraz*ine	151.08659	-0.29	108.06869	$[C_6H_8N_2]^+$	4.1	5.10
			92.05020	$[C_6H_6N]^+$	4.9	
4-Phenoxybenzoic acid	213.05572	-2.12	169.06526	$[C_{12}H_9O]^-$	-2.7	6.44
			93.03345	$[C_6H_5O]^-$	-0.4	

In **Publication X**, spectrometric characterization was carried out as follows: two characteristic fragments were monitored for propamocarb. One of them, *m/z* 102.05496, was obtained due to the cleavage of carbon-amide nitrogen linkage, losing an amine group ($-C_5H_{12}N$), whereas the other fragment, *m/z* 74.02365, corresponded to the loss of an ethyl group ($-C_2H_5$) from the previous fragment (**Table 6.19**). The acetophenone fragments were obtained by the loss of methyl group ($-CH_3$, *m/z* 82.04132) and methoxy group ($-CH_3CO$, *m/z* 77.03858) (**Table 6.19**).

For analytes determined by GC-Q-Orbitrap-MS, spectrometric characterization was performed using the following procedure: the most intense and selective peak was used as quantifier peak, and the second and the third ions were used as qualifiers (**Table 6.19**). However, for 5-methylhydantoin, only one qualifier ion was obtained, because low sensitivity was achieved for the other fragments. The spectral library NIST 2.0 was used to find out the ions for each analyte. The exact masses of each peak were selected with the criterion that mass errors were lower than 5 ppm for the target compounds.

Finally, for **Publication XII**, propamocarb and acetophenone were characterized previously in **Publication X** and the other compounds (fenamidone and metabolites) were characterized using the procedure described above. HRMS conditions are shown in **Table 6.19.**

Table 6.19. HRMS conditions developed for target compounds: a) Publication X and b) Publication XII.

a)	***Quantifier ion***		***Qualifier ions***			***RT (min)***
Compounds	***Exact mass (m/z)***	***Mass error (ppm)***	***Exact mass (m/z)***	***Molecular formula***	***Mass error (ppm)***	
Propamocarb (LC)	189.1597	1.45	102.0549	$[C_4H_8O_2N]^+$	-4.2	5.9
			74.0237	$[C_2H_4O_2N]^+$	5.3	
Acetophenone (LC)	121.0648	2.26	77.0386	$[C_6H_5]^+$	4.7	10.5
			82.0413	$[C_5H_5O]^+$	4.9	
Fenamidone (GC)	238.1101	0.51	268.0903	$[C_{15}H_{14}N_3S]^{\cdot}$	0.3	14.2
			237.1022	$[C_{15}H_{13}ON_2]^{\cdot}$	-0.8	
RPA 410193 (GC)	120.0808	0.72	237.1022	$[C_{15}H_{13}ON_2]^{\cdot}$	-0.4	14.6
			281.1159	$[C_{16}H_{15}O_2N_3]^{\cdot}$	0.1	
2-phenylpropionic acid (GC)	150.0675	-0.07	105.0704	$[C_8H_9]^{\cdot}$	-0.3	7.7
			77.0391	$[C_6H_5]^{\cdot}$	-0.5	
5-methylhydantoin (GC)	114.0429	0.38	105.0502	$[C_4H_7O_2N_2]^{\cdot}$	0.9	8.6
5-methyl-5-phenylhydantoin (GC)	175.0502	-0.72	119.0730	$[C_8H_9N]^{\cdot}$	0.7	11.3

b)

Compounds	**Precursor ion**		**Fragments**			**RT**
	Exact mass (m/z)	***Mass error (ppm)***	***Exact mass (m/z)***	***Molecular formula***	***Mass error (ppm)***	***(min)***
Fenamidone	312.11651	0.62	236.11822	$[C_{15}H_{14}N_3]^+$	3.2	12.2
			134.07127	$[C_7H_8N_3]^+$	2.9	
RPA-410193	282.12370	-1.38	120.08078	$[C_8H_{10}N]^+$	2.8	11.1
			92.04948	$[C_6H_6N]^+$	4.0	
5-methyl-5-phenylhydantoin	191.08150	3.49	120.08078	$[C_8H_{10}N]^+$	2.2	9.3
			65.03858	$[C_5H_5]^+$	4.9	

5.3. VALIDATION PROCEDURE

Validation of the proposed methods was carried out according to the parameters and criteria set by SANTE guidelines.

Table 6.20 shows the validation parameter values for famoxadone and their metabolites in vegetables and environmental matrices. It is worth mentioning that for both matrices there was a strong matrix effect: a signal enhancement was observed in vegetable matrices meanwhile for environmental matrices, both signal suppression (-52%) and enhancement (198%) were detected. Linearity, expressed as R^2, was higher than 0.9880 and trueness ranged from 77-111% in vegetable matrices and between 72-113% for environmental matrices. In the case of precision values, they ranged from 3-18% in vegetable matrices and 3-19% in environmental matrices.

LODs were 5 µg/kg and LOQs 10 µg/kg in vegetable matrices. In environmental matrices, LODs were set at 2-10 µg/kg in the case of soils and 0.05 µg/L for water matrices, and LOQs were estimated at 20 µg/kg in soils and 0.1 µg/L in water.

Table 6.20. Summary of the validation results for famoxadone and its metabolites in Publications IX and XI.

Validation parameters	***Vegetables***	***Soil and water***
Matrix effect[a]	28% to 50%	-52 to 198%
Linearity (R^2)	> 0.9941	> 0.9880
Trueness[b] *(%)*	77-111	72-113
Interday precision[b] *(%)*	3-18	7-19
Intraday precision[b] *(%)*	3-14	3-16
LODs	5 µg/kg	2-10 µg/kg (soil) and 0.05 µg/L (water)
LOQs	10 µg/kg	20 µg/kg (soil) and 0.1 µg/L (water)

[a] Equation used: $Matrix\,effect = \left(\frac{slope\,in\,matrix}{slope\,in\,solvent} - 1\right) x100\%$

[b] Concentrations tested: LOQs and 100 µg/kg (vegetables), 200 µg/kg (soils) or 1 µg/L (water): n=5

Performance characteristics of the method for fenamidone, propamocarb and their metabolites are shown in **Table 6.21**. Recoveries were between 50% and 116% in vegetable samples, whereas for water and soil samples they ranged between 56% and 120%. Intra and inter-day precision values were lower than 19% for both vegetables and environmental samples respectively. Finally, LODs were not defined in these studies and LOQs were 10 µg/kg (vegetables and soil) and 0.1 µg/L (water).

Table 6.21. Summary of the validation results for fenamidone, propamocarb and their metabolites in Publications X and XII.

Validation parameters	*Vegetables*	*Soil and water*
Matrix effect[a]	58% to 567%	-25 to 197%
Linearity (R^2)	> 0.9881	> 0.9900
Trueness[b] *(%)*	50-116	56-120
Interday precision[b] *(%)*	4-19	4-19
Intraday precision[b] *(%)*	1-17	2-17
LOQs	10 µg/kg	0 µg/kg (soil) and 0.1 µg/L (water)

[a] Equation used: $Matrix\,effect = \left(\frac{slope\,in\,matrix}{slope\,in\,solvent} - 1 \right) x100\%$

[b] Concentrations tested: LOQs and 100 µg/kg (vegetables and soils) or 1 µg/L (water): n=5

4.4. DISSIPATION STUDIES

Dissipation studies were carried out for the famoxadone, propamocarb and fenamidone fungicides in vegetables and environmental samples. The target vegetables were cucumber, courgette and tomato, meanwhile environmental samples were soils and water.

4.4.1. Vegetables studies

Famoxadone (**Publication IX**), fenamidone and propamocarb (**Publication XI**) were evaluated in vegetables under greenhouse conditions. Several variables were evaluated as the type of crop, and the applied dose. Vegetable samples were sprayed with the commercial product of each fungicide at normal and double dose and they were randomly collected from 2 hours until 40 days in the case of tomato, 30 days for cucumber and 15 days for courgette. Samples were analysed using the SFO kinetic model.

The SFO kinetic model parameters for famoxadone in cucumber and courgettes are shown in **Table 6.22**. Tomato studies cannot be fitted to any kinetic model ($r^2 < 0.70$), so dissipation parameters in tomato were not calculated. Tomato residues showed that the concentration of famoxadone increased up until 9 days after the application of commercial product for both doses, and slowly decreased until 41 days (**Figure 6.29**).

Table 6.22. SFO kinetic model parameters and vegetables dissipation (DT_{50}) of famoxadone

Matrix	***Cucumber***		***Courgette***	
Parameters	*Normal dose*	*Double dose*	*Normal dose*	*Double dose*
C_0 *(µg/kg)*	392	768	1097	1588
k $(days^{-1})$	0.07	0.07	0.25	0.14
DT_{50} *(days)*	9.42	10.66	2.78	5.01
R^2	0.856	0.905	0.920	0.951

In the case of cucumber, SFO kinetic model revealed that persistence of famoxadone was low, with DT_{50} values around 10 days for both doses, so application dose does not affect pesticide persistence and *k* values were 0.07 $days^{-1}$ for both conditions. Residues decreased until 30 days after application, except at day 5 after application of commercial product, where the concentration slightly increased (**Figure 6.29**).

For courgette, SFO kinetic model (**Table 6.23**) shows that the *k* value is different for both doses, and this can affect the dissipation of the compound. However, persistence was less than in cucumber in both cases, being 2.8 and 5.0 days at normal and double dose respectively. Residue concentrations increased up at 2 days after the application of commercial product and later quickly decreased until 12 days. For example, the maximum concentration was 1200 µg/kg at normal dose.

In summary, famoxadone dissipation follows the same pattern in all matrices. Thus, after the application of the compound, the pesticide was not totally absorbed and, after a few days, the whole amount of pesticide was absorbed. In addition, the main metabolites of famoxadone (4-phenoxybenzoic acid and 1-acetyl-2-phenylhydrazine) were not detected in vegetable samples at concentrations higher than LOQ.

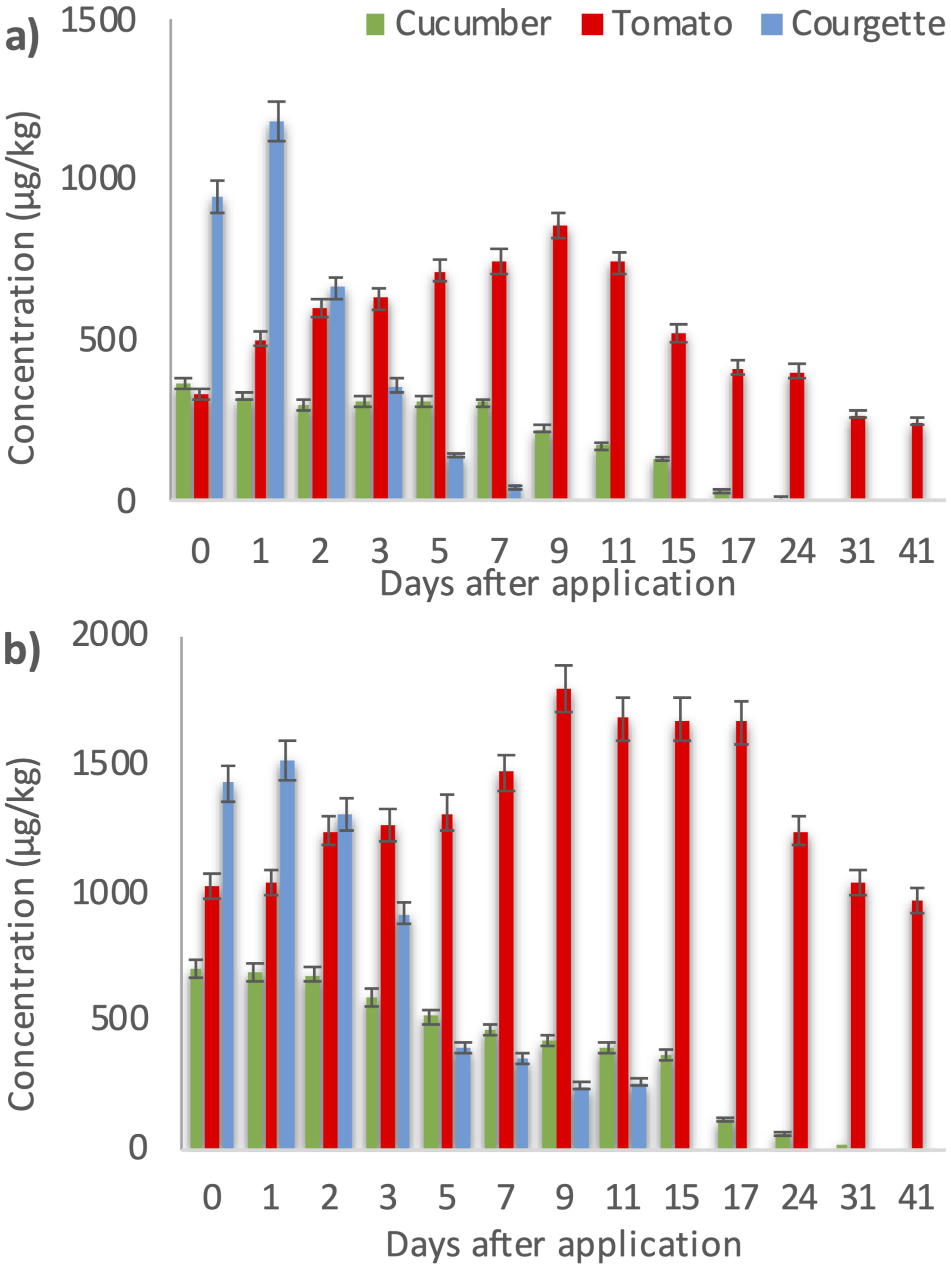


Figure 6.29. Evaluation of famoxadone concentrations in **cucumber**, **tomato** and **courgette** samples during studies at: a) normal dose and b) double dose. Error bars obtained for n=3.

The other two fungicides, propamocarb and fenamidone, were evaluated using the "biphasic" kinetic model (**Table 6.23**). For propamocarb, *k* values were different in each type of matrix (tomato, cucumber and courgette). Propamocarb degraded slower in tomato and cucumber than in courgette. In cucumber studies, k_1 and k_2 values show that the dissipation process (k_1) is slightly lower at the beginning, 0.15 days^{-1} than later, k_2 0.19 days^{-1}, indicating that the behaviour was independent of the dose applied. In tomato at normal dose, k_2 value was really small compared to double dose (2 days^{-1} and 23 days^{-1} respectively). Therefore, the parent compound degraded faster at normal dose than at double dose. Finally, its behaviour in courgettes was the opposite to tomato, and this can be explained because courgettes were affected by a plague of white fly and the low temperatures in February and March. Persistence (DT_{50}) in the three matrices was lower than 15 days. In relation to residues, propamocarb follows the same pattern in all matrices (**Figure 6.30**) except in courgette at normal dose, where concentration decreased throughout all the period monitored.

Fenamidone degraded quicker in courgette studies and its persistence was low, being DT_{50} lower than 30 days (**Table 6.23**). Residue behaviour was the same for all matrices, and its concentration increased after application of commercial product and then decreased (**Figure 6.30**).

On the other hand, metabolites as acetophenone and RPA 410193 were detected in all matrices at concentrations ranging from 10 to 150 µg/kg in the case of normal dose, and from 10 to 200 µg/kg for double dose (**Figure 6.30**). Moreover, 5-methyl-5-phenylhydantoin was only detected in cucumber and courgette samples, but only at double dose (lower than 20 µg/kg). The concentration of RPA 410193 and acetophenone slightly decreased in cucumber at the beginning (1 day) and then it increased up and finally decreased for both doses. For tomato and courgettes, concentrations increased and later decreased.

Table 6.23. Biphasic kinetic model parameters and vegetables dissipation (DT_{50}) of fenamidone and propamocarb

Matrix	***Tomato***		***Cucumber***		***Courgette***	
Parameters	*Normal dose*	*Double dose*	*Normal dose*	*Double dose*	*Normal dose*	*Double dose*
			Fenamidone			
C_0 **(µg/kg)**	1522	2565	3012	8600	1331	2780
C_1 **(µg/kg)**	-0.49	-0.20	-19.90	-19.20	-0.98	-0.50
k_1 **(days^{-1})**	0.03	0.05	0.14	0.21	0.14	0.18
k_2 **(days^{-1})**	0.79	22.90	0.17	0.25	14.49	1.69
DT_{50} **(k_1) (days)**	20.55	15.02	4.80	3.27	4.87	3.89
DT_{50} **(k_2) (days)**	0.87	0.03	4.10	2.78	0.05	0.41
			Propamocarb			
C_0 **(µg/kg)**	1041	1903	1418	2886	2850	6481
C_1 **(µg/kg)**	-0.99	-0.49	-9.25	-8.45	-0.61	-0.06
k_1 **(days^{-1})**	0.08	0.02	0.13	0.15	0.10	0.16
k_2 **(days^{-1})**	2.00	22.99	0.19	0.20	22.33	10.75
DT_{50} **(k_1) (days)**	8.55	29.56	5.28	4.51	7.15	4.20
DT_{50} **(k_2) (days)**	0.34	0.03	3.70	3.44	0.05	0.06

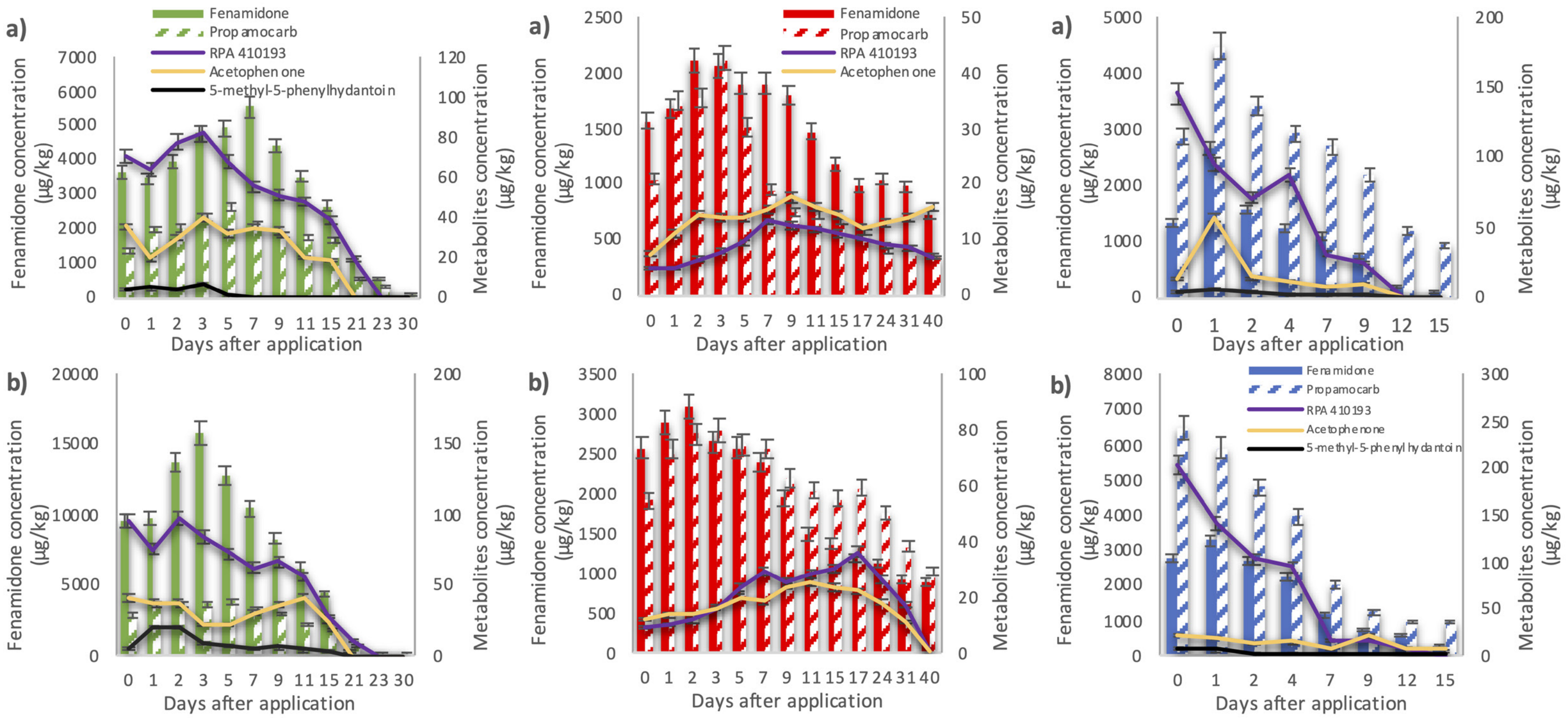


Figure 6.30. Evaluation of fenamidone and propamocarb (bars) and fenamidone metabolites (lines) concentrations in cucumber, tomato and courgette samples in studies at: a) normal dose and b) double dose. Error bars obtained for n=3.

4.4.2. Environmental studies

Dissipation studies in environmental samples were carried out in soils and water for famoxadone (**Publication IX**) and fenamidone and propamocarb (**Publication XI**). For famoxadone, dissipation data are shown in **Table 6.24**. For soils, dissipation of famoxadone was different for each type of soil, being this fungicide more persistent in loam soils than in sandy loam soils. For example, at double dose, DT_{50} was 20 days for sandy loam and 35 days for loam soils. In water, persistence (DT_{50}) of famoxadone was higher under sunny conditions than under dark ones, being DT_{50} value approximately the double in sunny than in dark conditions (22 days for sunny and 12 days for dark conditions).

Table 6.24. SFO kinetic model parameters and environmental dissipation (DT_{50}) of famoxadone

	Sandy loam		***Loam***	
Parameters	*Normal dose*	*Double dose*	*Normal dose*	*Double dose*
C_0 *(μg/kg)*	2045	4093	1916	4050
k $(days^{-1})$	0.040	0.030	0.057	0.018
DT_{50} *(days)*	17	23	20	35
R^2	0.981	0.979	0.962	0.938
	Water Sunny		***Water Dark***	
Parameters	*Normal dose*	*Fivefold dose*	*Normal dose*	*Fivefold dose*
C_0 *(μg/kg)*	912	5041	1916	4124
k $(days^{-1})$	0.032	0.030	0.058	0.049
DT_{50} *(days)*	22	23	12	14
R^2	0.889	0.918	0.913	0.937

Residue behaviours shown in **Figure 6.31** demonstrate that famoxadone behaviour was very similar in soils and water for both doses/conditions, and the concentration of famoxadone decreased during all monitored period.

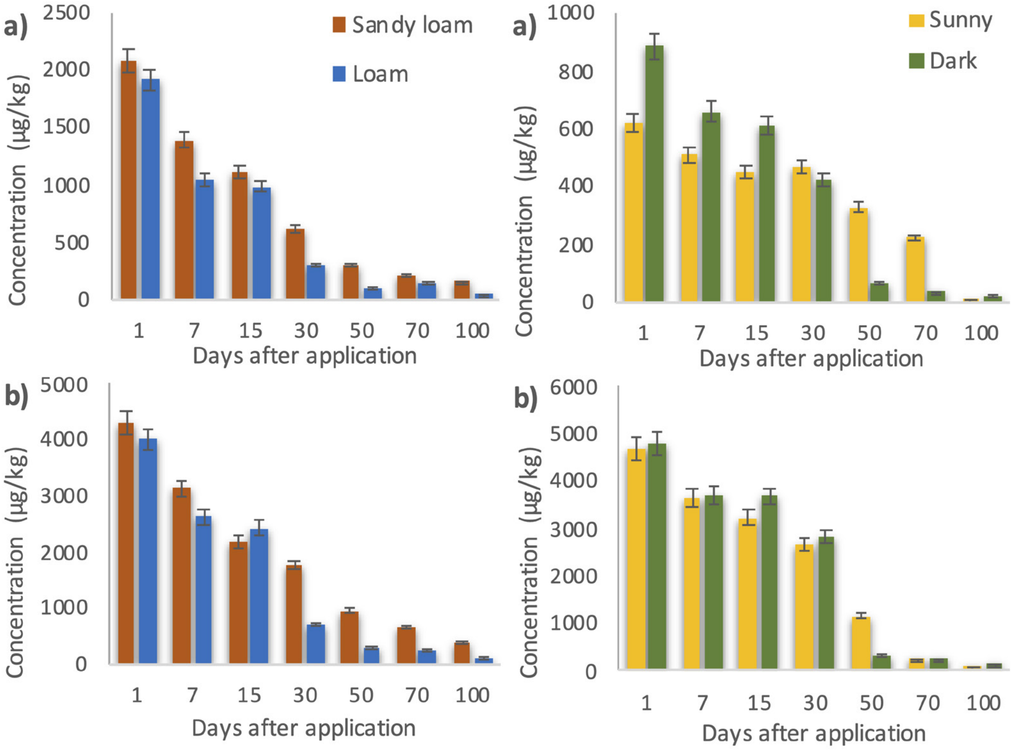


Figure 6.31. Evaluation of famoxadone concentrations in environmental samples in studies at: a) normal dose and b) double/fivefold dose. Error bars obtained for n=3.

Metabolites, as 4-phenoxybenzoic acid and 1-acetyl-2-phenylhydrazine, were not detected at concentrations higher than LOQ, as it was observed in vegetables.

For propamocarb, dissipation in sandy loam and loam soils was similar, as well as in water but it was different when both doses were evaluated. *k* value at double dose was twofold in relation to normal dose in soils (**Table 6.25**). In water, *k* value was slightly higher at normal dose experiments, and the persistence (DT_{50}) was similar for both conditions, but different between doses. In soils, propamocarb was less persistent, with DT_{50} values < 12 days, meanwhile in water, persistence was medium to high with values between 50 and 90 days. In relation to propamocarb residue concentrations, it decreased during the monitored period for both matrices (**Figure 6.32**).

Table 6.25. SFO kinetic model parameters and environmental dissipation (DT_{50}) of fenamidone and propamocarb

Matrix	***Soil***				***Water***			
Conditions	***Sandy loam***		***Loam***		***Sunny***		***Dark***	
Parameters	*Normal dose*	*Double dose*	*Normal dose*	*Fivefold dose*	*Normal dose*	*Fivefold dose*	*Normal dose*	*Fivefold dose*
				Fenamidone				
C_0 *(μg/kg)*	3149	4948	2882	5232	1129	5506	1184	5218
k (days)	0.014	0.018	0.037	0.052	0.011	0.013	0.009	0.013
DT_{50} *(days)*	48.69	39.44	18.78	13.30	62.66	51.46	73.32	57.02
R^2	0.850	0.904	0.902	0.958	0.803	0.834	0.805	0.801
				Propamocarb				
C_0 *(μg/kg)*	8461	14677	5562	11724	5849	14274	6215	12892
k (days)	0.060	0.113	0.075	0.109	0.013	0.010	0.013	0.008
DT_{50} *(days)*	11.57	6.13	9.20	6.36	52.73	66.21	52.49	86.58
R^2	0.912	0.850	0.986	0.992	0.923	0.816	0.914	0.877

Fenamidone dissipation behaviour was different for both types of soils (sandy loam and loam soils) and the concentration decreased during the monitored period (**Figure 6.32**). *k* values were different in both cases (**Table 6.26**). For sandy loam at normal dose, *k* value was 0.014 days^{-1} whereas in loam soils it was 0.037 days^{-1}. Consequently, persistence in sandy loam soils was higher than in loam soils as it can be observed by evaluating DT_{50} values, which were 49 days and 19 days in sandy loam and loam soils respectively. Finally, DT_{50} was higher at normal dose in both soils, and so it can be concluded that the higher fenamidone concentration, the faster dissipation rate.

In water, fenamidone dissipation was similar for both conditions, dark and sunny. When DT_{50} values (**Table 6.25**) were compared (63 days for sunny normal dose and 73 days for dark normal dose), persistence in water was the same under both conditions. In consequence fenamidone dissipation was not affected by the sun in this study and therefore, neither photodegradation nor photolysis affect the degradation of this pesticide. Comparing soils and water trials, fenamidone persistence in water was higher (DT_{50} > 50 days) than in soils (DT_{50} values < 50 days).

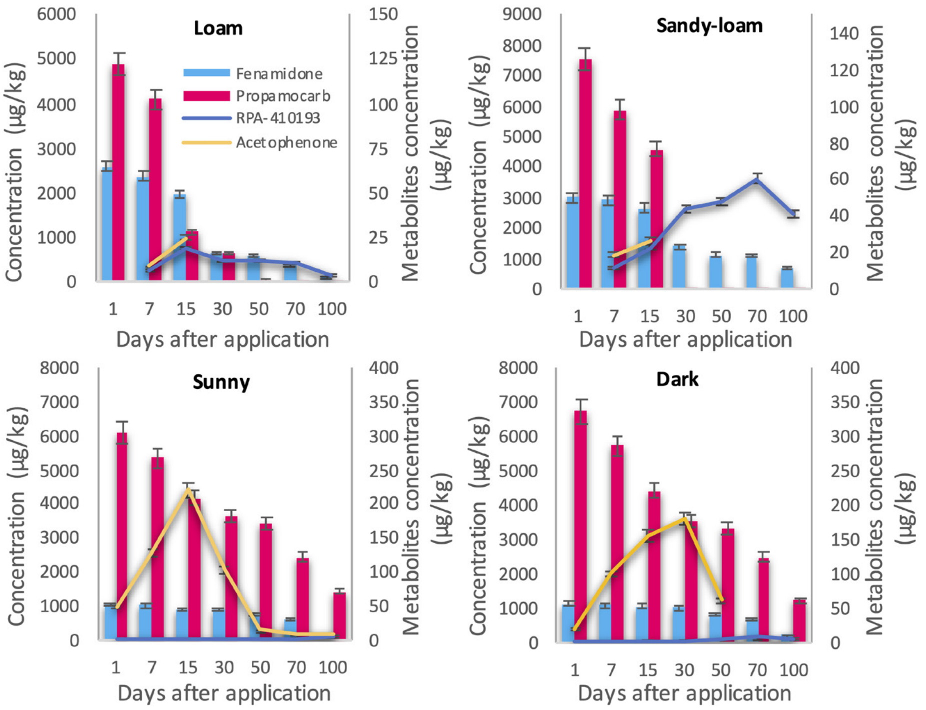


Figure 6.32. Evaluation of fenamidone and propamocarb (bars) and fenamidone metabolites (lines) concentrations in soils and water samples at normal dose studies. Error bars obtained for n=3.

The RPA-41093 and acetophenone metabolites were detected in soils and water samples and their behaviour was the same in both cases: increase their concentration and later decrease. Acetophenone was only detected in soils at 7 and 15 days, meanwhile in water it was detected throughout the study, being the main metabolite detected in water. The main metabolite detected in soils was RPA-41093, with a maximum concentration of 60 μg/kg.

4.5. METABOLITE IDENTIFICATION STUDIES

Identification studies were developed following the two pathways indicated for herbicides and insecticides, i. e. suspect screening and unknown analysis. A data base was built with the data provided by EFSA risk assessment studies. Metabolites used for suspect screening were described in **Figure 6.33**.

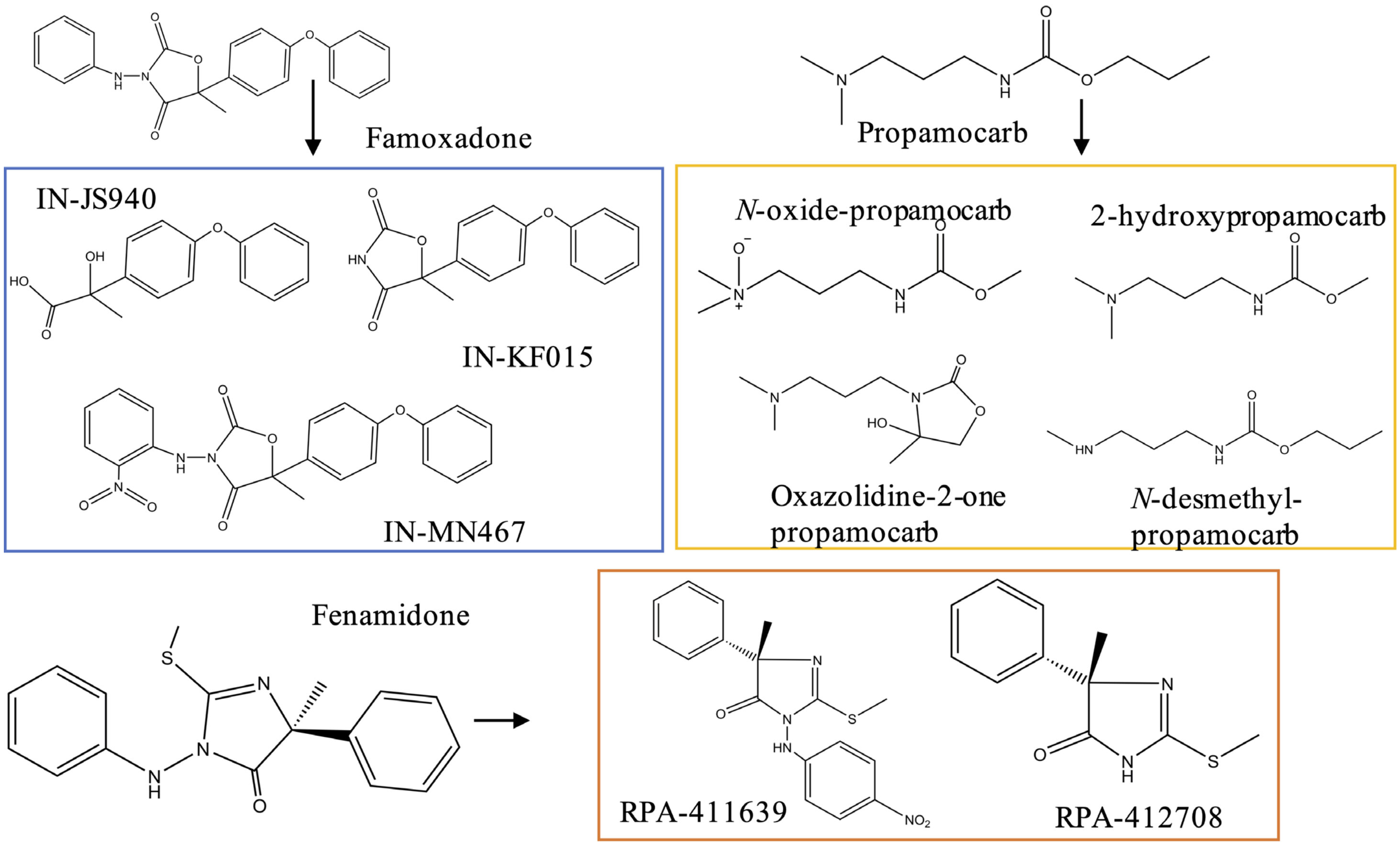


Figure 6.33. Famoxadone, propamocarb and fenamidone metabolites previously described by EFSA.

4.5.1. Vegetable studies

Famoxadone identification studies were carried out using suspect screening and unknown analysis (**Publication IX**). Suspect screening results are shown in **Figure 6.34,** where concentration of metabolites was expressed as the fraction referring to the initial famoxadone content (%). The three detected metabolites, IN-JS940, IN-MN467 and IN-KF015, were found in all vegetables, but in courgette, IN-MN467 was only detected at 2 days after application. In the other matrices, its concentration increased until 15 days and later decreased. The IN-KF015 metabolite was detected in all matrices at a higher concentration compared to the other metabolites and behaviour was the same in cucumber and courgette (decreasing during all period monitored), meanwhile in tomato it was different (increased and later decreased) (**Figure 6.34**). Finally, the IN-JL940 metabolite was detected at 15 days for cucumber and disappeared after 21 days. In tomato, it was detected at 7 days, then slightly increased until 11 days and later decreased. In courgette, it was detected at 9 days after application.

Unknown analysis using software tools aided to the identification of one metabolite of famoxadone. This new metabolite is generated by the reduction of the carbonyl groups present in the famoxadone structure to alcohol. Most likely, it was formed when famoxadone was combined with the matrix because it was not detected in the solution of commercial product with water.

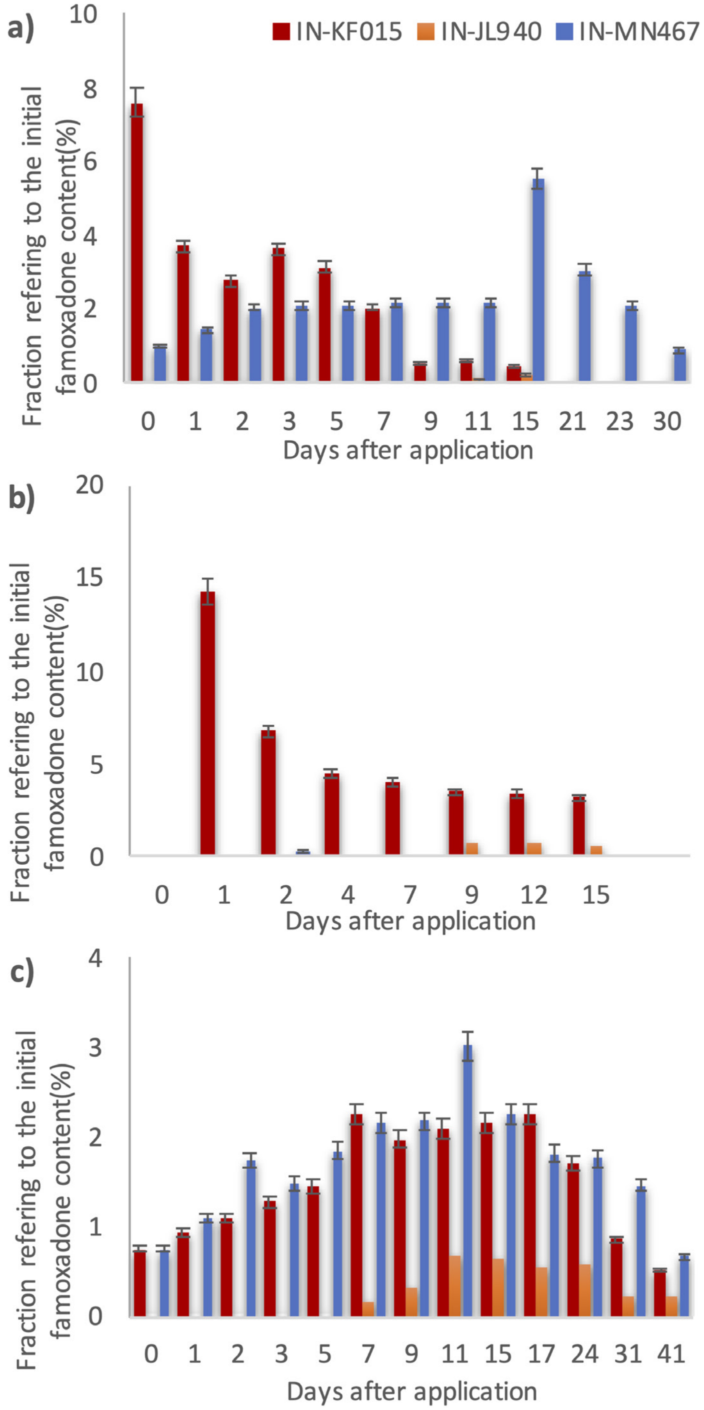


Figure 6.34. Evaluation of famoxadone metabolites concentrations in a) cucumber; b) courgette and c) tomato samples at double dose studies. Error bars obtained for n=3.

This new metabolite was detected with a mass error of 4.5 ppm and a retention time of 4.73 min in cucumber and tomato samples. The structure of the new compound (379 *m/z*) is proposed in **Figure 6.35**. The metabolite was detected 1 day after the application of the commercial product and the dissipation was similar for both doses but not for both matrices. In cucumber, the metabolite concentration increased after the application over the monitoring period, whereas, in tomato, the concentration increased and later decreased, and this was the same for both doses. Comparing the concentration of metabolite 379 *m/z* with the others, it was found to be higher than them. For these reasons, the MRL of famoxadone should include this new metabolite because the sum of the parent compound and the new metabolite would probably overcome the MRL of famoxadone for tomato (2 mg/kg) and cucumber matrices (0.2 mg/kg).

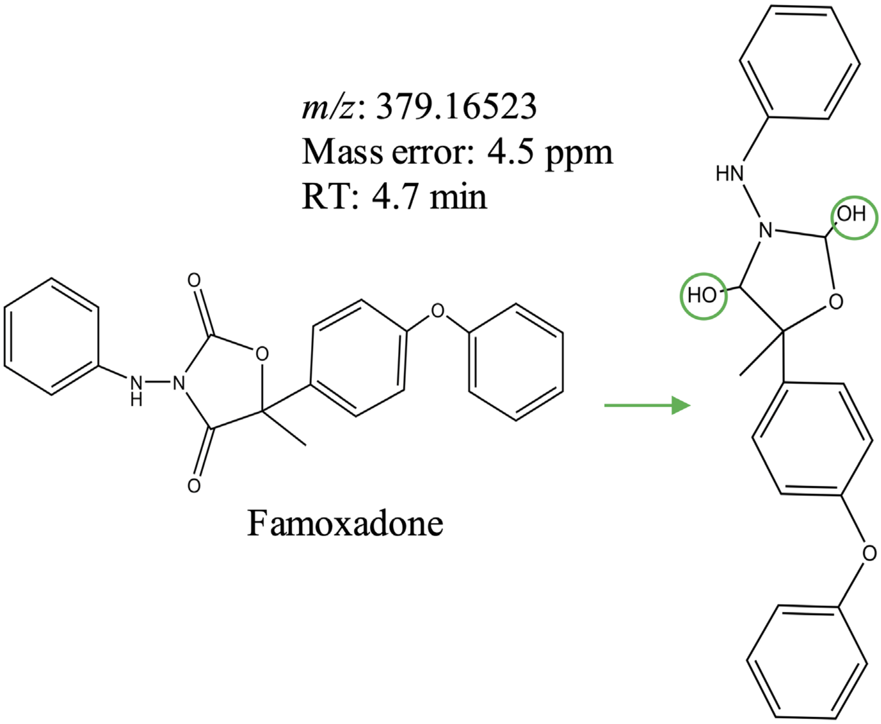


Figure 6.35. Metabolite 379 *m/z* tentatively elucidated from famoxadone in vegetables.

Metabolites of fenamidone (**Publication X**) were not detected at concentrations higher than LOQ of fenamidone, when suspect screening workflow was performed. Using unknown analysis, one metabolite was detected (**Figure 6.36**). This metabolite, 264 *m/z,* was formed from the cleavage of carbon-sulfur group (-CH_3S). It was detected at 11.37 min with a mass error of 0.48 ppm. According to Schymanski classification, different levels related to the identification of new compounds using HRMS can be set, and in this study, metabolite can be classified at level 2. Metabolite behaviour was shown in **Figure 6.37**. It was detected 1 day after the application and its concentration increased up and later decreased for all matrices (**Figure 6.37**).

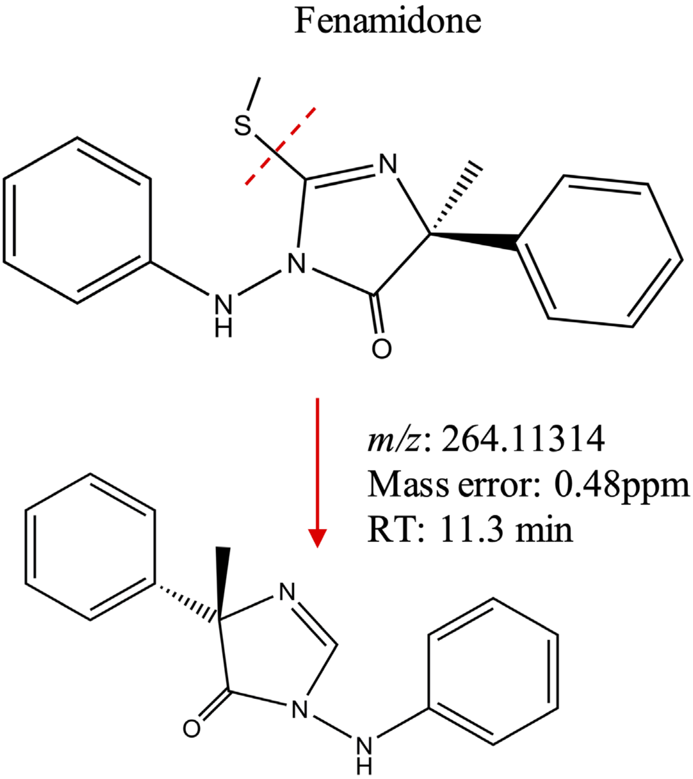


Figure 6.36. Metabolite 264 *m/z* tentatively elucidated from fenamidone in vegetables.

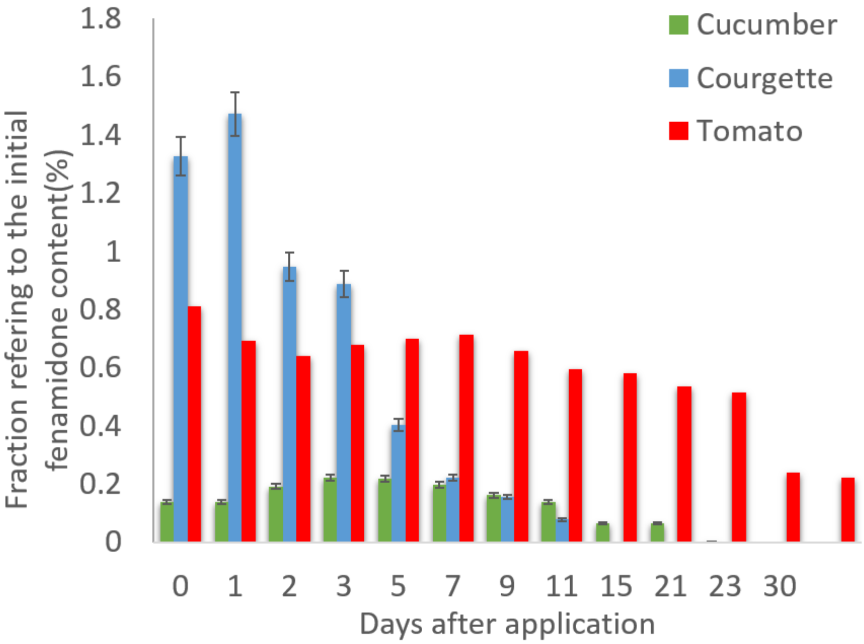


Figure 6.37. Evaluation of fenamidone metabolite 264 *m/z* concentrations in cucumber, tomato and courgette samples at double dose studies. Error bars obtained for n=3.

Two metabolites of propamocarb were detected by suspect screening, oxazoline-2-one propamocarb and *N*-desmethyl propamocarb. The others (**Figure 6.33**) were not detected at concentrations higher than LOQ. Metabolites were detected in all matrices after the first day of application at concentrations lower than 5% of propamocarb content. Metabolites' behaviour was the same for the different dosages and matrices evaluated, and their amount increased. Then their concentrations decreased. It can be observed in **Figure 6.38** that the highest molar mass ratio of metabolites in relation to the initial concentration of the parent compound was obtained in tomato, observing conversion factors higher than 10%, whereas in the other two matrices, this was lower than 1%.

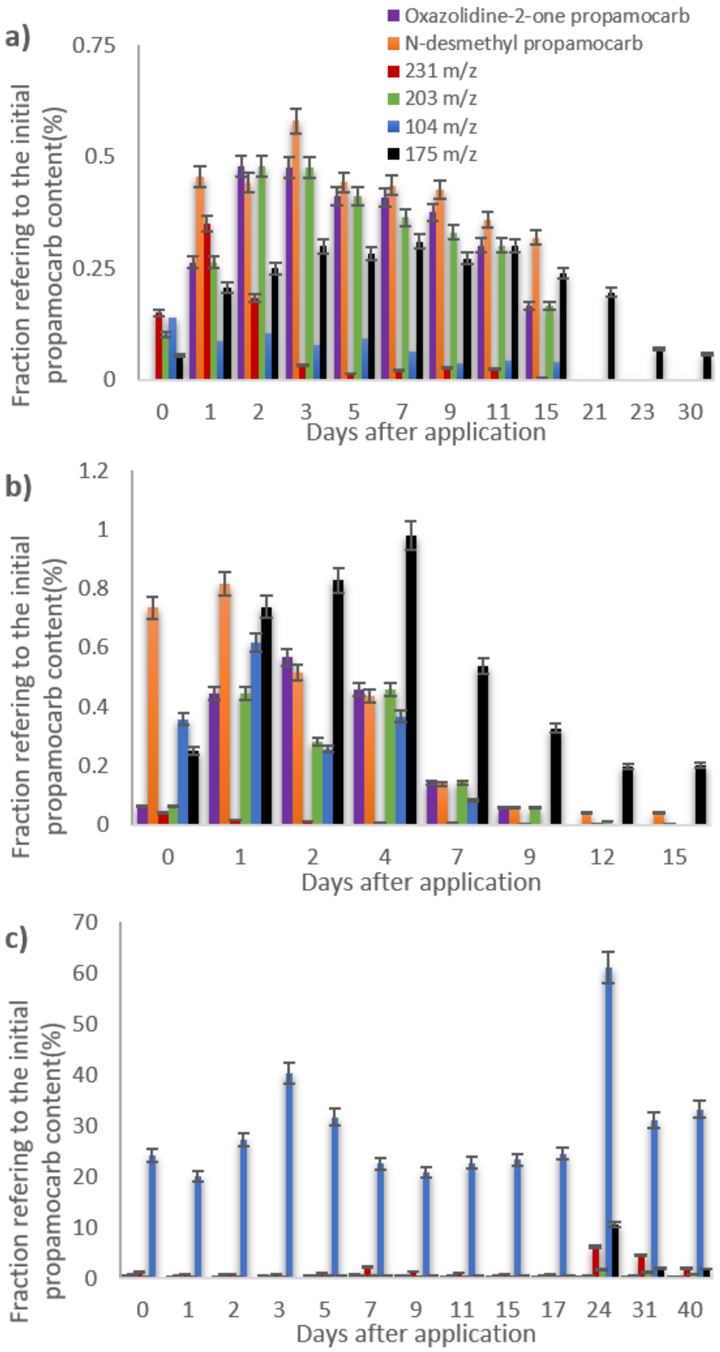


Figure 6.38. Evaluation of propamocarb metabolites concentrations in a) cucumber; b) courgette and c) tomato samples at double dose studies. Error bars obtained for n=3.

Four propamocarb metabolites were tentatively identified by unknown studies (**Figure 6.39**). Metabolite 104 *m/z* was originated from the successive cleavage of a carbon-carbon linkage and later the hydrogenation of nitrogen. It was detected with a mass error of 4.67 ppm and retention time of 1.40 min ($-C_5H_{11}N$). Metabolite 175 *m/z* was detected at 1.67 min (mass error -0.52 ppm) and it comes from the cleavage of a carbon-nitrogen linkage followed by the hydrogenation of nitrogen ($-CH_3$). For metabolite 203 *m/z*, two possible structures were elucidated. They involved the aliphatic N-hydroxilation and the dehydrogenation of two carbons to form a cis or trans double bond. It was detected with a mass error of -0.62 ppm and retention time of 6.94 min. Metabolite 231 *m/z* comes from the dehydrogenation of nitrogen and formation of an amide group (**Figure 6.39**).

In vegetable samples, behaviour of metabolites was the following: metabolite 104 *m/z* was detected at the highest concentration and its behaviour was slightly different in tomato than in cucumber, where its concentrations were constant or it was hardly detectable (**Figure 6.38**). Metabolite 175 *m/z* was detected in all matrices and their fraction was higher in tomato (10%) than other matrices (<1%). The other two metabolites, 203 *m/z* and 231 *m/z*, were detected 1 day after the application of commercial product and their concentration slowly decreased to values lower than LOQ.

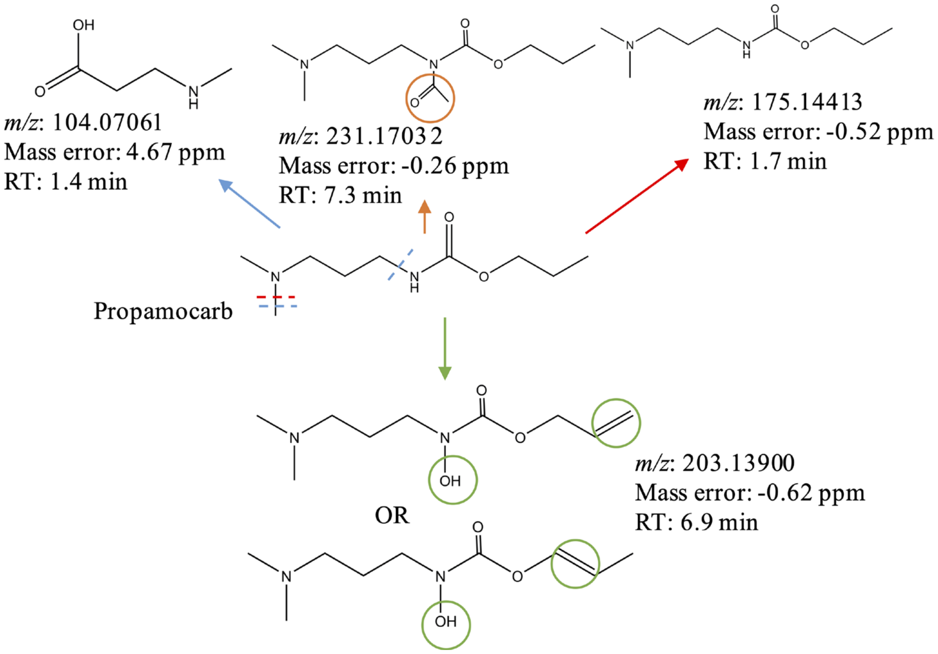


Figure 6.39. Metabolites tentatively elucidated from propamocarb in vegetables.

4.5.2. Environmental studies

Identification studies of new metabolites were also performed in environmental matrices. Famoxadone studies (**Publication XI**) were based on suspect screening analysis using databases developed for vegetables, and with the addition of the new metabolite identified by unknown analysis in vegetables.

In soils, the IN-KF015, IN-MN467 and IN-JS940 metabolites were detected. IN-JS940 was the main metabolite detected, and at a high percentage in relation to the parent compound (25%). Metabolites IN-MN467 and IN-KF015 were also detected in both types of soils but at a small percentage in relation to initial famoxadone content (<2%). Although they were detected in both soils, their behaviour was different. In the case of sandy loam soils, their concentration increased until 70 days and later quickly decreased until 100 days. In loam soils their concentration increased until 30 days and then, slowly decreased until 100 days (**Figure 6.40**).

In water, IN-KF015 and IN-JS940 were the only two metabolites detected. Their behaviour was the same for both conditions/doses. IN-JS940 was the main metabolite detected (from 0.4 to 5% of initial famoxadone content) as in soils. When the concentration of the parent compound decreased, its concentration increased until the end of the monitoring period (**Figure 6.40**). The IN-KF015 metabolite followed the same behaviour but its maximum fraction was 0.25% of the initial famoxadone content.

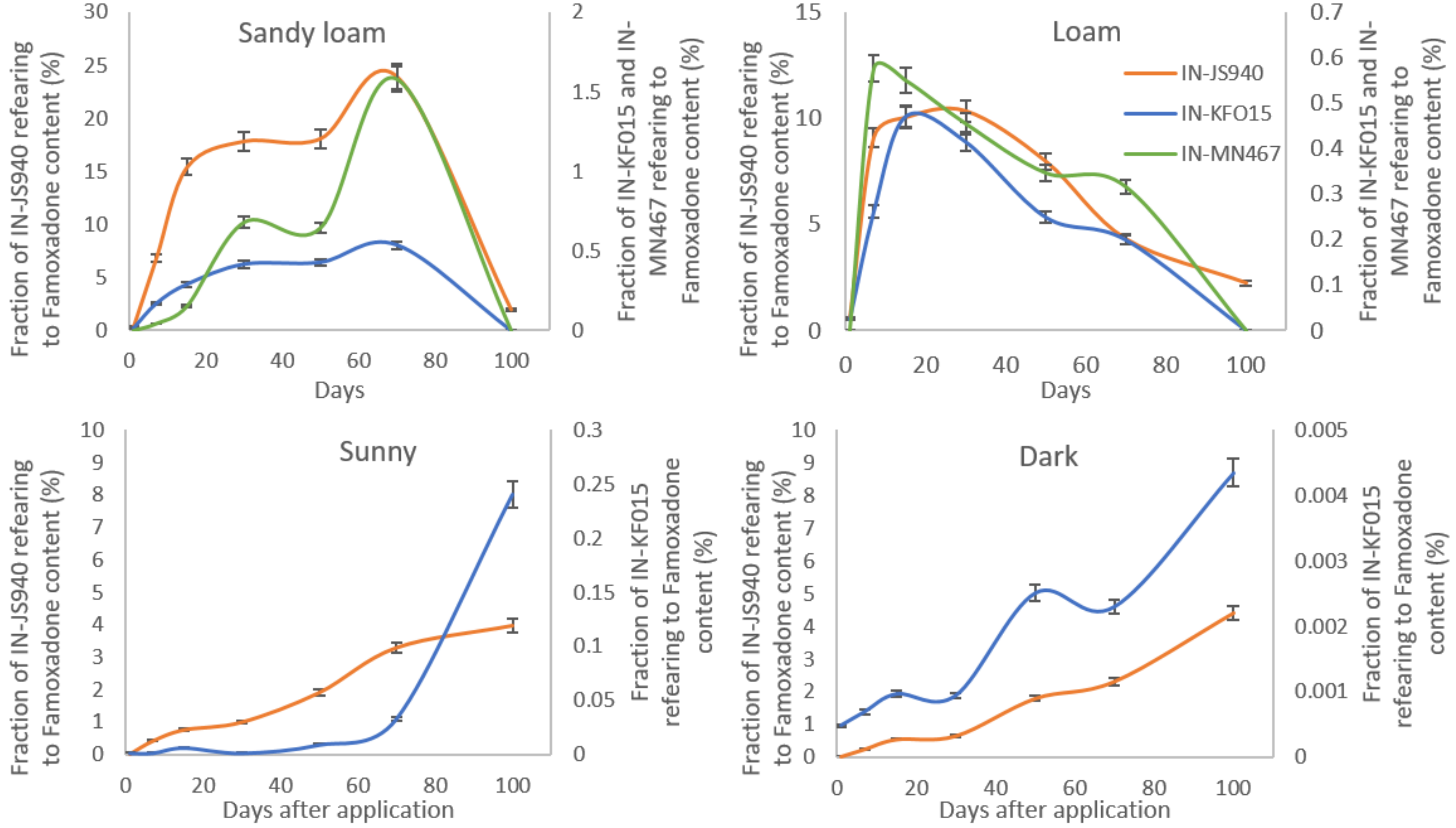


Figure 6.40. Evaluation of famoxadone metabolites concentrations in environmental samples at double dose studies. Error bars obtained for n=3.

Fenamidone studies (**Publication XII**) were carried out as famoxadone using a homemade database containing EFSA metabolites and unknown metabolites detected in vegetables.

RPA-412708, RPA-411639 and metabolite 264 *m/z* were detected in soils. Metabolite concentration increased up, while parent compound concentration decreased, and then decreased at 100 days. RPA-411639 was the metabolite detected at the highest concentration (23% of fenamidone initial content), and its concentration ranged from 0.5% of initial fenamidone content at 1 day to 23% at 70 days and later decreased until 10% (**Figure 6.41**). Metabolite behaviour is completely different in water trials compared with soil studies. In soils, RPA-411639 and RPA-412708 were the main metabolites but in water, metabolite 264 *m/z* was the main detected metabolite (**Figure 6.41**). However in this matrix, RPA-412708, RPA-411639 and metabolite 264 *m/z* were detected at percentages lower than 0.5% (**Figure 6.41**). The content of the metabolites RPA-412708 and RPA-411639 increased from 1 day to 100 days and metabolite 264 *m/z* increased its concentration till 15 days and later decreased until the end of monitored period.

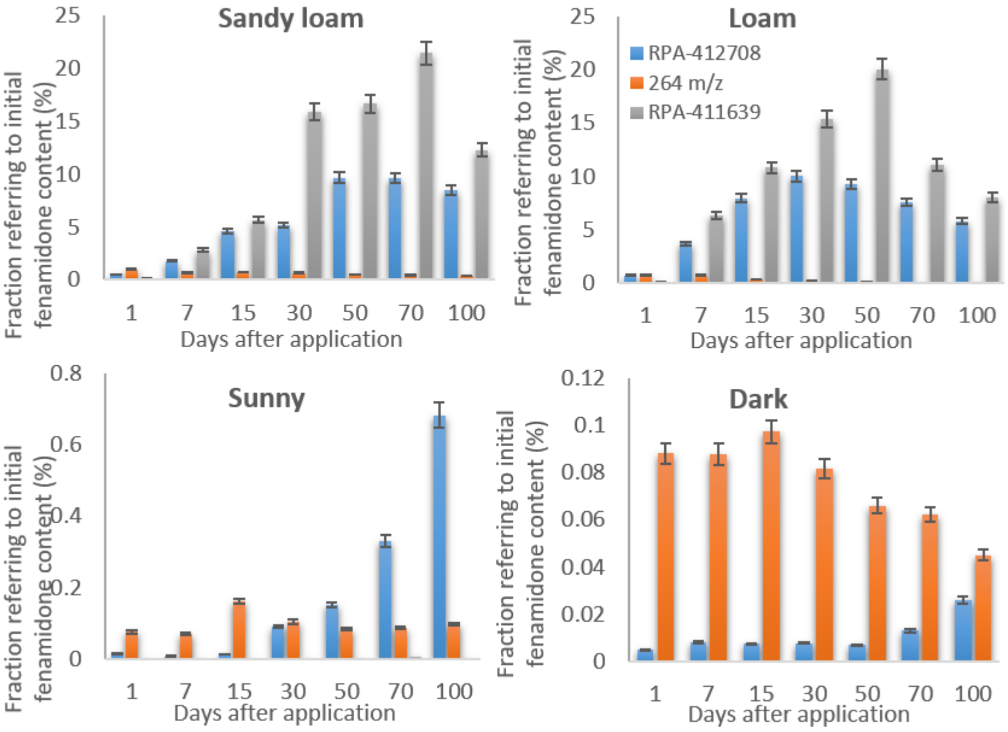


Figure 6.41. Evaluation of fenamidone metabolites concentrations in environmental samples at double dose studies. Error bars obtained for n=3.

Propamocarb studies involved suspect screening as well as unknown analysis (**Publication XII**). Propamocarb oxozoline, propamocarb n-desmethyl, 175 *m/z* and 203 *m/z* metabolites were monitored in soils (**Figure 6.42**). Their behaviour was the same in both soils/doses except for metabolite 175 *m/z*, which showed different behaviour when different doses were evaluated. The presence of propamocarb n-desmethyl decreased during the monitoring period as the parent compound and the other metabolites increased in concentration (from 0 to 30 days), after which they started decreasing until 100 days. The fraction of metabolites in relation to initial propamocarb content was not higher than 3% so the quantity of metabolites originated was not relevant when compared with the parent compound (**Figure 6.42**).

Metabolite behaviour for propamocarb oxozoline, propamocarb n-desmethyl, 175 *m/z* and 203 *m/z* were detected in water trials (**Figure 6.42**) but it was different than in soils. Propamocarb n-desmethyl was the main metabolite detected in water (0.7%). Propamocarb n-desmethyl behaviour content increased at the beginning (30 days) and later decreased. The other detected metabolites (propamocarb oxozoline, 175 *m/z* and 203 *m/z*) had similar behaviour to propamocarb n-desmethyl but their concentrations were very low (<0.06%) compared with propamocarb n-desmethyl content. As in soils, the total percentage of metabolites referring to the initial propamocarb content was lower than 1% during the whole monitored period, so metabolite behaviour was not very significant.

Unknown analysis allowed the tentatively elucidation of three new metabolites of propamocarb. Propamocarb metabolites were generated by simple reactions. Metabolite 160 *m/z* was originated from the cleavage of a carbon-nitrogen union ($-C_2H_6N$) followed by the oxygenation of the carbon (+OH). This compound eluted at 9.23 min and the mass error was 0.064 ppm (**Figure 6.43**). Metabolite 162 *m/z* ($C_7H_{15}NO_3$) was monitored at 8.96 min (mass error 0.309 ppm) which can be explained because of the cleavage of a carbon-nitrogen union ($-C_2H_6N$) followed by reduction of the carbon (C=O). Metabolite 205 *m/z* ($C_9H_{20}N_2O_3$) was formed by the oxygenation of a secondary carbon (+OH). It was monitored with a mass error of 0.590 ppm at 6.61 min (**Figure 6.43**).

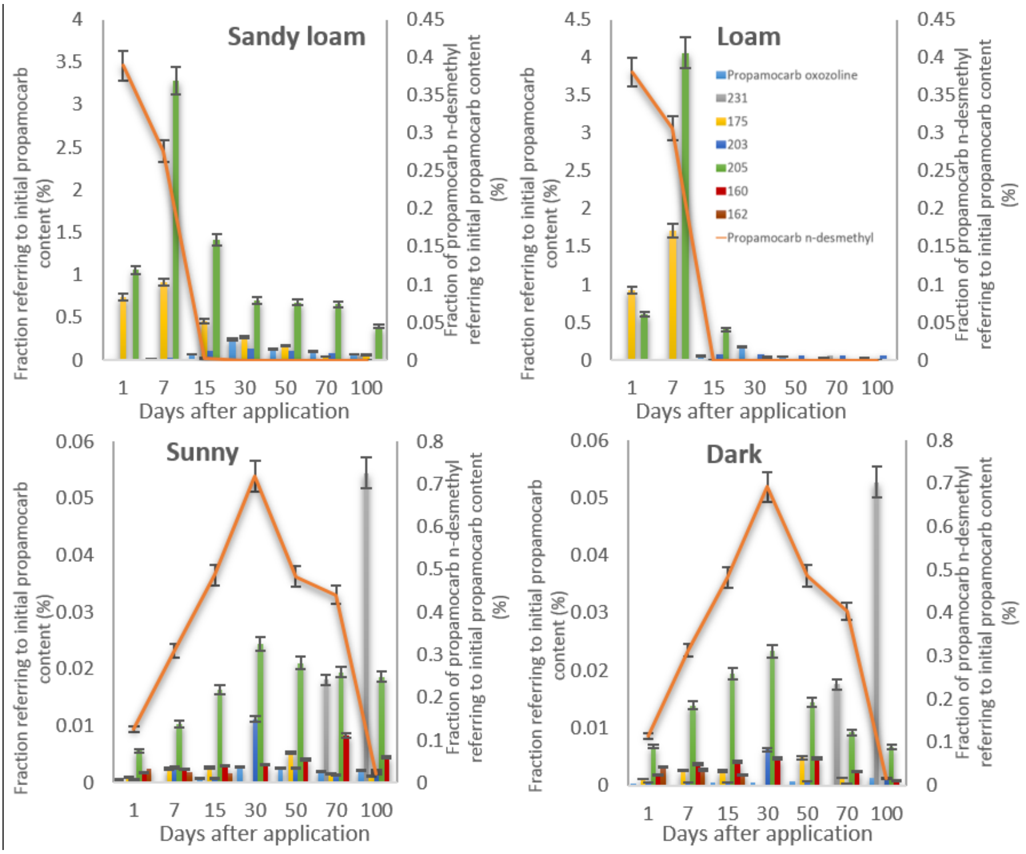
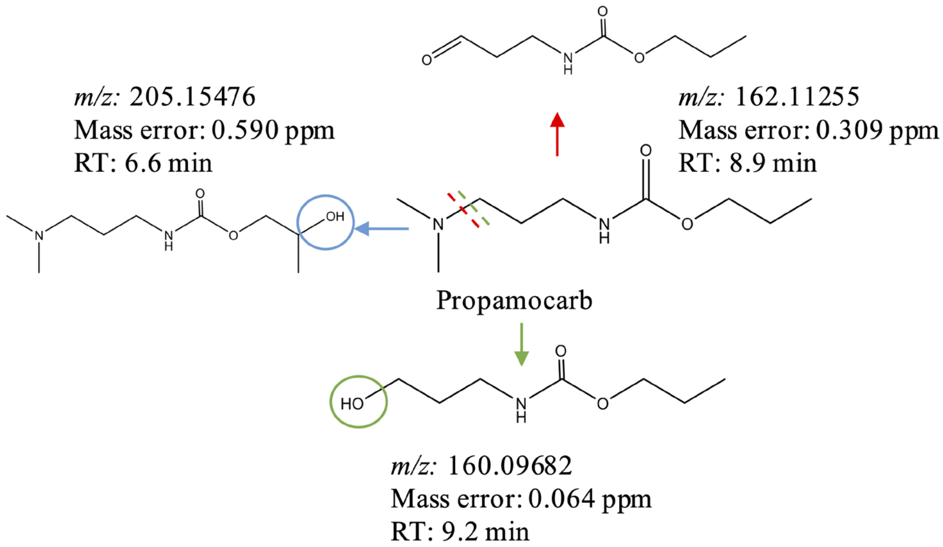


Figure 6.42. Evaluation of propamocarb metabolites concentrations in environmental samples at double dose studies. Error bars obtained for n=3.

These metabolites were studied in all the samples, monitoring their behaviour during the whole period (**Figure 6.42**). Metabolite 160 *m/z* was only monitored in water samples and its concentration increased up until 50 days and later decreased for both conditions/doses. Metabolite 162 *m/z* was only monitored in water and it was detected 1 day after applying the plant protection product and then the amount decreased until 30 days, when the fraction in relation to the initial propamocarb content became negligible. Finally, metabolite 205 *m/z* was monitored in soil and water trials and it was the tentative metabolite detected at the highest concentration, and this concentration increased in soils and water (**Figure 6.42**).



m/z: 205.15476
Mass error: 0.590 ppm
RT: 6.6 min

m/z: 162.11255
Mass error: 0.309 ppm
RT: 8.9 min

Propamocarb

m/z: 160.09682
Mass error: 0.064 ppm
RT: 9.2 min

Figure 6.43. Metabolites tentatively elucidated from propamocarb in environmental samples.

CHAPTER 7

Conclusions

1. CONCLUSIONS

The general objective of this Thesis was based on the study of the dissipation of pesticides after their application, as well as the detection of targeted and non-targeted metabolites in food and environmental matrices. From the results obtained throughout this Thesis, the following conclusions can be highlighted:

- A generic methodology was developed to carry out the dissipation study of pesticides in vegetable and environmental matrices under greenhouse and laboratory conditions. Studies were developed at two doses of the plant protection product to check whether the behavior of pesticides was influenced by the dose and to monitor the metabolite appearance.
- Dissipation kinetic models were performed for the pesticides studied in the current Thesis, evaluating the model the experimental data were fitted. Data provided by the models determined the behavior of pesticides in the different matrices.
    - Persistence of the pesticides in vegetables was low (DT_{50} < 30 days) in all cases (insecticides and fungicides), being famoxadone and thiocyclam non-persistent, with DT_{50} values lower than 10 days.
    - In soils, persistence was different depending on the pesticide type. For herbicides, persistence was high in the case of dimethachlor (DT_{50} > 50 days) and non-persistent for quizalofop related compounds (DT_{50} < 1 day). Fungicides persistence was medium to low (DT_{50} < 50 days) for the three compounds (fenamidone, propamocarb and famoxadone).
    - In water, persistence was similar in herbicides and fungicides with DT_{50} values higher than 50 days, except for

    the famoxadone fungicide whose persistence was medium to low (DT_{50} < 30 days).
  - Due to the higher persistence in environmental matrices, the toxicology effects of these pesticides to fauna and flora should be studied to ensure the environmental safety.
- The use of Orbitrap-MS and Q-Orbitrap-MS have been demonstrated to be suitable tools to perform retrospective analysis. Due to the use of the full scan mode, the identification of unknown compounds (unknown analysis) and the detection of metabolites previously described by home-made database (suspect screening) can be performed.
- A software workflow was developed with the purpose of carrying out unknown analysis easily and quickly. That based on common reactions as oxidation and reduction using the parent compounds as starting molecule. A total of 12 metabolites were tentatively elucidated from the studied pesticides: three metabolites of dimethachlor, one for fenamidone and famoxadone and seven for propamocarb.
- The detection of some metabolites in vegetables and environmental matrices highlights the necessity to review the legislation about the MRLs of the parent compound to evaluate the possibility of included some metabolites in the MRLs definition.

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Ultrahigh-pressure liquid chromatography-mass spectrometry: An overview of the last decade

Author: Rosalía López-Ruiz,Roberto Romero-González,Antonia Garrido Frenich

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Metabolomics approaches for the determination of multiple contaminants in food

Author: Rosalía López-Ruiz,Roberto Romero-González,Antonia Garrido Frenich

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Determination of flonicamid and its metabolites in bell pepper using ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (Orbitrap)

Author: Rosalía López-Ruiz, , Roberto Romero-González, et al

Publication: Food Additives & Contaminants

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The metabolic pathway of flonicamid in oranges using an orthogonal approach based on high-resolution mass spectrometry and nuclear magnetic resonance

R. López-Ruiz, A. B. Ruiz-Muelle, R. Romero-González, I. Fernández, J. L. Martínez Vidal and A. G. Frenich, *Anal. Methods*, 2017, **9**, 1718
DOI: 10.1039/C7AY00101K

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Degradation studies of quizalofop-p and related compounds in soils using liquid chromatography coupled to low and high resolution mass analyzers

Author:
Rosalía López-Ruiz,Roberto Romero-González,José Luis Martínez Vidal,Manuel Fernández-Pérez,Antonia Garrido Frenich

Publication: Science of The Total Environment

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Behavior of quizalofop-p and its commercial products in water by liquid chromatography coupled to high resolution mass spectrometry

Author:
Rosalía López-Ruiz,Roberto Romero-González,José Luis Martínez Vidal,Antonia Garrido Frenich

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Degradation studies of dimethachlor in soils and water by UHPLC-HRMS: putative elucidation of unknown metabolites

Author: Rosalía López-Ruiz, Roberto Romero-González, Elisabeth Ortega-Carrasco, et al

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Dissipation studies of famoxadone in vegetables under greenhouse conditions using liquid chromatography coupled to high-resolution mass spectrometry: putative elucidation of a new metabolite

Author: Antonia Garrido Frenich, Elisabeth Ortega-Carrasco, Roberto Romero-González, et al

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Dissipation kinetic studies of fenamidone and propamocarb in vegetables under greenhouse conditions using liquid and gas chromatography coupled to high-resolution mass spectrometry

Author: Rosalía López-Ruiz,Roberto Romero-González,Blanca Serra,Antonia Garrido Frenich

Publication: Chemosphere

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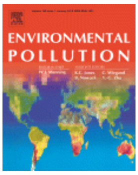

Residues and dissipation kinetics of famoxadone and its metabolites in environmental water and soil samples under different conditions

Author: Rosalía López-Ruiz,Roberto Romero-González,Antonia Garrido Frenich

Publication: Environmental Pollution

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Dissipation kinetics of fenamidone, propamocarb and their metabolites in ambient soil and water samples and unknown screening of metabolites

Author: Rosalía López-Ruiz,Roberto Romero-González,Antonia Garrido Frenich

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Artículos científicos:

1. López-Ruiz, R; Romero-González, R; Garrido-Frenich, A. Simultaneous determination of polar pesticides in human blood serum by liquid chromatography coupled to triple quadrupole mass spectrometer. Journal of Pharmaceutical and Biomedical Analysis. 190, pp. 113492. 2020.
2. Rivera-Pérez, A; López-Ruiz, R; Romero-González, R; Garrido-Frenich, A. A new strategy based on gas chromatography–high resolution mass spectrometry (GC–HRMS-Q-Orbitrap) for the determination of alkenylbenzenes in pepper and its varieties. Food Chemistry. 321, pp. 126727. 2020.
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5. Ruiz-Delgado, A; Martínez-Domínguez, G; Romero-González, R; López-Ruiz, R; Garrido-Frenich, A. Determination of polycyclic

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8. Título del trabajo: Determination of trans-anethole and myristicin in infusion products by ultrasound extraction and gas chromatography-mass spectrometry. Rivera-Pérez, A; Lopez-Ruiz, R; Garrido-Frenich, A; XVIII scientific meeting of the Spanish society of chromatography and related techniques – SECyTA 2018. Granada, Andalucía (España). 02/10/2018-04/10/2018.
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11. Título del trabajo: Monitoring of quizalofop-p metabolites in environmental samples using liquid chromatography coupled to high resolution mass spectrometry. López Ruiz, R; Romero-Gonzalez, R; Martinez-Vidal, JL; Garrido Frenich, A; 14th Annual

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14. Título del trabajo: Automated multiresidue analysis of neonicotinoid insecticides and their metabolites in human urine by on-line turbulent-flow liquid chromatography orbitrap high resolution mass spectrometry Lopez-Garcia, M; Romero-Gonzalez, R; Arrébola-Liébanas, FJ; Lopez-Ruiz, R; Dominguez-Perez, I; Garrido-Frenich, A; 2nd International Caparica Conference on Pollutant Toxic Ions and Molecules. Caparica (Portugal). 06/11/2017-09/11/2017.
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27. Título del trabajo: Dissipation studies of Flonicamid in its metabolites in orange samples by liquid chromatography - orbitrap mass spectrometry. Lopez-Ruiz, R; Romero-González, R; Martínez-Vidal, JL; Marín-Sáez, J; Garrido-Frenich, A; XXV RNE – IX CIE Espectroscopía. Alicante, Comunidad Valenciana (España). 20/07/2016-22/07/2016. Comunicación oral.
28. Título del trabajo: Enantiomeric separation of (-) and (+)-hyoscyamine by high performance liquid chromatography-tandem mass spectrometry. Marín-Sáez, J; Romero-González, R; Martínez-Vidal, JL; Lopez-Ruiz, R; Garrido-Frenich, A; XXV RNE – IX CIE Espectroscopía. Alicante, Comunidad Valenciana (España). 20/07/2016-22/07/2016. Comunicación oral.
29. Título del trabajo: Determination of bioactive compounds in “gazpacho” by ultra-high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry. Marín-Sáez, J; Ferrer-Aguirre, A; Lopez-Ruiz, R; Romero-González, R; Martínez Vidal, JL; Garrido-Frenich, A; IV Mini simposio de Investigación en Ciencias Experimentales. Almería, Andalucía (España). 13/11/2015.
30. Título del trabajo: Fast determination of Flonicamid and its metabolites in orange samples by ultra-high performance liquid chromatography – tandem mass spectrometry. Lopez-Ruiz, R; Marín-Sáez, J; Romero-González, R; Garrido-Frenich, A; IV Mini simposio de Investigación en Ciencias Experimentales. Almería, Andalucía (España). 13/11/2015.
31. Título del trabajo: Fast determination of Flonicamid and its metabolites in orange samples by ultra-high performance liquid chromatography - tandem mass spectrometry. López-Ruiz, R; Ruiz-Delgado, A; Marín-Sáez, J; Romero-González, R; Garrido-Frenich, A; XV Reunión Científica de la Sociedad Española de Cromatografía y Técnicas Afines (SECyTA 2015). Castellón, Comunidad Valenciana (España). 28/10/2015-30/10/2015.
32. Título del trabajo: Determination of bioactive compounds in “gazpacho” by ultra-high performance liquid chromatography

coupled to triple quadrupole tandem mass spectrometry. Marín-Sáez, J; Ferrer-Aguirre, A; Lopez-Ruiz, R; Romero-González, R; Garrido-Frenich, A; XV Reunión Científica de la Sociedad Española de Cromatografía y Técnicas Afines (SECyTA 2015). Castellón, Comunidad Valenciana (España). 28/10/2015-30/10/2015.

33. Título del trabajo: Análisis de contaminantes polares en productos nutraceúticos derivados de soja mediante cromatografía de líquidos acoplada a un analizador de triple cuadrupolo. Romera-Torres, A; Domingos-Alves, R; Romero-González, R; López-Ruiz, R; López-García, M; Garrido-Frenich, A; XV Graseqa 2016. Almería, Andalucía (España). 30/06/2015-01/07/2015.
34. Título del trabajo: Control de más de 500 residuos de plaguicidas en muestras vegetales mediante LC-MS/MS y GC-MS/MS. Lopez-Ruiz, R; Lopez-Martínez, JC; Diaz-Moreno, L; Hernández-Torrez, ME; Ferrer-Aguirre, A; Martínez-Vidal, JL; Garrido-Frenich, A; XV Graseqa 2016. Almería, Andalucía (España). 30/06/2015-01/07/2015.

Actividades I+D+i

1. II AmBioBlitz; Universidad de Almería; Almería, Andalucía, España. 25/04/2019
2. La información química sobre los alimentos; La noche de los investigadores, Almería, Andalucía, España. 28/09/2018.
3. La química de los alimentos; La noche de los investigadores, Almería, Andalucía, España. 29/09/2017.
4. Química Analítica: ¿Hay vida después del CSI?; La noche de los investigadores, Almería, Andalucía, España. 30/09/2016.
5. Reunión de la Sociedad Andaluza de Química Analítica (GRASEQA); Congreso, Almería, Andalucía, España. 30/06/2016-01/07-2016.

Patente

1. Título propiedad industrial registrada: Compuestos deuterados de Flonicamida. Procedimiento de preparación y uso de los mismos; Tipo de propiedad industrial: Patente de invención; Inventores/autores/obtentores: López-Ruiz, R; Romero-González, R; Ruiz-Muelle, AB; Fernández, I; Martínez-Vidal, JL; Garrido-Frenich, A; Nº de solicitud: P201600466; País de inscripción: España, Andalucía; Fecha de registro: 13/06/2016; Patente española: Si

Estancias de investigación:

1. Estancia en el centro nacional de la investigación científica (Centre National de la Recherche Scientifique, CNRS), Duración: 3 meses, Lyon, Francia.

Docencia impartida:

1. Asignatura: Experimentación en Química Analítica (Grado en Química). Tipo de docencia: Laboratorio. Horas: 60. Curso 2020-2021.
2. Asignatura: Experimentación en Química Analítica (Grado en Química). Tipo de docencia: Laboratorio. Horas: 56. Curso 2019-2020.
3. Asignatura: Presiones humanas e impactos en el litoral andaluz. Curso teórico-práctico (Curso de extensión universitaria). Tipo de docencia: Teórico-Laboratorio. Horas: 4. Curso 2018-2019.
4. Asignatura: Química general (Grado en Química). Tipo de docencia: Laboratorio. Horas: 30. Curso 2018-2019.

Trabajos fin de estudios supervisados:

1. Título del trabajo: Determinación de fosetil-al, ácido fosfónico y MPPA en suero sanguíneo mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem. Codirector/a tesis: López-Ruiz, R; Domínguez-Pérez, I. Entidad de realización: Universidad de Almería; Alumno/a: Gallego-Reche, J; Fecha de defensa: 06/07/2020.
2. Título del trabajo: Determinación de glifosato, etefón y su metabolito en suero sanguíneo mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem. Codirector/a tesis: Garrido-Frenich, A; López-Ruiz, R; Entidad de realización: Universidad de Almería; Alumno/a: Rodríguez-López, E; Fecha de defensa: 06/07/2020.
3. Título del trabajo: Determinación de surfactantes en tomate mediante cromatografía de líquidos acoplada a espectrometría de masas de alta (Orbitrap-Exactive) y baja resolución (QqQ). Codirector/a tesis: López-Ruiz, R; Romero-González, R. Entidad de realización: Universidad de Almería; Alumno/a: Maldonado-Reina, A; Fecha de defensa: 16/07/2020.
4. Título del trabajo: Determinación simultánea de quizalofop-p y sus derivados en cebolla, naranja y pimiento mediante cromatografía de líquidos acoplada a espectrometría de masas de alta resolución. Codirector/a tesis: Garrido-Frenich, A; López-Ruiz, R; Entidad de

realización: Universidad de Almería; Alumno/a: Hergueta-Castillo, E; Fecha de defensa: 16/07/2020.

5. Título del trabajo: Determinación de aflatoxinas en pimentón mediante cromatografía de líquidos de ultra alta eficacia acoplada a espectrometría de masas en tándem. Codirector/a tesis: López-Ruiz, R; Romero-González, R. Entidad de realización: Universidad de Almería; Alumno/a: Maldonado-Reina, A; Fecha de defensa: 09/09/2019.
6. Título del trabajo: Determinación de alquenilbencenos en pimienta mediante extracción con ultrasonidos y cromatografía de gases-espectrometría de masas de alta resolución. Codirector/a tesis: Garrido Frenich, A; López-Ruiz, R; Entidad de realización: Universidad de Almería; Alumno/a: Rivera-Pérez, A; Fecha de defensa: 23/07/2019.
7. Título del trabajo: Aplicación de técnicas de cromatografía-espectrometría de masas a la determinación de compuestos orgánicos en muestras alimentarias o ambientales. Codirector/a tesis: Garrido-Frenich, A; Lopez-Ruiz, R; Entidad de realización: Universidad de Almería; Alumno/a: Rivera-Pérez, A; Fecha de defensa: 11/07/2018.