UNIVERSIDAD DE GRANADA
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NUEVAS ESTRATEGIAS ANALÍTICAS PARA LA DETERMINACIÓN DE RESIDUOS DE INSECTICIDAS Y ALCALOIDES ERGÓTICOS EN MUESTRAS AMBIENTALES Y ALIMENTARIAS

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NEW ANALYTICAL STRATEGIES FOR THE DETERMINATION OF INSECTICIDE RESIDUES AND ERGOT ALKALOIDS IN ENVIRONMENTAL AND FOOD SAMPLES

PROGRAMA DE DOCTORADO EN QUÍMICA

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AIMS AND OBJETIVES

The aims of this Thesis are framed in several research projects carried out over the last years and included within the research lines of the research group FQM-302 in which this Thesis has been carried out.

The main goal of this Thesis has been to develop new, sensitive, selective and efficient analytical methodologies to determine currently relevant residues and contaminants. For this purpose, we have evaluated miniaturized analytical techniques, powerful detection systems and novel instrumental platforms combined with new green sample treatments. The aim has been to apply these strategies in the monitoring of several insecticides (neonicotinoids (NNIs), fipronil (FPN) and its metabolites, and the fungicide boscalid (BCL)) and a class of mycotoxins known as ergot alkaloids (EAs), with the purpose to propose alternative analytical methods to be used in environmental control and food safety.

The following specific goals of this Thesis must be highlighted:

- Application of capillary liquid chromatography with UV detection (CLC-UV) for the determination of NNIs in food samples such as cereals (rice, oat, barley, wheat and maize) and honeys from different floral origins (multi-flower, rosemary, eucalyptus, and orange tree honeys) using fast and simple extraction techniques.

- Evaluation of the use of nanofibers as novel sorbents in an on-line SPE procedure, using a Lab-In-Syringe (LIS) system coupled to HPLC-UV for the determination of NNI residues in environmental water samples.

- Evaluation of the applicability of green solvents called natural deep eutectic solvents (NADESs) as novel dispersive solvents in a dispersive liquid-liquid microextraction based on a solid floating organic droplet (DLLME-SFO) instead of the toxic organic solvents commonly employed for this purpose. Validation of this methodology is proposed for the extraction of FPN and its metabolites as well as BCL from white wine and environmental water samples.

- Study of the potential of capillary electrophoresis (CE) in the mode of micellar electrokinetic chromatography (MEKC) coupled to UV, to determine NNI residues in different water samples (river, well and spring waters) as well as in soil samples using off-line preconcentration techniques to overcome the lack of sensitivity attributed to this technique.

- Application of the coupling MEKC to tandem mass spectrometry (MEKC-MS/MS) using a volatile surfactant for the determination of NNI and BCL residues in pollen and honeybee samples, considering the identification capacity of the selected detection.

- Application of UHPLC-MS/MS for the simultaneous determination of the main EAs together with its corresponding epimers in oat-based products considered food supplements.

- Study of the natural occurrence of EAs in cereal samples (wheat and barley) from Algeria using UHPLC-MS/MS, including the study of the health risks attributed to the found concentration levels.

- Evaluation of the potential of ion mobility spectrometry (IMS) coupled to LC-MS workflows for the determination of EAs in cereal samples using the collision cross section (CCS) as an identification parameter together with the exact mass and retention time.

- Proposal of new, simple, efficient and selective sample treatments for the extraction and isolation of both insecticide residues and ergot alkaloids prior their analysis by the analytical techniques above mentioned.

OBJETIVOS

Los objetivos de esta tesis se enmarcan dentro de varios proyectos de investigación desarrollados en los últimos años e incluidos en las líneas de investigación del grupo FQM-302 en el cual se ha desarrollado esta tesis.

El objetivo principal de esta Tesis ha sido desarrollar nuevos métodos de análisis, selectivos y sensibles para la determinación de residuos y contaminantes de gran importancia en la actualidad. Para ello, se han evaluado técnicas analíticas de separación miniaturizadas, distintos sistemas de detección y novedosas plataformas instrumentales combinadas con nuevos tratamientos de muestra respetuosos con el medio ambiente. El objetivo ha sido aplicar estas técnicas tanto para el control de insecticidas, incluyendo los neonicotinoides (NNIs), el fipronil (FPN) y sus metabolitos, y el fungicida boscalid (BCL), como para la determinación de una clase de micotoxinas conocidas como alcaloides ergóticos (EAs), con el propósito de proponer métodos analíticos alternativos en control medioambiental y seguridad alimentaria.

Como objetivos específicos de esta Tesis destacan los siguientes:

- Aplicar la cromatografía líquida capilar con detección ultravioleta (CLC-UV) para determinar NNIs en muestras alimentarias como cereales (arroz, avena, trigo, cebada, y maíz) y mieles de diferentes orígenes florales (mil flores, de romero, eucalipto y de naranjo) usando técnicas de extracción rápidas y sencillas.

- Evaluar el uso de nanofibras como sorbentes novedosos en un procedimiento basado en la SPE *on-line* usando un sistema automatizado multijeringa acoplado a HPLC-UV para la determinación de residuos de NNIs en muestras de aguas naturales.

- Evaluar la aplicabilidad de ciertos disolventes “verdes”, conocidos como disolventes eutécticos profundos naturales (NADESs) como nuevos disolventes dispersivos aplicados en la técnica de micro-extracción liquido-liquido dispersiva mediante la solidificación de una gota orgánica flotante (DLLME-SFO), en sustitución de otros disolventes orgánicos más tóxicos empleados normalmente para este fin. Además, validar esta metodología aplicada a la extracción de FPN y sus metabolitos junto con BCL en muestras de vino blanco y agua.

- Estudiar el potencial de la electroforesis capilar (CE), concretamente la cromatografía capilar electrocinética micelar (MEKC) acoplada a detección ultravioleta para determinar residuos de NNIs en diferentes muestras de agua (agua de río, pozo y manantial), así como en muestras de suelo, usando técnicas *off-line* de extracción y preconcentración para contrarrestar la menor sensibilidad asociada a esta técnica.

- Aplicar el acoplamiento de la MEKC y la espectrometría de masas en tándem (MEKC-MS/MS) a la determinación de residuos de NNIs y BCL en muestras de polen y abejas considerando la capacidad de identificación del sistema de detección usado.

- Aplicar de la cromatografía líquida de ultra-alta eficacia a espectrometría de masas en tándem (UHPLC-MS/MS) para la determinación simultánea de los principales EAs junto con sus correspondientes epímeros en productos a base de avena considerados como suplementos alimenticios.

- Estudiar la presencia de EAs en muestras de cereales (cebada y trigo) procedentes de Argelia usando UHPLC-MS/MS así llevar a cabo el análisis de los riesgos para la salud atribuidos a los niveles de concentración encontrados.

- Evaluar el potencial la espectrometría de movilidad iónica (IMS) incorporada a los flujos de trabajo basados en LC-MS para la determinación de EAs en muestras de cereales, usando la sección transversal de colisión (CCS) como parámetro de identificación junto con la masa exacta y el tiempo de retención.

- Proponer nuevos tratamientos de muestra, simples, eficientes y selectivos para la extracción de residuos de insecticidas y alcaloides ergóticos antes de su análisis por las técnicas analíticas anteriormente mencionadas.

SUMMARY

In this Thesis, different analytical strategies have been proposed as efficient alternatives for the monitoring of several insecticides and ergot alkaloids, considered as relevant chemical hazards in food and environmental samples.

Neonicotinoids (NNIs) are a class of insecticides widely used in the last decades to protect mainly plants (i.e., crops, fruits, and vegetables) from pest insect attack acting selectively on the nicotinic acetylcholine receptors (nAChRs) in the central nervous system (CNS) of insects. Due to their high solubility and relatively small molecular weight, they can be easily incorporated into the plant tissues via xylem staying for a long time after application. Thus, NNIs can be found in nectar and pollen, being easily available for non-target and beneficial insects such as pollinators. For these reasons, they have being related with the rapid lost of entire beehives phenomenon known as Colony Collapse Disorder (CCD). Although the action mode of fipronil (FPN) and its metabolites (FPN-sulfide, FPN-sulfone) is different, they have been also related to the death of honeybees. They act as potent disrupters of the insect CNS interfering with the passage of chloride ions through the γ-aminobutyric acid- (GABA-) regulated chloride ion channel, which triggers in a severe paralysis and death of the insect. In addition, recent studies have revealed that the fungicide boscalid (BCL) can interact with other pesticides such as NNIs reducing the lethal time and lethal doses (LD_{50}) for bees, as well as decreasing the ATP concentration, pollen consumption and protein digestion in bees. Due to this effects, the use of these pesticides has been banned or restricted in several European regulations.

In addition, due to their systemic nature, these compounds can be found in food samples and pose a health risk in humans. For this reason, the EU has established limitations of their use and maximum residue levels (MRLs) have been set in a great number of food commodities in order to guarantee food safety. In light of these concerns, analytical methods for the determination of these insecticides are required in environmental and food safety fields, that must present high efficiency, selectivity and sensitivity in order

to fulfill the European legislation. In this sense, novel analytical methods accomplishing these conditions to determine the above-mentioned insecticides in environmental and food samples have been developed in this Thesis.

On the other hand, ergot alkaloids (EAs) are mycotoxins produced mainly by fungi of the *Claviceps* genus, as *Claviceps purpurea*, which parasitize the seeds of plants, especially in cereals (i.e rye, triticale, wheat, oat, and barley) replacing the developing grain with fungal structures known as sclerotia that contain toxic alkaloid substances. The sclerotia are harvested together with the cereals leading to their contamination with EAs which can cause intoxications and ergotism in humans and animals, illness characterized by symptoms such as vomiting, burning sensation of the skin, insomnia, and hallucinations, and in some cases gangrenous limbs or even death. Ergotism, whose effects have been known since the fifth century AD, was historically known as St. Anthony's Fire or holy fire.

Cleaning improvements in grain processing have reduced the presence of sclerotia in cereals; however, EAs are still found in food and feed samples. Consequently, the European Commission has established a maximum level for ergot sclerotia and a tolerable daily intake for total EAs. Multiple factors affect EA production; the EA pattern and contents in sclerotia vary with the fungal strain, geographical region, host plant, and whether conditions. For that reason, the European Food and Safety Authority (EFSA) has asked for more studies including the analysis of EAs in cereal samples especially in processed foods destined for human consumption. In this regard, the determination of the more predominant EAs together with their epimers, in such samples using different analytical methods has been carried out in this Thesis.

In general, to face with the analytical challenges above mentioned, the last technical advances in terms of miniaturization, increased efficiency, high sensitivity, high resolution, high capacity of identification and low consumption of solvents and sample have been taken into consideration. Different miniaturized separation techniques such as capillary electrophoresis (CE) and capillary liquid chromatography (CLC) have been selected because these involve low solvent consumption as it is recommended by the trends in green analytical chemistry. Moreover, the use of high efficient techniques such ultra-HPLC (UHPLC) provides lower solvent consumption than traditional LC methods, and also shorter analysis time. In addition to UV-Vis detection, mass spectrometry (MS) has been considered because of its high sensitivity and the capability for the unequivocal identification of compounds. Finally, the coupling of ion mobility spectrometry (IMS) to a LC-MS workflow has been evaluated to provide complementary information to mass spectra and retention time by means of the so-called collision cross section (CCS).

It is important to emphasize that this Thesis presents for the first time the application of CLC as well as the use of MEKC-MS/MS technique for the determination of NNIs. Furthermore, it is the first time that the main EAs have been characterized in terms of CCS using travelling wave ion mobility (TWIM)-time of flight high resolution mass spectrometry (TOF-HRMS).

In addition, different sample treatments involving on-line and off-line preconcentration methodologies and miniaturized extraction techniques have been proposed. These strategies have allowed a sensitivity and efficiency enhancement in the extraction and preconcentration of the target compounds in environmental and food samples. Moreover, these sample treatments are environmental-friendly being in accordance with current trends in Green Chemistry.

The present Thesis has been divided into two different parts. **Part I** consists of an introduction including important aspects related with the insecticides under study and compiling the experimental works carried out in this Thesis. In this part the following chapters are included:

- **Chapter 1** presents a new analytical method based on capillary liquid chromatography with diode array detection (CLC-DAD) for the determination of seven NNIs. Two different sample treatments such as solid-liquid extraction (SLE) and dispersive liquid-liquid microextraction (DLLME) were established for cereal and honey samples, respectively.

- **Chapter 2** involves the evaluation of a variety of polymeric nano- and microfibers as potential sorbents for the extraction of five NNIs from environmental water samples (river and lake). A Lab-In-Syringe system was connected to a modified in-line filter containing nanofibers which was coupled to the chromatographic column. This work was carried out in collaboration with the Department of Analytical Chemistry of the Faculty of Pharmacy (Charles University, Hradec Králové).

- **Chapter 3** consists of the evaluation of a natural deep eutectic solvent (NADES) as dispersive solvent in a dispersive liquid-liquid microextraction based on a solid floating organic droplet (DLLME-SOF) procedure for the extraction of FPN and its metabolites together with BCL from white wine and water samples, using HPLC-UV for their determination. This work was carried out during a short predoctoral stay at the group of "Green Analytical Chemistry" at the "Instituto de Biología Agrícola de Mendoza" (IBAM) at the University of Cuyo (Argentine).

- **Chapter 4** shows a micellar electrokinetic chromatography coupled to DAD detection (MEKC-DAD) method for the determination of seven NNIs and the main metabolite 6-chloronicotinic acid (6-CNA) using "sweeping" as on-line preconcentration strategy for improving the sensitivity of the method. In addition, SPE as off-line preconcentration methodology for environmental water samples and a SLE procedure for soils were proposed.

- **Chapter 5** presents for the first time the use of MEKC coupled to MS/MS for the determination of eight NNIs together with BCL, using a volatile surfactant as background electrolyte and applying the "sweeping" as on-line preconcentration technique. A scaled-down QuEChERS (quick, easy, cheap, effective, rugged, and safe) procedure was optimized for the extraction and isolation of the analytes from pollen and honeybee samples.

Part II consists of an introduction providing useful information about EAs and their analysis and includes the works developed in this Thesis for their determination. In this part the following chapters are included:

- **Chapter 6** comprises the optimization of a modified-QuEChERS for the extraction and clean-up of six main EAs and its corresponding epimers from oat-based products including food supplements, avoiding sample dilution, and therefore, improving sensitivity. A selective UHPLC-MS/MS method was used in order to provide the unequivocal identification of EAs and their epimers.

- **Chapter 7** consists of a study of occurrence over 60 cereal samples (wheat and barley) from Algeria. The optimized modified-QuEChERS-UHPLC-MS/MS method was used to evaluate the presence of EAs in such samples founding twelve positives. Moreover, the risk of exposure of the adult population to the found concentration levels was evaluated.

- **Chapter 8** presents the research carried out at the "Laboratoire d' Étude des Résidus et Contaminants dans les Aliments" (LABERCA, Nantes, France) during the predoctoral stay. It consists in the building of a CCS database as a complementary parameter to characterize twelve EAs, which was inter-laboratory cross-validated with the collaboration of the Department of Food and Drug located at the University of Parma (UNIPR, Italy). Moreover, the advantages offered by the integration of TWIM into traditional LC-TOF-MS workflow were evaluated. Finally, the proposed LC-TWIM-TOF-MS method was applied to the analysis of EAs in cereal samples and compared with the results found with the UHPLC-MS/MS method in order to reduce possible false positive results.

RESUMEN

En esta tesis se han propuesto diferentes estrategias analíticas como alternativas eficientes para la determinación de diferentes insecticidas y alcaloides ergóticos, considerados como peligros químicos de gran importancia en muestras alimentarias y medioambientales.

Los neonicotinoides (NNIs) son una clase de insecticidas ampliamente utilizados en las últimas décadas para proteger principalmente a las plantas (es decir, cultivos, frutas y verduras) del ataque de plagas de insectos que actúan selectivamente sobre los receptores nicotínicos de acetilcolina (nAChR) en el sistema nervioso central (SNC) de los insectos. Debido a su alta solubilidad y peso molecular relativamente bajo, pueden incorporarse fácilmente a los tejidos de la planta a través del xilema y permanecer durante mucho tiempo después de la aplicación. Por lo tanto, los NNIs se pueden encontrar en el néctar y el polen, y están fácilmente disponibles para insectos beneficiosos que no constituyen su objetivo, como los polinizadores. Por esta razón, estos insecticidas se han relacionado con el trastorno denominado "colapso de colonias apícolas (CCD)" por el cual se produce una pérdida rápida de colmenas. Aunque el modo de acción del fipronil (FPN) y sus metabolitos (FPN-sulfuro y FPN-sulfona) es diferente, también se han relacionado con la muerte de las abejas. Estos actúan como potentes disruptores del SNC de los insectos, interfiriendo con el paso de los iones de cloruro a través del canal de iones de cloruro regulado por el ácido γ-aminobutírico (GABA), lo que desencadena una parálisis severa y la posterior muerte del insecto. Además, estudios recientes han revelado que el fungida boscalid (BCL) puede interactuar con otros pesticidas como los NNIs reduciendo el tiempo letal y la dosis letal (LD50) para las abejas, así como disminuyendo la concentración de ATP, el consumo de polen y la digestión de proteínas en éstas. Debido a esta problemática, el uso de estos pesticidas ha sido prohibido o restringido en varias regulaciones europeas.

Además, debido a su naturaleza sistémica, estos compuestos se pueden encontrar en muestras alimentarias suponiendo un riesgo para la salud de los seres

humanos. Por este motivo, la UE ha establecido limitaciones en cuanto al uso y se han propuesto límites máximos de residuos (LMRs) permitidos para estos compuestos en un gran número de productos alimenticios de origen animal y vegetal con el fin de garantizar la seguridad alimentaria. Ante esta problemática, se requiere de metodología analítica para la determinación de estos insecticidas con objeto de garantizar la seguridad alimentaria y medioambiental. Estos métodos deben presentar alta eficiencia, selectividad y sensibilidad para cumplir con los requisitos de la legislación europea. En este sentido, en esta Tesis se han desarrollado nuevos métodos analíticos que cumplen estas condiciones para su aplicación en el control de los insecticidas antes mencionados en muestras ambientales y alimentarias.

Por otro lado, los alcaloides del cornezuelo del centeno o alcaloides ergóticos (EAs) son micotoxinas producidas principalmente por hongos del género *Claviceps,* como *Claviceps purpurea,* que parasitan las semillas de las plantas, especialmente en los cereales (centeno, triticale, trigo, avena y cebada) reemplazando el grano en desarrollo por unas estructuras fúngicas conocidas como esclerocios que contienen las sustancias alcaloides tóxicas. Los esclerocios se recolectan junto con los cereales provocando su contaminación con EAs y causando intoxicaciones y ergotismo en humanos y animales, enfermedad caracterizada por síntomas como vómitos, sensación de quemazón en la piel, insomnio y alucinaciones, y en algunos casos, gangrena en las extremidades o incluso la muerte. El ergotismo, cuyos efectos se conocen desde el siglo V d.C., se conocía históricamente como Fuego de San Antonio o fuego sagrado.

La mejora de los mecanismos de eliminación durante el procesado de los granos han reducido la presencia de esclerocios en cereales; sin embargo, todavía se siguen encontrando EAs en muestras alimentarias y piensos. En consecuencia, la Comisión Europea ha establecido un nivel máximo para los esclerocios del cornezuelo de centeno y una ingesta diaria tolerable para el total de EAs.

Múltiples factores afectan la producción de EA; el patrón y el contenido de EA en los esclerocios varían con la cepa fúngica, la región geográfica, la planta hospedante y las condiciones medioambientales. Por esa razón, la Autoridad Europea de Seguridad Alimentaria (EFSA) ha solicitado más estudios, incluyendo el análisis de EAs en muestras de cereales, especialmente en alimentos procesados destinados al consumo humano. En este sentido, en esta Tesis se ha realizado la determinación de los EAs más predominantes en dichas muestras, junto a sus epímeros, utilizando diferentes métodos analíticos.

En general, para afrontar los retos analíticos antes mencionados, se han tenido en cuenta los últimos avances técnicos en cuanto a miniaturización, aumento de eficiencia, alta sensibilidad, alta resolución, alta capacidad de identificación y bajo consumo de disolventes y muestra. Se han seleccionado diferentes técnicas de separación miniaturizada como la electroforesis capilar (CE) y la cromatografía líquida capilar (CLC) ya que implican un bajo consumo de disolventes tal y como recomiendan las tendencias en química analítica. El uso de técnicas de alta eficiencia como la ultra-HPLC (UHPLC) proporciona un menor consumo de disolventes y corto tiempo de análisis si los comparamos con los métodos LC tradicionales. Además de la detección UV-Vis, se ha considerado la espectrometría de masas (MS) debido a su alta sensibilidad y capacidad para la identificación inequívoca de compuestos. Finalmente, se ha evaluado el acoplamiento de la espectrometría de movilidad iónica (IMS) a un flujo de trabajo basado en LC-MS para proporcionar información complementaria a los espectros de masas y el tiempo de retención mediante la denominada sección transversal de colisión (CCS).

Es importante destacar que en esta Tesis se presenta por primera vez la aplicación de CLC y de la modalidad de cromatografía capilar electrocinética micelar acoplada a espectrometría de masas en tándem (MEKC-MS/MS) para la determinación de NNIs. Además, es la primera vez que los principales EAs se han caracterizado en términos de

CCS utilizando la movilidad iónica de ondas de desplazamiento (TWIM) junto con la espectrometría de masas de alta resolución con analizador de tiempo de vuelo (TOF-HRMS).

Además, se han propuesto diferentes tratamientos de muestras que involucran metodologías de preconcentración *on-line* y *off-line* y técnicas de extracción miniaturizadas. Estas estrategias han permitido mejorar la sensibilidad y la eficiencia de la extracción de los compuestos estudiados en muestras ambientales y alimentarias. Además, estos tratamientos de muestras son respetuosos con el medio ambiente y cumplen con las tendencias actuales de la Química Verde.

La presente Tesis se ha dividido en dos partes diferenciadas. La **Parte I** consiste en una introducción que brinda información relevante sobre los insecticidas objeto de estudio, así como recopila los trabajos experimentales llevados a cabo en esta Tesis. En esta parte se incluyen los siguientes capítulos:

- El **Capítulo 1** presenta un nuevo método analítico basado en la cromatografía líquida capilar con detección por batería de diodos (CLC-DAD) para la determinación de siete NNIs. Se desarrollaron dos tratamientos de muestra diferentes; la extracción sólido-líquido (SLE) y la microextracción líquido-líquido dispersivo (DLLME) para muestras de cereales y miel, respectivamente.

- El **Capítulo 2** incluye la evaluación de diferentes nano y microfibras poliméricas como sorbentes para la extracción de cinco NNIs de muestras de aguas naturales (río y lago). Se utilizó un sistema automatizado multijeringa acoplado a un filtro en cuyo interior se encontraban las nanofibras y que fue acoplado a la columna cromatográfica. Este trabajo se llevó a cabo en colaboración con el Departamento de Química Analítica de la Facultad de Farmacia de la Universidad Charles de Praga (Hradec Králové).

- El **Capítulo 3** consiste en la evaluación de un disolvente eutéctico profundo natural (NADES) como disolvente dispersivo empleado en la microextracción líquido-líquido dispersiva, usando un procedimiento de solidificación de gota orgánica flotante (DLLME-SOF) para la extracción de FPN y sus metabolitos junto con BCL en muestras de vino blanco y agua, mediante HPLC-UV. Este trabajo se realizó durante una estancia predoctoral breve en el grupo de "Química Analítica Verde" del Instituto de Biología Agrícola de Mendoza (IBAM) de la Universidad de Cuyo (Argentina).

- El **Capítulo 4** presenta un método de cromatografía capilar electrocinética micelar acoplada a DAD (MEKC-DAD) para la determinación de siete NNIs y el metabolito principal ácido 6-cloronicotínico (6-CNA) utilizando la técnica de *sweeping* (barrido) como estrategia de preconcentración *on-line* para mejorar la sensibilidad del método. Además, se propuso la SPE como metodología de preconcentración *off-line* para muestras de agua naturales y un procedimiento SLE para muestras de suelo.

- El **Capítulo 5** presenta por primera vez el uso de MEKC acoplado a MS/MS para la determinación de ocho NNIs junto con BCL utilizando un surfactante volátil como el pefluorooctanato de amonio (APFO) como electrolito de fondo y la técnica de *sweeping* como estrategía de preconcentración *on-line*. Se optimizó un procedimiento QuEChERS reducido (rápido, fácil, económico, eficaz, resistente y seguro) para la extracción y aislamiento de los analitos de muestras de polen y abejas.

La **Parte II** consiste en una introducción que proporciona información útil sobre los EAs y su análisis, además de incluir los trabajos desarrollados en esta Tesis para su determinación. En esta parte se incluyen los siguientes capítulos:

- El **Capítulo 6** muestra la optimización de un procedimiento QuEChERS modificado para la extracción y limpieza de seis EA principales y sus correspondientes epímeros, en productos a base de avena, incluyendo complementos alimenticios, evitando la dilución de la muestra y, por lo tanto, mejorando la sensibilidad. Se utilizó un método selectivo de UHPLC-MS/MS para proporcionar la identificación inequívoca de los EAs y sus epímeros.

- El **Capítulo 7** consiste en un estudio de presencia de EAs en 60 muestras de cereales (trigo y cebada) procedentes de Argelia. El método QuEChERS-UHPLC-MS/MS previamente optimizado se utilizó para evaluar la presencia de EAs en dichas muestras, encontrándose doce positivas. Además, se evaluó el riesgo de exposición de la población adulta a los niveles de concentración encontrados.

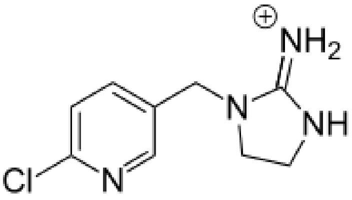
- El **Capítulo 8** presenta la investigación llevada a cabo en el "Laboratoire d'Étude des Résidus et Contaminants dans les Aliments" (LABERCA, Nantes, Francia) durante la estancia predoctoral. Este capítulo consiste en la construcción de una base de datos para el parámetro denominado "sección transversal de colisión (CCS), usado como parámetro complementario para caracterizar doce EAs. Dicha base de datos fue validada en un estudio interlaboratorio con la colaboración del Departamento de Alimentos y Medicamentos de la Universidad de Parma (UNIPR, Italia). Además, se evaluaron las ventajas que ofrece la integración de TWIM en el flujo de trabajo LC-TOF-MS tradicional. Finalmente, el método propuesto LC-TWIM-TOF-MS se aplicó al análisis de EAs en las muestras de cereales procedentes de Argelia y se comparó con los resultados encontrados con el método UHPLC-MS/MS con el fin de reducir posibles falsos positivos.

PART I

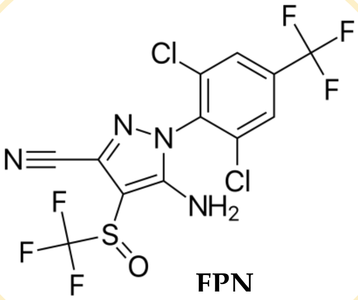
DETERMINATION OF INSECTICIDE RESIDUES

- **Introduction**
- **Chapter 1**
  Determination of neonicotinoids residues in honey and cereal samples by capillary liquid chromatography
- **Chapter 2**
  Nanofibrous on-line solid-phase extraction coupled with liquid chromatography for the determination of neonicotinoid residues in environmental waters
- **Chapter 3**
  Evaluation of natural deep eutectic solvents as novel dispersive solvents in DLLME-SFO for the determination of pesticide residues.
- **Chapter 4**
  Determination of neonicotinoids in environmental samples by micellar electrokinetic chromatography
- **Chapter 5**
  Determination of neonicotinoids and boscalid in pollen and honeybee samples by micellar electrokinetic chromatography tandem mass spectrometry

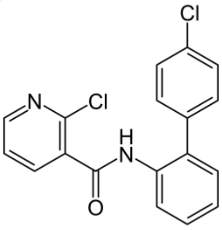
INTRODUCTION



NNIs



FPN



BCL

1. GENERAL ASPECTS ON NEONICOTINOIDS, FIPRONIL AND BOSCALID

Since neonicotinoid insecticides (NNIs) were discovered in early 1990s and introduced to the global insecticide market, they have quickly become in the most widely used insecticides worldwide. They are registered in more than 120 countries counting for more than 20% of the global insecticide market [1]. NNIs are a class of neuro-active insecticides which derive from the nicotine moiety with a novel action mode. They act selectively on the nicotinic acetylcholine receptors (nAChRs) in the central nervous system (CNS) of insects by interrupting the synaptic transmission, and therefore, leading to paralysis and ultimately death of the organism [2]. They are considered as broad spectrum insecticides which act against numerous sucking and biting pest insects, including aphids, leafhoppers, whiteflies, beetles and some *Lepidoptera* species as well. Fundamental differences between the nAChRs of insects and mammals confer remarkable selectivity for the NNIs [3]. The main compounds belonging to this family available commercially can be classified into one of these three chemical groups, the N-nitroguanidines (imidacloprid, thiamethoxam, clothianidin, dinotefuran, imidaclothiz), N-nitromethylenes (nitenpyram) and N-cyanoamidines (acetamiprid, flonicamid and thiacloprid) [4]. NNIs which have a common chemical structure of chloropyridinyl such as imidacloprid, nitenpyram, thiacloprid and acetamiprid, are metabolized into 6-chloronicotinic acid (6-CNA), whereas thiamethoxam and clothianidin who have a common structure of chlorothiazole are metabolized into 2-chloro-1,3-thiazole-5-carboxylic acid (2CTC), both metabolites conjugated with glycine or glucuronic acid. Dinotefuran is metabolized into 3-furoic acid (3FA) conjugated with glycine [5]. The chemical structures of the studied NNIs in this Thesis are shown in **Figure 1**.

During the last decades many pests have developed resistance to the existing insecticides such as organophosphorus, carbamates and pyrethroids, which has decreased their effectiveness. Therefore, due to their high selectivity and efficacy, NNIs have replaced them in the agricultural industry for plant protection (i.e., crops, vegetables, fruits) [6].

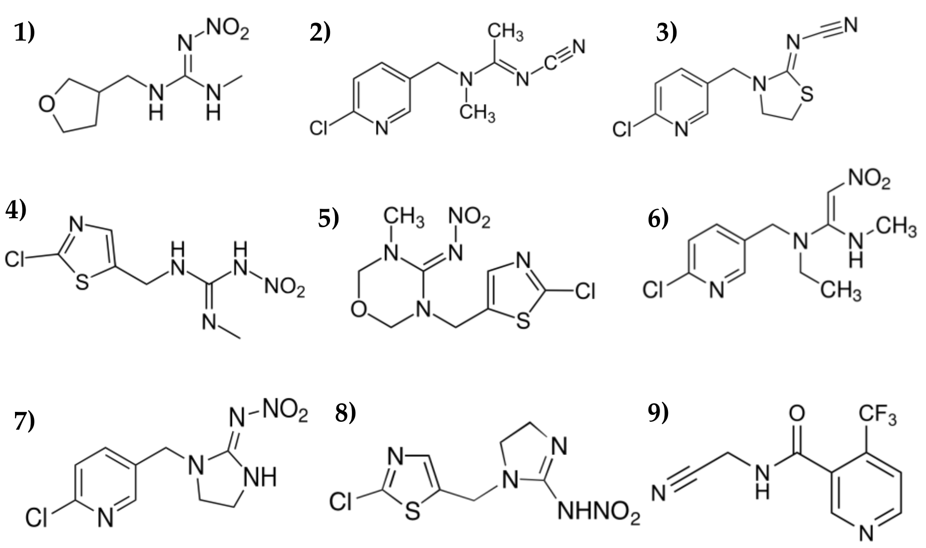


Figure 1. Chemical structures of NNIs. 1) dinotefuran; 2) acetamiprid; 3) ahiacloprid; 4) clothianidin; 5) thiamethoxam; 6) nitempyran; 7) imidacloprid; 8; imidaclothiz; 9) flonicamid.

Furthermore, they are widely used in veterinary medicine for livestock and pest protection, being part of veterinary products as well as of biocides to invertebrate pest control in fish farming [7]. Other reasons that make them successful in agriculture are their lost lasting effects and versatility in application. They can be applied to the crop fields by foliar spraying, soil treatment, drip and drench irrigation systems, or more frequently, through seed dressing and seed pilling [8]. Due to their physicochemical properties such as relatively small molecular weight, high solubility in water and systemic nature, they can be easily taken up through the roots and leaves, being distributed by the xylem pathway to the entire plant protecting it during growth [9,10]. However, the same properties may have led to potential environment and health risks. Specifically, their widespread and persistence, so that these substances can be found in all environmental compartments including soil, water, and air, staying for a long time after application without being degraded [11]. Their long biological half-lives increase the probability of environmental contamination that can have serious adverse impacts in ecosystems. These hazard effects were published into a Worlwide Integrated Assessment (WIA) in 2015 which has been recently updated including alternatives to the use of NNIs and other systemic insecticides [12,13]. In addition, their ecological risks have been also discussed by the Environmental Protection Agency`s (EPA) [14].

Moreover, as it was mentioned before, due to their prophylactic use and systemic behavior in plants, they can be present by themselves or converted into new toxic metabolites, in leaves, flowers, pollen and nectar. This fact favors their contact with beneficial and non-target organisms feeding on the plant or even by direct contact, including insect predators, vertebrates and invertebrate species having potential adverse effects for them [15]. In this framework, NNIs can be a risk for pollinators since they have been associated with the intoxication of adult worker bees, and therefore, with the colony collapse disorder (CCD), syndrome which is characterized by a rapid depopulation of honeybees due to the loss of their motor capacities making difficult their return to the beehive [16,17].

On the other hand, fipronil ((±)-5-amino-1-(2,6-dichloro-α,α,α-trifluoro-p-tolyl)-4trifluoromethylsulfinylpyrazole-3-carbonitrile) is a highly effective phenyl-pyrazole insecticide which act as a potent disrupter of the insect CNS interfering with the passage of chloride ions through the y-aminobutyric acid- (GABA-) regulated chloride ion channel, which triggers in an excessive neural excitation, severe paralysis and death of the insect [18]. Due to its action mode, fipronil (FPN) and its metabolites have been also related with CCD [19,20]. Consequently, research studies analyzing both families of insecticides altogether have been recently reported as we shall discuss in more detail later.

In addition, recent studies have also revealed that some fungicides such as boscalid (2-chloro-N-(4′-chloro-[1,1′-biphenyl]-2-yl) nicotinamide) can interact with other pesticides such as NNIs, reducing the lethal time and LD_{50} for bees, and therefore, increasing their toxicity in growing regions [21,22]. Boscalid (BCL) has a novel action mode inhibiting the succinate ubiquinone reductase (Complex II) of the mitochondrial electron transport chain what affect to the growth of the fungi and the production of energy [23]. This lead to a decrease in ATP concentration, pollen consumption and protein digestion in bees [24,25]. For that reason, it is also of a great interest to considerer this sort of compounds together with NNIs.

Furthermore, NNI residues could be present in plant products such as food from vegetal origin (i.e., fruits, vegetables, cereals…) or honeybee products (i.e., honey, royal jelly, propolis), having potential health hazards in humans. There are some studies about the risks that the use of NNIs in agriculture, forestry and veterinary could pose to human health since they could come into contact though different potential pathways of intoxication such as inhalation and ingestion exposure. For instance, thiacloprid has been designated as likely to be carcinogenic to humans by the EPA due to the fact that thyroid tumors in male rats and, uterine and ovarian tumors in female mice were observed after the exposition of NNIs [26,27]. In any case, scientific information about NNI exposure and epidemiological studies in humans are relatively lack, so this is an important issue, which is currently being evaluated [28, 29, 30].

As a consequence of the increasing public concern about NNIs, 2013, the European Commission restricted the use of plant protection products and treated seeds containing clothianidin, imidacloprid and thiamethoxam, to protect honeybees [31], based on a risk assessment of the European Food Safety Authority (EFSA). These NNIs were banned in bee-attractive crops (including maize, oilseed rape and sunflower) with the exception of uses in greenhouses, in the treatment of some crops after flowering and in winter cereals. However, considering the worrying exposure of pollinators to NNIs and its consequences, in May 2018 the European Commission restricted the application of imidacloprid, clothianidin and thiamethoxam to greenhouse uses [32]. Also, on February 2020 the approval of thiacloprid was not renewed following scientific advice by EFSA that the substance presents health and environmental concerns [33]. However, some EU countries have repeatedly granted emergency authorizations for their use in different crops, such as sugar beets. In this sense, maximum residues levels (MRLs) for different commodities or lower limit of analytical determination (in such matrixes for which their use is forbidden, including apiculture products) have been established [34]. In addition, due to their toxicity, the Worldwide Integrated Assessment (WIA) has recently reported alternatives to systemic insecticides such NNIs in pest control [35].

In light of these concerns, analytical methods for NNI determination are required in environmental and food safety fields. In order to fulfill the legislation requirements it is mandatory to propose highly efficient, selective and sensitive methods. A wide range of methods have been propose to this purpose, which involves novel and efficient sample pretreatment methods and analytical techniques for their determination at trace level.

In this introduction, an overview including a critical selection of the novel analytical methods for the control of NNIs based on different analytical techniques such as liquid chromatography (LC) and capillary electrophoresis (CE) coupled to ultraviolet (UV), mass spectrometer (MS) or high resolution detectors is presented. Similarly, the most interesting samples submitted to several sample pretreatment reported in the last decade for the determination of NNI residues will be commented. Furthermore, the most relevant methods involving the analysis of FPN and BCL together with NNIs will be considered. Thus, in the next sections, only most recent or relevant contributions in this field will be discussed.

2. ANALYTICAL METHODS FOR THE DETERMINATION OF NNIs

A wide number of analytical methods have been proposed for the determination of NNI residues and their metabolites at trace level in different matrices such as environmental, food and biological samples. Among the analytical techniques used for their separation and quantification, LC coupled to UV or MS detection modes are the most widely used, however, other techniques including CE have been also reported.

Typically, sample treatments are required prior to the instrumental analysis of pesticide residues which are often carried out in some steps mainly including solvent extraction, clean-up, and finally a preconcentration or enrichment step. The majority of residue analyses require clean-up procedures for avoiding or minimizing matrix interferences, although they may result in the partial loss of target compounds as well as in the increase of labour and cost demands. Moreover, analyte preconcentration is usually crucial for improving the sensitivity of the method, and therefore, for achieving low detection limits (LODs) which allow fulfilling legislation requirements. Due to the complexity of the samples chosen for the determination of NNI residues, a great variety of sample treatment methods have been developed and techniques such as solid-liquid extraction (SLE), liquid-liquid extraction (LLE), QuEChERS (Quick, Easy, Cheap, Effective, Rugged & Safe) technique, dispersive liquid-liquid microextraction (DLLME) or solid phase extraction (SPE) have been widely used.

Most relevant analytical techniques employed for the determination of NNIs as well as the methodologies selected for their extraction and clean-up of matrices such as environmental, food and biological samples, are discussed below.

2.1 Methods based on Liquid chromatography

Taking into account the physic-chemical properties of NNIs, such as thermolability, low volatility and high polarity, LC and specifically high-performance liquid chromatography (HPLC) is the most commonly applied technique for their determination. Despite the fact that during the last decades, MS and MS/MS detection began to become popularly used for the determination of pesticides, LC-UV and LC-DAD are still contributing to the analysis of NNIs in different matrices. Far less frequent is the use of other detection systems such as electrochemical (ECD) and fluorescence (FLD) detections.

Although LC-MS or LC-MS/MS provide higher sensitivity and selectivity than UV detection, it is instrumentally far expensive and complex than UV detectors, which may be not affordable to every control laboratory for routine analysis. LC-UV methods are useful because they are ease of operation and involve lower operating costs, allowing the detection of the target compounds at the first level of interest. However, to overcome the limitations of using UV detection, clean-up and preconcetration steps are indispensable during the pretreatment procedures of complex matrices. Among the most commonly pretreatment procedures employed when LC is coupled to UV detection, it can be found DLLME and SPE, normally applied to food samples. As a consequence, high selectivity and sensitivity are achieved in some studies in spite of using UV detection, being even comparable with the results obtained by using MS detection. The wavelengths selected depend on the analyte and its maximum of absorbance, but overall, 254 nm is the most used wavelength for the UV detection of NNIs.

During the last decade most of the reported methods in NNI residue analysis employed HPLC [36] and ultra high-resolution LC (UHPLC), which usually provides better resolution and sensitivity in shorter running times than conventional HPLC due to the use of analytical columns packed with small particles [37, 38]. Recently, the use of nanoflow LC has been also reported showing a sensitivity enhancement if compared with UHPLC [39]. In the same way, the use of capillary liquid chromatography (CLC) has been recently investigated [40,41]. Regarding to the mass spectrometers, most of the LC instruments have been equipped with triple quadrupole MS (QqQ), being possible to operate in tandem MS [42], and time-of-flight MS (TOF-MS) [43,44]. Less frequently, some works using ion trap mass spectrometer (IT-MS) [45] have been also reported.

MS enables a highly sensitive detection of trace pesticide residues and their unequivocal identification fulfilling EU regulation requirements for confirmatory methods. In this regard, the Commission Decision 2002/657/EC establishes the identification points needed in order to achieve the unambiguous identification of the legislated residues that are monitored [46]. In addition, MS/MS provides great chemical information by obtaining product ions from the precursor ion reducing the interferences troubles produced by matrix components. For this reason and unlike LC-UV, LC-MS/MS enable the quantification of the analyzed compounds at trace levels using straightforward sample treatment procedures. Thus, QuEChERS based extraction is the main sample treatment used in combination with this technique. Moreover, in order to compensate the matrix effects most methodologies include procedural or matrix-matched calibration curves during method validation.

Regardless the detection mode, reverse phase-based chromatography is the mode of choice for the separation of NNIs, mainly using C8 and C18-sorbents. Moreover, several C18 fused-core analytical columns, which are an alternative for improving separation efficiencies and speed without reducing particle size, have been also used [47,48]. Different types of LC columns were evaluated by Hao et al., and most of the columns provided acceptable separation for the eight NNIs studied. The biphenyl stationary phase retained target compounds with higher efficiency if compared with straight-chain alkyl phases and other phenyl phases [49]. Several phenyl-hexyl columns were also compared with C8 columns and selected for the separation of some NNIs [37,50]. In addition, capillary columns of narrow internal diameter (100-500 µm) have been used for increasing the sensitivity and reducing the volume of organic solvents involved in the chromatographic separation as it is shown in **Chapter 1** [40,41].

Regarding the mobile phase selected, the best results in terms of peak resolution and efficiency have been obtained when acetonitrile (MeCN) as organic solvent, and ultrapure water as aqueous solvent were employed. Thus, these solvents are the most commonly used for the chromatographic separation of NNIs. It is remarkable to notice the great number of studies developed by using LC-UV which opted for an isocratic mode for the separation of these compounds. In all cases, NNIs separation involved a low content of organic solvent (from 25 to 30%) as mobile phase obtaining good results in a short analysis time [51,52,53,54,55,56,57,58]. On the other hand, when MS/MS is used as detection system, the mobile phase consisted of MeCN and ultrapure water have been additionally acidified with formic acid and gradient elution mode have been used instead of an isocratic mode. There are just a few cases that employed MeOH as organic solvent that normally match with the use of phenyl-hexyl or biphenyl columns [50,51,54]. In order to acidify the mobile phase consisted of MeOH/ultrapure water, ammonium acetate, acetic acid, or ammonium hidroxide have been chosen for that purpose instead of formic acid.

The majority of the proposed LC-MS methods have employed electrospray ionization (ESI) as ionization mode for being the most universal, and rarely other ionization modes such as atmospheric pressure chemical ionization (APCI) have been used [45,59]. Formic acid has been the most common acid added to the mobile phase to facilitate the ionization and aid the protonation of the sample molecules, since identification of the ions has been done in positive ion mode. Normally the content of formic acid selected has been 0.1%, which was demonstrated to be enough for allowing satisfactory ionization. Taira et *al.*, evaluated both positive and negative modes, and reported the data obtained from each ionization mode. The intensity values obtained for NNIs were higher when positive mode was used [60], however, in the case of NNIs analysis together with FPN, both modes must be considered since FPN and its metabolites are only observed in negative ion mode [61,62,63]. In general, under ESI^+ mode multiple reaction monitoring (MRM) transitions and associated acquisition parameters have been optimized for the maximum abundance of fragmented ions. The protonated molecular ion $(M+H)^+$ has been determined and chosen as the precursor ion, while loss of NO_2 and Cl radical are also observed in fragmentation patterns of NNIs and some metabolites [64,65,66]. The Commision Decission 2002/657/EC includes the requirements for corfirmatory with the concept of identification points in order to achieve the unambiguous identification of the legislated residues that are monitores. Three identification points are required for the identification of group B substances (veterinary drugs and contaminants) whereas four identification points are claimed for the unequivocal determination of group A substances (substances having anabolic effect and unauthorized substances) [46]. Thus, in this case three points of identification are needed.

Recently, there has been an increase in the number of NNIs analyzed simultaneously by LC-MS/MS considering also their metabolites, which have been proved to show nearly equal or even higher toxicity than NNIs themselves [67]. The metabolite 6-CNA, which is considered to be a NNI synthetic precursor, final transformation product and also an intermediate of NNI decomposition, has been the most investigated metabolite [36,64]. During the last decade more metabolites have been also included for their analysis by LC-MS/MS mainly in food samples [42,50,65,68,69]. In addition, the growing concern about human exposure to NNIs has led to pay more attention to the study of NNI metabolites in biological samples such as urine [60,70,71], hair [59] or even tooth samples [72].

With the aim of satisfying the MRLs established for NNIs by EU, satisfactory results in terms of sensitivity have been generally achieved in most reported works. For instance, an ultrasensitive and effective clean-up analytical method for the quantification of ten NNIs in complex food samples by LC-HMRS with LOQs between 0.02 and 0.025 $\mu g\ kg^{-1}$ have been reported [44], as well as a very sensitive UHPLC-MS/MS method applied to milk samples where the LOQs ranged from 5 to 10 pg mL^{-1} [73]. The established MRLs are constantly been updated, and have become more and more stringent because of the new evidences provided by numerous studies about the presence and risks of these pesticides.

2.1.1 Solid phase extraction

SPE have been widely used as sample treatment for multiresidue pesticide determination in water and food samples prior to LC since it offers some advantages such as robustness and high versatility. Generally, analytes are extracted from a liquid phase using a solid stationary phase, which normally is packed into a SPE cartridge. Therefore, in most cases target analytes are extracted into a solvent using SLE or LLE methodologies prior to be load through the cartridge. Once the analytes are retained in the sorbent, a wash step can be added to elute and eliminate other matrix components, and finally the target analytes are eluted using a suitable solvent. In this framework, SPE has been especially employed for the extraction and clean-up of NNI residues from honeybee products such as honey, royal-jelly, propolis, and beeswax, among others. Off-line SPE has been the most usual mode used and a great variety of different sorbents have been studied.

Beeswax could be considered a contaminant reservoir and NNIs present in it could directly affect the bee colony or be transmitted to other honeybee products, so the determination of NNI residues in this matrix is valuable for evaluating the exposure of honeybees to NNIs. To obtain a homogenous beeswax sample it is necessary to heat and dissolved it before to pass it through the SPE cartridge. Yánez et al. used a n-hexane- isopropanol (8:2, v/v) mixture to dissolve the beeswax, and then, a LLE with water was performed followed by a clean-up on diatomaceous earth cartridges (Isolute® HM-N) [47]. Other types of cartridges were also tested such as Florisil®, Oasis® HLB, and Strata® C18-E SPE cartridges, but Isolute® HM-N was chosen since it provided the best retention for most of the studied NNIs. However, it should be said that although diatomaceous-based cartridges do not require as many steps as other cartridges, organic solvent consumption is much higher since 20 mL of acetone was employed as elution solvent. Afterwards, a diatomaceous-based cartridge was also compared with a reverse phase polymeric sorbent such as Strata® X for the extraction of the same NNIs from a liquid dietary supplement containing freeze-dried royal jelly. Results showed that Strata® X provided highest recoveries in most cases, which was eluted with 2 mL of the mixture methanol-ethyl acetate (70:30, v/v) [48].

Strata® X 33 µ polymeric reversed-phase cartridges was firstly used for the analysis of NNIs in environmental water samples in 2015 [49], and then, it was also applied in honey and pollen samples from sunflower and maize seed dressing crops [74]. A variant of this cartridge, known as Strata® X-CW cartridge, was evaluated together with Oasis® HLB, C18, and Strata® X cartridges and compared in terms of recoveries for seven NNIs and six metabolites in honey samples [65]. Results showed that the best clean-up effect was achieved with the Strata® X-CW cartridge whose elution was performed with a mixture of MeCN and ethyl-acetate (80:20, v/v) under neutral conditions.

In an effort to reduce the presence of high sugar and protein content in royal jelly and honey samples, C18, MCX and Oasis® HLB cartridges were studied to investigate their clean-up effect and influences on recovery by Hou *et al.* [68]. In this case, Oasis® HLB cartridges were chosen since it provided the best clean-up conditions for the simultaneous determination of ten NNIs and two metabolites. The sample was previously extracted with MeOH, and then, the proteins were precipitated by centrifugation. The supernatant was loaded into the cartridge, washed with 5 mL of MeOH-water (10:90, v/v), and eluted by 5 mL of MeOH. The efficiency of HLB cartridges has been also compared with styrene divinyl benzene-reverse phase sulfonate (SDB-RPS) and C18 SPE disks for the simultaneous enrichment and cleaning of seven common NNIs together with FPN and its metabolites: fipronil desulfinyl (FPN-desulfinyl), fipronil sulfide (FPN-sulfide), and fipronil sulfone (FPN-sulfone) in seawater and river water samples [62]. Although, Oasis® HLB cartridges provided the highest recoveries, SDB-RPS SPE disk was selected instead because it allowed the cleaning of a larger volume of sample (2000 mL), and therefore, low LOQs, ranging from 0.05- 0.50 ng L^{-1} ,were achieved. Afterwards, this disk was also compared with C8 SPE disk for the extraction of the two families of compounds obtaining satisfactory recoveries with SDB-RPS disk for all of them except for dinotefuran [75]. Recently, a new method combining a continuous solvent extraction (CSE) and a SPE has been also established for the simultaneous determination of NNIs and FPN and its metabolites in river and marine sediments using Oasis® HLB cartridges for enrichment and obtaining recovery rates from 75.5 to 98.5%, however, the whole method involved a high amount of organic solvents [63].

Also, Oasis® HLB cartridge has been efficiently on-line connected to a Florisil clean-up cartridge for the concentration of four NNIs in different kind of wines [76]. Activated carbon cartridge has been also used together with the Oasis® HLB cartridge obtaining cleaning eluents from different samples such as chestnut, shallot, ginger and tea. However, the elution solvent from the first cartridge had to be dried and reconstituted into water before the loading into the second one [77].

The presence of NNI residues in honeybee bodies has been also checked. SPE Florisil column was employed after the extraction of NNIs from honeybees for the first time, however, the volume of chlorinated solvents used was quite high [78]. The extraction of 13 NNIs from honeybee samples with a mixture of MeCN and ethyl acetate (8:2, v/v) followed by clean-up step using Sep-Pak Alumina N Plus Long cartridges has been also proposed. As no preconditioning step was needed, this method becomes in a less time-consuming and environmental-friendly approach [65].

Bee pollen is a challenging sample due to the complexity of its composition and the high diversity of potential interferences present in this matrix. In addition, very low LODs are required considering the low MRLestablished by EU. López-Fernández *et al.*, tested different sorbents such as silica, C18, primary–secondary amine (PSA), and Envi-Carb II/PSA for the clean-up of the extracts obtained by a previous SLE with hexane in dietary bee pollen. Envi-Carb II/PSA cartridge eluted with a MeCN with 1% acetic acid was selected as optimun since some co-eluted interferences were removed and colorless extracts were obtained when it was employed [79].

In addition, in attempt to reduce interference matrix, SPE has been further combined with other methodologies. For instance, although high enrichment factors (EF) are obtained with DLLME, the complexity of certain matrices prevents from high sensitivity if no cleaning step is included in the procedure. To overcome that limitation, Campillo *et al.*, proposed the introduction of a previous SPE using DSC-18Lt as sorbent, followed by a DLLME in the monitoring of NNIs in honey samples [45]. In the same way, a SPE step was carried out after a salting-out liquid-liquid extraction (SALLE) procedure in honey products. It was reported that the use of C18 SPE cartridges instead of PSA sorbent for cleaning the sample was crucial for the recovery of the metabolites. Moreover, the addition of triethylamine (TEA), a strongly polar base, to the elution solvent was needed to aid the elution of the metabolites from the cartridge, so 2% of TEA in MeCN allowed their efficient desorption [42]. Xiao *et al.* reported a sample preparation procedure based on a high-automated pressurized solved extraction (PSE) combined with a SPE clean-up for bovine tissues samples. The supernatant obtained from the extraction was loaded onto an Oasis® HLB cartridge, washed with 5 mL of methanol–water (20:80, v/v) and eluted with 3 mL of MeOH [80].

The use of automated techniques for sample preparation such as turbulent flow chromatography (TurboFlow™) and on-line SPE have been also evaluated. TurboFlow™ provided better peak sensitivity than SPE for all tested analytes, as a result, TurboFlow™ system was coupled to UHPLC-Orbitrap to inject urine samples without any off-line sample treatment [71]. Afterwards, an automated on-line enrichment coupled to UHPLC-MS/MS was reported for the first time by Montiel-León *et al.*, for the quantification of NNIs and FPN [61]. Under optimized conditions, a HyperSep Retain PEP on-line SPE column was used in conjunction with HCOOH-amended on-line mobile phases. This on-line column was selected for its low backpressure, short equilibration time, and suitable signal intensity for the targeted analytes if compared with BetaBasic C18 column. This approach allowed to improve the high LODs obtained for the analysis of water samples when direct aqueous injection (DAI) without a preconcentration step was employed [49]. In addition, in **Chapter 2** the evaluation of different micro and nano-fibers as novel sorbents in an on-line SPE procedure, using a Lab-In-Syringe (LIS) system coupled to HPLC-UV for the determination of NNI residues in environmental water samples have been developed.

SPE has been also used for the purification of biological matrices such as urine samples. Ueyama *et al.*, reported that after phosphate-induced acidification of a urine sample, NNIs were trapped by a Bond Elut PCX column with recoveries ranging from 89 to 103% [81]. Based on this work, a method for the determination of NNI biomarkers from disposable diapers has been recently reported [70]. The urine absorbed in the diapers was extracted using acetone, and then, it was cleaned-up by a SPE composed of two steps, a Bond Elut PCX cartridge followed by an ISOLUTE SLE+. The latter is a porous inert diatomaceous earth column that provided the cleanest samples in comparison with other columns. However, 20 mL of ethyl acetate was necessary to elute dinotefuran with high recoveries.

2.1.2 Dispersive liquid-liquid microextraction

DLLME is based on the generation of a cloudy solution (fine droplets of the extracts) when an organic solvent acting as extraction solvent (mainly chlorinated solvents immiscible in water) is rapidly injected into the aqueous phase in presence of another organic solvent acting as a disperser solvent (partially soluble in both phases). After extraction equilibrium, the ternary system is centrifuged and the extraction solvent is drawn out from the bottom of the centrifuge tube with a syringe because of its heavier density. In some cases, the addition of a salting-out agent to favor phase separation is required. Finally the enriched extract is injected into the chromatographic system for analysis. Due to the large contact surface area on the two immiscible phases, high extraction efficiency is achieved in a relatively short time. Among other advantages are its simple operation, low cost, high preconcentration factors and the use of small volumes of solvents being environmentally friendly if compared with traditional sample treatment techniques.

DLLME has been successfully applied to the extraction and preconcentration of a wide variety of organic compounds and metal ions, mainly from water samples [82]. Regarding to NNIs isolation, Jovanov *et al.,* described a DLLME method employing 0.5 mL of MeCN (dispersant solvent) and 2 mL of dichloromethane (extraction solvent) for the extraction of seven NNIs from 5 mL of a honey dilution. The sediment phase was collected and analyzed by LC-MS/MS obtaining low LODs [83]. Afterwards, this DLLME method was compared with a QuEChERS method developed for the same analytes in honey liqueur [84]. When using DLLME, most of NNIs exhibited lower matrix influence as well as lower LOQ values despite of using UV detection [85].

Nevertheless, the use of a honey solution instead of directly spiked honey samples would not be appropriate since the interferences present in the matrix would be diluted without representing the real sample composition. In this regard, a DLLME avoiding honey dilution was later proposed and included in this Thesis [41]. In the same way, Valverde *et al.*, tried different sample treatments in royal-jelly products and they concluded that a DLLME procedure using MeCN as dispersive solvent and chloroform as extraction solvent should be employed for fresh royal jelly since it provided better extraction recovery values overall [48]. Also, DLLME was used to preconcentrate five NNI residues from fruit and vegetable matrices [86]. Different chlorinated solvents such as carbon tetrachloride, chloroform, dichloromethane, 1,2-dichloroethane and different mixtures of them were tested as extraction solvents. First, NNIs were extracted from the solid matrix with MeCN which was then used as dispersive solvent in the DLLME procedure together with chloroform as extraction solvent. Moreover, the ionic strength of the aqueous phase was adjusted by adding sodium chloride (NaCl). EFs between 15-52 were obtained, decreasing the LODs which were between 0.025 and 0.5 ng g^{-1}.

Furthermore, DLLME has been combined with other methodologies especially after the use of those which have clean-up ability. These combinations are utilized not only to reduce the interference compounds, but also for increasing concentration efficiencies, thereby, increasing the sensitivity of the analysis especially when UV detection was employed. As it was mentioned before, Campillo *et al.,* proposed a SPE-DLLME sample treatment; the elution solvent containing the target compounds collected from the SPE was used as dispersive solvent in DLLME (1.5 mL MeCN) which was rapidly injected into the aqueous solution containing 10 % (w/v) NaCl in presence of 100 μL of chloroform. The lower layer was collected and analyzed by LC-DAD [45]. In other work, a dispersive solid-phase extraction (dSPE)-DLLME-HPLC-DAD method was proposed for the determination of NNIs in grain samples including brown rice, maize, millet and oat for the first time [52]. The effect of dichloromethane and chloroform as well as its combination in different ratios using MeCN as dispersive solvent was tested. Finally, a mixture of chloroform and dichloromethane (1:1) was selected as extraction solvent in order to achieve higher recoveries for nitenpyram and dinotefuran.

However, this procedure involved numerous stages, and therefore, the time and solvent consumption increased.

In general, no significant differences were found when the pH of the aqueous phase or the extraction time were evaluated in most of the proposed DLLME methods. So it can be said that the extraction of NNIs using this technique is not significantly influenced by these variables.

In order to overcome the drawbacks of DLLME such as the use of organic dispersive and chlorinated extraction solvents some variations have been reported to avoid them. For instance, Vichapong *et al.,* reported a microextraction procedure based on vortex-assisted surfactant-enhanced-emulsification liquid–liquid microextraction with solidification of floating organic droplet (VSLLME-SFO) coupled to HPLC-UV for the preconcentration and determination of five NNI residues in surface water and fruit samples [53]. The aqueous sample solution containing sodium sulfate (Na_2SO_4) and hydrochloric acid (HCl) for favoring the phase separation, was mixed with the surfactant sodium dodecyl sulfate (SDS) which acted as an emulsifier. The extraction solvent was octanol (considered as a green solvent), which was rapidly injected into the mixture. After centrifugation, the extraction solvent floating on the top of the tube was collected and injected into the system. The method showed good recoveries in the range of 85-105% without using any organic dispersive or chlorinated solvents. In the same way, an ionic liquid based cold-induced aggregation micro-extraction (RTIL-VALLME) procedure coupled to HPLC-UV was developed for the same group for the preconcentration and determination of four NNI residues in honey samples [87]. Different types of surfactants including cationic (CTAB, TBABr), anionic (SDS) and non-ionic (Triton X-114, Triton X-100) were compared. The extraction solvent was a room temperature ionic liquid (RTIL) such as 1-butyl-3-methylimidazolium hexafluorophosphate ($[C_4MIM][PF_6]$), while the emulsifier selected for favoring the dispersion of the RTIL in the aqueous phase was SDS. Both were mixed with an aqueous sample of honey containing sodium carbonate (Na_2CO_3) to induce salting-out effect. After vortex and centrifugation, the target analytes were extracted into the RTIL and collected from the bottom of the tube. A high EF of 200 was achieved allowing a high sensitivity (LOD of 0.01 $\mu g\ L^{-1}$).

Other alternative to produce a dispersion of an extraction solvent into an aqueous sample is the use of a Ringer tablet. This is constituted by a mixture of several inorganic salts which dissolves easily giving rise to a cloudy solution. In this sense, Farajzadeh *et al.*, who developed a Ringer tablet-based ionic liquid phase microextraction (RT-ILME-HPLC-DAD) [54]. In this work, 63 μL of 1-hexyl-3-methylimidazolium hexafluorophosphate ([HMIM][PF_6]) as extraction solvent and 0.9 g of a Ringer tablet were selected for the extraction of NNIs from 10 mL of several fruit juices and vegetable samples. Despite the fact that the recoveries were not so high (66-84%), the EF was in the range of 655-843%. Similarly, a work using a sodium chloride tablet acting both as disperser and as salting-out agent together with toluene (extraction solvent) in a ultrasound-assisted liquid phase microextraction (USA-LPME)-HPLC-DAD method has been recently reported to determine NNI residues in fruit juice samples [58]. Very high EF values ranging from 3400 to 4000 were obtained, allowing LOQs between 0.27-0.92 μg L^{-1}.

In **Chapter 3**, the applicability of green solvents called natural deep eutectic solvents (NADESs) as novel dispersive solvents in a DLLME-SFO procedure instead of the toxic organic solvents commonly employed for this purpose have been evaluated. The NADES so-called "LGH" composed by lactose, glucose and water was chosen as optimum. This solvent was able to dispersive the extraction solvent in fine droplets leading to a cloudy solution characteristic of DLLME-based methods without the need of employing toxic organic solvents.This sample treatment was proposed for the extraction of FPN and its metabolites as well as BCL from white wine and environmental water samples prior their analysis by HPLC-UV [88].

2.1.3 QuEChERS

QuEChERS stands for quick, easy, cheap, effective, rugged, and safe sample preparation method that has been successfully assessed for the extraction and clean- up of pesticide residues, especially in food samples. The main advantages of the QuEChERS method include high recoveries for a wide range of polarities of pesticides and high sample throughput, requiring little labor and low organic solvents consumption. Since the original QuEChERS was described in 2003 [89], it has been modified to optimize the effectiveness depending on both target compounds and matrices to be analyzed. In general, this method is based on an liquid extraction/partitioning step from liquid or solid samples, or a salting-out assisted liquid-liquid extraction (SALLE) normally using MeCN as extraction solvent followed by a clean-up based on dispersive SPE (d-SPE). In this step a dispersive sorbent is employed to remove impurities present in the matrix and extracted together the analytes in the first step. The most commonly used are PSA to remove sugars and fatty acids, C18 to remove non-polar interferences, such as lipids, and graphitized carbon black (GCB) which removes pigments. QuEChERS have been the most employed sample treatment for the determination of NNI insecticides prior to the analysis by LC-MS/MS.

In the original procedure, MeCN was employed as extraction solvent which has the advantage of being able to precipitate proteins and limit lipid solubility. A mixture of salts composed by $MgSO_4$ and NaCl was also used to induce phase separation. Subsequently, an aliquot of MeCN was cleaned-up with PSA to reduce interferent compounds. Based on this protocol, some works for the determination of NNIs by LC-MS/MS have been reported in different matrices such as sugarcane juice [90] and pistachio samples [91], since PSA gave the cleanest extracts and the highest recoveries in all cases.

Although the original procedure is still widely used, many different modifications have been carried out. The main modification involves the use of a different sorbent in the d-SPE step which selection is closely related to the kind of matrix studied and the interfering compounds present in it. For instance, C18 has been proposed as dispersive sorbent instead of PSA in cucumber and soil samples [36,55]. When PSA was tested, the metabolite 6-CNA was completely unrecovered because it was bound to the PSA, which is known to retain pesticides containing carboxylic acid. In addition, in some cases, due to the complexity of the matrixes, a mixture of several sorbents is needed.

For instance, a mixture of GCB and PSA has been reported for the clean-up of spinach, cucumber, apple and pomelo samples [92], and for rice straw samples [93], as well as a mixture of C18 and PSA has been employed in the case of pollen samples [38].

Jiao *et al.,* proposed a QuEChERS method for the extraction of eight NNIs using Polyvinylpolypyrrolidone (PVPP) and GCB clean-up in combination with a dilution method in order to diminish the complex and varied matrix interferences in six different types of tea [94]. PVPP was previously investigated for the same group and verified that PVPP and PSA yielded nearly identical reduction of polar interfering compounds (especially polyphenols) in tea matrix [66,95]. In the same way, PVPP in combination with a strong cationic exchange adsorbent (PCX) has been recently employed as dispersive sorbent to clean NNIs and FPN from tea extracts. The use of PCX to clean tea alkaloids (caffeine and theophylline) lead to reduce considerably the matrix effects (between 2.9-8.4%) [96] if compared with those obtained by the dilution of QuEChERS extracts in the same kind of matrices proposed by Jiao *et al.* [80].

Furthermore, the addition of acetate or citrate salts to buffer the extraction step and to improve the recovery of pesticides which are pH sensitive has been studied. Paradis et *al.*, compared the original QuEChERS procedure with other two protocols for the extraction of three chemical families of insecticides including NNIs from honey samples [97]: the EN 15662 method, which is performed with anhydrous magnesium sulfate, sodium acetate and citrate salts [98] and the AOAC method, which is performed with anhydrous magnesium sulfate and sodium acetate [99]. No significant differences in terms of recoveries were obtained, being slightly better when using citrate buffer. Afterwards, this buffer has been also reported for the extraction of NNIs from bee pollen samples [38,100]. However, Giroud et *al.*, revealed that acetate buffer was generally more efficient in extracting target compounds from beebread samples obtaining higher recoveries overall than using citrate, with the exception of thiacloprid [50]. On the contrary, there are other works where the presence of buffering salts was not needed, so it seems that the selection of a buffer or the absence of it should be evaluated, depending on the matrix under study.

Less frequently, a triple partitioning extraction step has been also considered. In this sense, Giroud et *al.*, studied the use of heptane together with MeCN-water solution in the extraction step to allow the extraction of lipids, and thus, to limit its presence in the MeCN phase. In this case the use of TEA to recover 6-CNA in presence of PSA was again considered [50]. Similarly, the triple partitioning extraction step using hexane has been proposed for the extraction of NNIs from honey, honeybees and bee pollen samples [101]. Nevertheless, Valverde et *al.*, reported that the addition of hexane for the precipitation of lipids can provoke a decrease in the recovery percentages of NNIs. For this reason, as an alternative, they proposed the use of a freeze-out step obtaining recoveries in the range of 91-105% [38]. Moreover, it was reported that a freeze-out step after the extraction of NNIs from pistachio samples was able to remove the pigments present in this matrix without the need of using GCB [57].

A scaled-down QuEChERS methods reducing significantly the extraction solvent volume (until 500 μL of MeCN) have been also proposed for the extraction of NNIs and some fungicides including boscalid from pollen and bumblebees [102]. A mixture of PSA/C18/GCB was employed as dispersive sorbent, which was then extracted with MeCN/toluene to desorb planar pesticides. This additional step is normally required when GCB is used for clean-up. Recently, the scaled-down QuEChERS was also applied for the extraction of NNIs from pollen and nectar samples [39], as well as NNIs together with FPN and FPN-sulfone from honeybee samples [103].

2.2 Methods based on Capillary Electrophoresis

CE in its different separation modes, mainly capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC), using different detection systems (UV, DAD, MS, LIF detection, etc) has been increasingly used in the last decade. This methodology is in developing in many different areas, becoming an attractive approach for determination of pesticide residues [104]. This is mainly due to its high separation efficiency, short separation time, low sample and reagent consumption, ease of operation and low operation cost. However, it suffers from low sensitivity because of the small sample volumes typically injected and the short optical length of the capillary when coupled with UV-Vis detectors. To overcome this limitation, CE can be used in combination with off-line sample pretreatments or on-line preconcentration techniques providing high EF [105]. Therefore, CE has emerged as an alternative to LC and gas chromatography (GC) reaching equal or even lower LODs, and as a result, overcoming its low sensitivity for the application in trace analysis. The application of CE methods for NNIs separation has been reviewed (**Table 3)**, showing that, until now all the contribution applied MEKC mode with UV detection and only one use CZE coupled to MS. Few articles have been published so far, most of them employing UV detection. Normally, sample pretreatment methods carried out prior to CE analysis involve an extraction procedure that isolates the analytes of interest from the matrix and preconcentrate them in the extract prior to the analysis. It must be pointed out that some off-line sample pretreatments such as SPE and DLLME as well as great variety of on-line preconcentration strategies been employed in most of the reported articles in order to improve the sensitivity of the method.

For instance, among on-line preconcentration strategies, field amplification, large-volume sample and isotachophoretic stacking, as well as dynamic pH junction and sweeping have been evaluated [106]. When MEKC mode is used, sweeping can be applied. This phenomenon of sweeping the analytes inside the capillary occurs when the sample hydrodinamically injected is prepared in a buffer solution devoid of micelles with lower, similar or higher conductivity than the pseudostationary phase. As a consequence of the application of a voltage, charged micelles will penetrate the sample zone and the analytes will be picked up and accumulated in narrow bands due to a partitioning mechanism which allow their preconcentration [107].. Taking advantage of this, EFs in the range of 4000 to 10.000 were obtained with a DLLME-sweeping-MEKC-DAD method for the analysis of NNIs [108]. Furthermore, the use of an extended light-path capillary (48.5 cm of total length × 50 μm i.d., 150 μm of optical path length) has been also reported in order to increase the sensitivity of a MEKC-DAD method for the determination of eight NNIs including the metabolite 6-can, which was reported in **Chapter 4**. [109].

It should be highlighted that all the CE-UV reported methods for NNIs determination were developed in MEKC mode, where SDS was used as the pseudostationary phase, due to its high aqueous solubility, low critical micelle concentration (CMC=25 mM), low ultraviolet molar absorptivity at low wavelengths, availability and low cost. It is also usual the addition of an organic modifier such as MeCN or MeOH which may influence both the resolution and migration time of the analytes. For instance, Ettiene et *al.*, used a background electrolyte (BGE) consisted of 5 mM sodium tetraborate, 40 mmol L^{-1} SDS and 5% (v/v) of MeOH [110].

Normally, basic buffers with pH above 10 have been employed because the voltage applied for the electrophoretic separation of NNIs was positive. On the contrary, if the voltage used is negative, an acid buffer would be selected as it was reported by Zhang et *al.*, who used 50 mmol L^{-1} of boric acid, 80 mmol L^{-1} SDS and 25% MeOH with a pH of 2 as a BGE [108]. In some cases, an ion pair reagent such as tetrabutylammonium phosphate (TBAP) which affects the selectivity of the separation was also added [111,112].

The sensitivity of CE can also be enhanced by using a more sensitive detection method such as laser-induced fluorescence (LIF) which can be further divided into direct and indirect laser-induced fluorescence (ILIF). Taking into consideration that NNIs are non-fluorescent analytes, ILIF detection was considered and evaluated by Chen et *al.*, who proposed a MEKC-ILIF method for the determination of thiamethoxam, acetamiprid and imidacloprid [113]. In the ILIF, a fluorescent substance should be added into the BGE solution, so that, cadmium telluride quantum dots (CdTe QDs) was synthesized for that purpose and matched with the operation conditions of a LIF detector. The running buffer was composed of 4.4 μM CdTe QDs, 40 mM borate, and 60 mM SDS. When NNIs passed through the separation channel, the CdTe QDs located in the analyte zones was replaced, resulting in lower background fluorescence and producing the reversal peaks of the analytes. Although LIF presents a higher sensitivity than UV detection, the results obtained in terms of LODs were worse if compared with the obtained with other MEKC-UV methods using off-line preconcentration techniques. In light of this, the use of these techniques together with LIF detection could be considered in next studies in order to improve the sensitivity obtained so far.

The advantages of CE techniques together with the high sensitivity, selectivity and capacity for identifying unknown compounds offered by MS detection, makes the coupling CE-MS an attractive alternative to those previously described by LC-MS. It should be mentioned that when coupling CE to MS detection there is an additional drawback. Commonly used surfactants such as SDS are non-volatile and can cause analyte signal suppression and contamination of the ionization source being incompatible with MS detection. That fact may explain that most of the studies found for the determination of NNIs residues carried out using CE were coupled to UV detectors. For the first time, a CZE-MS/MS method for the determination of NNIs residues was developed by Sánchez-Hernández et *al*., [114]. The operating conditions in CZE were compatible with MS detection since no surfactants are needed, so 0.5 M ammonia solution (pH>10) was selected as BGE. As a result, just a slight separation between the seven NNIs was achieved; this partial co-elution might be due to that some of the compounds were neutral at basic pHs, and therefore, they eluted with the electro-osmotic flow (EOF). Moreover, when ESI is used in CE-MS, a sheath-liquid flow interface is needed to establish electrical contact at the electrospray needle tip and to maintain a stable electrospray. In this work, the sheath-liquid consisted of a mixture of isopropanol/water (50:50, v/v) containing 0.2% formic acid in water (v/v) since ESI was set in positive mode. Low LOQs were achieved despite the fact that the use of a sheath-liquid entails dilution of the analyte with the subsequent decrease of sensitivity. However, in **Chapter 5**, a CE method based on micellar electrokinetic chromatography coupled to tandem mass (MEKC-MS/MS) offers shorter analysis time, higher resolution and higher selectivity and sensitivity than the above-mentioned method for the control of NNIs using CZE-MS. The coupling between MEKC and mass detection was possible by using a non-volatile surfactant such as 50 mM ammonium perfluorooctanoate (APFO) which was considered as both BGE and micelar medium. Moreover, this method was validated for a higher number of NNIs including also BCL as target compound in pollen and honeybee samples.

Overall, if we focus on the results reported by using MEKC-UV it can be seen that this methodology produces similar results in terms of repeatability, sensitivity, and retention times as other HPLC-UV methods. Moreover, due to the lack of works reported by CE-MS, this technique should be explored further providing improvements in terms of sensitivity and selectivity for the determination of NNIs and other pesticide residues, which has been one of the aims of this Thesis.

Regarding to the sample treatments, SPE is the preferred technique for the isolation and clean-up of NNIs before their analysis by CE. In this Thesis (**Chapter 4**), Oasis® HLB cartridges have been selected for the extraction of NNI residues from environmental waters from different origins prior their analysis by MEKC-UV achieving an off-line analyte pre-concentration of 250-fold [109]. This cartridge had been before reported in a multi-residue method for pesticides analyzed by MEKC-UV but just including imidacloprid [111]. Other kind of sorbents have been also employed, such as diatomaceous earth cartridges for cleaning-up the extraction solvent obtained previously in a SLE to extract seven NNIs from beeswax samples [114]. Moreover, a polymeric hydrophobic sorbent (Strata®-X) has been reported for the enrichment of NNIs from tap and river water, achieving a preconcentracion factor fold of 40 [110]. In other cases, no clean-step was necessary and only a SLE was carried out to extract NNIs from soil samples obtaining a preconcentration factor of 25, which is included in **Chapter 4** [109]. SLE was also employed for the analysis of NNIs in vegetable samples [113].

There is very little literature about the combination of DLLME with CE for the analysis of NNIs in real samples. Zhang et *al.*, proposed a DLLME-MEKC-UV method for the monitoring of NNIs in cucumber samples [108]. A mixture of 0.8 mL of MeCN (as dispersive solvent) and 100.0 µL of chloroform (as extraction solvent) was rapidly added into 5 mL of aqueous sample, achieving EFs from 111 to 171.

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CHAPTER 1

DETERMINATION OF NEONICOTINOID RESIDUES IN HONEY AND CEREAL SAMPLES BY CAPILLARY LIQUID CHROMATOGRAPHY

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“**A novel approach based on capillary liquid chromatography for the simultaneous determination of neonicotinoid residues in cereal samples.”** L. Carbonell-Rozas, F.J. Lara, M. del Olmo Iruela, A.M. García-Campaña. Microchemical Journal 161 (2021) 105756. DOI: 10.1016/j.microc.2020.105756. Journal Impact Factor: 4.821 (Q1, JCR Chemistry, Analytical).

1. SUMMARY

For the first time, capillary liquid chromatography with diode array detection (CLC-DAD) has been proposed for the determination of seven neonicotinoid insecticides (NNIs) commercially available (imidacloprid, thiacloprid, clothianidin, thiamethoxam, acetamiprid, nitenpyram and dinotefuran) in food samples. Chromatographic separation was performed in a Zorbax XDB-C18 column (150 mm × 0.5 mm i.d, 5 μm) at 25 °C, with a mobile phase consisting of ultrapure water and acetonitrile at a flow rate of 10 μL min^{-1}. Detection wavelengths of 254 or 270 nm were used, depending on the analyte. Two different sample treatments were optimized depending on the matrix to be analyzed. For cereal samples such as wheat, corn, rice, barley and oat, solid-liquid extraction (SLE) was proposed as simple and fast technique using a mixture of 1:3 (v/v) acetonitrile/dichloromethane as extraction solvent. In the case of honey samples, a dispersive liquid-liquid microextraction (DLLME) was evaluated choosing acetonitrile and dichloromethane as dispersive and extraction solvents, respectively. Under the optimum conditions, the proposed SLE-CLC-DAD and DLLME-CLC-DAD methods were satisfactorily characterized in terms of linearity ($R^2 \geq 0.9901$), repeatability (RSD ≤ 7.4 %), reproducibility (RSD ≤ 10 %), and extraction efficiency (recoveries ≥ 80 %). The limits of detection and quantification were lower than 5 and 16.7 μg kg^{-1} respectively, for cereal samples, and below 6.6 and 22.0 μg kg^{-1} respectively, for honey samples. Thus, both methods allow the determination of NNIs below the maximum residue limits (MRLs) established by the European legislation.

2. EXPERIMENTAL

2.1 Materials and reagents

All reagents used through this work were analytical reagent grade and solvents were HPLC grade. Chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), and 1,1,2,2-tetrachlorethane ($C_2H_2Cl_4$) were obtained from Panreac-Química (Madrid, Spain). Methanol (MeOH) and acetonitrile (MeCN) were purchased from VWR International (West Chester, PA, USA) while carbon tetrachloride (CCl_4) and 1,2-dichloroethane ($C_2H_4Cl_2$) were supplied by Sigma-Aldrich (St. Louis, MO, USA). EtOH was supplied from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work. Teflon (PTFE) syringe filters (0.2 µm x 13 mm) by VWR (Center Valley, PA, USA) were used to filter the sample extracts before injection into the CLC system.

Analytical standards of dinotefuran (DNT), thiamethoxam (TMT), clothianidin (CLT), nitenpyram (NTP), imidacloprid (IMD), thiacloprid (TCP) and acetamiprid (ACT) were supplied by Sigma-Aldrich (St.Louis, MO, USA). Individual standard solutions were obtained by dissolving the appropriate amount of each NNI in MeOH, reaching a final concentration of 500 µg mL^{-1}. They were kept in the freezer at -20 °C avoiding exposure to light. Intermediate stock standard solutions containing 50 µg mL^{-1} of each compound were obtained by mixing the appropriate amount of each individual standard solution, followed by drying and subsequent dilution with ultrapure water. Working standard solutions were freshly prepared by dilution of the intermediate stock standard solution with ultrapure water at the required concentration. Both intermediate and working solutions were stored at 4 °C avoiding exposure to direct light.

2.2 Instrumentation

CLC experiments were performed in a 1200 Series Capillary LC System Agilent Technologies (Waldbronn, Germany) containing an online degasser, capillary pump (maximum flow rate 20 µL min^{-1}), autosampler (8 µL full loop), column thermostat and diode array detector (DAD). Data acquisition and processing were collected with HP ChemStation (version A.09.01) software.

A high-speed solid crusher (Hukoer, China), a centrifuge Universal 320 R model (Hettich Zentrifugen, Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH, Bielefeld, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) and a multi-tube vortexer BenchMixer™ XL (Sigma-Aldrich, St. Louis, MO, USA) were also used.

2.3 Chromatographic separation

NNI separation was performed in a Zorbax XDB-C18 capillary column (150 mm x 0.5 mm i.d, 5 µm and 80 Å pore size) from Agilent Technologies (Waldbronn, Germany) employing a mobile phase consisted of ultrapure water (solvent A) and MeCN (solvent B) at a flow rate of 10 µL min $^{-1}$. Gradient mode was used as follows: 10 % B (0 min), 10 % B (6 min), 80 % B (15 min) and 80 % B (20 min). Afterwards, mobile phase composition was back to initial conditions in 5 min. In order to guarantee column equilibration and achieve a reproducible and stable separation, the initial conditions were maintained for 20 min. The temperature of the column was 25 °C and the UV-detection was performed at 254 and 270 nm, depending on the analyte; 254 nm for thiamethoxam, acetamiprid and thiacloprid, and 270 nm in the case of dinotefuran, nitenpyram, clothianidin and imidacloprid. The optimized CLC-DAD method was able to separate seven NNIs in less than 19 min.

2.4 Sample treatment procedure

2.4.1 Solid-liquid extraction for cereal samples

Some of the cereal samples used in this work, such as barley, wheat and corn, were gently supplied by a local farm located in Fuente Vera (Granada, Spain). Rice and oat samples were purchased from a local market in the same city. Portions of 20 g from each cereal sample were crashed for 10 s in a high-speed solid crusher, packaged and kept at ambient temperature for their daily use.

Then, the SLE procedure was performed as follows: portions of 1 g of each crushed cereal sample were placed in a 15 mL centrifuge tube with conical bottom and then, they were fortified at the desired NNI concentration, waiting 15 min for equilibration. Each portion was mixed with 3 mL of a mixture 1:3 (v/v) MeCN/dichloromethane. The tube was vortexed for 4 min and centrifuged at 9000 rpm for 10 min. Liquid phase was carefully collected with a syringe and placed into a 6 mL glass vial and then, the extraction procedure was repeated with 3 mL of fresh extraction mixture. Once the second extraction was completed, both extracts were combined, and they were evaporated to dryness under a gentle stream of nitrogen at 35 °C. Finally, the residue was reconstituted in 500 µL of ultrapure water by vortex agitation for 1 min and filtered through a 0.22 µm PTFE filter before injection into the CLC system.

The optimized sample procedure for the analysis of different kind of cereal samples is shown in **Figure 1**.

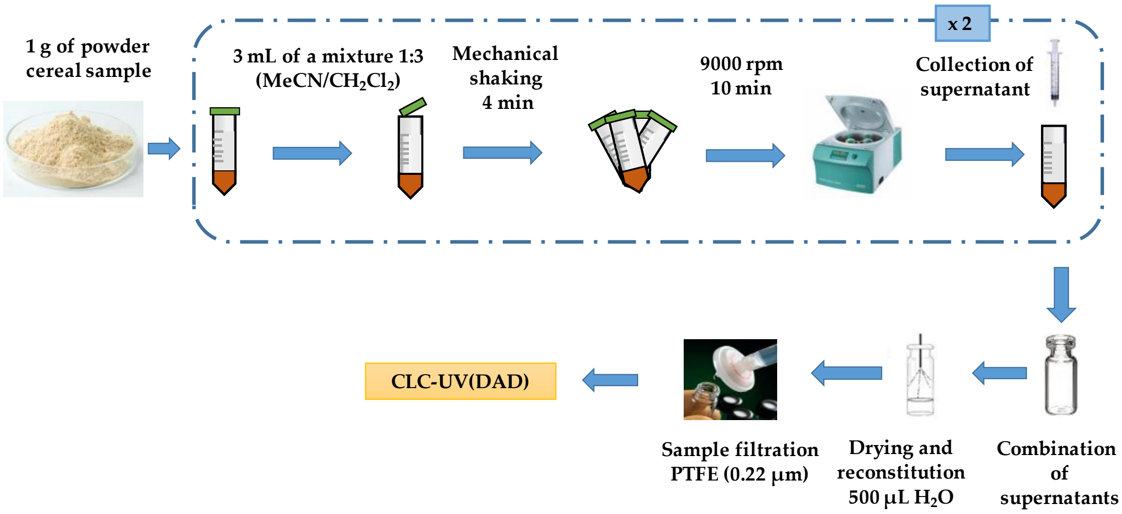


Figure 1. SLE procedure for the analysis of cereal samples.

2.4.2 Dispersive liquid-liquid microextraction for honey samples

Honey samples of different floral origins, such as multi-flower, orange tree, eucalyptus, and rosemary, were purchased from local markets (Granada, Spain). All samples were kept in their original packaging at ambient temperature for their daily use. Portions of 1 g of each kind of honey were weighed into a 15 mL falcon tube with conical bottom and 10 mL of ultrapure water was added. The mixture was immersed in an ultrasonic bath at 30 °C for 2 min to make it more fluid, and afterwards, it was shaken by vortex for 1 min until a proper homogenization was achieved. Then, the homogeneous sample was used for DLLME as follows: An aliquot of 5 mL of the diluted honey solution was placed in an extraction tube; 1 g of $MgSO_4$ was added and mechanically agitated for 1 min to induce the later separation between the organic and aqueous phases. Subsequently, the organic phase, consisted of a mixture of 3 mL of MeCN as dispersive solvent and 2 mL of dichloromethane as extraction solvent, was injected into the aqueous sample solution with a syringe coupled to a needle with a flat point, causing a cloudy solution. Then, the ternary system was vigorously shaken for 10 min by mechanical agitation and centrifuged for 5 min at 9000 rpm for phase separation. The dichloromethane phase was carefully collected with a syringe and placed into a 4 mL vial where the extraction solvent was evaporated to dryness under a gentle stream of nitrogen at 35 °C. Finally, the residue was reconstituted with 500 µL of ultrapure water by vortex agitation for 1 min and filtered through a 0.2 µm PTFE filter before injection. The optimized DLLME procedure for the analysis of honey samples is shown in **Figure 2.**

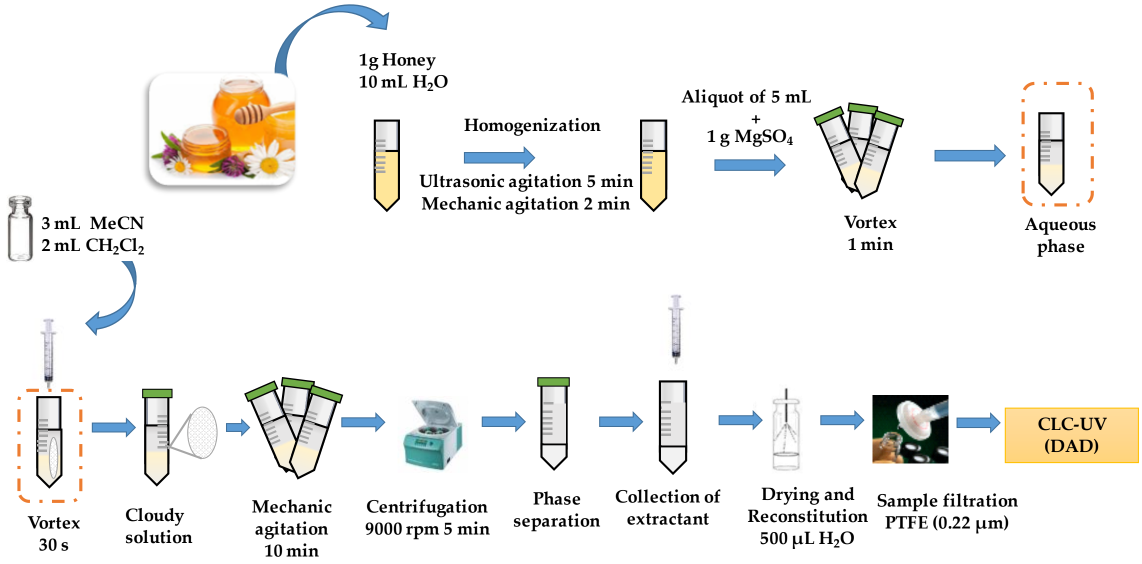


Figure 2. DLLME procedure for the analysis of honey samples.

3. RESULTS AND DISCUSSION

3.1 Optimization of chromatographic separation

In order to achieve an optimum chromatographic separation of NNIs, several parameters such as the influence of the mobile phase composition, gradient program, mobile phase flow rate, and separation temperature were investigated using a Zorbax XDB-C18 capillary column (150 mm x 0.5 mm i.d, 5 μm). This column was used because the combination of extra-dense surface coverage by the bonded phase and double end-capping produces a highly, deactivated stationary phase that removes undesirable interactions between polar solutes and the silica surface, achieving superior peak shape, high efficiency, and long-term chromatographic reproducibility at both intermediate and low pH. Initially, several mobile phases were tested, including MeCN, MeOH or mixtures of MeCN/MeOH at different ratios (3:1, 1:1, 1:3, v/v), as solvent B, and ultrapure water as solvent A. The flow rate was set at 8 μL min^{-1}, temperature at 25 °C and initial gradient conditions were as follows: 5 % B (0 min), 5 % B (5 min), 80 % B (15 min), and 80 % B (20 min). Afterwards, mobile phase composition was back to the initial conditions in 5 min and stated for 20 min for column equilibration. The use of MeCN provided the best resolution between peaks allowing the separation of the seven NNIs to baseline, so it was selected as organic solvent in the mobile phase. Then, the addition of 0.1 % formic acid to both solvents was also tested, but the peak shape worsened especially for dinotefuran and thiamethoxam. Thus, the best results in terms of resolution and peak efficiency were obtained when MeCN (solvent B) and ultrapure water (solvent A) were used as the mobile phase. Afterwards, various gradient programs were tested. In order to improve the peak efficiency of the first peaks; nitenpyram and dinotefuran, the content of MeCN was increased from 5 to 20 % at the beginning of the gradient. The best compromise in terms of resolution and separation efficiency was obtained when 10 % of MeCN was used, so the gradient conditions chosen were as follows: 10 % B (0 min), 10 % B (5 min), 80 % B (15 min), and 80 % B (20 min). Subsequently, the effect of the mobile phase flow rate was evaluated in the range of 8 to 12 μL min^{-1}. Above 10 μL min^{-1}, peak efficiency of most analytes worsened, therefore, as a compromise between peak efficiency and analysis time, a flow rate of 10 μL min^{-1} was chosen as optimum throughout the analysis. The effect of the injection volume was then investigated from 3 to 8 μL, selecting a full loop injection volume (8 μL) as it provided the best sensitivity without affecting the peak shape; no band-broadening or loss of peak resolution were observed. Furthermore, column temperature was evaluated from 20 to 35 °C, selecting 25 °C as optimum since it provided the best results in terms of peak resolution without extending the analysis time. The monitoring wavelengths were set at 254 and 270 nm, depending on the maximum absorbance of each studied analyte. Finally, when real samples were injected, the presence of interferences at the beginning of the analysis, particularly with honey samples, forced us to modify the gradient conditions, keeping constant the initial percentage of B (10 %) for 1 min more, ergo until 6 min. To sum up, separation was carried out using the following gradient program: 10 % B (0 min), 10 % B (6 min), 80 % B (15 min), and 80 % B (20 min) using ultrapure water as solvent A and MeCN as solvent B.

3.2 Optimization of sample treatments

3.2.1 Optimization of SLE for the analysis of cereal samples

For the purpose of determining NNI residues in cereal samples by the optimized CLC-DAD method, a very simple and fast SLE procedure was developed as sample treatment based on our previous study concerning the determination of NNIs by MEKC-UV in soil samples which employed MeCN/dichloromethane (1:3, v/v) as extraction solvent [1]. Several parameters that influence the extraction efficiency were optimized, using barley sample as representative of the commodity group. The studied parameters were the extraction solvent volume, extraction time and centrifugation time. The selection of the optimum value in each case was made with the aim to maximize the recovery values. As initial experimental conditions for the study, 1 g of barley sample and 3 mL of a mixture MeCN/dichloromethane (1:3, v/v) as extraction solvent volume were used.

Selection of the extraction solvent volume

To evaluate the effect of the extraction solvent volume, 3 and 6 mL of the mixture MeCN/dichloromethane (1:3, v/v) were checked. With 3 mL the recoveries were below 60% in almost all cases. When 6 mL were used, despite doubling the volume, the recovery for all compounds increased except for thiamethoxam. A consecutive extraction with 3 mL of fresh extraction solvent was also tried and both extracts were combined for their later evaporation. As can be seen in **Figure 3a,** a great improvement in recoveries for all analytes was obtained, achieving around 80% for all of them. Therefore, the extraction was accomplished in two steps, with 3 mL of the mixture MeCN/dichloromethane (1:3, v/v) every time.

Selection of the centrifugation time

In order to obtain a clean extract and a reproducible volume of organic phase after centrifugation, the effect of the centrifugation time on the extraction efficiency was evaluated firstly from 5 to 15 min, at 9000 rpm. There was a significant increase in the recovery of thiamethoxam when 10 min was used, while the recoveries for the other analytes remained practically constant, as can be seen in **Figure 3b**. Furthermore, using 10 min as centrifugation time, the solid sample remains more compacted resulting in a cleaner liquid organic phase, which was crucial for getting reproducible conditions and also for helping the filtration of the extract after its reconstitution at the end of the sample treatment and before the injection. In the light of these facts, 10 min was selected as centrifugation time in subsequent experiments.

Selection of the extraction time

Subsequently, the extraction time was evaluated from 2 to 8 min keeping the vortex agitator at the highest rotational speed (2500 rpm). The recoveries were increased with the extraction time, but above 4 min no improvements or very slight increases were observed, and even the recoveries for some analytes were lower **(Figure 3c).** Therefore, 4 min was selected as the optimum extraction time, significantly improving extraction efficiency mainly for nitenpyram, thiamethoxam and thiacloprid.

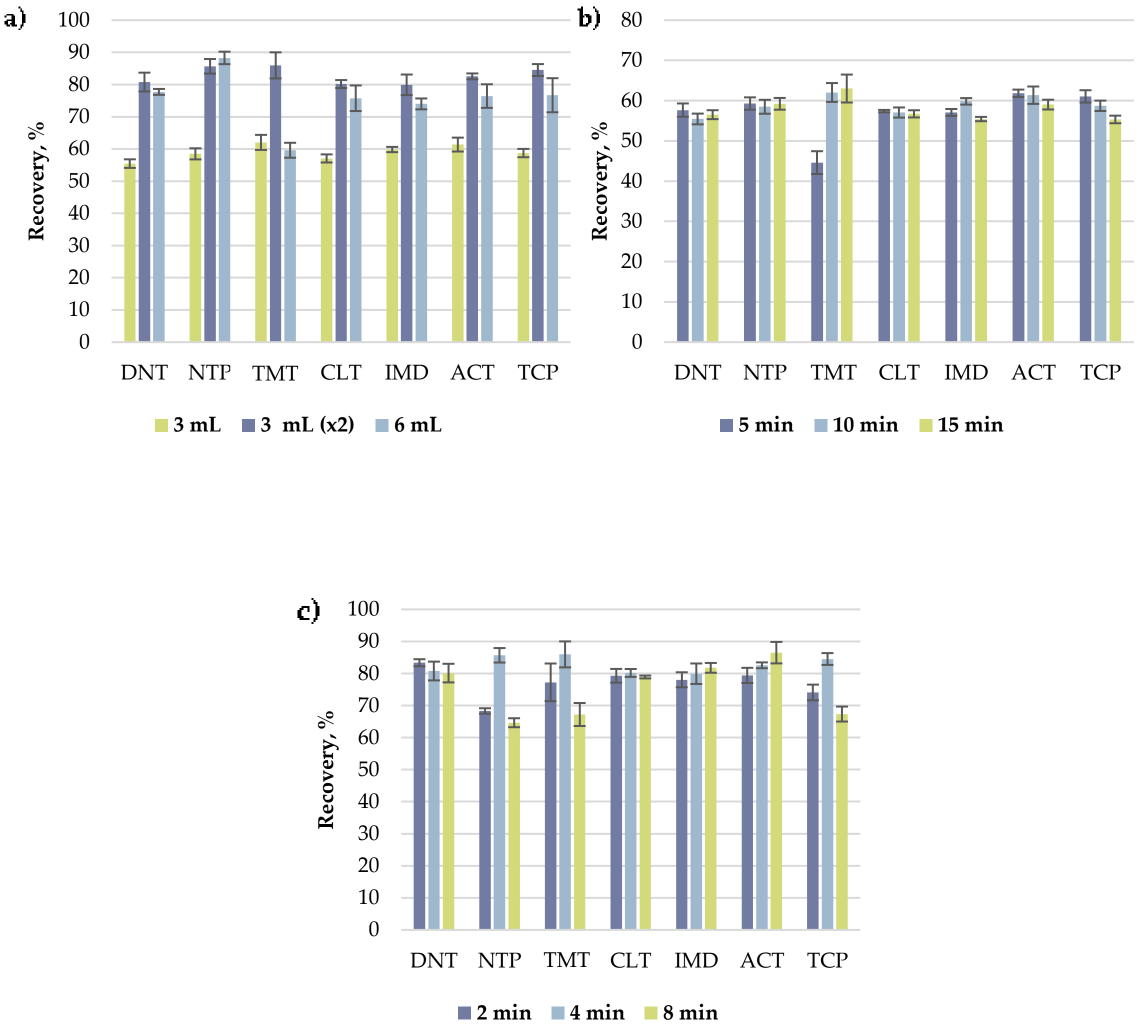


Figure 3. Optimization of the SLE procedure for cereal samples. Effect of the **a)** extraction solvent volume; **b)** centrifugation time; **c)** extraction time. Error bars represent the standard error (n=4).

3.2.2 Optimization of DLLME for the analysis of honey samples

For the optimization of the sample treatment, portions of 1 g of a blank honey sample were spiked with the different solutions of NNIs at room temperature and extracted after 20 min of rest. Several parameters that influence the extraction efficiency in a DLLME procedure were studied, including type and volume of extraction and dispersive solvents, the extraction time, pH of the sample solution, and the effect of the salting-out agent. The selection of the optimum in each case was made in terms of extraction recovery.

Selection of the extraction and dispersive solvents

The selection of an appropriate extraction solvent is highly important for the DLLME process. This is typically denser than water and it must be immiscible with it; these are mainly chlorinated solvents. For NNI extraction, five different organic solvents, such as CCl_4 (1.59 g mL^{-1}), $CHCl_3$ (1.47 g mL^{-1}), CH_2Cl_2 (1.32 g mL^{-1}), $C_2H_2Cl_4$ (1.54 g mL^{-1}), and $C_2H_4Cl_2$ (1.25 g mL^{-1}), were studied. On the other hand, dispersive solvent must be miscible both with aqueous phase and with organic solvent. These solvents can disperse the extraction solvent as very fine droplets in the aqueous phase. In this case, MeOH, MeCN, and EtOH were tested as dispersive solvents. Initially, 5 mL of acidified ultrapure water (pH 3) fortified at 1 mg L^{-1} of each NNI was used as aqueous phase and each extraction solvent (2 mL) was evaluated using MeCN (2 mL) as dispersive solvent. There were no big differences when CH_2Cl_2, $CHCl_3$, and $C_2H_2Cl_4$ were used. As CH_2Cl_2 presented slightly higher recoveries for most analytes, it was chosen as extraction solvent (**Figure 4a**). Then, each dispersive solvent (2 mL) was studied employing a constant volume of CH_2Cl_2 (2 mL). Under these conditions, the use of MeCN as dispersive solvent brought the best recoveries, as can be seen in **Figure 4b**, so MeCN was chosen as dispersive solvent.

Selection of the extraction and dispersive solvent volume

Then, extraction and dispersive solvent volumes were studied. For the extraction solvent, volumes of CH_2Cl_2 from 1 to 3 mL were tested. As dinotefuran and nitenpyram, both with poor recoveries (less than 50%), presented a slight improvement with 2 mL without affecting the other analytes negatively, it was selected as optimum (**Figure 4c**). Subsequently, the dispersive solvent volume was evaluated between 0.5 and 4 mL. Values higher than 3 mL produced a decreased in the recoveries, so 3 mL was set as optimum.

Selection of sample pH

In order to evaluate the effect of the pH of sample solution, experiments were carried out by diluting 1 g of the honey sample directly with ultrapure water or adjusting the pH with 1 M HCl or 5 M NH_3(aq) solutions, in order to obtain pH values from 3 to 8.

Sample pH had no significant effect on the extraction efficiency, obtaining slightly better results when ultrapure water was used to dilute the honey sample.

- **Selection of the shaking mode and extraction time**

Due to the formation of microdroplets because of the extraction solvent dispersal into the aqueous phase, a large contact surface is achieved between the two phases, resulting in a fast mass transfer process. For that, it is necessary to evaluate the effect of the shaking step after the injection of the DLLME mixture into the sample. Two different shaking modes were studied: ultrasonic bath and mechanical agitation. The extraction efficiency was remarkably better using mechanical agitation, obtaining recoveries around 20% higher for most analytes than using ultrasonic bath, so it was selected. Subsequently, the mechanical agitation time was examined in the range of 5–20 min, achieving the highest recoveries around 10 min. No significant improvements were observed for higher extraction times.

- **Selection of the salting-out agent**

Under the above-optimized conditions, recoveries were higher than 70% for all analytes except for dinotefuran and nitenpyram. Therefore, in order to improve the extraction efficiency for these analytes, the addition of salts was evaluated. First, three different types of salts, such as ammonium sulfate ($(NH_4)_2SO_4$), sodium chloride (NaCl) and anhydrous magnesium sulfate ($MgSO_4$), were evaluated as salting-out agents using 0.5 g of each one. The highest recoveries were obtained with $MgSO_4$, so it was selected, and subsequently the amount of this salt was optimized testing 0.5, 1, and 2 g. The addition of this salt produced a positive salting-out effect, increasing the recovery values for all analytes, mainly when 1 g of $MgSO_4$ was added, especially for dinotefuran and nitenpyram, which finally reached recoveries up to 80%, as it is shown in **Figure 4d**; so, 1 g of $MgSO_4$ was selected.

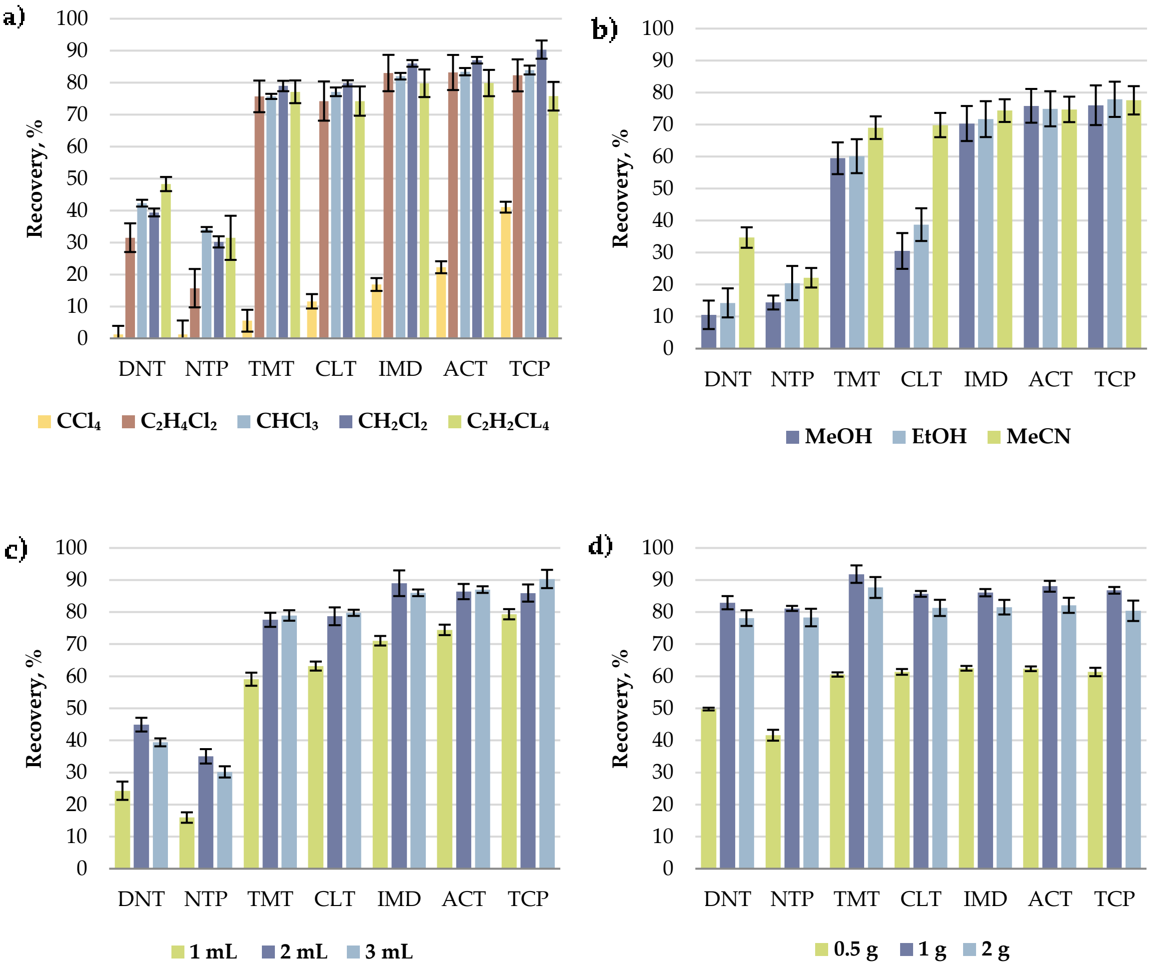


Figure 4. Optimization of the DLLME procedure for honey samples**.** Effect of the **a)** extraction solvent type; **b)** dispersive solvent type; **c)** extraction solvent volume, and **d)** amount of $MgSO_4$. Bars represent the standard error (n = 4).

4. METHOD CHARACTERIZATION

SLE-CLC-DAD and DLLME-CLC-DAD methods were characterized in terms of linearity, limits of detection and quantification (LODs and LOQs, respectively), precision (i.e., repeatability and intermediate precision), and recovery efficiency. SANTE/12682/2019 guideline specifies that for characterization of a method in samples belonging to the same commodity group, only one representative sample can be selected in the validation study [2]. According to the guide above mentioned, cereal grains and products thereof characterized by a high starch and/or protein content and low water and fat content, belong to the same group. This group includes barley, rice, wheat, corn and oat among others, so barley was chosen as representative commodity for the characterization of the optimized SLE-CLC-DAD method in cereal samples. Similarly, for the characterization of the optimized DLLME-CLC-DAD method, multi-flower honey was selected as representative commodity for the rest of honey samples (orange tree, rosemary and eucalyptus honey).

4.1 Calibration curves and performance characteristics

For the characterization of the SLE-CLC-DAD method, procedural calibration curves were established using crushed barley samples, which were spiked at different NNI concentration levels (20, 50, 100, 150, 200 μg kg^{-1}), and submitted to the whole proposed analytical method. Two samples per each concentration level were processed following the developed SLE method and analyzed in duplicate. In the same way, for the characterization of the DLLME-CLC-DAD method, procedural calibration curves were established using multi-flower honey samples, which were fortified at different NNIs concentration levels (40, 80, 120, 160, 200, and 240 μg kg^{-1}). Two samples per each concentration level were processed following the DLLME method and analyzed in duplicate.

In both cases, peak area was considered as a function of the analyte concentration on the sample, and LODs and LOQs were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten, respectively. Statistical parameters, calculated by least-square regression, and performance characteristics of both proposed methods are shown in **Table 1**. For the SLE-CLC-DAD method, LODs and LOQs were lower than 5 and 17 μg kg^{-1} respectively, meaning that the proposed method allows the determination of NNIs in cereal samples at levels below their MRLs established by the European legislation [3]. In addition, these results belong among the lowest reported in such kind of samples, being comparable and even lower than those obtained by using MS/MS detection [4,5]. In the case of the DLLME-CLC-DAD method, LODs and LOQs were below 6.6 and 22.0 μg kg^{-1}, respectively, what implies that the proposed method allows determining NNIs at levels below the MRLs established by the European legislation in honeybee products [3].

Table 1. Statistical and performance characteristics of the proposed SLE-CLC-DAD and DLLME-CLC-DAD methods for NNIs determination in cereal and honey samples, respectively.

SLE-CLC-DAD in cereal samples

NNI	MRLs (μg kg^{-1})	Linear range (μg kg^{-1})	Intercept	Slope	LOD (μg kg^{-1})	LOQ (μg kg^{1})	R^2
DNT	8000	16.7-200	-13.427	2.824	5.0	16.7	0.9924
NTP	*	9.8-200	5.378	2.011	3.7	9.8	0.9927
TMT	400	14.7-200	21.119	1.6605	4.4	14.7	0.9930
CLT	40	13.7-200	-4.225	3.4267	4.1	13.7	0.9940
IMD	100	12.9-200	-37.445	3.5953	3.9	12.9	0.9937
ACT	50	9.5-200	-17.694	3.5351	2.9	9.5	0.9964
TCP	900	9.3-200	6.7034	2.1358	4.9	9.3	0.9901

DLLME-CLC-DAD in honey samples

NNI	MRLs (μg kg^{-1})	Linear range (μg kg^{-1})	Intercept	Slope	LOD (μg kg^{-1})	LOQ (μg kg^{1})	R^2
DNT	*	9.6-240	-18.633	1.529	3.1	9.6	0.9975
NTP	*	10.0-240	157.28	2.047	3.2	10.0	0.9969
TMT	50	10.9-240	38.900	1.735	3.3	10.9	0.9948
CLT	50	14.1-240	-17.583	1.800	4.3	14.1	0.9964
IMD	50	17.5-240	-13.967	1.387	5.2	17.5	0.9957
ACT	50	12.9-240	-12.333	1.710	3.9	12.9	0.9959
TCP	200	22.0-240	-3.1000	1.001	6.6	22.0	0.9957

*Non-established. Default value of 10 μg kg^{-1}.

4.2 Repeatability and intermediate precision assays

Precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by the application of the proposed methods to real samples. First, SLE-CLC-DAD method was applied to barley samples spiked at three different concentration levels in the linear range such as 20, 100 and 200 μg kg^{-1}). Repeatability was evaluated over two samples prepared and injected by triplicate on the same day, under the same conditions. Intermediate precision was evaluated over two samples injected by triplicate during three consecutive days. In addition, precision of the DLLME-CLC-DAD method was evaluated by its application to multi-flower honey samples at two different concentration levels of NNIs in the linear range (50 and 200 μg kg^{-1}). Repeatability and intermediate precision assays were carried out as it was mentioned before.

The obtained results, expressed as RSD (%) of peak areas are summarized in **Table 2.** Satisfactory results were obtained in terms of precision for both methods, achieving RSDs lower than 10 % in barley samples and below 6.3 % in multi-flower honey samples.

Table 2. Precision of the SLE-CLC-DAD method for spiked barley samples (level 1: 20 μg kg^{-1}, level 2: 100 μg kg^{-1}, level 3: 200 μg kg^{-1}), and DLLME-CLC-DAD method for spiked multi-flower honey samples (level 1: 50 μg kg^{-1}, level 2: 200 μg kg^{-1}).

		DNT	**NTP**	**TMT**	**CLT**	**IMD**	**ACT**	**TCP**
Repeatability RSD (%) n=6								
Barley	**Level 1**	6.6	7.4	7.1	3.1	6.6	6.3	7.1
	Level 2	2.9	5.9	5.1	3.5	6.1	4.5	4.6
	Level 3	2.5	5.2	2.3	3.1	2.9	0.4	2.4
Multi-flower honey	**Level 1**	2.9	0.9	2.3	2.7	4.5	3.7	4.5
	Level 2	2.3	1.4	0.6	1.9	2.5	1.5	1.4
Intermediate precision RSD (%) n=18								
Barley	**Level 1**	6.7	9.0	7.5	8.7	10.0	7.4	9.8
	Level 2	4.8	7.5	7.2	4.4	5.6	5.2	5.6
	Level 3	2.7	5.5	2.2	2.8	2.7	1.1	5.6
Multi-flower honey	**Level 1**	5.3	6.3	5.9	5.2	5.6	5.6	4.9
	Level 2	4.2	5.3	3.3	5.0	4.3	4.3	4.1

4.3 Recovery studies

In order to check the extraction efficiency of the proposed methods, recovery experiments were carried out. On the one hand, three samples of each kind of cereal (barley, wheat, corn, rice and oat) were spiked at three different concentration levels (20, 100 and 200 μg kg^{-1}), treated and analyzed following the proposed SLE-CLC-UV method. A blank sample for each type of cereal was also processed and no matrix interferences were detected at the retention times for the studied NNIs, except for the case of thiamethoxam in rice samples. Thus, the determination of this analyte in this matrix was discarded. The data, in terms of peak area, were compared with those obtained by analyzing extracts of blank samples submitted to the sample treatment and spiked with NNIs just before the injection. In general, recoveries over 80 % were obtained for all analytes, with RSDs lower than 15 % (**Table 3**). These results were better than those obtained with the sample treatment proposed by Wang *et al.*, which was applied to similar samples, and whose extraction recoveries ranged from 60 to 116 % [6]. These results suggest that the proposed SLE-CLC-DAD method could be satisfactorily applied not just to barley but also to other cereals, such as wheat, corn, rice and oat.

On the other hand, recovery experiments were carried out in honey samples of different floral origins (multi-flower, orange tree, eucalyptus, and rosemary) spiked at two different concentration levels (50 and 200 μg kg^{-1}). Three samples of each type of honey were fortified at each concentration level, treated, and analyzed following the proposed DLLME-CLC-DAD. The data were obtained as it was stated before. In addition, a blank simple of each type of honey was processed to check the absence of NNIs, and none of them gave signals corresponding to concentrations higher than the LODs. Recoveries over 80% were obtained, with RSDs lower than 15% (**Table 4**). These results suggest that the proposed DLLME-CLC-DAD method could be satisfactorily applied for honey samples of different floral origins.

Table 3. Recovery studies for the proposed SLE-CLC-DAD method in cereal samples (level 1: 20 μg kg^{-1}, level 2: 100 μg kg^{-1}, level 3: 200 μg kg^{-1}) (n=6).

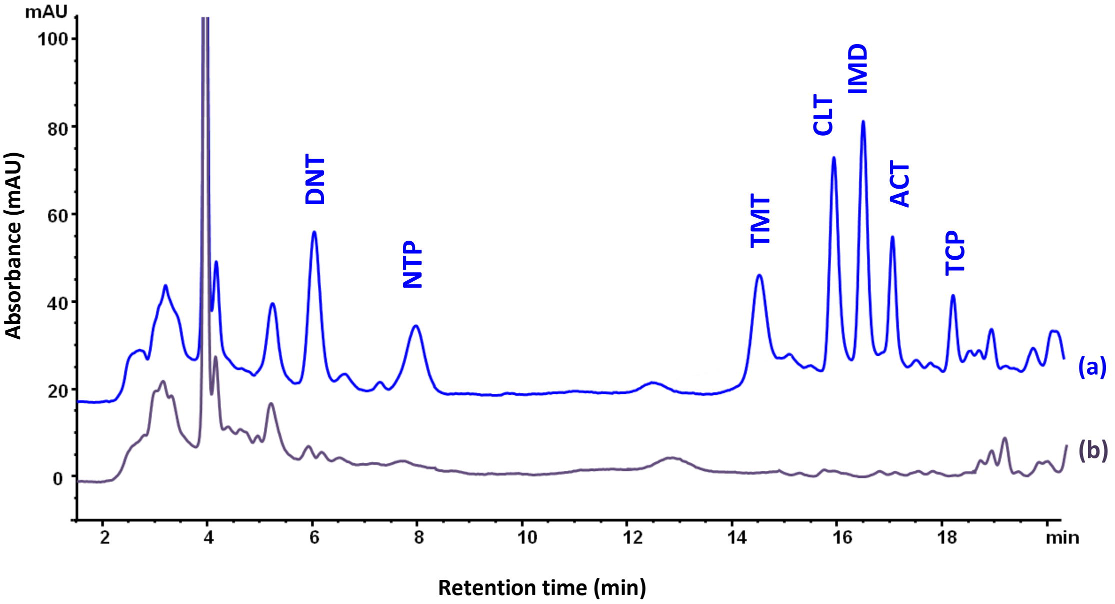
			DNT	NTP	TMT	IMD	CLT	ACT	TCP
Barley	**Level 1**	**R %**	85.5	88.2	98.9	86.3	89.2	104.5	96.5
		RSD	6.6	5.0	6.4	8.58	13.6	12.8	11.4
	Level 2	**R %**	92.8	105.0	87.8	86.4	104.5	91.0	93.8
		RSD	5.3	3.6	4.6	6.3	4.9	6.5	4
	Level 3	**R %**	95.5	107.4	88.1	90.5	102.3	102.6	87.7
		RSD	4.5	3.7	4.2	4.6	5.8	5.5	3.31
Oat	**Level 1**	**R %**	101.4	82.8	81.2	85.4	89.2	83.0	80.3
		RSD	8.4	9.8	9.2	7.2	8.5	13.9	8.8
	Level 2	**R %**	85.7	84.9	81.4	80.4	81.4	93.0	92.4
		RSD	7.8	6.4	8.2	7.4	6.7	6.3	6.7
	Level 3	**R %**	85.4	103.7	107.4	81.0	91.5	95.2	81.6
		RSD	7.6	5.3	4.5	7.8	4.1	5.5	2.7
Wheat	**Level 1**	**R %**	86.2	84.2	83.9	84.5	90.0	94.6	83.0
		RSD	8.7	14.1	12.1	12.4	11.5	8.3	7.9
	Level 2	**R %**	98.8	99.0	83.9	81.7	90.2	92.2	93.3
		RSD	7.12	9.4	6.4	5.7	8.3	4.9	7.8
	Level 3	**R %**	95.6	96.3	89.1	91.8	90.2	101.5	102.6
		RSD	4.3	8.7	5.1	6.6	7.5	4.7	5.8
Maize	**Level 1**	**R %**	85.5	82.2	98.9	86.3	89.2	96.5	86.9
		RSD	7.7	6.4	7.4	8.5	11.8	12.1	7.8
	Level 2	**R %**	87.5	85.7	84.6	96.4	94.8	81.4	94.0
		RSD	6.1	3.2	6.7	5.8	7.6	6.1	8.1
	Level 3	**R %**	82.5	96.9	86.0	86.6	83.5	86.5	87.0
		RSD	6.2	3.9	6.4	7.3	3.9	6.1	7.2
Rice	**Level 1**	**R %**	84.7	103.4	-	95.7	83.5	83.4	82.3
		RSD	14.5	8.5		10.2	7.5	9.0	6.5
	Level 2	**R %**	97.1	98.2	-	100.0	87.7	95.6	84.4
		RSD	10.6	5.4		4.7	6.3	7.3	2.7
	Level 3	**R %**	94.5	93.1	-	95.4	89.2	95.5	89.7
		RSD	9.4	6.4		4.8	5.1	5.2	2.4

Table 4. Recovery studies for the proposed DLLME-CLC-DAD method in honey samples of different floral origins (level 1: 50 μg kg^{-1}, level 2: 200 μg kg^{-1}) (n=6).

			DNT	NTP	TMT	IMD	CLT	ACT	TCP
Multi-flower honey	**Level 1**	**R %**	80.5	81.5	93.4	83.0	92.3	81.5	90.4
		RSD	3.4	11.3	8.2	6.5	6.9	7.3	9.6
	Level 2	**R %**	89.7	85.4	99.1	95.2	91.1	83.4	88.6
		RSD	5.9	3.6	6.2	2.7	7.1	5.6	5.3
Orange tree honey	**Level 1**	**R %**	87.3	90.8	88.3	89.3	85.4	87.5	82.8
		RSD	8.2	3.9	6.9	4.6	9.1	12.1	9.4
	Level 2	**R %**	89.4	86.3	94.2	85.4	87.6	90.2	86.0
		RSD	6.4	3.6	6.7	5.4	4.7	6.5	4.4
Eucalyptus honey	**Level 1**	**R %**	88.4	81.9	81.4	86.7	92.7	90.5	83.7
		RSD	7.8	14.5	9.1	8.7	12.1	8.3	9.2
	Level 2	**R %**	86.0	81.5	96.7	87.4	87.9	87.4	84.1
		RSD	4.3	10.6	10.3	13.8	9.2	7.8	7.2
Rosemary honey	**Level 1**	**R %**	84.7	81.7	94.4	86.4	82.3	90.3	86.8
		RSD	10.3	11.1	6.7	8.6	7.6	6.2	4.9
	Level 2	**R %**	84.1	85.9	90.0	84.5	80.4	85.1	84.4
		RSD	5.7	9.5	5.6	6.1	6.3	3.3	9.4

Finally, chromatograms of a spiked barley sample and its corresponding blank analyzed by SLE-CLC-DAD are shown in **Figure 5.1,** as well as chromatograms of a spiked multi-flower honey sample and its corresponding blank analyzed by DLLME-CLC-DAD are shown in **Figure 5.2.**

1)



2)

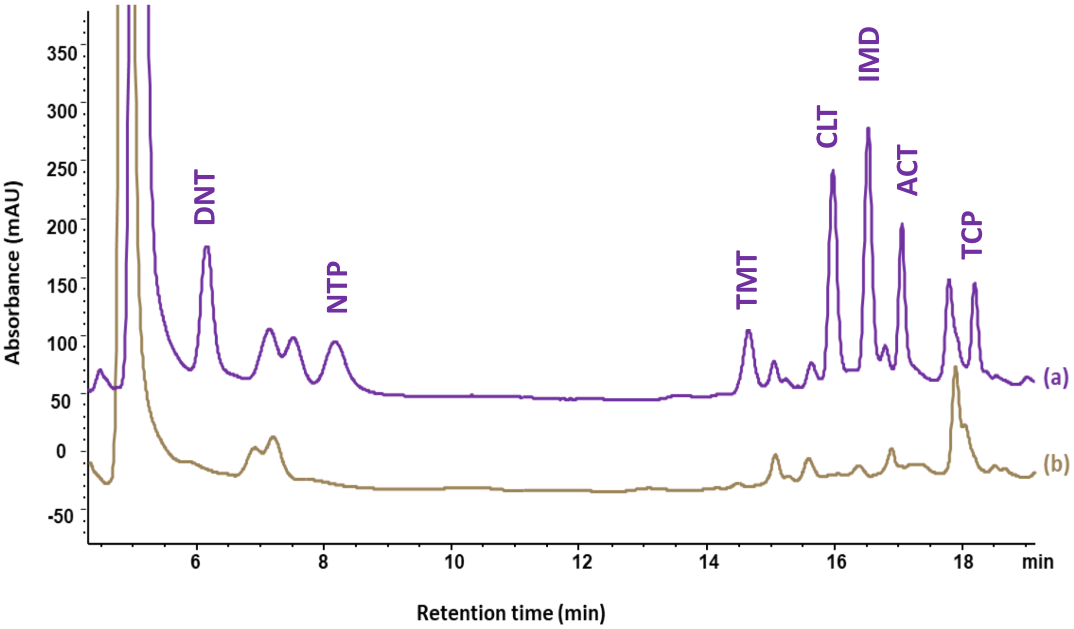


Figure 5. 1) Chromatograms obtained by the proposed SLE-CLC-DAD method for a barley sample spiked with the seven NNIs at 200 µg kg^{-1} (a) together with its blank (b). **2)** Chromatograms obtained by the proposed DLLME-CLC-DAD method for a multi-flower honey sample spiked with the seven NNIs at 200 µg kg^{-1} (a) together with its blank (b).

5. CONCLUSIONS

To the best of our knowledge, this is the first proposed application of CLC for the determination of NNIs. This approach involves lower solvent consumption compared with conventional HPLC. The applicability of the CLC-DAD method was achieved through its combination with two different sample treatments. Firstly, a SLE procedure was proposed as sample treatment for different kind of cereal samples. This is an easy, fast and inexpensive procedure in which a clean-up step was not required in contrast with other sample treatments reported for this type of food matrices. Then, a DLLME procedure was also optimized, being a simple and effective procedure for the extraction of NNIs from honey samples of different floral origins. Both SLE-CLC-DAD and DLLME-CLC-DAD methods presented satisfactory extraction recoveries (above 80 %), and high selectivity and sensitivity in spite of using UV detection. These methods were fully characterized for the determination of seven NNIs obtaining, in both cases, LOQs lower than their corresponding MRLs in each matrix, with good linearity and precision. Therefore, these procedures could be applied in routine analysis, being a powerful, simple, and fast alternative for screening and quantification of NNI residues in cereal and honey samples, previous to confirmation by MS detection.

6. REFERENCES

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CHAPTER 2

NANOFIBROUS ON-LINE SOLID-PHASE EXTRACTION COUPLED WITH LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF NEONICOTINOID RESIDUES IN ENVIRONMENTAL WATERS

This work has been sent for publication.

1. SUMMARY

A variety of polymeric nano- and microfibers were tested as potential sorbents for the on-line solid-phase extraction (SPE) of five neonicotinoids (NNIs) from river water samples. Nanofibers prepared from polycaprolactone, polyvinylidene fluoride, polystyrene, polyamide 6, polyacrylonitrile, and polyimide, as well as microfibers comprising polyethylene, a conjugate of polycaprolactone nano and microfibers, and polycaprolactone microfibers combined with polyvinylidene fluoride nanofibers were included in the screening. Polyimide nanofibers were selected as the most suitable for these analytes and type of matrix. An automated Lab-In-Syringe system enabled preconcentration via the on-line solid phase extraction of a large sample volume at low pressure with posterior analyte separation by HPLC. The sorbent consisted of several layers of mat disc housed in a solvent filter holder that was integrated in the injection loop of the HPLC. After loading 2 mL in-system buffered sample on the sorbent via a Lab-In-Syringe system, the retained analytes were eluted using the mobile phase and transferred directly in the chromatographic column. Extraction efficiencies of 68.8-83.4 % were achieved. Large preconcentration factors ranging from 70 for thiamethoxam to 82 for imidacloprid allowed reaching limits of detection and determination of 0.4 to 1.7 and 1.2 to 5.5 μg L^{-1} respectively. Analyte recoveries from spiked river waters ranged from 53.8 % to 113.3 % at 5 μg L^{-1} level and from 62.8 % to 119.8 % at 20 μg L^{-1} level. The polyimide nanofibers and the developed methodology proved suitable for the determination of thiamethoxam, clothianidin, imidacloprid, and thiacloprid while acetamiprid could not be quantified due to matrix peak overlaying the peak of the compound.

2. EXPERIMENTAL

2.1 Materials and reagents

Methanol (MeOH) and acetonitrile (MeCN) both LC-MS grade, as well as formic and acetic were obtained from VWR International s.r.o. (Stříbrná Skalice, Czech Republic). Millipore direct-Q generated ultrapure water (18.2 MΩ × cm) was used throughout the experiments.

Analytical standards of thiamethoxam (TMT), clothianidin (CLT), imidacloprid (IMD), thiacloprid (TCP) and acetamiprid (ACT) were purchased from Merck (Darmstadt, Germany). Individual standard solutions of the five NNIs were obtained by dissolving the appropriate amount of each one in MeOH at a concentration of 500 μg mL^{-1}. They were kept in the freezer at -20 °C avoiding exposure to light. Intermediate stock standard solutions were obtained from these standards by appropriate dilution to a final concentration 10 μg mL^{-1}. Working standard solutions were daily prepared by dilution of the intermediate stock standard solution with ultrapure water at the required concentration. Both intermediate and working solutions were stored in the dark at 4 °C when not used.

The buffer solutions of the following components and pH values were prepared at a concentration 0.1 mol L^{-1} to determine the loading conditions on the nanofibrous sorbent: formic acid (pH 2.0, 3.0, and 4.0), acetic acid (pH 5.0 and 6.0), and tris(hydroxymethyl)aminomethane (TRIS) (pH 7.0, 8.0, 9.0, 10.0) that were adjusted with 0.3 mol L^{-1} NaOH. In addition, 0.1 mol L^{-1} HCl was tested for sample acidification.

3D printed auxiliary materials were produced by fused deposition modelling (FDM) using a DeltiQ, size M, printer from TriLAB Group s.r.o. (Hradec Králové, Czech Republic) as well as polypropylene and polylactic acid filaments.

Mobile phases were filtered through a 0.45 μm hydrophilic PTFE filter (Millipore Corporation, Bedford, MA) while samples were filtered through a standard filter paper, stored in the dark at 4 °C, and then used without further modification.

2.2 Instrumentation

Lab-In-Syringe (LIS) system was used for liquid handling. It was assembled from an automatic Cavro XC3+ syringe pump (Tecan Trading AG, Männedorf, Switzerland) equipped with a 2.5 mL glass syringe and a 3-way head valve, an 8-port selection valve for the selection of solutions, and a 6-port high pressure injection valve that acted as interface between the LIS system and the HPLC system used for analyte separation and quantitation. Both valves (drive EMMA, head 4468 and drive ETMA, head C2-2346D, respectively) were purchased from Vici Valco Instruments Co. Inc. (Schenkon, Switzerland). An AIM 3200 autosampler from AIM Lab Inc. (Virginia, Queensland, Australia) was connected to the flow system during sample measurements to allow automatic exchange of sample solutions. All low-pressure connections consisted of 0.8 mm i.d. PTFE tubing while the high pressure connections were PEEK capillary. **Figure 1** shows the entire instrumental setup including tubing dimensions.

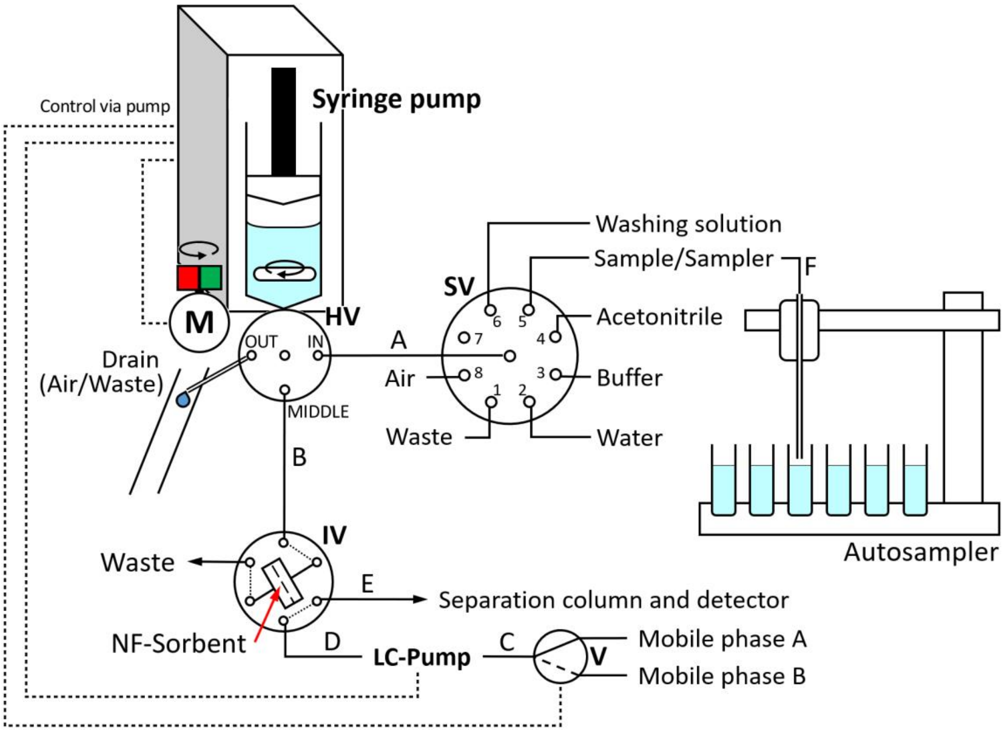

Figure 1. Scheme of Lab-In-Syringe system for large-volume SPE on nanofibrous (NF) sorbent membranes. HV – Head valve of syringe pump, IV – Injection valve, M – Motor, SV – Selection valve, V – Solenoid valve. Tubes: A – PTFE, 25 cm, 0.8 mm i.d., B – PTFE, 40 cm, 0.5 mm i.d., C – PTFE, 15 cm, 1.5 mm i.d., D – PEEK, 40 cm, 0.2 mm i.d., E – PEEK, 33 cm, 0.2 mm i.d.

A magnetic stirring bar (10 mm long, 3 mm in diameter) was placed inside the void of the syringe pump to enable in-syringe homogenous mixing of sample and loading buffer and for in-system preparation of washing solutions. A DC motor adapted from a pulse-width modulated computer fan was positioned closely to the syringe [1]. The motor held a stack of neodymium magnets (25 mm, 4 mm in diameter) on top. Upon initiating the motor, the magnetic stirring bar inside the syringe followed the rotating magnetic field thus forcing a synchronized rotation. Velocity was controlled via a simple analogue control board.

A stainless-steel PREP column in-line filter (AF0-7866, Phenomenex Int., Torrance, CA, USA) equipped with a 2 µm porosity stainless-steel filter disk (3 mm x 21.2 mm in diameter) was used for holding several layers of nanofibrous sorbent mat that were cut with preparation scissors to fit the size of the frit. After initial observation of pressure increase due to the stainless-steel filter, a disc made of a commercial felt pad purchased from a local hardware store (3 mm thickness, 22 mm in diameter), was used instead. The glue from the adhesive side was removed by soaking the felt disk repeatedly in ethyl acetate under sonication. The felt pad was inserted into a 3D printed support ring of polypropylene before placing it in the holder to obtain a hard rim that would allow high pressure sealing against the rubber rings of the in-line filter holder (**Figure 2**). This implementation also allowed adapting the felt pad diameter to the holder dimensions. The in-line filter, in the following referred to as “fibre holder”, was integrated in the injection loop (**Figure 1**).

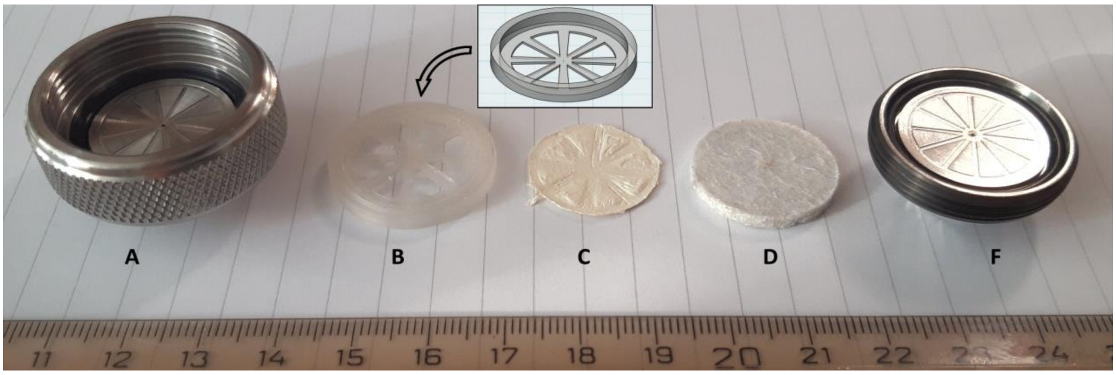

Figure 2. Assembly of the fibre holder consisting of a commercial in-line filter (A, F), nanofibrous sorbent (C), a fused deposition modelling-3D-printed holder (B) allowing the insertion of the nanofiber mats and a commercial felt pad as a support of low flow resistance

An LC-20AD pump and SPD-20A UV detector from Shimadzu Inc. (Tokyo, Japan) were used for on-line coupling of nanofibrous SPE automated via LIS technique. All separations were carried out using a reversed phase fused-core Kinetex® column (Phenomenex RP-C18 150 x 4.6, 2.1 µm, 100 Å). Gradient mode was enabled by adding a 3-way solenoid valve type MTV-3-1 UKGH from Takasago Electronics Inc. (Nagoya, Japan) on the aspiration side of the LC pump that switched proportionally between the reservoirs of mobile phases A and B to form the optimized gradient. The solenoid valve was controlled via a Trinket M0 circuit (Adafruit) and a program written in Python programming language reported earlier [1].

The syringe pump was controlled by an external hardware via three TTL contacts that were used for relay activation/deactivation of the stirring motor, the HPLC trigger, and initialization of the Trinket M0 chip for gradient operation of HPLC.

The LabSolutions software (Shimadzu Inc., Tokyo, Japan) was used for data evaluation and control of the chromatographic system. CocoSoft 5.11 [2] cared of the procedures on the flow system, i.e. sample mixing with buffer, loading, fibres washing as well as initial conditioning, cleaning of tubes, valve switching, and to trigger the chromatographic method and initiation of the gradient by pre-programmed switching protocol of the solenoid pump.

2.3 Chromatographic separation

NNI separation was performed using a reversed phase fused-core Kinetex® column (Phenomenex RP-C18 150 x 4.6, 2.1 µm, 100 Å pore size) from Phenomenex (Waldbronn, Germany) employing a mobile phase consisted of 10 % MeCN (v/v) in 0.05 % (v/v) aqueous formic acid (solvent A) and B 70 % MeCN (v/v) in 0.05 % (v/v) formic acid (solvent B) at a flow rate of 1 mL min^{-1} during the optimization of the gradient conditions and 0.8 mL min $^{-1}$ when on-line SPE was coupled to the HPLC with the added in-line filter in order to counteract the increased flow resistance.

Gradient mode was used as follows: 15 % B (6 min), 45 % B (6.5 min), 45 % B (7.5 min), 60 % B (8.5 min) and 0 % (11 min). Afterwards, mobile phase composition was back to initial conditions in 3 min to guarantee column equilibration.

Addition of 0.05 % (v/v) formic acid to the aqueous part of the mobile phase improved the peak shape, resolution, and column efficiency compared to no acid containing mobile phase counterpart. Doubling the formic acid concentration did not bring any further improvement. Addition of 5 mmol L^{-1} ammonium acetate was also tested but no improvements in peak resolution and average peak symmetry values were observed. Thus, addition of 0.05 % (v/v) formic acid in the mobile phase was adopted in the final procedure.

The temperature of the column was 25 °C and the UV-detection was performed 270 nm corresponding to the maximum absorbance for clothianidin and imidacloprid. The optimized method allowed baseline separation of all compounds in 8 min.

2.4 Preparation of nanofibers

Fibrous sorbents included polycaprolactone nanofibers (nPCL) and a combination of micro- and nanofibers (μ/nPCL), polycaprolactone microfibers in combination with polyvinylidene fluoride nanofibers (μPCL/nPVDF), polyethylene microfibers (μPE), and polyvinylidene fluoride (nPVDF), polystyrene (nPS), polyamide 6 (nPA6), polyacrylonitrile (nPAN), and polyimide (nPID) nanofibers. Production of these fibers except nPID is detailed elsewhere [3,4,5]. Briefly, nPVDF, nPA6, nPS, nPAN, and nPID nanofibers were produced by electrospinning from polymer solutions in an organic solvent or solvent mixture at a final concentration ranging from 7 to 16 wt %. Fibres μ/nPCL and μPCL/nPVDF were produced via a novel combination of the electrospinning and melt blown technology [3]. nPE fibres were produced by melt blown technology that comprised extrusion of the melted polymer through a spinning head with gaps, each 0.4 mm in diameter, resulting in the formation of microfibers in a hot airstream [4].

The nPID fibers eventually chosen for this work were prepared via electrospinning from a 16 wt % solution of polyimide pellets P84TM SG (HPpolymer Inc., Austria) in

N,N-dimethylacetamide (99.8 %; PENTA Chemicals, Czech Republic). The solution was stirred for 24 h at 250 rpm and 22 °C and then filled in a 15 mL cartridge attached to the spinning electrode. A Nanospider extruder, type NS 1WS500U from Elmarco Ltd. (Liberec, Czech Republic), was used for the electrospinning process.

2.5 On-line solid-phase extraction for natural water samples

Surface water samples were collected in glass bottles in the surroundings of Hradec Králové, (Czech Republic) in August 2020, from one lake and three rivers, two of those from intensively agriculturally used areas. The samples were filtered through a standard filter paper and stored, avoiding direct light exposure, at 4°C until their use.

The details of the operation are described in **Anexo I**. In short, all operation consisted of the aspiration of the required solutions from the selection valve in the syringe and propelling them slowly through the head valve in position "MIDDLE" towards the nanofibrous sorbent with the injection valve in position "LOAD" or in case of cleaning, rapidly through the head valve position "OUT" to waste.

The nanofibrous sorbent was cleaned with 1 mL MeCN and 1 mL water before aspiration of 2 mL sample and 0.3 mL buffer in the syringe void with activated stirring to achieve homogeneous mixing, and then loaded on the nanofibers. Afterwards, the syringe was cleaned twice with water to wash away any remnants of the sample. Then, the fibres were washed with 1 mL in-syringe diluted loading buffer. Finally, the injection valve was switched to position "INJECT" and the gradient, HPLC pump operation, and data acquisition were triggered.

Next, the analytes were eluted from the fibres in the separation column using the mobile phase with steadily increasing elution strength and their separation occurred within 14 min. The injection valve was switched 300 s after the injection back to position "LOAD" and the preconcentration of the next sample was carried out in the LIS system in parallel to the running separation.

3. RESULTS AND DISCUSSION

3.1 Optimization of mat holder

The in-line filter, selected to accommodate the nanofibers, was chosen for its large cross-section area of approximately 350 mm^2. The holder featured a 2 μm pore width stainless-steel frit. However, after filling it with the nanofibrous sorbent placed on top of the frit, we monitored an unacceptably high back pressure inhibiting sample loading by the low pressure LIS system. Besides, improper sealing of the nanofiber sheets with the integrated rubber O-rings was observed causing leaks during HPLC operation. Moreover, this configuration only allowed co-direction of loading and elution. The use of a commercial felt pad (see section 2.2) as a frit of higher porosity and low flow resistance combined with the polypropylene cover allowed firm holding of the nanofiber layers. This implementation enabled both adequate sealing without using the additional filter paper as well as counter-direction of loading (nanofibers-> frit) and elution (frit -> nanofibers) by the mobile phase. This mode proved superior to the co-directional operation, particularly because of the significantly reduced dead volume.

3.2 Optimization of nanofibers

The key parameter of method development was finding a suitable nanofibrous sorbent. Nine fibrous materials listed in section 2.2 were examined using the LIS system for automated and reproducible loading and elution in co-direction (use of stainless steel frit) for off-line analysis by HPLC. We opted for offline measurement in this early stage of method development to prevent any bias by uncomplete analyte elution due to unoptimized conditions.

The experiment was carried out using three layers of the respective fibre mat. The sorbent layers were placed between two layers of laboratory filter paper for easy handling avoiding layer folding by electrostatic charge, for mechanical protection, and for improved sealing. The effect of the two filter paper sheets was evaluated as blank. The fibres were washed inside the holder by the LIS system with 1 mL ACN and then 1 mL water for cleaning and conditioning. Mixed NNI standard solution (2 mL) at a concentration of 50 μg L^{-1} for each analyte, was mixed in-syringe with 200 μL 0.1

mol L^{-1} HCl to yield an acidic pH as learned from the previous studies [6,7]. NNI metabolite, 6-chloronicotinoic acid (pK_a = 3.73), was primarily included in our experiments, which was another reason to aim at an acidic loading pH. After loading, the retained analytes were eluted with 1 mL ACN that was previously aspirated in the syringe and then pushed through the fibre holder. In this step, the eluate was manually collected and analysed off-line using HPLC with an injection volume of 25 µL. Experiments were conducted in triplicate and the peak areas were compared to those obtained via direct injections of a 100 µg L^{-1} mixed standard to calculate the extraction efficiencies. The results presented in **Figure 3** demonstrate that the highest extraction efficiencies were achieved with PID nanofibers.

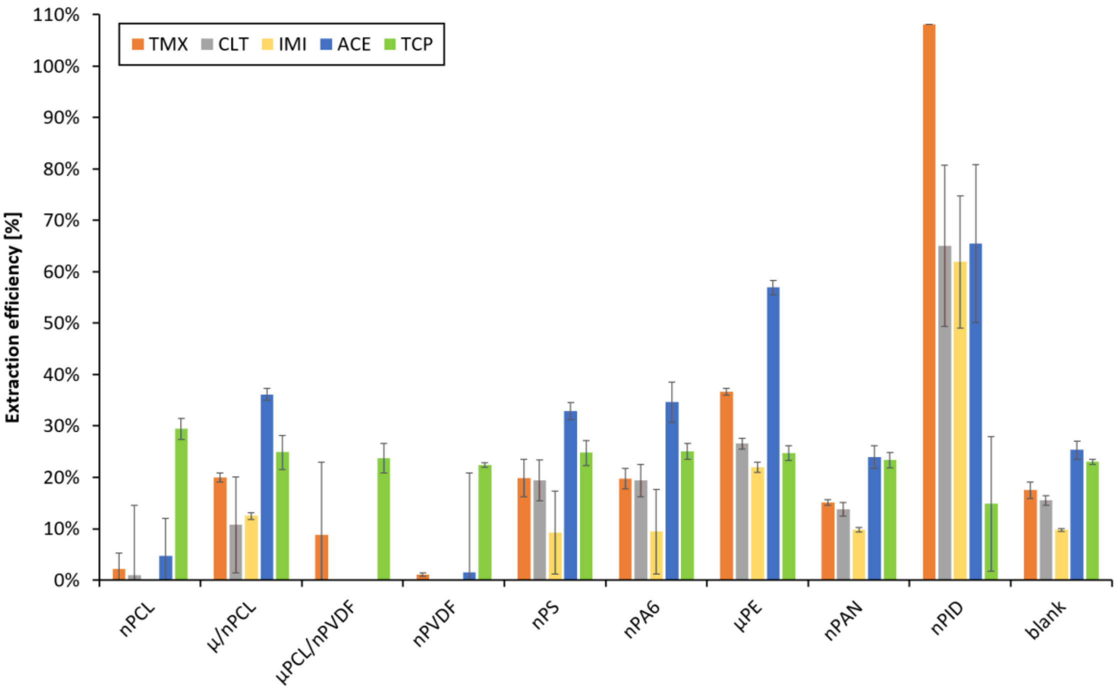

Figure 3. Suitability of nanofibrous sorbents for NNI compounds. Loading: 2 mL mixed standards, 50 µg L^{-1} each, acidified with 200 µL HCl, pH 3. Elution: 1 mL MeCN. Loading and elution flow rates: 500 µL min-1. Off-line HPLC measurement, injection volume: 25 µL.

Extraction efficiencies between 20 and 60 % were achieved with µPE. In contrast, nPS, nPA6, nPAN, and µPCL/nPVDF were not suitable for extraction of NNI at all, as the extraction efficiencies were less than 30 %. Even worse results were achieved with nPCL and nPVDF nanofibers in which only less than 10 % of the target analytes were retained. The filter paper itself exhibited no significant extraction capacity for the NNI. We further verified that also the felt material used in the following experiments did not display any significant extraction capacity for the target analytes. nPID was selected as the extracting material for further experiments.

3.3 Optimization of on-line SPE conditions

Sample volume was examined in offline mode after selecting the extraction sorbent. The preconcentration system was coupled to HPLC and the experiments concerning the SPE conditions were conducted online.

3.3.1 Selection of sample volume

In a preliminary experiment, the extraction capacity of the fibres was determined using a mixed standard featuring a high analyte concentration of 1 ppm per analyte. The syringe size used in this experiment was 2.5 mL. Thus, repeated execution of the loading step was carried out when volumes exceeding 2 mL were needed before elution in online mode. The results confirmed that the response for most analytes increased linearly with the loaded volume of standard over a range of at least up to 6 mL. The extraction efficiencies remained stable at around 80 % for thiacloprid, acetamiprid, imidacloprid and clothianidin. However, they decreased for the most polar compound thiamethoxam. Details are presented in Supplementary material 6. These experiments verified that the sensitivity can be easily improved by simply increasing the sample volume and that the extraction capacity of the sorbent is sufficient even for absolute analyte amounts of at least 30 μg.

3.3.2 Selection of flow rate of sample loading

The flow rate for sample loading was studied at 250, 500, and 750 μL min^{-1}. **Figure 4a** shows the parameters and results. We found that the slowest flow rate, providing the longest contact time of the analyte with the nanofibers, did not provide any benefit compared to a flow rate of 500 μL min^{-1} but even decreased the reproducibility. On the other hand, a decrease in the extraction efficiency was observed after an increase in the flow rate. Therefore, an intermediate value of 500 µL min^{-1} was chosen for further work that led to both acceptable extraction efficiency and time of analysis.

3.3.3 Selection of number of PID layers

An increase in the extraction efficiency was the main objective of all following experiments. The amount of the sorbent or rather the number of nanofiber layers was considered to have a substantial effect on the method sensitivity and extraction efficiency particularly in terms of the extraction capacity considering the short contact time of the analytes with the sorbent. Results achieved with stacks of 3, 6, and 9 PID layers were compared to an extraction carried out with the felt pad alone, i.e. without any PID layer (**Figure 4b**). The extraction recovery increased to 6 layers achieving values between 58 % (clothianidin) and 85 % (thiacloprid), whereas 9 layers yielded only similar results to those using mere 3 layers. We assume that reason for this phenomenon is a reduced penetration of both sample and eluent through the fibrous layers that decreases the accessibility of the fibre surface as well as delayed elution of the analytes from the larger mass of fibres. Therefore, 6 layers were used.

3.3.4 Selection of loading pH

Based on previous findings partly using SDS as ion-pairing reagent [8], the loading sample pH was acidic at pH 3. The analytes of interest are neutral between pH 2 and 9 with exception of imidcloprid and acetamiprid. Nonetheless, we decided to study the effect of the loading pH over a range of pH 2-10 using 300 µL buffer solution mixed inside the syringe with 2 mL sample. **Figure 4c** shows that the extraction efficiency increased slightly with an increase in the loading pH to pH 8 and then decreased for higher values. We decided to use TRIS buffer pH 8, which yielded the highest extraction efficiency, for all further work.

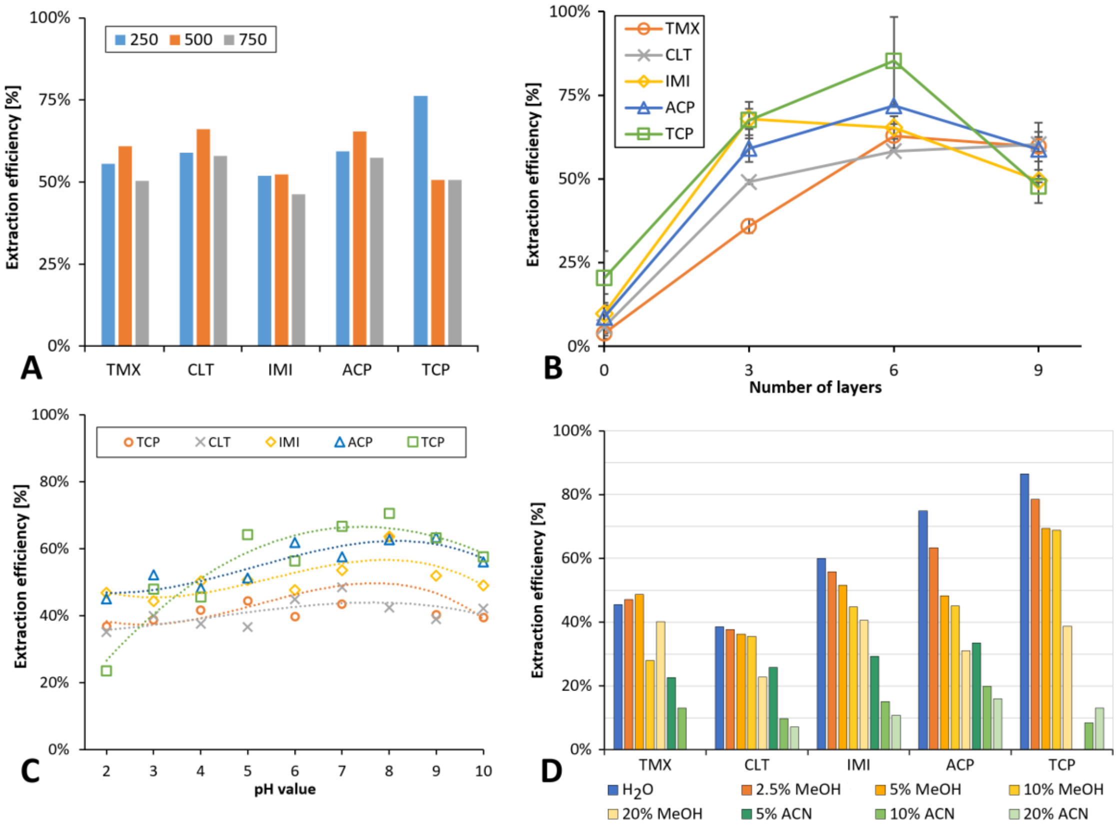

Figure 4. Effect of conditions on online SPE. **A:** Flow rate at sample loading: 3 layers of PID nanofibers, loading 2 mL mixed standard, 50 µg L^{-1} ,pH adjusted to 3, washing with 2 mL water. **B:** Number of PID nanofibers layers: loading 2 mL mixed standard, 50 µg L^{-1}, pH adjusted to 1.3, washing with 2 mL water. **C:** Loading pH value: loading 2 mL mixed standard, 10 µg L^{-1}, with in-syringe addition of 300 µL buffer (pH 2-4 - formate, pH 5 and 6 - acetate, pH 7-10 - TRIS-HCl). Washing with 2 mL water mixed in-syringe with 50 µL buffer. **D:** Composition of washing solution: 6 layers PID nanofibers, loading 2 mL mixed standard, 10 µg L^{-1}, pH adjusted to 1.3.

3.3.5 Selection of salt addition

In addition, we examined reducing the water solubility of the analytes and increasing their affinity to the fibrous sorbent via increasing the ionic strength of the loaded sample solution. For this, standards were prepared in water as well as in 30 % (w/w) NaCl solution. No significant salting-out effect was observed (data not shown). Thus, this strategy to increase the extraction efficiency of the NNI analytes was not adopted.

3.3.6 Selection of washing solution

The effect of the type and quantity of the washing solution used after sample loading on the analyte recovery was assessed. Diluted MeOH and MeCN were considered as washing solutions at concentrations of 5, 10, and 20 % (v/v), and were compared to the neat water (**Figure 4d**). A MeOH content of 2.5% (v/v) did not cause any significant loss of analytes. At 5 % (v/v) MeOH, a signal decrease of about 20 % was observed only for thiacoplid. MeCN eluted part of the analytes even at the lowest tested concentration. Therefore, a washing solution of 2.5 % (v/v) was used further on.

The volume of the washing solution had to be large enough to eliminate remains of sample from the injection loop including the dead-volume of the sorbent holder, yet, as small as possible to minimize waste, time of analysis, and to avoid untimely elution of retained analytes. For the related experiment, a two times higher concentration of the standard (20 μg L^{-1} of each analyte) was used to facilitate verifying that the washing solutions sufficed to wash out any residues of the non-retained analytes. In addition, the washing solution was adjusted to the optimal loading pH by adding 2.5 % (v/v) of the loading buffer to the MeOH solution.

Peak areas decreased on average by 25 % after increasing the washing solution volume from 750 μL to 2000 μL accounting for 5 % with each additional 250 μL. Therefore, 1 mL washing solution (2.5 % MeOH in 2.5 mmol L^{-1} buffer) was chosen for following experiments. By decreasing the volume of washing solution and using a flow rate of 750 μL min^{-1} for sorbent washing, the time of this step was reduced from 2.6 to 1.3 min.

4. METHOD CHARACTERIZATION

The proposed method was characterized in terms of signal linearity, reproducibility, and applicability of the optimized method. **Table 1** summarizes the obtained analytical figures of merit. The values of limits of detection and quantitation were calculated from the threefold and tenfold amplitude of the baseline divided by the slope. LOD values were between 0.4 and 1.7 μg L^{-1}, thus meeting with the requirements on methods sensitivity for neonicotinoids in water [9]). Repeatability of the entire procedure was typically below 5%. Preconcentration factors (PFs) were 70 to 82 corresponding to an equal percent extraction efficiency considering a theoretical preconcentration factor of 100 (injection loop size for direct injection: 20 µL vs. online preconcentration of 2 mL sample).

Table 1: Analytical figures of merit

Analyte	Sensitivity / calibration slope (n = 3)	Linear range (µg L^{-1})	LOD (µg L^{-1})	LOQ (µg L^{-1})	Repeatability (% RSD, n = 3, 10 µg L^{-1} level)	PF
TMT	$2.16 \cdot 10^3 \pm 24$	2-100	0.55	1.82	6.8	70
CLT	$6.50 \cdot 10^3 \pm 240$	2-100	0.56	1.88	0.4	76
IMI	$7.00 \cdot 10^3 \pm 56$	2-100	0.67	2.25	2.8	82
ACT	$2.90 \cdot 10^3 \pm 225$	5-100	1.65	5.49	4.4	81
TCP	$2.35 \cdot 10^3 \pm 119$	1-100	0.36	1.21	4.2	76

The average extraction efficiency was 77.0 ± 4.8 %. Extraction efficiencies were calculated comparing the peak areas of 2 mL 10 µg L^{-1} standards undergoing the online SPE-HPLC procedure and peak areas of 1 µg L^{-1} standards after direct injection of 20 µL.

The observed validated parameters of the separation method, i.e. the peak width at 5 % peak height and the tailing factor, were somewhat affected by online SPE yet to a justifiable degree. The observed dispersion was caused by the dead volume of the holder and felt pad that caused peak broadening by about 30 % (0.212 min on average compared to 0.165 min for direct injection) and a peak symmetry/tailing factor of 1.7 on average with the extraction step compared to 1.2 for the direct injection. The nanofibers contribution to this increase was negligible and was mainly caused by the holder itself. In contrast, the felt pad did not contribute due to applying the counter-directional elution. On the other hand, repeatability values of retention times were adequate for sample analysis with an average value of 4.9 % RSD (n = 6) and peak resolution ranged from 2.9 to 8.3. HPLC validation parameters are listed in **Table 2.**

Table 2. HPLC validation parameters (5 μg L^{-1}, n=6)

Analyte	t_R ± RSD%	Tailing factor	R_S	Width (5%) [min]
TMT	7.73±4.59%	1.79	-	0.242
CLT	8.49±6.03%	1.78	4.45	0.235
IMI	8.85±6.13%	1.57	2.14	0.235
ACT	9.34±5.31%	1.41	2.94	0.203
TCP	10.58±2.57%	1.41	8.29	0.192

5. APPLICATION TO REAL SAMPLES

The analysed surface waters were collected in August 2020 in the area of Hradec Králové, Czech Republic. Sample 2 was collected at a former excavation lake. The other three samples were collected from rivers and ditches in the same area. The only sample treatment carried out was the filtration through the standard filter paper to eliminate sedimented and suspended particulate matter. Neonicotinoids were not found in any of the collected samples. To estimate the method applicability, the samples were spiked with 5 and 20 μg L^{-1} mixed standard solutions, respectively, to mimic contaminated surface waters. The results are listed in **Table 3**.

Table 3: Recoveries of analytes in spiked samples.

R (%)	**Sample 1**		**Sample 2**		**Sample 3**		**Sample 4**	
Analyte	5 μg L^{-1}	20 μg L^{-1}	5 μg L^{-1}	20 μg L^{-1}	5 μg L^{-1}	20 μg L^{-1}	5 μg L^{-1}	20 μg L^{-1}
TMT	101.7±12.8	97.3±4.5	113.3±0.9	95.4±4.6	n. e.	83.3±4.9	90.2±10.0	103.6±22.6
CLT	74.1±6.0	91.7±6.8	59.0±5.7	82.6±2.0	37.6±12.8	65.1±0.7	54.1±4.8	84.8±8.3
IMI	85.0±1.4	91.7±1.0	81.2±7.2	81.5±0.9	83.1±5.1	80.0±7.3	83.3±7.3	92.9±5.7
TCP	60.7±14.5	76.1±8.0	60.5±3.0	62.8±7.1	53.8±15.8	66.7±3.5	53.9±5.8	68.5±2.4

We observed that quantifying acetamiprid was not possible due to overlap of the compound and matrix peaks that could not be resolved. On the other hand, analyte recovery values for the other analytes calculated from the calibration curves ranged from 53.8 to 113.3 % for a 5 μg L^{-1} spike level with the exception for clothianidin in

Sample 3 and from 62.8 to 119.8% for samples spiked to 20 μg L^{-1}. Repeatability values were slightly higher than in the standards measurement but generally did not exceed 10 % RSD.

6. COMPARISON WITH OTHER METHODS

LOD or LOQs values obtained in our work are in the same range as previously reported by other authors including SPE preconcentration of NNIs followed by HPLC-UV. However, PFs calculated from the peak areas of directly injected and on-line preconcentrated standards as well as gain in sensitivity of the SPE performed on-line were better reaching 70 (thiamethoxam) and 82 (imidacloprid). This relatively high factor was achieved due to the large ratio of sample volumes and effective sorbent volume and confirms the benefit of the preconcentration using nanofibrous sorbent. However, to achieve the full benefit of the very thin yet efficient sorbent, such as the nanofibers layers used in this research, the dead volume should be further minimized via optimization of the hardware.

Most of the preparation methods for HPLC- UV determination of NNIs rely on QuEChERS or other combinations of SPE and SLE. This is explained by the complexity of sample matrices including honey, fruits, grain, and vegetables. On the other hand, a lasting procedure, typically dispersive SPE, is generally required including addition of buffers, solvent, vortexing, centrifugation, collecting the supernatant, supernatant evaporation and reconstitution, and a secondary clean-up. For instance, Campillo *et al.*, used a C-18 functionalized sorbent with preceding DLLME. MeCN used as an eluent in the first step was further used in the DLLME step as a dispersant of chloroform, which acted as extractant [10]. Wang *et al.* even combined QuEChERS with three sorbents for extract clean-up followed by DLLME for further preconcentration that reached only a factor of 5 [11]. SPE alone appears insufficient for clean-up of those matrices when using HPLC-UV, and only a few authors reported on this technique. For example, Moyakao *et al*. used montmorillonite as a green and novel sorbent in dispersive SPE [12]. Despite numerous steps carried out manually, PFs range below values we achieved in the present work [6,13,14,15]. In fact, their analyte recovery values calculated from spiking experiments as well as values of LOD/LOQ and procedural reproducibility were in the same range, often even inferior to those obtained by our proposed system and method.

Given the relatively high polarity of NNI, alternative extraction media such as ionic liquids [16] micelles [17], and anionic surfactant SDS as ion-pairing reagent [8] have been used to enhance the extraction efficiency. Possible dynamic coating of the HPLC column induced peak tailing and the need for precise collection of the small volumes of extractant can be considered as drawbacks of these approaches that we could omit in this work. To the best of our knowledge, no method so far has reported use of flow automation of the sample preparation step, application of nanofibrous sorbents, and online SPE with perpendicularly permeated sorbent mat in HPLC methods.

It should be pointed out that LOD/LOQ values using MS detection have generally not been better than by a factor of 10 compared to those achieved in this work. On the other hand, Zhang *et al*. [18] and Iancu *et al.* [19] achieved far lower LOD values using LC-MS. Yet, they needed about 200- and 100-times larger sample volumes, respectively, to accomplished preconcentration by SPE.

Clearly, the performance of the present method is comparable, and in part even superior to previous reports, in terms of sensitivity, reproducibility, procedural time, and analyte recovery. Successful application to surface waters confirmed that the system and method were simple and effective, using solely online-SPE in microscale format. Carrying out the sample preparation procedure in a fully automatic fashion and coupled online to HPLC is an unprecedented feature among the methodologies developed for NNI analysis.

7. CONCLUSIONS

Polymer nanofibers were for the first time investigated as novel sorbents for neonicotinoid pesticides with polyimide being the most suitable material. On-line SPE with preconcentration of large sample volume using Lab-In-Syringe technique was then developed and successfully applied to the determination of these NNI insecticides in surface waters. The developed method enabled automated sorbent conditioning, in-system sample mixing with loading buffer, analyte preconcentration, and finally their separation and detection via a compact analyser system. Using the nanofibers in disc format, reproducible cartridge filling, low back pressure and preconcentration factors exceeding 70 were achieved that enabled determining NNI at nanomolar concentrations just with UV spectrometry as a readily available detection technique. This sensitivity fulfilled the requirements on NNI analysis in water bodies.

Time efficiency of separation in the gradient mode accomplished via instrument modification and parallel operation of preconcentration and online coupled analyte separation, as well as adequate analyte recovery and sensitivity were demonstrated. The viability of the proposed system and method were confirmed by surface water analysis. Our method was comparable or superior in terms of analytical performance including sample throughput, sensitivity, reproducibility, and recovery to those using HPLC-UV and reported elsewhere. It is also comparable to SPE methodologies with LC-MS quantification of NNI in aqueous samples in terms of sensitivity, even if lower number of target molecules is analysed. Further improvement of the extraction efficiency via modification of surface of the fibres and widening the application to other analyte groups is foreseen in future continuation of the work.

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CHAPTER 3

EVALUATION OF NATURAL DEEP EUTECTIC SOLVENTS AS NOVEL DISPERSIVE SOLVENTS IN DLLME-SFO FOR THE DETERMINATION OF PESTIDICE RESIDUES

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1. SUMMARY

Current trends in analytical chemistry encourage the use of innocuous solvents to develop modern methods aligned with Green Chemistry. In this sense, Natural Deep Eutectic Solvents (NADESs) have emerged as a novel generation of green solvents which can be employed in sample treatments as an alternative to the toxic organic solvents commonly used so far. In this work, a new extraction method employs dispersive liquid-liquid microextraction based on a solid floating organic droplet (DLLME-SFO), by using a mixture composed by a less dense than water extraction solvent, 1-dodecanol, and a novel dispersive solvent, which belongs to NADES. The methodology was proposed to extract and preconcentrate some pesticide residues (fipronil, fipronil-sulfide, fipronil-sulfone and boscalid) from environmental water and white wine samples before analysis by liquid-chromatography coupled to ultraviolet detection (HPLC-UV). Limits of quantification (LOQs) lower than 4.5 μg L^{-1}, recoveries above 82% and precision, expressed as RSD, below 12.4% were achieved in both samples showing that the proposed method is a powerful, efficient and a green alternative for the determination of these compounds, and therefore, demonstrating a new application for NADES in sample preparation. In addition, the DLLME-SFO-HPLC-UV method was evaluated and compared with other reported approaches using the Analytical GREEnness metric approach, which highlighted the greenness of the proposed method. This work was carried out in collaboration with the group of Prof. M.F. Silva at the National University of Cuyo (Mendoza, Argentina).

2. EXPERIMENTAL

2.1 Materials and reagents

All reagents used through this work were analytical reagent grade and solvents were high performance liquid chromatography (HPLC) grade, unless otherwise specified.

Analytical standards of fipronil (FPN), fipronil-sulfone (FPN-sulfone), fipronil-sulfide (FPN-sulfide), and boscalid (BCL) (≥ 99% pure) were supplied by Sigma-Aldrich (Darmstadt, Germany). Methanol (MeOH) and acetonitrile (MeCN) were obtained from J. T. Baker (USA), while 1-dodecanol for microextraction step was obtained from Sigma-Aldrich (St. Louis, USA). Compounds for NADES preparation, including glucose anhydrous (≥99 %), citric acid anhydrous (≥99%), l(+)lactic acid (85–90%), were purchased from Biopack (Bs. As., Argentina). Formic acid (85%) (FA), glycerol and the salts involved in sample treatment such as sodium sulfate (Na_2SO_4), sodium chloride (NaCl) and ammonium chloride (NH_4Cl) were acquired from Sintorgan (Bs. As., Argentina). Ultrapure water used throughout the work was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Stock standard solutions were obtained by dissolving the appropriate amount of each analyte in MeOH, reaching a final concentration of 100 mg L^{-1}. Stock standard solutions were kept in the freezer at -20 °C avoiding exposure to light. Intermediate standard solutions containing 10 mg L^{-1} of each compound were obtained by mixing an appropriate volume of each stock standard solution and its subsequent dilution with MeOH. They were stored in dark at 4 °C and equilibrated to room temperature before their use. Working standard solutions were freshly prepared every day by the dilution of an intermediate standard solution aliquot with MeOH according to the desired concentration.

2.2 Instrumentation

HPLC experiments were carried on an HPLC system with a Shimadzu (LC-2030 C, Prominence-i, Japan) equipped with a vacuum degasser unit, autosampler, quaternary pump and chromatographic oven and an UV-Vis detector. Data acquisition and processing were collected with a Shimadzu LC LabSolution Lite software.

An electronic microbalance with a readability of ≤ 0.1 mg (Acculab, Sartorius), magnetic stirrer with temperature control Fisatom (model 752A, Brasil) was used in the preparation of NADES. Moreover, a centrifuge (Eppendorf 5804-R, Germany), an ultrasonic bath (Testlab, model TB-04 TA, Buenos Aires, Argentina), and a vortex (Arcano, Buenos Aires, Argentina) were used through the sample preparation procedure.

2.3 Chromatographic separation

The optimum separation conditions were as follows: HPLC separations were performed in a Hypersil GOLD C18 analytical column (150 x 2.1 mm, 3 μm particle size, Thermo Fisher) using an isocratic elution of 65 % (v/v) MeOH in ultrapure water at a flow rate of 0.35 mL min^{-1}. The autosampler vial tray was maintained at 25 °C and the temperature of the column was set at 30 °C. A volume of 10 μL was selected as injection volume and the UV-detection was performed at 220 nm. Under these conditions, no sample contamination or sample-to-sample carryover was observed.

2.4 Preparation of NADES

Several natural eutectic systems combining sugars and organic acids (hydrogen bond acceptor with hydrogen bond donors) were prepared by using the heating and stirring method with the addition of a known amount of water following a previously method described by Dai et al. [1]. Water was added in order to reduce the viscosity of the eutectic systems. Three different NADESs namely LGH, CGLH, and LGLH characterized [2], and used in previous studies [3,4] were prepared. LGH was synthesized by mixing lactic acid, glucose and ultrapure water at a molar ratio of 5:1:3, CGLH by mixing citric acid, glycerol and ultrapure water at a molar ratio of 1:1:3 and finally, LGLH by mixing lactic acid, glycerol and ultrapure water at a molar ratio of 1:1:1. Each component was accurately weighed and placed in a 50 mL amber glass bottle. The component mixture was heated in a magnetic stirrer with temperature control at 40 °C for 45 min until forming a homogeneous transparent liquid.

2.5 DLLME-SFO for environmental water and white wine samples

Environmental water samples were collected from an area with high agricultural activity near vineyards located in Tupungato, which is a wine district in the northern Uco Valley region in Mendoza (Argentina). These samples were collected in 1-L glass bottles and kept at 4 °C for their daily use. On the other hand, the white wine samples were purchased from local markets (Mendoza, Argentina) whose production was carried out in the same region. After their degasification by agitation in a vortex, white wine samples were also kept at 4 °C until their use.

The extraction of target analytes from environmental water and white wine samples was accomplished by the developed DLLME-SFO procedure, which was performed under the optimum conditions as follows: A mixed solution containing 2 mL of the NADES so-called LGH, which is composed by lactose, glucose, and water at a molar ratio of 5:1:3 (dispersive solvent), and 100 μL of 1-dodecanol (extraction solvent) was rapidly injected into a glass tube containing 5 mL of a sample solution saturated with 0.75 g of Na_2SO_4, causing a cloudy solution due to the formation of dispersed fine droplets of 1-dodecanol. Then, the ternary system was vigorously shaken for 1 min by mechanical agitation and subsequently centrifuged for 5 min at 9000 rpm. After that, the organic droplet was observed on the surface of the aqueous solution because of its lower density. Afterward, the glass tube was placed into an ice bath for 10 min, which allowed solidification of the organic droplet because of the low melting point of 1-dodecanol (24 °C). The solid floating organic droplet (SFO) was transferred into a glass vial using a small spatula and 400 μL of MeOH were added to help the re-dissolution of the extraction solvent. Finally, the glass vial was vortex for 1 min until a proper homogenization and injected into the HPLC-UV system.

The optimized sample procedure for the analysis of environmental water and white wine samples is shown in **Figure 1**.

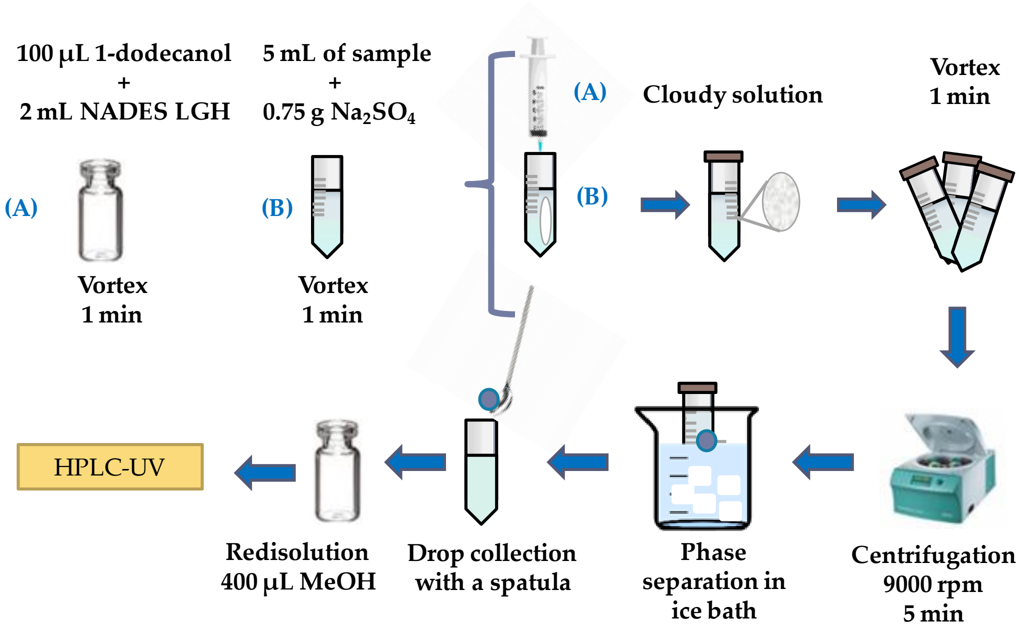

Figure 1. DLLME-SFO procedure for the analysis of environmental water and white wine samples.

3. RESULTS AND DISCUSSION

3.1 Optimization of chromatographic separation

All the parameters involved in the chromatographic separation such as gradient or isocratic mode, column temperature, injection volume, mobile phase and flow were evaluated.

Initially, separations were carried out using a mobile phase consisted of ultrapure water (eluent A) and MeCN (eluent B) supplied at 0.3 mL min^{-1} under gradient conditions as follows: 30% B (0 min), 30% B (2.5 min), 80% B (23 min) and 80% B (25 min). Afterwards, mobile phase composition was back to initial conditions in 5 min. In order to guarantee column equilibration, initial conditions were maintained for 2 min. An injection volume of 10 µL was considered and ultrapure water was used as injection solvent. Under these conditions, the metabolites FPN-sulfone and FPN-sulfide co-eluted, so the use of MeOH as eluent B instead of MeCN was considered, and for the first time it was possible to resolve all the peaks to baseline. In addition, peak resolution between boscalid and FPN was slightly improved. Therefore, MeOH was selected as mobile phase organic solvent. The addition of formic acid to the mobile phase was also evaluated, from 0.05 to 0.2% (v/v), and no improvement in terms of peak resolution was observed, so the addition of formic acid was discarded. However, under gradient conditions it was not possible to reduce the time of analysis because slight variations in the gradient led to co-elution of the metabolites. Then, with the aim to reduce analysis time, an isocratic elution of 60% (v/v) MeOH in ultrapure water was also tested. Baseline separation of all analytes was achieved in less than 7 min under these conditions, so isocratic mode was selected for further analysis.

Subsequently, the percentage of MeOH was studied from 50 to 70% obtaining the best results in terms of peak resolution when 65% of MeOH was used. Therefore, the isocratic elution selected was 65% (v/v) MeOH in ultrapure water. Afterwards, mobile phase flow rate was ranged from 0.2 to 0.8 mL min^{-1}. A flow rate of 0.35 mL min^{-1} was established as optimum because higher flow rates resulted on a decrease on peak resolution between FPN-sulfone and FPN-sulfide. Moreover, column temperature was studied between 20 °C and 40 °C. No significant influence was observed and 30 °C was selected as optimum because it provided slightly better resolution between peaks without extending the analysis time. Finally, the injection volume was also investigated from 5 to 20 μL selecting a volume of 10 μL as it provided the best sensitivity without negatively affecting the peak shape or resolution between peaks.

The monitoring wavelength was set at 220 nm taking into consideration the maximum absorbance of the target compounds. To sum up, under optimum conditions all the target compounds were fully separated in less than 7 min.

3.2 Optimization of sample treatment

A DLLME-SFO method was proposed as a sample treatment using NADES as a novel dispersive solvent prior to the analysis of boscalid, FPN, FPN-sulfone, and FPN-sulfide in environmental water and white wine samples by the optimized HPLC-UV method. Several parameters such as type and volume of NADES as dispersive solvent, type and volume of extraction solvent, extraction time, and effect of salting-out agent were studied through a univariate approach in order to achieve the best conditions that maximize the extraction efficiency. The selection of the optimum value in each case was made in terms of highest recoveries.

Selection of the type and volume of dispersive solvent

LGH, CGLH, and LGLH were assessed as dispersive solvents in order to evaluate their potential for dispersing the extraction solvent (1-dodecanol) in fine drops in a DLLME-SFO procedure. Initially, 100 μL of 1-dodecanol were mixed with 2 mL of each NADES and subsequently, the mixture was rapidly injected into 5 mL of an aqueous solution sample containing 1g of Na_2SO_4 to favor phase separation. Satisfactory recoveries were achieved, being approximately 70% or higher in all cases, what means that the evaluated NADES could act as dispersive solvents replacing toxic organic solvents used so far in DLLME. The most effective dispersion was achieved with LGH, which provided recoveries above 90% for all target compounds **(Figure 2a).** This could be due to the lower viscosity and higher polarity of LGH being essential for improving the interaction yield between the ternary system composed by the aqueous sample, NADES and extraction solvent. Then, different volumes of LGH ranged from 1 to 2.5 mL were studied. As can be seen in **Figure 2b**, as the volume increased, the recovery increased to a volume of 2 mL above which the recovery was significantly worse. Thus, 2 mL of LGH were selected as dispersive solvent for further experiments.

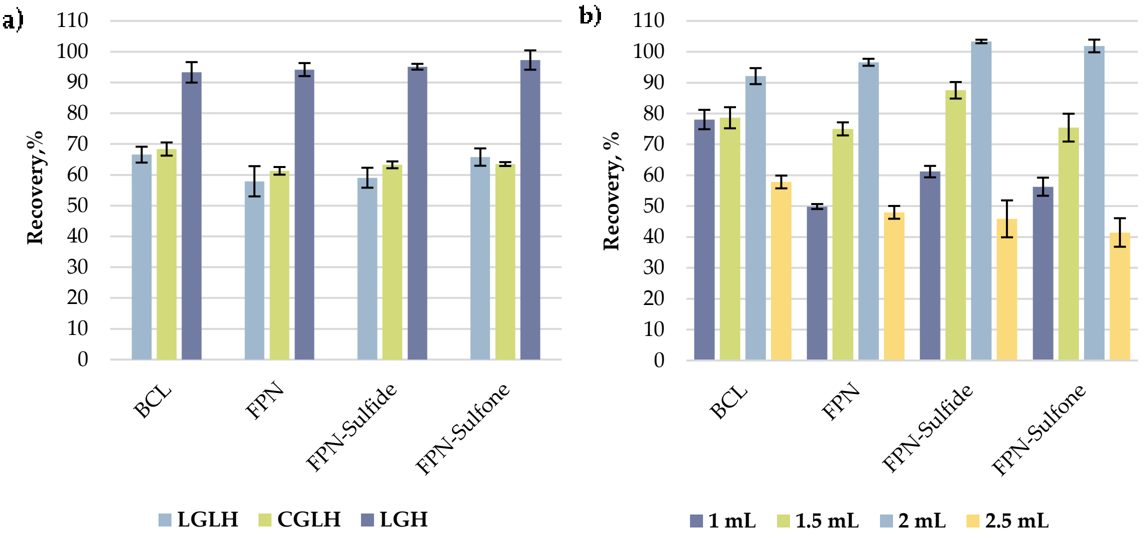

Figure 2. Optimization of the DLLME-SFO procedure. Effect of the **a)** dispersive solvent type; **b)** dispersive solvent volume. Error bars represent the standard error (n=4).

Selection of the type and volume of extraction solvent

The extraction solvent used in a DLLME-SFO procedure should accomplish several requirements such as high extraction efficiency for target compounds, lower density than water, low solubility in water, melting point (MP) close to room temperature, and, preferably, low toxicity, volatility and cost. Based on these requirements, 1-undecanol (MP ranges: 13–15 °C), 1-dodecanol (MP ranges: 22–24 °C) and 2-dodecanol (MP ranges: 17–18 °C) were taken into consideration. Firstly, the solvent 1-octanol was excluded from testing due to its low melting points (MP ranges: (-14)–(-16) °C), which made difficult the solidification of the droplet using an ice bath or even in a refrigerator. On the other hand, the main disadvantage of 1-undecanol and 2-dodecanol is the much longer solidification time needed if compared it with the corresponding to 1-dodecanol which has a higher melting point. Therefore, due to practical convenience, easy availability and lowest cost, 1-dodecanol was selected as extraction solvent. In addition, the selection of an appropriate extraction solvent volume is a major consideration in the DLLME-SFO procedure in order to favor drop formation. Thus, the effect of the volume of 1-dodecanol was evaluated from 75 to 120 μL with 2 mL of LGH as dispersive solvent. As can be seen in **Figure 3,** employing a volume of 100 µL slightly higher recoveries for all analytes were obtained, so 100 µL of 1-dodecanol were chosen as extraction solvent.

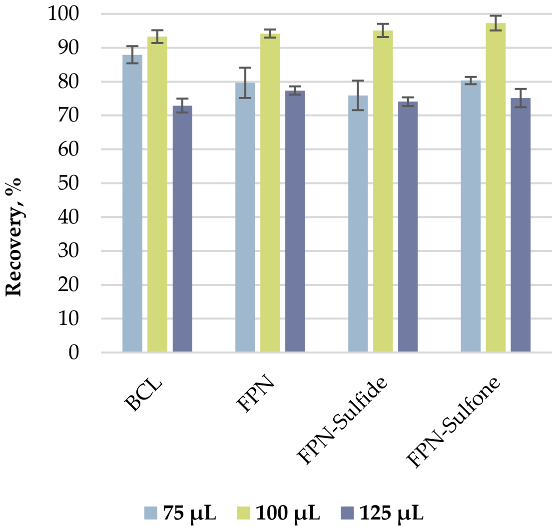

Figure 3. Optimization of the DLLME-SFO procedure. Effect of the extraction solvent volume. Error bars represent the standard error (n=4).

Selection of the extraction time

In DLLME, extraction time is defined as the interval time between the injection of the mixture of dispersive and extraction solvents in the aqueous phase and the centrifugation step. Therefore, the vortex-assisted extraction time after the injection of this mixture was evaluated from 0 to 5 min. The extraction efficiency did not change within the tested range which can be explained because of the quick mass transfer of the analytes from the dispersive solution to the extraction solvent to reach the extraction equilibrium. This is due to the large surface of contact between the two characteristic phases of the DLLME technique [5,6]. Nevertheless, a short agitation time of 1 min was selected to ensure the formation of a homogeneous emulsion.

Selection of the temperature of extraction

The effect of the temperature was observed both, in the extraction step and in the collection of the organic droplet. An increase in the temperature of extraction may facilitate the dispersion among solvents because of the higher solubility of the organic solvent in water. Thus, before injecting the extraction mixture into the aqueous phase tube, it was placed during 5 min in a water bath maintained at 30, 35, and 40 °C, but no significant differences were observed. An extraction temperature of 30 °C was selected instead of room temperature to avoid being so close to the melting point of 1-dodecanol which could hinder the emulsion phenomenon between phases [7]. Furthermore, after centrifugation step, the solidification of 1-dodecanol was achieved by cooling in an ice bath. The mixture tube was immediately placed into an ice bath for solidification of the organic phase giving rise to a floating solid organic droplet. It was observed that after 5 min the 1-dodecanol was completely solidified being possible its transference to a vial using a small spatula. An ice bath time lower than 5 min negatively affected droplet collection since some aqueous phase could be collected together with the drop and therefore, impairing the efficiency of the extraction. The collection of the extraction solvent with a syringe without using the ice bath step was discarded as it was difficult to differentiate the inter-phase between the floating organic phase and the aqueous phase, as it had been previously reported [8].

Centrifugation time

The effect of centrifugation time on the extraction performance of the proposed method was evaluated by varying the time from 5 to 15 min at 9000 rpm. An adequate separation was achieved after 5 min leading to a well-formed organic drop floating at the surface of a totally transparent aqueous phase, so it was selected as centrifugation time.

Selection of the salting-out agent

The ionic strength was studied because the addition of salts to the aqueous phase may have different effects on the extraction efficiency (salting out, salting-in, or no effect). The salting-out can decrease the analyte solubility in water and facilitate their transference to the organic phase [9]. Therefore, the influence of ionic strength on the performance of DLLME-SFO was investigated by adding three different types of salts such as sodium sulfate (Na_2SO_4), sodium chloride (NaCl), and ammonium chloride

(NH_4Cl). As a result, as can be seen in **Figure 4a**, a great enhancement in extraction efficiency was achieved in presence of salts, especially with Na_2SO_4, which was selected as salting-out agent. Consequently, the amount of this salt was evaluated testing 0.5, 0.75, and 1 g (**Figure 4b**). A slight decrease in the recovery for most analytes was observed when 1 g was employed, so 0.75 g was chosen as the optimum amount of Na_2SO_4.

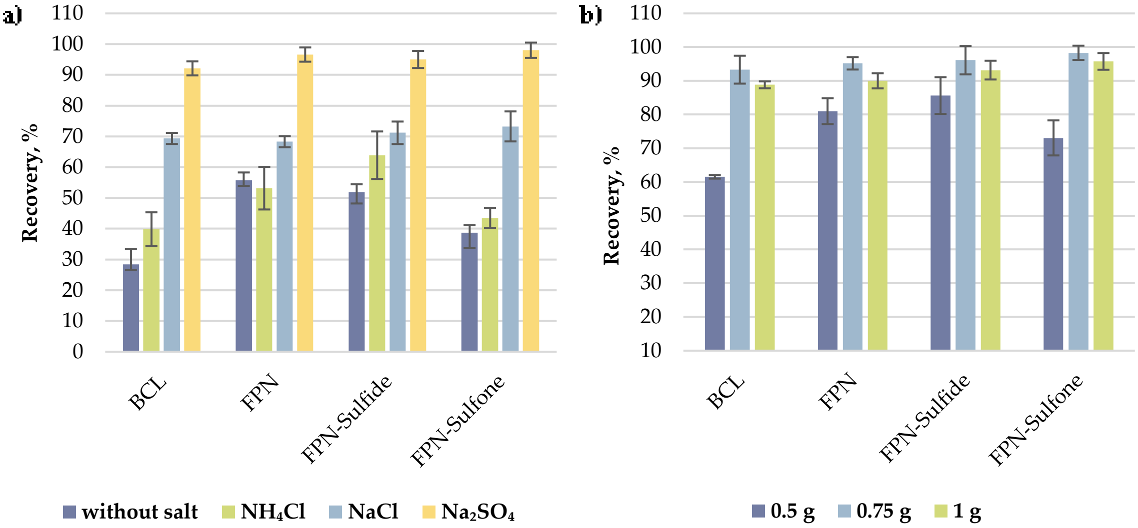

Figure 4. Optimization of the DLLME procedure. Effect of the **a)** addition of a salting-out agent; **b)** amount of Na_2SO_4. Bars represent the standard error (n = 4).

4. METHOD CHARACTERIZATION

The analytical method was characterized in terms of linearity, limits of detection and quantification (LODs and LOQs, respectively), precision (repeatability and intermediate precision) and recoveries.

4.1 Calibration curves and performance characteristics

Under the optimum conditions described above, the analytical performance characteristics of the proposed DLLME-SFO-HPLC-UV method were obtained. Procedural calibration curves were established in environmental water samples and white wine samples fortified with the pesticides under study at the following concentration levels: 5, 10, 25, 75, 100, 125, 150, and 200 μg L^{-1}. Two samples per each concentration level were processed following the developed method and analyzed in duplicate. Peak area was considered as a function of the analyte concentration on the sample. A blank sample was also processed and no matrix interferences were found at any analyte retention time. Statistical parameters, calculated by least-square regression, and performance characteristics obtained are shown in **Table 1.** LODs and LOQs were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to 3 and 10, respectively. LODs and LOQs were lower than 1.3 μg L^{-1} and 4.5 μg L^{-1} respectively, for all analytes in both samples. The results obtained are lower than the previously reported for the determination of fipronil in similar samples using UV-detection (LOQ = 4.7 μg L^{-1}) [10], and are comparable and even lower than those obtained by using MS/MS detection in other kind of matrices (LOQ = 10 μg kg^{-1}) [11,12]. In addition, LOQs are below the maximum residue limits (MRLs) established for fipronil and its metabolites, and for boscalid in wine grapes (5 μg kg^{-1} and 5 mg kg^{-1}, respectively) by the European Commission [13,14], what highlighted that the proposed method allows the determination of these pesticides in accordance with the current legislation.

Table 1. Statistical and performance characteristics of the proposed DLLME-SFO-HPLC-UV method for pesticide residue determination in environmental water and white wine samples.

	Analyte	Linear regression equation	Linear range (μg L^{-1})	R^2	LOD (μg L^{-1})	LOQ (μg L^{-1})
Environmental water	BCL	y=128.86x-198.64	4.4-200	0.9968	1.3	4.4
	FPN	y=219.18x-453.21	4.0-200	0.9964	1.2	4.0
	FPN-sulfide	y=127.03x+269.50	3.4-200	0.9963	1.0	3.4
	FPN-sulfone	y=70.68x+188.30	2.9-200	0.9981	0.9	2.9
White wine	BCL	y=124.12x-87.09	4.5-200	0.9962	1.3	4.5
	FIP	y=212.03+414-42	4.1-200	0.9975	1.2	4.1
	FPN-sulfide	y=125.09x+597.16	3.4-200	0.9985	1.0	3.4
	FPN-sulfone	y=74.24x+35.35	2.7-200	0.9990	0.8	2.7

4.2 Repeatability and intermediate precision assays

Precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by the application of the proposed method to environmental water and white wine samples spiked at three different concentration levels of the linear range (5, 25, and 75 μg L^{-1}). On the one hand, repeatability was evaluated by analyzing two samples fortified at each concentration level, which were injected in triplicate on the same day under the same conditions. On the other hand, intermediate precision was assessed for three consecutive days using a similar procedure but treating one sample each day. The obtained results, expressed as RSD (%) of peak areas, are summarized in **Table 2.** Satisfactory results were obtained; achieving RSDs lower than 6.4% and 11.5% for repeatability and intermediate precision, respectively in environmental water samples, and lower than 9.5% and 12.4% respectively, in white wine samples.

Table 2. Precision of the proposed DLLME-SFO-HPLC-UV method for the studied pesticides in environmental water and white wine samples (level 1: 5 μg L^{-1}, level 2: 25 μg L^{-1}, level 3: 75 μg L^{-1}).

		BCL	FPN	FPN-sulfide	FPN-sulfone
Environmental water				**Repeatability, RSD (%) n=6**	
	Level 1	4.2	5.7	6.4	4.5
	Level 2	3.1	1.8	0.9	3.3
	Level 3	1.9	0.9	1.0	2.5
				Intermediate precision, RSD (%) n=9	
	Level 1	11.5	11.4	7.2	8.4
	Level 2	7.7	2.7	3.7	6.4
	Level 3	3.9	2.2	2.9	2.7
White wine				**Repeatability, RSD (%) n=6**	
	Level 1	9.5	7.4	5.5	8.3
	Level 2	6.4	3.2	3.1	2.7
	Level 3	7.0	6.2	1.1	1.5
				Intermediate precision, RSD (%) n=9	
	Level 1	11.5	11.8	5.5	12.4
	Level 2	8.0	7.7	5.1	5.1
	Level 3	6.7	2.5	3.0	3.0

4.3 Recovery studies

In order to check the extraction efficiency of the proposed method, samples spiked at three different concentration levels of the linear range (5, 25, and 75 μg L^{-1}) were considered. Three samples of each matrix were treated following the DLLME-SFO procedure and finally, they were analyzed in duplicate. Recoveries were estimated by the comparison of the obtained signal for each sample with the signal obtained for a blank sample spiked after the sample treatment and prior its analysis by HPLC-UV. In general, as can be seen in **Table 3,** recoveries above 82% were obtained for all compounds, demonstrating the satisfactory results from the application of the proposed procedure for the extraction of boscalid, fipronil and its metabolites from environmental water and white wine samples.

Table 3. Recovery studies for the proposed DLLME-SFO-HPLC-UV method for the determination of the studied pesticides in environmental water and white wine samples (level 1: 5 μg L^{-1}, level 2: 25 μg L^{-1}, level 3: 75 μg L^{-1}) (n=6).

			BCL	**FPN**	**FPN-sulfide**	**FPN-sulfone**
Environmental water	**Level 1**	**R (%)**	84.2	84.8	88.1	85.6
		RSD(%)	4.9	5.9	3.5	4.1
	Level 2	**R (%)**	83.8	97.6	94.0	86.0
		RSD(%)	3.9	2.3	1.2	1.6
	Level 3	**R (%)**	95.3	94.1	93.8	94.2
		RSD(%)	2.6	2.7	3.8	4.0
White wine	**Level 1**	**R (%)**	95.8	84.2	88.5	82.1
		RSD(%)	8.1	3.7	8.1	8.8
	Level 2	**R (%)**	95.8	102.7	93.9	94.0
		RSD(%)	7.6	3.8	2.8	3.6
	Level 3	**R (%)**	112.1	91.4	92.6	96.3
		RSD(%)	1.2	2.8	1.7	1.8

Chromatograms of both, an environmental water sample and a white wine sample, spiked with each pesticide at 50 µg L^{-1} and their corresponding blanks analyzed by DLLME-SFO-HPLC-UV, are shown in **Figure 5.**

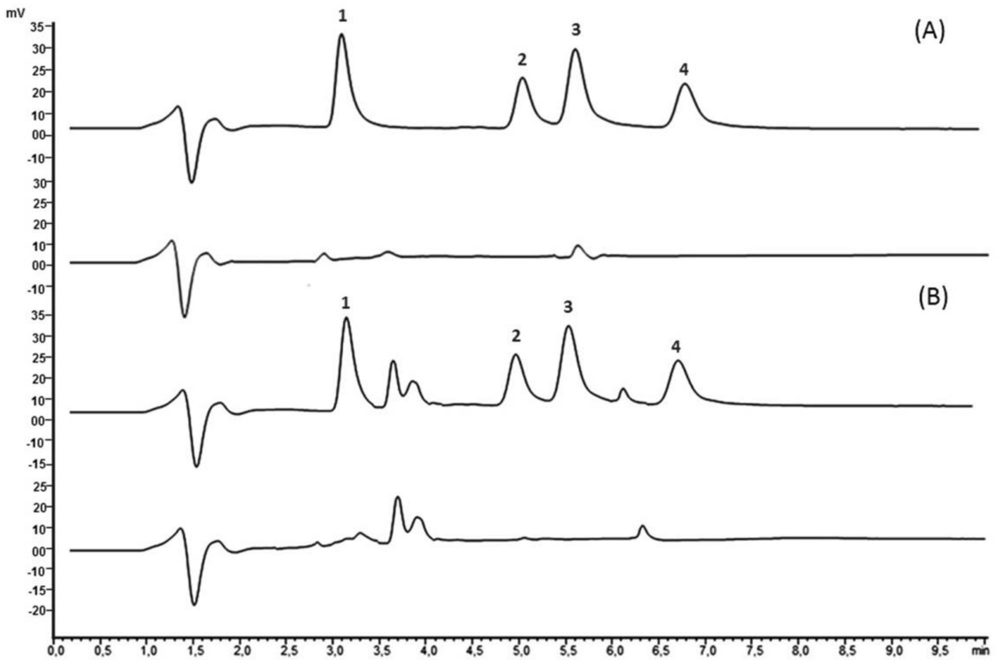

Figure 5. Chromatograms obtained by the proposed DLLME-SFO-HPLC-UV method for (A) an environmental water sample spiked with each pesticide at 50 µg L^{-1} and its corresponding blank; (B) a white wine sample spiked with each pesticide at 50 µg L^{-1} and its corresponding blank. 1) boscalid; 2) fipronil; 3) fipronil-sulfide; 4) fipronil-sulfone.

5. GREENNES OF THE PROPOSED METHODOLOGY

Recently, an analytical greenness calculator called AGREE (Analytical GREEness) has been developed by Pena-Pereira et al [15]. AGREE is a metric approach and software that allows the assessment of the greenness of analytical procedures. This metric is based on the 12 principles of Green Chemistry (significance), which have been transformed into a unified 0-1 scale and presented with a final score in a pictogram. Moreover, it is freely downloadable software, easy to perform and interpret.

To evaluate and compare the proposed DLLME-SFO-HPLC-UV method with other methods reported for the analysis of the target pesticides, especially for fipronil, the AGREE metric was applied. Among the reports found for the analysis of fipronil, only those which employed miniaturized sample treatment techniques such as DLLME were considered. Furthermore, in order to make a fair comparison, only the methods that had been applied to similar samples, such as environmental water samples, were selected. Thus, the works that accomplished these requirements such an IL-DLLME-HPLC-DAD method [16] and a DLLME-SFO-MS/MS method [17], were chosen for their comparison with the proposed method in this study, in terms of analytical greenness. The main characteristics of the three compared methods are shown in **Table 4.**

Table 4. Summary of reported studies for the determination of FPN in water samples using sample treatments based on DLLME.

Extraction method	**IL-DLLME**	**DLLME-SFO**	**DLLME-SFO**
water samples (volume, mL)	5	10	5
Extraction solvent (volume, µL)	$[C_6MIM][PF_6]$ (40)	1-dodecanol (100)	1-dodecanol (100)
Dispersive solvent (volume, µL)	MeOH (500)	MeOH (500)	LGH (2000)
TET/Tª (min /°C)	15/25	15/25	15/30
Separation and detection technique	HPLC-DAD	LC-ESI-MS/MS	HPLC-DAD
Separation time (min)	5	30	7
Reference	Liu, Y. et *al*,. [16]	Cardoso, L. et *al*.,[17]	This chapter

Although the software presents the possibility of assign different weights (from 1 to 4) to the different evaluating criteria, an equal weight of 2 was fixed for all of them, which means that all the principles had the same importance for the final score.

Regarding to the developed sample treatment procedure (principle 1), in all cases off-line analysis were carried out. Moreover, sample size should be considered (principle

2). The sample volume employed was 5 mL for the DLLME-SFO proposed method (A) as well as for the IL-DLLME sample procedure reported by Liu et al. (B) [16], while a higher volume of 10 mL was used in the DLLME-SFO method proposed for Cardoso et al. (C) [17]. In any case it was not possible to measure in situ, so off-line measurements were performed (principle 3). The number of major and distinct steps involved in the sample preparation procedure was fixed as 3 or less for all evaluated methods since the techniques used have the same main steps (principle 4). In the same way, all methods were semi-automated and miniaturized (principle 5) and no derivatization agents were necessary for the analysis (principle 6). The amount of waste generated through the whole method in terms of solvent extraction volume and mobile phase volume was lower in the proposed DLLME-SFO-HPLC-UV (principle 7). It was mainly due to the use of a fast and isocratic separation employing a small flow rate of 0.35 mL min $^{-1}$ if compared with the gradient programs and flow rates used in the other works being also more time-consuming. Multi-analyte methods are preferred and the number of analytes determined in one hour is based on the number of compounds analyzed in a single run and the sample throughput (principle 8). In this sense, all the methods were multi-analyte; the DLLME-SFO-LC-MS/MS method analyzed 26 analytes in 30 min, the IL-DLLME-HPLC-DAD method, 4 analytes in 13 min, and our DLLME-SFO-HPLC-UV method 4 analytes in 7 min. Despite the fact of having far fewer target analytes, due to the short time of analysis of our method, this reached a similar score in this principle to the method proposed by Cardoso et al. [17]. The quantification of the energy employed was done based on the selection of the most energy-intensive technique used during the procedure (principle 9). In all cases this corresponds to the instrument employed for the separation and quantification of the target compounds, being, therefore, the LC instrument coupled to mass spectrometry detection the most energy-demanding analytical technique. Reagents obtained from renewable sources should be preferred (principle 10). The proposed method is the only one that employs a reagent derived from bio-based sources since the traditional dispersive organic solvent was replaced by a NADES (LGH) being fully in compliance with this principle. Similarly, toxic reagents should be eliminated or replaced (principle 11). In all procedures, toxic reagents were employed, so it was estimated the amount of them in each case. As it was mentioned before, because of the use of NADES and the short time of analysis, the amount of toxic reagents needed in the proposed method were quite low (2.55 mL) if compared with the amount used in the DLLME-SFO-LC-MS/MS (11.6 mL) and the IL-DLLME-HPLC-DAD (18.54 mL) for a single run. Finally, the safety of the operator and environmental hazards should be taken into account. The number of threats that are not avoided is considered (principle 12). MeOH used in all procedures as mobile phase (solvent B) in the chromatographic separation is considered highly flammable and corrosive.

The pictograms obtained by using AGREE software to evaluate them are represented in **Figure 6**. Analyzing the results, the AGREE report ranged from 0.49 to 0.64 being the proposed DLLME-SFO-HPLC-UV method the greener one since it achieved the highest score. The replacement of toxic solvents by NADES at the sample treatment or its minimization during the chromatographic separation leads to an eco-friendlier analytical procedure. Therefore, not only the greenness of the proposed method using NADES as a dispersive solvent is demonstrated, but also the reduction of waste production and consumption of energy, decreasing the impact on the environment.

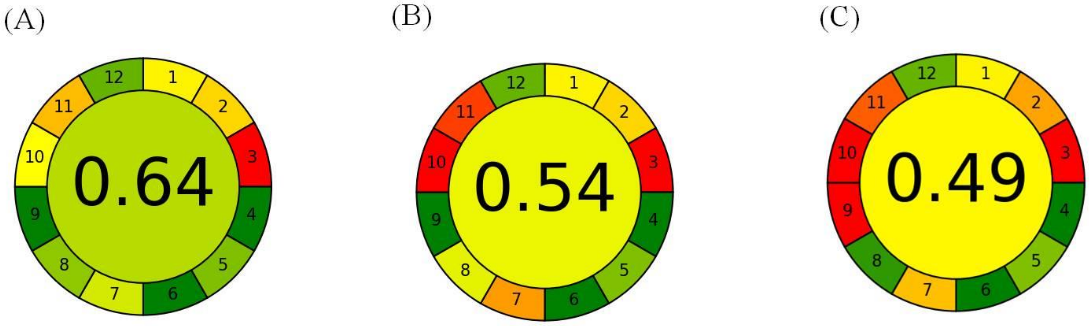

Figure 6. Comparative AGREE analysis for (A) DLLME-SFO-HPLC-UV using NADES; (B) IL-DLLME-HPLC-UV [16]; (C) DLLME-SFO-MS/MS [17].

6. CONCLUSIONS

A new DLLME-SFO method using a NADES namely LGH as an innovative dispersive solvent has been developed and applied for the rapid and efficient extraction of boscalid, fipronil, fipronil-sulfide, and fipronil-sulfone from environmental water and white wine samples followed by their determination by HPLC-UV in less than 7 minutes. To the best of our knowledge this is the first time that a NADES has been employed as a dispersive solvent in a DLLME-SFO procedure. The analytical method was satisfactorily characterized in both samples. In spite of using UV-detection, high sensitivity and selectivity were achieved, with LODs and LOQs in compliance with the current European legislation. Moreover, precision (below 12.4%) and recoveries (above 82%) were obtained. On the other hand, from the point of view of Green Chemistry, the use of NADES as a dispersive solvent, instead of the organic solvents traditionally used, provided a more sustainable alternative. The greenness of the proposed DLLME-SFO-HPLC-UV method was demonstrated by its evaluation through the recent Analytical GREEnness metric approach and its comparison with other methods previously reported for the same matrices. To sum up, the proposed method is a powerful, fast, eco-friendly, and sensitive alternative which highlighted the potential of using NADES in sample preparation.

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CHAPTER 4

DETERMINATION OF NEONICOTINOIDS IN ENVIRONMENTAL SAMPLES BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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1. SUMMARY

A simple, sensitive and efficient method has been developed for the determination of seven neonicotinoid insecticides (NNIs) commercially available (imidacloprid, thiacloprid, clothianidin, thiamethoxam, acetamiprid, nitenpyram and dinotefuran) and the main metabolite 6-chloronicotinic acid. Micellar electrokinetic chromatography (MEKC) mode was applied, using a 48.5 cm of total length capillary (50 µm i.d.) with extended light-path (150 µm). The running electrolyte consisted of 25 mM sodium tetraborate buffer (pH 9.2) containing 120 mM of sodium dodecyl sulfate and 15 % of methanol (v/v). A voltage of 27 kV and a temperature of 25 °C were applied. Samples dissolved in ultrapure water were hydrodynamically injected at 50 mbar for 12 s, achieving the analysis in less than 12 min. Diode array detection (DAD) was performed at 220, 254 and 270 nm, depending on the analyte. Two different methodologies as sample treatments were developed; for water samples, solid phase extraction (SPE) was checked using different cartridges (C18, Oasis® HLB, Oasis® HLB Prime and Strata-X), being the best option Oasis® HLB for preconcentration and clean-up. In the case of soil samples, a simple solid-liquid extraction (SLE) was applied using a mixture of 1:3 (v/v) acetonitrile/dichloromethane. Satisfactory linearity, trueness, and precision were achieved, with detection limits in the range of 0.1-0.4 µg L^{-1} for river water and 1.0-2.9 µg kg^{-1} for soil samples. Recoveries ranging 80-107 % for all of the assayed NNIs in water samples of different origin and 73–92 % for soil samples were achieved.

2. EXPERIMENTAL

2.1 Materials and reagents

All reagents used through this work were analytical reagent grade and solvents were HPLC grade, unless otherwise specified. NaOH, HCl and MeCN were obtained from Panreac-Química (Madrid, Spain). MeOH was purchased from VWR International (West Chester, PA, USA) while dichloromethane and sodium dodecyl sulfate (SDS) were supplied by Sigma-Aldrich (St.Louis, MO, USA). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Analytical standards of dinotefuran (DNT), thiamethoxam (TMT), clothianidin (CLT), nitenpyram (NTP), imidacloprid (IMD), thiacloprid (TCP), acetamiprid (ACT) and 6-chloronicotinic acid (6-CNA) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Individual standard solutions were obtained by dissolving the appropriate amount of each neonicotinoid in MeOH, reaching a final concentration of 500 μg mL^{-1}. They were kept in the freezer at -20 °C avoiding exposure to light. Intermediate stock standard solutions containing 50 μg mL^{-1} of each compound were obtained by mixing the appropriate amount of each individual standard solution, followed by drying and subsequent dilution with ultrapure water. Working standard solutions were freshly prepared by dilution of the intermediate stock standard solution with ultrapure water at the required concentration. Both intermediate and working solutions were stored at 4 °C avoiding exposure to direct light.

Oasis® HLB cartridges (60 mg, 3 mL, 30 μm of particle size) were supplied by Waters (Mildford, MA, USA). Glass microfiber filters with polypropylene housing of 13 mm of diameter and 0.7 μm pore size were supplied by Whatman™ – GE Healthcare Life Sciences (Buckinghamshire, UK).

2.2 Instrumentation

CE experiments were carried out with an Agilent 7100 CE System (Agilent Technologies, Waldbronn, Germany) equipped with a DAD. Data were collected using the software supplied with the HP ChemStation (Version B.02.01). Separations were performed in fused silica capillaries. Method optimization was accomplished in a standard capillary (48.5 cm of total length x 50 μm i.d., 40 cm of effective length) whereas water and soil sample analyses were carried out in an extended light-path capillary, which presented 150 μm of optical path length. Solid phase extraction (SPE) was carried out on a Visiprep™ DL vacuum manifold for 12 cartridges from Supelco (Bellefonte, PA, USA). A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH, Bielefeld, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) and a multi-tube vortexer BenchMixer™ XL (Sigma-Aldrich, St. Louis, MO, USA) were also used. A pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ± 0.01 pH unit was used for the pH adjustment.

2.3 Capillary electrophoresis separation

New capillaries were conditioned with 0.1 M NaOH solution for 15 min, followed by ultrapure water for 10 min and then with the running buffer for 20 min at 1 bar and 25 °C. This procedure was also used at the beginning of each day. In order to obtain an adequate repeatability between runs, capillary was subsequently rinsed with the background electrolyte (BGE) for 2 min at 1 bar and 25 °C. At the end of the working day, capillary was cleaned with ultrapure water for 2 min, followed by MeOH for 1 min and afterwards, it was dried with air for 1 min at 1 bar and 25 °C. Separation was performed in MEKC mode using a BGE that consisted of 25 mM sodium tetraborate buffer (pH 9.2) containing 120 mM of SDS and 15 % of MeOH (v/v) as organic modifier. A voltage of 27 kV and a temperature of 25 °C were applied for NNI separation. Standard solutions and samples dissolved in ultrapure water were hydrodynamically injected at 50 mbar for 12 s. UV-detection was performed at 220, 254 and 270 nm depending on the analyte with a bandwidth of 20 nm. Teflon (PTFE) syringe filters (0.2 μm x 13 mm) (VWR, Radnor, PA, USA) and fiberglass syringe filters (0.72 μm x 13 mm) were used for filtration of buffer solutions and sample extracts, respectively, prior to the injection into the CE system.

2.4 Sample treatment procedures

2.4.1 Solid phase extraction for water samples

Environmental water samples from different sources (river, spring, and well) were considered in this study. River water samples were collected from Castril River (Fuente Vera, Granada, Spain), spring water samples were obtained from a natural source located close to the previously mentioned river. In addition, water samples from a well, used in irrigation activities (Almontaras, Granada, Spain) were also analyzed. In all cases, the sampling points were located near an agricultural area. All samples were collected in 1 L-amber glass bottles and kept at 4°C for their daily use.

A sample treatment was developed for analyte extraction and preconcentration using Oasis® HLB cartridges. An aliquot of each type of water sample (50 mL) was placed in a beaker and fortified at the desired NNIs concentration level. Then the sample was acidified to pH 3 with 1M HCl. Afterwards, the sample was loaded onto an Oasis® HLB (60 mg, 3 mL) cartridge which was previously conditioned with 1 mL of MeOH and equilibrated with 2 mL of ultrapure water at pH 3. The sample was passed through the cartridge at 3 mL min^{-1} applying vacuum. After sample loading, cartridge was washed with 2 mL of a mixture 95:5 (v/v) water/MeOH. Cartridge was vacuum dried prior to sample elution. Finally, the analytes were eluted with 1 mL of MeOH. The obtained extract was collected in a new 4 mL-vial and dried under a gentle stream of nitrogen at 35 °C. Finally, it was reconstituted in 200 µL of ultrapure water by vortex agitation for 1 min and filtered through a 0.7 µm fiber glass filter. Consequently, an off-line analyte preconcentration of 250-fold was achieved by the application of the optimized SPE procedure. Finally, the resulting solution was analyzed by the developed MEKC-UV procedure. The optimized sample procedure for the analysis of environmental water samples is shown in **Figure 1**.

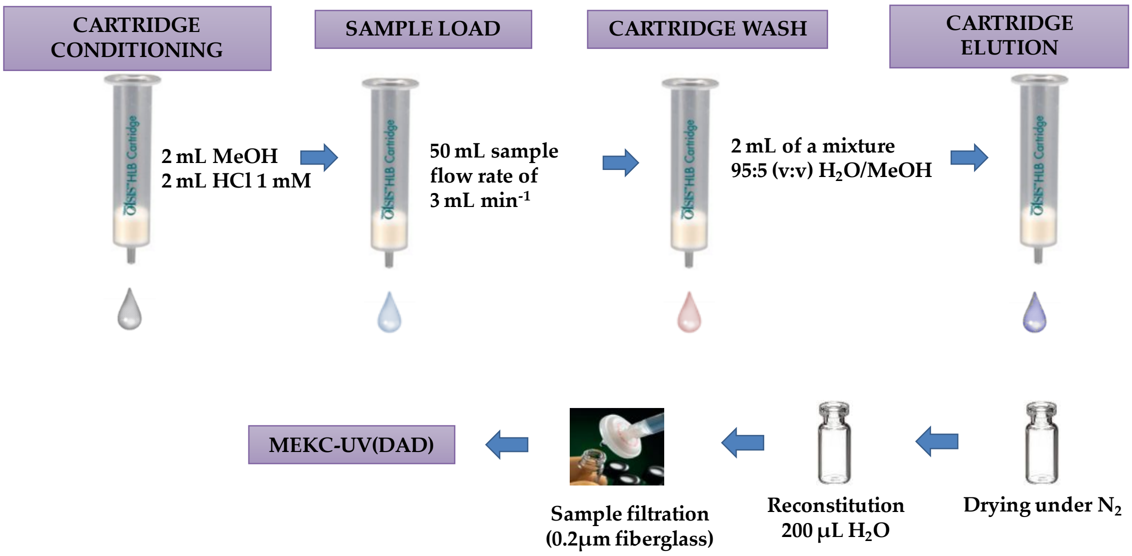


Figure 1. SPE procedure for the analysis of NNIs in environmental waters.

2.4.2 Solid liquid extraction for soil samples

Soil samples were obtained from an area with high agricultural activity in Fuente Vera (Granada, Spain). The collected soil samples were cleaned of leaves, stones, roots and others exogenous objects, and sieved through a 0.2 mm mesh. For each sample, a portion of 5 g sieved soil was placed in a 50 mL centrifuge tube and fortified at the desired concentration levels of NNIs. Then, the soil was mixed with 12 mL of a mixture 1:3 (v/v) MeCN/dichloromethane. The tube was vortexed for 4 min and centrifuged at 5000 rpm for 10 min. The whole supernatant was taken into a 12 mL glass vial and evaporated to dryness under a gentle stream of nitrogen at 35 °C. Finally, the residue was reconstituted in 200 μL of ultrapure water by vortex agitation for 1 min and filtered through a 0.7 μm fiber glass filter. The resulting solution was analyzed by the developed MEKC-UV procedure. Consequently, a 25-fold preconcentration factor was achieved by the application of the optimized procedure. The optimized SLE procedure for the extraction of NNIs from soil samples is shown in **Figure 2.**

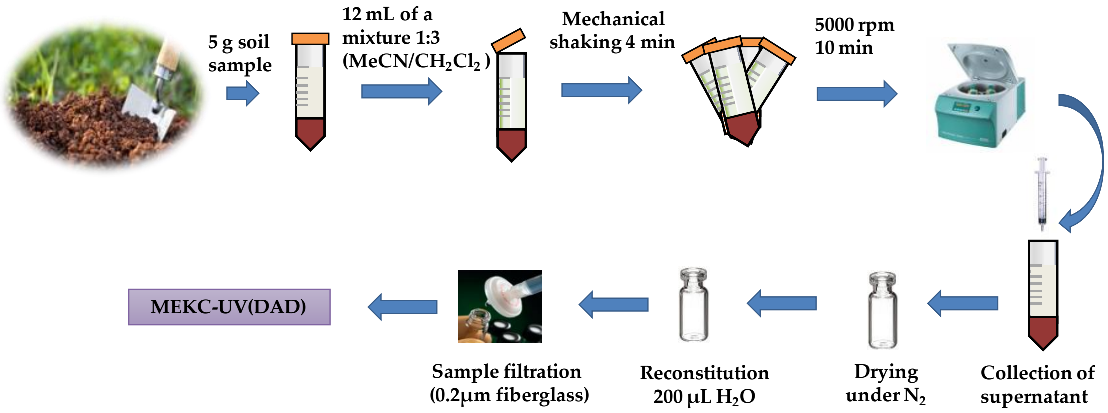


Figure 2. SLE procedure for the analysis of NNIs in soil samples

3. RESULTS AND DISCUSSION

3.1 Optimization of electrophoretic separation

In this study, MEKC was applied for the simultaneous separation and identification of seven NNIs (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam) and the metabolite 6-CNA. UV detection was used, selecting the wavelengths of maximum absorption: 220 nm for nitenpyram and 6-CNA, 254 nm for thiamethoxam, acetamiprid and thiacloprid, and 270 nm for dinotefuran, imidacloprid and clothianidin. In a previous paper, Sánchez-Hernández *et al.* have carefully studied different pH values, nature and concentration of BGE, capillary length and voltage in order to check the separation of the seven NNIs by CZE considering their pK_a values [1]. At the best conditions, acetamiprid, thiacloprid, thiamethoxam and imidacloprid migrated at the same time, and dinotefuran was separated from these four NNIs, although full separation was not achieved. Baseline separation was obtained only for nitenpyram and clothianidin. At that case, it was possible to use CZE mode in spite of its limited resolution because of the coupling with MS but the applicability of the method in beeswax samples was verified only for clothianidin, imidacloprid and thiamethoxam. For this reason, and with the aim to achieve satisfactory resolution of the eight studied compounds, MEKC has been proposed as separation mode in this work, coupled with UV-detection, using SDS in the BGE.

The optimization of the main variables affecting the separation and simultaneous quantification of the NNIs was carried out, in order to maximize the resolution and sensitivity (peak area). The generated current was kept lower than 100 μA in each experiment to minimize the Joule effect. First of all, the influence of the SDS surfactant concentration in the BGE (20 mM sodium tetraborate, pH 9.2) was investigated between 60 mM and 120 mM. In this range, resolution was satisfactory for all compounds, except for thiamethoxam and nitenpyram, for which resolution improved at 120 mM, so it was selected as optimum value. Higher concentrations were not tested in order not to exceed the maximum current previously indicated. Then, sodium tetraborate buffer concentration was evaluated between 15 and 25 mM (pH 9.2) keeping the concentration of SDS at 120 mM. It was observed that, with a concentration of 25 mM, resolution for thiamethoxam and nitenpyram was slightly improved.

Then, the addition of an organic modifier to the BGE was also considered. Methanol addition was studied in the range of 10 to 20%, producing an increase in the analysis time with the increase of percentage, from 10 to 15 min, but also a significant improvement in resolution, especially between the peaks of thiamethoxam and dinotefuran (resolution changed from 1.7 to 2.2). Moreover, a decrease in the electrophoretic current (from 95 μA to 76 μA) was observed, so 15% MeOH was selected as optimum, as it provided the best compromise in terms of resolution, electrophoretic current, and analysis time. Finally, using the optimized buffer, the pH was studied in the range from 8 to 10. No significant changes in the resolution were observed, so a pH 9.2 was maintained.

Once the running buffer had been optimized, capillary temperature during the separation was also studied in the range of 20-30 °C. An increase in the temperature resulted in a decrease in the migration times (due to lower electrolyte viscosity) and also in the resolution, as well as an increase in the generated current. According to these effects, 25 °C was selected as the most appropriate temperature. Then, the separation voltage was modified between 20 and 30 kV. A voltage of 27 kV was selected as optimal, as a compromise between running time, resolution, and electrophoretic current.

The previous studies were carried out using a 48.5 cm of total length capillary (50 μm i.d., 40 cm of effective length). A longer one was tested (64.5 cm of total length x 50 μm i.d., 56 cm of effective length) but although the electrophoretic current decreased slightly, the analysis time increased in 10 min, so finally it was discarded.

In order to check if some kind of stacking preconcentration was possible, sample solvent and sample injection volume were studied. In the case of sample solvent, ultrapure water and BGE were checked. The best results in terms of peak efficiency and sensitivity were obtained using ultrapure water, so it was set as sample solvent. Furthermore, the hydrodynamic injection time was studied from 10 to 20 s. An increase in the time resulted in an increase of the sensitivity but also in a decrease in resolution and peak efficiency. In accordance with this, 12 s at 50 mbar were used as optimum values, approximately 2% of the total volume of the capillary.

Once all the experimental conditions were optimized, NNIs separation was carried out in an extended light-path capillary (48.5 cm of total length x 50 μm i.d., 150 μm of optical path length) in order to increase sensitivity. As a result, signal intensity (peak area) was increased approximately three-fold.

3.2 Optimization of sample treatment

3.2.1 Optimization of SPE for the analysis of natural waters

In order to determine the selected compounds in environmental water samples by the optimized MEKC-UV method, a SPE method was proposed as a sample treatment, considering its operational flexibility and simplicity for preconcentrating and cleaning-up water samples. Several cartridges such as Supelco C18 (6 mL, 500 mg), Oasis® HLB (3 mL, 60 mg) and Strata-X (6 mL, 200 mg) were checked using a blank water sample volume of 2 mL, free of analytes, which was spiked with the studied analytes at a concentration of 1 μg mL^{-1}. Manufactures' recommendations were initially followed. C18 cartridges were preconditioned with 6 mL of MeOH, followed by an equilibration with 6 mL of ultrapure water and the elution was carried out with 2 mL of MeOH.

Oasis® HLB cartridges were preconditioned with 2 mL of MeOH and equilibrated with 2 mL of ultrapure water and the elution step was carried out with 2 mL of MeOH, and finally Strata-X cartridges were preconditioned with 4 mL of MeOH and equilibrated with 4 mL of ultrapure water and the elution step was carried out with 4 mL of a mixture MeOH/MeCN (50:50) (v/v). In order to ensure that 6-CNA was in its neutral form (pK_a 3.24) and to improve its recovery, the sample was previously acidified using 1M HCl (pH=3). The recovery increases considerably for 6-CNA without affecting the other analytes. The comparison of analyte recoveries for the different studied SPE cartridges is shown in **Figure 3a**. The best results were obtained when Oasis® HLB was used, achieving recoveries around 80 % for all the studied compounds except for 6-CNA, which was around 60 %.

Subsequently, different volumes of MeOH (1, 2, 3 and 4 mL) as elution solvent were tested. There were no significant differences on analyte recoveries, so 1 mL was enough to elute them from the sorbent. Then, the washing step was evaluated, checking ultrapure water with MeOH and MeCN in different percentages. Even with a low amount of MeCN, some analytes, such as dinotefuran, nitenpyram and 6-CNA, were lost at this stage, so it was discarded. Above 10% MeOH, recoveries decreased for all of them, so 5% MeOH was chosen in order to assure the adequate retention of the compounds (mainly for 6-CNA and thiacloprid) (**Figure 3b**).

Moreover, different Oasis® HLB cartridges were assayed (60 mg, 3 mL; 200 mg, 6 mL; 500 mg, 6 mL) using a volume of water sample of 50 mL in the preconcentration step. Cartridges containing 60 mg, which had been used until now, provided satisfactory recoveries for most of the analytes, so they were selected. Also, Oasis® HLB PRiME cartridges (3 mL, 60 mg), which do not need the preconditioning steps, were tested using the same water sample volume, however, better results were obtained with Oasis® HLB, providing the highest retention efficiency for all the studied NNIs, and especially for dinotefuran.

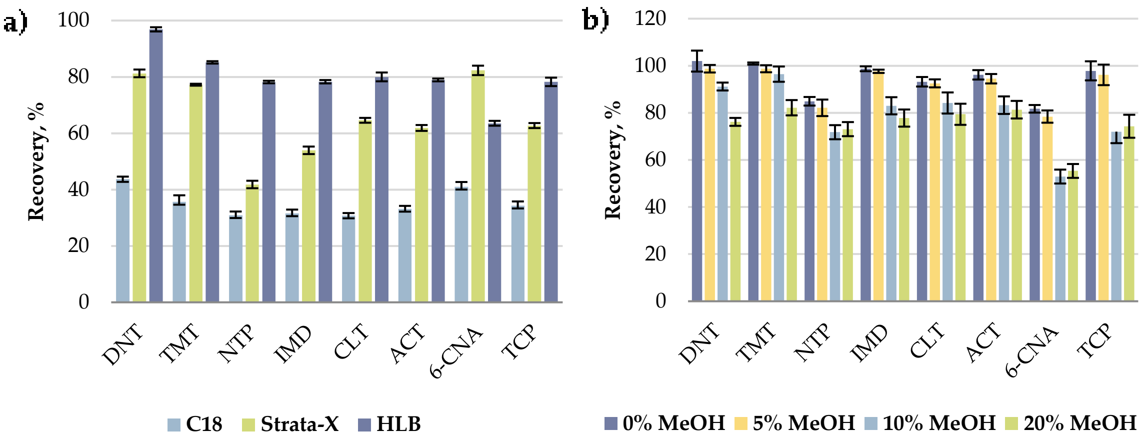


Figure 3. Optimization of SPE for water samples. **a)** Effect of different sorbents on the extraction recovery of the studied compounds. **b)** Effect of washing solvent on the extraction recovery. Error bars represent the standard error (n=4).

3.2.2 Optimization of SLE for the analysis of soil samples

A simple solid-liquid extraction was optimized for this NNIs determination by MEKC-UV in environmental soil samples, based on previous works in LC, in which a mixture of MeCN and dichloromethane was used without any clean-up step [2] or followed by a clean-up with primary secondary amine (PSA) [3]. This sample treatment was initially proposed for the determination of the seven NNIs and the 6-CNA metabolite, however it was not possible to recover 6-CNA. First of all, using an amount of 5 g of soil sample, we checked the extraction solvent, so dichloromethane and mixtures of MeCN with dichloromethane were evaluated in different proportions (1:1, 1:2, 1:3 (v/v); MeCN /dichloromethane) with a final volume of 7.5 mL. As shown in **Figure 4a**, the addition of dichloromethane was crucial for the recovery of nitenpyram, so a mixture MeCN /dichloromethane (1:3, v/v) was chosen. Once the composition of the extraction solvent was selected, different volumes of this mixture (7.5, 12 and 15 mL) were evaluated. Using 12 mL, a recovery up to 70% was reached in all cases whereas higher volumes did not provide any improvement. In order to evaluate extraction time, different values, from 1 to 8 min, were evaluated using mechanical agitation. The results showed that the extraction efficiency increased with the extraction time up to 4 min without any significant effect for longer extraction times (**Figure 4b**). Therefore, 4 min was selected. In this case, sample clean-up was not necessary since no interferences were co-eluting with the target compounds.

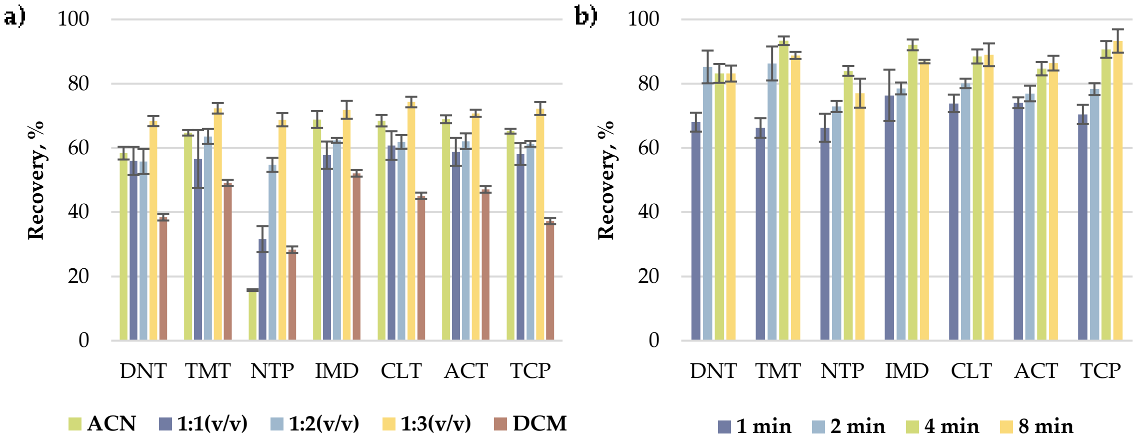


Figure 4. Optimization of SLE for soil samples**. a)** Effect of extraction solvent type on the extraction recovery of the studied compounds. (v/v); MeCN/dichlorometane (DCM). **b)** Effect of the extraction time on the extraction recovery. Error bars represent the standard error (n=4).

4. METHOD CHARACTERIZATION

4.1 Calibration curves and performance characteristics

Slopes from external standard calibration curves and procedural calibration curves were firstly compared for each analyte in order to check systematic errors due to the nature of the matrix and losses in the extraction procedure. Statistically significant differences were found for all the analytes, so in order to correct systematic errors, procedural calibration curves in presence of matrix were established in the three different environmental water samples selected (spring, river and well water). Volumes of 50 mL of each sample were fortified at different NNI concentration levels (1, 2.5, 5, 10, 20 and 40 μg L^{-1}). Two samples per each concentration level were processed following the developed SPE method and analyzed in duplicate. Peak area was considered as a function of the analyte concentration on the sample.

Statistical parameters, calculated by least-square regression, and performance characteristics of the proposed SPE-MEKC-UV method for river, spring and well water samples are shown in **Table 1**. A blank sample in each case was also processed, and no matrix interferences were detected at the migration times of the studied compounds. LODs and LOQs were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three (3 S/N) and ten (10 S/N), respectively.

Table 1. Statistical and performance characteristics of the proposed SPE-MEKC-UV methods for NNI determination in river, spring, and well water samples, respectively.

	Analyte	**Linear range (μg L^{-1})**	**Intercept**	**Slope**	**LOD (μg L^{-1})**	**LOQ (μg L^{-1})**	**R^2**
River water	**DNT**	0.6-40	-0.6808	2.9917	0.2	0.6	0.9960
	TMT	0.6-40	-0.5951	3.0352	0.2	0.6	0.9929
	NTP	1.4-40	-0.9435	3.6610	0.4	1.4	0.9966
	IMD	0.4-40	-2.1726	7.9873	0.1	0.4	0.9960
	CLT	0.6-40	-1.9332	6.3476	0.2	0.6	0.9958
	ACT	0.3-40	-2.5514	9.3482	0.1	0.3	0.9958
	6-CNA	0.1-40	-1.6074	7.1654	0.3	0.1	0.9951
	TCP	0.4-40	-2.4750	8.6264	0.1	0.4	0.9945
Spring water	**DNT**	0.6-40	0.1806	2.8467	0.2	0.6	0.9945
	TMT	1.0-40	0.6783	2.8767	0.3	1.0	0.9907
	NTP	9.1-40	-0.3218	3.3018	2.7	9.1	0.9905
	IMD	0.3-40	-2.7413	7.0449	0.1	0.3	0.9892
	CLT	0.4-40	-1.4273	5.5855	0.1	0.4	0.9889
	ACT	4.5-40	-2.8849	7.8639	1.3	4.5	0.9889
	6-CNA	4.4-40	-0.3808	6.8853	1.3	4.4	0.9934
	TCP	0.7-40	-0.7370	5.4049	0.2	0.7	0.9854
Well water	**DNT**	0.9-40	-0.1806	2.8467	0.3	0.9	0.9962
	TMT	1.4-40	-0.6783	2.8767	0.4	1.4	0.9939
	NTP	1.3-40	-0.3218	3.3018	0.4	1.3	0.9959
	IMD	0.6-40	-2.7413	7.0449	0.2	0.6	0.9913
	CLT	0.7-40	-1.4273	5.5855	0.2	0.7	0.9947
	ACT	0.8-40	-2.8849	7.8639	0.2	0.8	0.9924
	6-CNA	1.1-40	-0.3808	6.8853	0.3	1.1	0.9960
	TCP	1.0-40	-0.7370	5.4049	0.3	1.0	0.9960

Furthermore, in the same way, procedural calibration curves in presence of matrix were established by the optimized SLE-MEKC-UV method, using soil samples from farmland. A previous comparison between the slopes from external standard calibration curves and procedural calibration curves were carried out and statistically significant differences were found for all the analytes. Portions of 5 g of soil samples were fortified at different concentration levels (10, 25, 50, 100, 2000 and 400 μg kg^{-1}). Two samples per each concentration level were processed following the developed SLE method and analyzed in duplicate. Peak area was considered as a function of the analyte concentration on the sample. A blank sample was also processed, and no matrix interferences were comigrating at the NNIs migration times. Statistical parameters and performance characteristics of the proposed SLE-MEKC-UV method for soil are shown in **Table 2**.

Table 2. Statistical and performance characteristics of the proposed SLE-MEKC-UV method for NNI determination in soil samples.

Analyte	Linear range (μg kg^{-1})	Intercept	Slope	LOD (μg kg^{-1})	LOQ (μg kg^{1})	R^2
DNT	4.4-400	0.3632	1.8994	1.3	4.4	0.9945
TMT	9.1-400	0.5582	1.8157	2.7	9.1	0.9921
NTP	9.5-400	0.5744	2.3417	2.9	9.5	0.9940
IMD	3.2-400	1.2598	4.125	1.0	3.2	0.9941
CLT	3.2-400	0.9054	4.1947	1.0	3.2	0.9958
ACT	3.5-400	1.0733	5.9787	1.0	3.5	0.9963
TCP	4.8-400	1.5597	6.0477	1.4	4.8	0.9973

4.2 Repeatability and intermediate precision assays

Precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by the application of the proposed SPE-MEKC-UV method to three different environmental water samples (spring, river and well water) and by the application of the SLE-MEKC-UV method to soil samples, both spiked at two different concentration levels in the linear range (2.5 and 20 μg L^{-1}) for water samples and (25 and 200 μg kg^{-1}) for soil samples. Repeatability was evaluated over two samples prepared and injected by duplicate on the same day, under the same conditions. Intermediate precision was evaluated with a similar procedure, but the samples were analyzed in three consecutive days. The obtained results, expressed as RSD (%) of peak areas for river, spring and well water, and for soil samples are summarized in **Table 3.** Satisfactory results were obtained in terms of precision, achieving RSDs lower than 12% in water samples and below 9% in soil samples.

Table 3. Precision of the SPE-MEKC-UV method for spiked water samples (level 1: 2.5 μg L^{-1}, level 2: 20 μg L^{-1}) and SLE-MEKC-UV method for spiked soil samples (level 1: 25 μg kg^{-1}, level 2: 200 μg kg^{-1}).

		DNT	TMT	NTP	IMD	CLT	ACT	6-CNA	TCP
Repeatability RSD (%) n=4									
River water	**Level 1**	6.2	9.1	2.1	7.8	4.3	3.9	9.5	6.3
	Level 2	1.8	4.9	2.6	2.0	2.1	3.4	4.4	4.3
Spring water	**Level 1**	4.4	6.7	3.0	4.2	3.4	6.3	8.2	5.2
	Level 2	3.3	5.7	6.6	3.5	3.7	3.5	1.6	2.2
Well water	**Level 1**	3.5	8.9	9.4	3.6	8.0	11.7	7.9	8.5
	Level 2	2.7	1.7	4.1	0.8	3.6	2.0	2.3	6.4
Soil	**Level 1**	6.1	4.4	8.7	5.8	2.5	5	-	3.4
	Level 2	2.5	0.8	2.7	3.1	4.4	4.5	-	4.3
Intermediate precision RSD (%) n=12									
River water	**Level 1**	11.1	10.7	9.3	12.7	9.3	12.6	9.3	9.4
	Level 2	0.9	0.8	1.1	0.8	0.8	1.4	3.0	2.5
Spring water	**Level 1**	8.2	8.2	6.7	7.3	6.4	7.8	9.2	5.5
	Level 2	0.8	1.3	2.1	3.7	3.3	4.7	4.0	2.7
Well water	**Level 1**	7.1	12.0	8.3	12.9	13.2	14.0	7.9	9.7
	Level 2	0.8	1.0	0.6	4.8	4.1	4.0	2.5	3.3
Soil	**Level 1**	5.7	9.3	8.5	6.9	6.1	6.7	-	3.2
	Level 2	1.1	0.8	1.2	2.9	2.3	2.5	-	2.1

4.3 Recovery studies

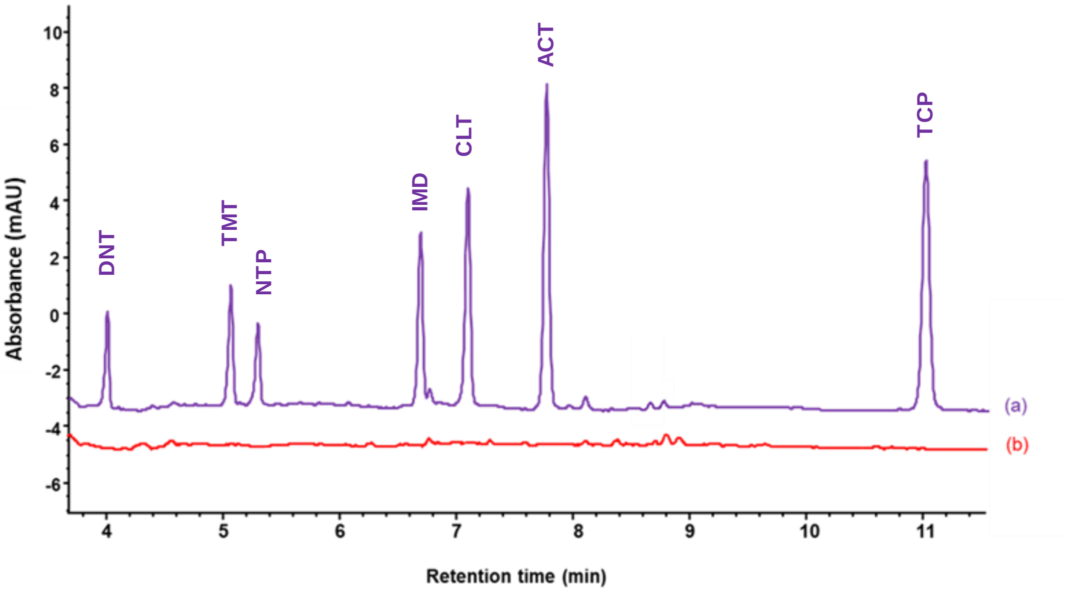
Recovery experiments were carried out in three different environmental water samples (spring, river and well water) spiked at two different concentration levels (2.5 and 20 μg L^{-1}). Three samples of each kind of water were fortified at each concentration level, treated and analyzed following the proposed SPE-MEKC-UV method. The data, in terms of peak area, were compared with those obtained by analyzing extracts of blank samples submitted to the sample treatment and spiked with NNIs and 6-CNA just before the measurement. In the same way, three samples of soil were fortified at two different concentrations levels (25 and 200 μg kg^{-1}), treated and analyzed following the proposed SLE-MEKC-UV method. A blank of each type of water and a blank of soil were processed to check the absence of NNIs and 6-CNA and none of them gave signals corresponding to concentrations higher than the LODs. In general, recoveries over 80% were achieved for environmental water samples and over 70 % for the soil samples, as it can be seen in **Table 4.**

Table 4. Recovery studies for the proposed SPE-MEKC-UV method in water samples (level 1: 2.5 µg L^{-1}, level 2: 20 µg L^{-1}) and for SLE-MEKC-UV method in soil samples (level 1: 25 µg kg^{-1}, level 2: 200 µg kg^{-1}) (n=6).

			DNT	TMT	NTP	IMD	CLT	ACT	6-CNA	TCP
River water	**Level 1**	**R %**	100.3	87.0	85.8	91.7	99.0	82.3	97.7	84.3
		RSD	6.7	13.2	12.6	17.0	11.7	13.6	12.4	17.5
	Level 2	**R %**	100.1	90.6	87.6	82.7	87.7	82.0	88.0	81.7
		RSD	5.4	5.9	2.4	7.1	4.7	3.7	2.6	7.2
Spring water	**Level 1**	**R %**	83.8	85.2	80.6	80.1	81.1	82.9	85.5	86.4
		RSD	7.9	15.9	9.0	12.4	8.8	10.4	10.4	20.3
	Level 2	**R %**	99.2	92.4	98.2	91.4	95.9	88.9	106.0	86.9
		RSD	9.3	10.7	5.2	17.9	18.8	13.5	6.3	23.3
Well water	**Level 1**	**R %**	106.9	100.5	103.3	99.4	96.3	97.5	95.0	96.2
		RSD	9.8	9.3	9.6	9.6	7.1	8.3	9.7	9.3
	Level 2	**R %**	89.9	85.2	87.8	79.6	82.5	84.0	89.5	85.3
		RSD	6.9	9.0	6.3	8.5	6.9	8.9	4.5	13.0
Soil	**Level 1**	**R %**	82.2	80	73.2	83.4	77.1	78.1	-	78.3
		RSD	8.0	17.0	16.2	7.7	8.0	9.8	-	10.3
	Level 2	**R %**	91.5	90.1	75.7	78.5	80.3	85.8	-	79.0
		RSD	8.8	16.4	10.2	6.6	5.4	5.8	-	4.1

Finally, electropherograms of a spiked soil sample and its corresponding blank analyzed by SLE-MEKC-UV are shown in **Figure 5.1** as well as electropherograms of a spiked river water sample and its corresponding blank analyzed by SPE-MEKC-UV are shown in **Figure 5.2.**

1)



2)

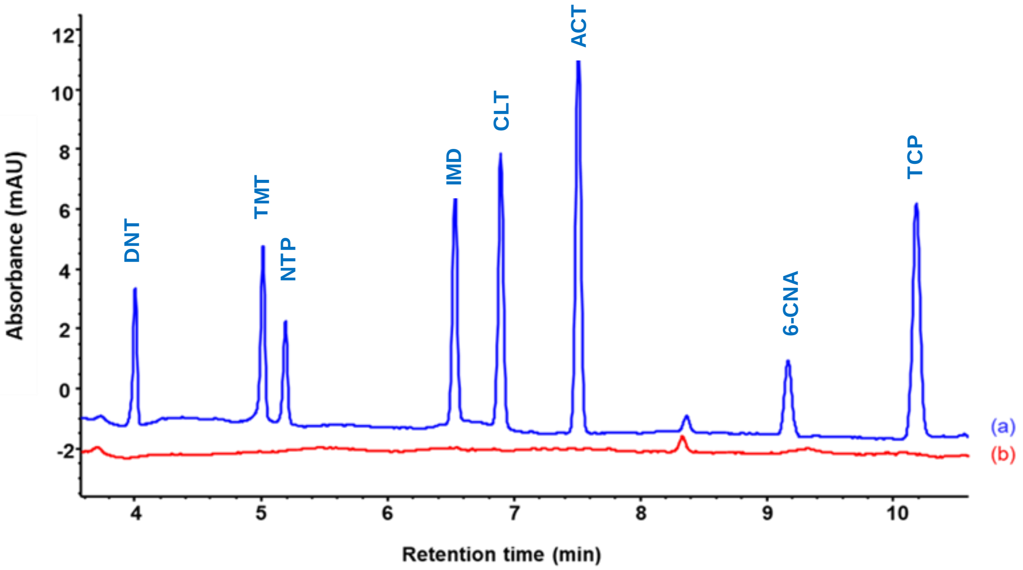


Figure 5. 1) Electropherograms obtained by the proposed SLE-MEKC-UV method for a soil sample spiked with the seven NNIs at 200 μg kg^{-1} (a) together with its blank (b). **2)** Electropherograms obtained by the proposed SPE-MEKC-UV method for a river water sample spiked with the seven NNIs and the metabolite 6-CNA at 20 μg L^{-1} (a) together with its blank (b).

5. CONCLUSIONS

This is the first time that a MEKC-UV method is used for the simultaneous determination of the seven commercially used neonicotinoid insecticides and the main metabolite 6-CNA. The optimized MEKC method is able to separate the eight compounds in less than 12 min. The applicability of the proposed method was successfully verified by characterizing the analytical methods for environmental water and soil samples. Thus, the efficiency of the two sample treatments studied was checked. On the one hand, SPE using Oasis HLB® has been proposed as sample treatment for environmental waters, being an efficient methodology for the extraction, achieving enrichment factors of 250-fold for the eight compounds. Recoveries higher than 80 % were obtained for all the studied analytes. On the other hand, SLE has been optimized as a simple treatment for soil samples, achieving an easy and fast procedure for the extraction of seven NNIs with recoveries above 70%, obtaining extracts compatible with the CE separation even without any clean-up step. Good linearity, satisfactory trueness, precision and sensitivity were obtained in both cases, with LODs in the low $\mu g\ L^{-1}$ or $\mu g\ kg^{-1}$ levels. In comparison with previous CE or LC based methods for the monitoring of these residues (**Table 5**), the proposed method is able to extract a higher number of compounds with high efficiency and easy sample treatments, avoiding extra clean-up, showing even lower LODs in relation to other methods using UV-detection and even comparable with some LC-MS methods. To sum up, it can be concluded that the proposed method could be considered as real alternative to LC-based methods. It is characterized by being relatively fast, simpler and with a lower cost. It also requires a small volume of sample, a lower consumption of organic solvents, and reduces the amount of contaminant waste.

Table 5. Comparison of the proposed MEKC method with other methods for the determination of neonicotinoids and 6-CNA.

Analyte	Sample	Extraction method	Technique	LOD	R (%)	Rf
TMT, CLT, IMD, ACT, TCP	Honey	SPE (DSC-18)-DLLME	LC-UV LC-MS/MS	0.2-1.0 µg kg^{-1} 0.02-0.1 µg kg^{-1}	-- 90-104	4
IMD, ACT, CLT, TCP, TMT	Fruit juice and natural water	VA-D-µ-SPE	LC-UV	0.01-0.07 µg L^{-1}	70-138	5
NTP, DNT, CLT, THT, ACT, IMD, TCP	Grain	SLE-DSPE-DLLME	LC-UV	2-5 µg kg^{-1}	76-123	6
ACT, CLT, NTP, IMD,TMT	Water and fruit juice	VSLLME-SFO	LC-UV	0.1-0.5 µg L^{-1}	85-105	7
DNT, NTP, TMT, CLT, IMD, ACT, TCP	Honey	DLLME QuEChERS	LC-UV	1.5-2.5 µg kg^{-1} 2.0-2.5 µg kg^{-1}	73-118 74-90	8
ACT, IMD, TMT	Fruit juice and vegetables	RTIL-LPME	LC-UV	0.12-0.33 µg L^{-1}	85-99	9
ACT, IMD, TCP, TMT	Drinking water	SPE (LiChrolut-EN)	LC-MS	0.01 µg L^{-1}	95-104	10
ACT, CLT, TMT, IMD, DNT, TCP	Honey	DLLME	LC-MS/MS	0.5-1 µg kg^{-1}	74-114	11
TMT, IMD, TCP, ACT, CLT	Sugarcane juice	QuEChERS	LC-MS/MS	0.7–2 µg kg^{-1}	62-130	12
TMT, CLT, IMD, ACT, TCP	Surface water	CPE	LC-UV	0.3–2 µg L^{-1}	60-120	13
TCP, ACT, IMZ, IMD	Cucumber	DLLME	MEKC-UV	0.8-1.2 µg kg^{-1}	78-98	14
TMT, IMD, ACT, 6-CNA	Drinking and river water Soil	SPE (Strata X) MSPD (C18)	MEKC-UV	103-810 µg L^{-1} 166-375 µg kg^{1}	86-99 63-99	15
CLT, IMD, TMT	Beeswax	SLE-SPE	CZE-MS	2.2 – 4.7 µg kg^{1}	88-98	1
NTP, DNT,CLT, TMT, ACT, IMD, TCP, 6-CNA	Spring, well and river water Soil	SPE (Oasis® HLB) SLE	MEKC-UV	0.1-0.4 µg L^{-1} 1.0-2.9 µg kg^{-1}	80-107 73-92	*

* The SPE-MEKC-UV and SLE-MEKC-UV methods proposed in this work.

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CHAPTER 5

DETERMINATION OF NEONICOTINOIDS AND BOSCALID IN POLLEN AND HONEYBEE SAMPLES BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

1. SUMMARY

In this chapter, it is proposed for the first time an electrophoretic approach based on micellar electrokinetic chromatography coupled with tandem mass spectrometry (MEKC-MS/MS) for the simultaneous determination of nine neonicotinoids (NNIs) together with the fungicide boscalid in pollen and honeybee samples. The separation was performed using ammonium perfluorooctanoate (50 mM, pH 9) as both volatile surfactant and electrophoretic buffer compatible with MS detection. A stacking strategy for accomplishing the on-line pre-concentration of the target compounds, known as sweeping, was carried out in order to improve peak efficiency and sensitivity. Furthermore, a scaled-down QuEChERS was developed as sample treatment, involving a lower organic solvent consumption and using Z-Sep+ as dispersive sorbent in the clean-up step. Regarding the detection mode, a triple quadrupole mass spectrometer was operating in positive ion electrospray mode (ESI^+) under multiple reaction monitoring (MRM). The main parameters affecting MS/MS detection as well as the composition of the sheath-liquid (ethanol/ultrapure water/formic acid, 50:49.5:0.5 v/v/v) and other electrospray variables were optimized in order to achieve satisfactory sensitivity and reproducibility. Procedural calibration curves were established in pollen and honeybee samples with LOQs below 11.6 $\mu g\ kg^{-1}$ and 12.5 $\mu g\ kg^{-1}$, respectively. Precision, expressed as RSD, lower than 15.2% and recoveries higher than 70 % were obtained in both samples. Two positive samples of pollen were found, containing imidacloprid and thiamethoxam. Imidacloprid was also found in a sample of honeybees. The obtained results highlight the applicability of the proposed method, being an environmentally friendly, efficient, sensitive and useful alternative for the determination of NNIs and boscalid in pollen and honeybee samples.

2. EXPERIMENTAL

2.1 Materials and reagents

Unless otherwise specified, analytical grade reagents and HPLC grade solvents were used in this work. Acetonitrile (MeCN), formic acid (FA), isopropanol (IPA) and methanol (MeOH) were supplied by VWR International (West Chester, PA, USA), while ethanol (EtOH) and ammonia solution, (NH_3 (aq), 30 % w/w) were obtained from Merk (Darmstadt, Germany). Sodium hydroxide (NaOH) as well as salts such as magnesium sulfate anhydrous ($MgSO_4$), sodium sulfate (Na_2SO_4), and sodium chloride (NaCl) were obtained from PanReac-Química (Madrid, Spain), while ammonium sulfate ($(NH_4)_2SO_4$) was obtained from VWR Chemicals (Barcelona, Spain). Dispersive sorbents such as Primary Secondary Amine (PSA) and C18 endcapped sorbent were supplied by Agilent Technologies (Waldbronn, Germany) while activated carbon and Z-Sep+ were obtained from Sigma-Aldrich (St. Louis, MO, USA) as well as the perfluorooctanoic acid (PFOA) (96 % w/w).

Analytical standards of dinotefuran (DNT), thiamethoxam (TMT), clothianidin (CLT), nitenpyram (NTP), imidacloprid (IMD), thiacloprid (TCP), acetamiprid (ACT), imidaclothiz (IMZ), flonicamid (FNC), and boscalid (BCL) were supplied by Sigma Aldrich. Individual standard solutions were obtained by dissolving the appropriate amount of each compound in MeOH, reaching a final concentration of 500 $\mu g\ mL^{-1}$, which were kept in dark at -20 °C. Intermediate stock standard solutions containing 50 $\mu g\ mL^{-1}$ of each compound were obtained by mixing individual stock standard solutions, followed by a solvent evaporation step under a current of N_2, and subsequent dilution with ultrapure water. Working standard solutions were freshly prepared by dilution of the intermediate stock standard solutions with ultrapure water at the required concentration. These solutions were stored at 4 °C avoiding exposure to direct light.

Solutions of 50 mM ammonium perfluorooctanoate (APFO) at pH 9 used as background electrolyte (BGE) were prepared weekly dissolving the necessary amount of PFOA in ultrapure water and adjusting the pH with 5 M NH_3(aq).

Polytetrafluoroethylene (PTFE) syringe filters (0.22 µm x 13 mm) such as CLARIFY-PTFE (hydrophilic) obtained from Phenomenex (California, USA) were used for sample filtration, and PTFE filters from VWR international (West Chester, PA, USA) were employed for filtration of NaOH and BGE.

2.2 Instrumentation

MEKC experiments were performed with an Agilent 7100 CE system coupled to a triple quadrupole 6495C mass spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionization source operating in positive ionization mode (ESI^+). Sheath liquid was supplied with a 1260 Infinity II Iso Pump. MS data were collected and processed by MassHunter software (version 10.0).

Separations were carried out in bare fused-silica capillaries (70 cm of total length, 50 µm i.d., 375 o.d.) from Polymicro Technologies (Phoenix, AZ, USA).

A pH meter (Crison model pH 2000, Barcelona, Spain), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), a multi-tube vortexer BenchMixer XL (Sigma-Aldrich, St. Louis, MO, USA), and a nitrogen dryer EVA-EC System (VLM GmbH, Bielefeld, Germany) were also employed.

2.3 Capillary electrophoresis separation

New capillaries were conditioned with 1 M NaOH for 15 min, followed by ultrapure water for 10 min and then, with the running BGE for 15 min at 1 bar and 25 °C. At the beginning of each day, this procedure was repeated but using 0.1 M NaOH. In order to obtain an adequate repeatability between runs, capillary was rinsed with the BGE for 3 min at 1 bar and 25 °C at the beginning of each run. At the end of the working day, capillary was cleaned with ultrapure water for 5 min, followed by MeOH for 2 min, and finally dried with air for 1 min at 1 bar and 25 °C.

Electrophoretic separation was performed in MEKC mode using a BGE consisted of an aqueous solution of 50 mM PFOA at pH 9, which gave a stable electric current of 25 μA. The capillary was kept at 25 °C and a voltage of 25 kV (normal polarity) was applied. Samples were hydrodynamically injected for 50 s at 50 mbar using ultrapure water as injection solvent in order to induce sweeping.

2.4 MS/MS conditions

Sheath-liquid consisting of a mixture of ethanol/ultrapure water/formic acid, 50:49.5:0.5 (v/v/v) was provided at a flow rate of 5 μL min^{-1} (0.5 mL min^{-1} with a 1:100 splitter). The mass spectrometer was operated in positive ionization mode (ESI^{+}) under multiple reaction monitoring (MRM) conditions. 2000 V were applied in both capillary and nozzle. Other electrospray parameters at optimum conditions were: nebulizer pressure, 10 psi; dry gas flow rate, 11 L min^{-1}; and dry gas temperature, 200 °C. MS/MS experiments were performed by fragmentation of the molecular ions $[M+H]^{+}$, which were selected as the precursor ions. Collision energies (V) were adjusted in a range of 9-25 depending on the analyte, and product ions were analyzed in the range of 114-307 *m*/*z*.

2.5 Sample treatment procedure

2.5.1 Sample collection and preparation

Commercially available pollen from a local market (Granada, Spain) was used for method optimization. The pollen was kept in its original packaging at ambient temperature until further handling. Fresh pollen samples used to monitor the presence of the target compounds were gathered from almond blossoms at three different farmlands located in Fuente Vera (Granada, Spain), and immediately stored at -20 °C until their use. Flowers were defrosted and dried at 30 °C for 24 hours to extract the pollen from the anthers. Afterwards, flowers were carefully sieved through a 0.2-mm mesh to separate pollen from them. Lab tweezers were also needed to release the pollen in some cases. The obtained pollen samples from each farmland were kept in a dry place until their analysis.

In order to characterize the method in blank honeybee samples, approximately 500 dead specimens were carefully collected from an organic farmland in which the use of beehives is common. In addition, about 200 dead honeybees were collected in an area located close to the almond fields above mentioned. This sampling point was selected because hundreds of dead adult worker bees were found there, so the analysis of these samples was particularly interesting in order to prove the usefulness of this method. All samples were rapidly stored at -20 °C until their use. Then, honeybees were lyophilized at -109 °C in order to guarantee the proper crushing and homogenization of the sample.

2.5.2 Scaled-down QuEChERS procedure

The sample treatment for pollen and honeybee samples was carried out as follows: a representative 200 mg portion of each sample was placed into a 15 mL centrifuge tube and 1 mL of ultrapure water was added to hydrate the samples, which were subsequently vortexed for 1 min. Then, 2.5 mL of MeCN were added as well as 200 mg of $MgSO_4$ to favor salting-out effect. The tube was mechanically shaken for 2 min and centrifuged for 5 min at 9000 rpm and 4 °C. Then, the whole supernatant was transferred to another 15 mL centrifuge tube containing 30 mg of Z-Sep+ as dispersive sorbent and 100 mg of $MgSO_4$. The tube was stirred in vortex for 2 min and centrifuged for 5 min at 9000 rpm and 4 °C. Afterwards, an aliquot of 2 mL of supernatant was transferred to a glass vial and dried under a gentle N_2 stream at 35 °C. Finally, the dried residue was reconstituted with 200 µL of ultrapure water, shaken in an ultrasonic bath and filtered through a 0.22 µm hydrophilic-PTFE filter before its injection into the CE-MS/MS system.

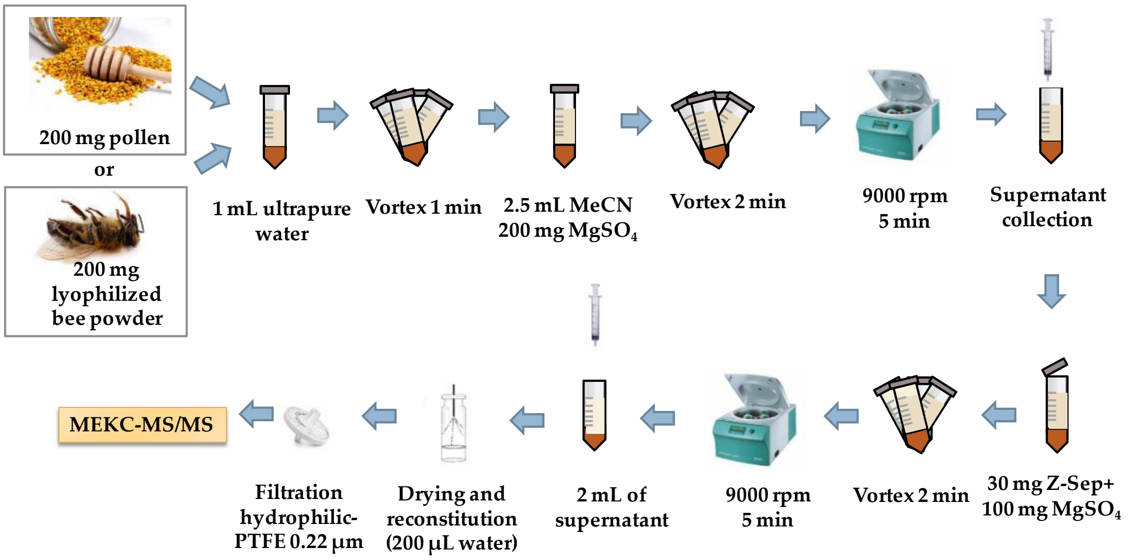


Figure 1. Scaled-down QuEChERS procedure for the analysis of NNIs and boscalid in pollen and honeybee samples by MEKC-MS/MS.

3. RESULTS AND DISCUSSION

3.1 Optimization of electrophoretic separation

Capillary electrophoretic separations were performed using MEKC mode, in which neutral analytes show different electrophoretic mobilities due to their different interaction with the micelles. Optimization of the main variables affecting the separation of the target compounds by MEKC was carried out considering different response variables such as S/N ratio, migration time and peak resolution. In addition, the generated current was kept lower than 30 µA to minimize the Joule effect.

Surfactants such as the commonly used sodium dodecyl sulfate (SDS) are not recommended when MS detection is used because they are non-volatile and can contaminate the ion-source of the mass spectrometer, leading to an analyte signal suppression and a marked decrease of sensitivity. Therefore, the use of a volatile surfactant such as APFO was considered as both BGE and micellar medium. Firstly, basic pH conditions are needed to dissolve PFOA in ultrapure water. In addition, target compounds are neutral at basic conditions. Therefore, the effect of pH in the separation was investigated between 8 and 10 using 75 mM PFOA. There were no significant differences in this pH range, so pH 9 was selected.

Subsequently, taking into consideration that the critical micelle concentration (CMC) of APFO is 25 mM, different concentrations of APFO between 30 and 100 mM were investigated at pH 9. As the concentration increases so does the resolution between peaks as well as the migration time. However, the intensity of the signal for most analytes was higher at lower concentrations and the migration time was significantly shorter (10 min). Thus, a concentration of 50 mM APFO was selected as a compromise between migration time, signal intensity and resolution. In order to reduce the total analysis time, capillary was shortened from 80 to 70 cm. Peak efficiency, especially for acetamiprid, was slightly better and the total analysis time was reduced in 2 min when this capillary was used, so a length of 70 cm was selected as optimum for further experiments.

Afterwards, the effect of the temperature on the MEKC separation was studied in the range of 20-35 °C. It was observed that the total analysis time decreased when the temperature increased up to 30 °C. Nevertheless, the electrophoretic current increased with the temperature, so in order to avoid this, a temperature of 25 °C was selected. Moreover, the separation voltage was also studied in the range of 20-30 kV. The best results as a compromise between the analysis time and the electrophoretic current were obtained when 25 kV was used, so it was selected for further analysis.

In order to improve sensitivity, an on-line preconcentration of the analytes was performed using a solvent devoid of micelles as injection solvent. This approach, known as "sweeping" is designed to focus the analytes into a narrow band within the capillary, thereby increasing the sample volume that can be injected without any loss of efficiency. It is based on the interactions between an additive (i.e., a pseudostationary phase or micellar media) in the separation buffer and the sample in a matrix that is free of the additive used. It involves the accumulation of neutral analytes by the pseudostationary phase that penetrates the sample zone and "sweeps" the analytes, producing a focusing effect. In this case, ultrapure water was used as injection solvent, since it allowed the stacking of the analytes when they were swept by the BGE containing APFO micelles [1,2]. The use of an organic solvent as injection solvent was discarded since this negatively affected the formation of micelles and had an adverse impact on peak shapes as it was also previously reported [3]. Finally, the effect of the hydrodynamic injection time on peak height was checked in the range from 20 to 60 s at 50 mbar. There was an increase in the peak height up to 50 s without significantly affecting peak efficiency. In this regard, an injection time of 50 s was set. This injection time corresponds to a sample volume of 55 nL approximately (4 % of the total capillary volume).

Sensitivity enhancement factors (SEFs) for NNIs and boscalid were estimated comparing peak heights of standard solutions dissolved in water (sweeping) with standard solutions of the same concentration dissolved in BGE (no sweeping):

$$\mathrm{SEF_{height}} = \frac{\text{Analyte peak height using sweeping}}{\text{Analyte peak height without using sweeping}}$$

SEF ranging from 1.6 to 5.6 were achieved for the studied analytes using sweeping, as can be seen in **Table 1**. In addition, peak efficiencies (theoretical plate number) with and without sweeping were checked for each analyte. Significantly better results were obtained when ultrapure water was employed as injection solvent (**Table 2**). In view of these results, the use of sweeping as on-line preconcentration led to an improvement in sensitivity as well as in peak efficiency for the studied compounds.

Table 1. Sensitivity enhancement factors (SEFs) using sweeping for on-line preconcentration of the target analytes in the proposed MEKC method.

Analyte	SEF $_{\text{height}}$	RSD, % (n=3)
DNT	1.6	3.2
TMT	1.8	4.5
FCM	2.1	6.4
CLT	2.1	3.4
NTP	3.2	5.0
IMZ	4.4	6.7
IMD	4.5	5.4
TCP	5.6	6.8
ACT	2.4	3.9
BCL	5.0	7.3

Table 2. Analyte peak efficiency, expressed as number of theoretical plates (N), obtained when sweeping or no sweeping is used.

Analyte	N (with sweeping)	N (without sweeping)
DNT	6311	2513
TMT	8490	2776
FCM	12878	2676
CLT	10380	2704
NTP	45988	3129
IMZ	44337	2809
IMD	73767	2867
TCP	52678	2879
ACT	12582	1975
BCL	62554	2290

3.2 Optimization of MEKC-ESI-MS/MS conditions

The sheath-liquid must be carefully selected in order to have a stable electrospray and good sensitivity. Thus, different parameters affecting the electrospray such as composition and flow of the sheath-liquid, dry gas flow rate and temperature, and nebulizer pressure have been optimized considering the S/N ratio obtained as response variable.

At the beginning, the composition of the sheath-liquid was evaluated considering different organic solvents such as MeOH, EtOH, IPA and MeCN. The sheath-liquid in all cases consisted of a 50:50 organic solvent/ultrapure water mixture containing 0.5% (v/v) of formic acid (50:49.5:0.5; organic solvent/ultrapure water/formic acid). For most compounds, similar S/N ratios were obtained when MeOH and EtOH were used, except in the case of thiacloprid and acetamiprid that showed an increase in the S/N ratio when EtOH was employed. With MeCN and IPA the S/N was lower in all cases **(Figure 2a)**. Considering also that EtOH is more environmentally friendly, it was selected as the organic solvent for the sheath-liquid. Subsequently, as can be seen in **Figure 2b** the percentage of EtOH was studied from 40 to 60 %. An increase in the S/N ratio was achieved using 50 %, so it was chosen as optimum. Formic acid was added to ensure the adequate positive ionization of the analytes. The percentage added was checked from 0.1 to 1 %. It was observed that percentages higher than 0.5 did not improve the S/N ratio, therefore, this value was selected as optimum (**Figure 2c**). Because of these results, sheath-liquid composition was 50:49.5:0.5 (v/v/v), EtOH/ultrapure water/formic acid. Sheath-liquid flow rate plays an important role to ensure electrospray stability, and therefore, it has an influence in the analysis reproducibility. Consequently, it was studied in the range 2.5-15 µL min^{-1} **(Figure 2d).** A reduction of the S/N ratio was observed when the flow rate increased, which may be explained because of the dilution of the CE effluent. A flow rate below 5 µL min^{-1} led to an unstable electrospray, so it was discarded. Ergo, 5 µL min^{-1} were selected as optimum for further analysis.

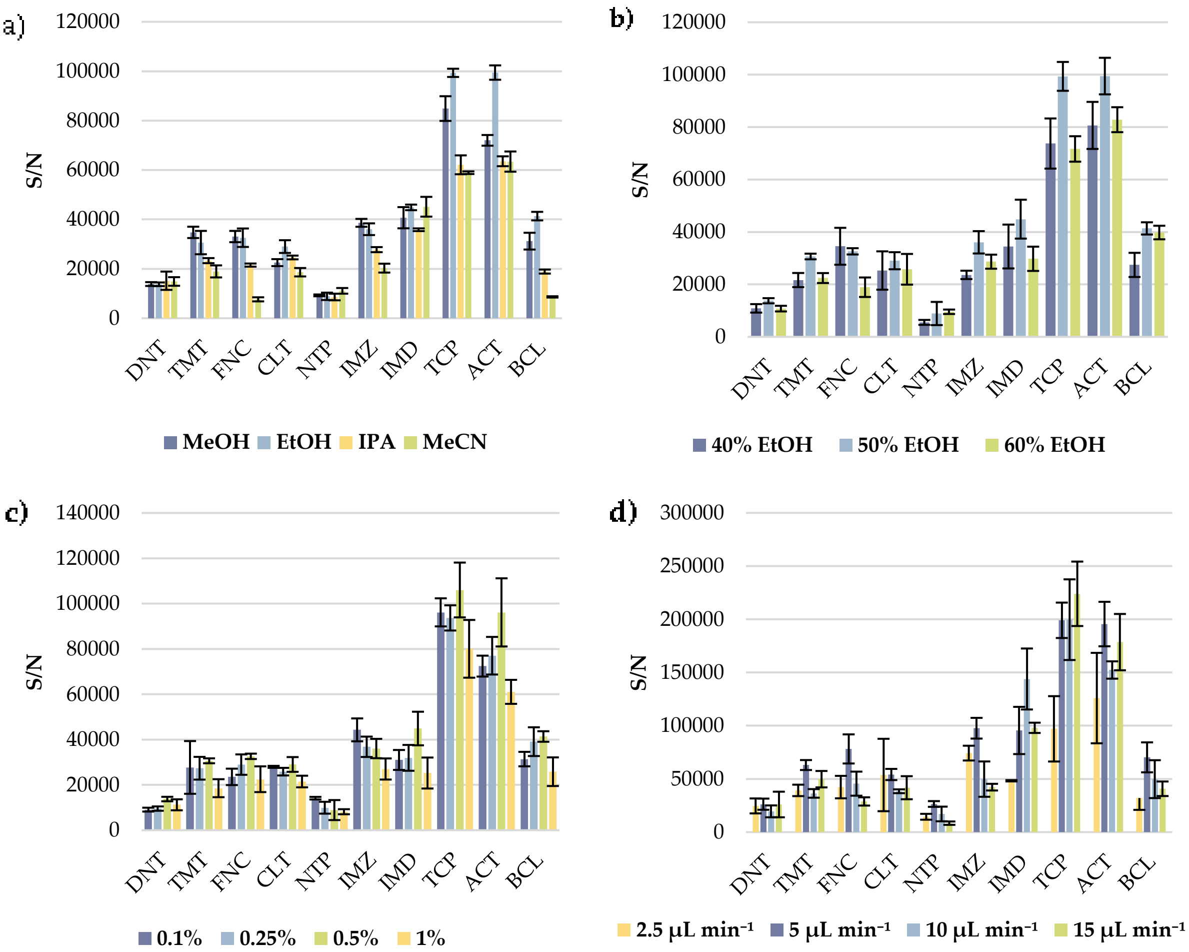


Figure 2. Optimization of the sheat-liquid composition. Effect of the **a)** type of organic solvent; **b)** percentage of EtOH; **c)** percentage of formic acid; **d)** flow rate. Error bars represent standard error (n=4).

After optimizing the sheath-liquid, the nebulizer pressure was evaluated between 6 and 12 psi. Above 10 psi, the spray stability decreased, inducing irreproducibility in the migration. The best compromise between reproducibility and S/N ratio was obtained when a nebulizer pressure of 10 psi was used. Regarding the dry gas, temperature and flow rate were evaluated. Firstly, the dry gas temperature was tested from 200 to 300 °C taking into consideration that APFO can be used as volatile surfactant at temperatures above 150 °C at which this surfactant decomposes. An increase in the temperature did not improve the S/N ratio, so 200 °C was selected. Then, the dry gas flow rate was studied from 11 to 20 L min^{-1}, obtaining the best S/N ratio when 11 L min^{-1} was employed.

Finally, the capillary voltage which affects the sensitivity of MS detection was also studied. The voltage was varied from 1000 to 3000 V keeping the nozzle at 2000 V. With a voltage of 1000 V a significant reduction of the S/N ratio was observed, however, for the rest of the tested voltages no significant differences were noticed. Thus, 2000 V was chosen as capillary voltage.

In order to get optimum selectivity, the main MS/MS parameters were studied. First of all, using the SCAN mode, it was observed that the protonated molecules $[M+H]^+$ were the most abundant ions for all analytes. Once the precursor ion was fixed for each compound, the main fragments were investigated by collision induced dissociations selecting the optimum collision energy to be applied in order to obtain the highest signal in each case. Finally, an MRM method was developed taking into consideration the previously mentioned data as well as the migration time of the target analytes. In this method, dwell time for each transition was also optimized varying from 40 to 80 ms depending on the analyte to guarantee a minimum data acquisition of 10 points per peak. Optimized MS/MS parameters are summarized in **Table 3**.

Table 3. MS/MS parameters for target compounds.

	Migration time (min)	Precursor ion (*m*/*z*)	Molecular Ion	Product Ions[a]	CE[b]	Dwell time (ms)
TMT	5.25	292.0	$[M+H]^+$	210.9 (Q)	10	50
				131.7 (I)	10	50
DNT	5.29	203.1	$[M+H]^+$	129.2 (Q)	9	50
				114.0 (I)	9	50
FCM	5.40	230.1	$[M+H]^+$	202.8 (Q)	15	40
				173.9 (I)	15	40
CLT	5.42	250.0	$[M+H]^+$	168.9 (Q)	10	80
				132.0 (I)	10	80
NTP	5.88	271.1	$[M+H]^+$	189.0 (Q)	15	50
				237.3 (I)	15	50
IMZ	6.00	262.0	$[M+H]^+$	180.9 (Q)	15	50
				131.7 (I)	15	50
IMD	6.47	256.1	$[M+H]^+$	209.1 (Q)	15	50
				175.0 (I)	15	50
TCP	6.52	253.0	$[M+H]^+$	125.9 (Q)	25	50
				90.0 (I)	25	50
ACT	6.77	223.1	$[M+H]^+$	126.0 (Q)	15	80
				56.1 (I)	15	80
BCL	7.18	343.0	$[M+H]^+$	307.0 (Q)	20	60
				140.0 (I)	20	60

[a] Product ions: (Q) Transition used for quantification, (I) Transition used for identification.
[b] Collision Energy (CE). All values expressed in volts.

3.3 Optimization of sample treatment

In this work, a scaled-down QuEChERS procedure has been developed for the extraction and clean-up of nine NNIs and boscalid from pollen and honeybee samples. In a scaled-down QuEChERS, the amount of sample is reduced as well as the volume of MeCN required for the extraction of the analytes, reducing the organic solvent consumption and avoiding the dilution of the analyte concentration.

No satisfactory recoveries were obtained when a previously published protocol for determination of NNIs by LC-MS was applied [4], probably due to a higher matrix effect in CE-MS. In consequence, the main variables affecting the scaled-down QuEChERS were optimized to achieve the highest extraction recoveries.

To begin with, a representative pollen sample (200 mg) was placed in a 15-mL centrifuge tube and spiked with the desired concentration of the target analytes. Then, the sample was hydrated with 1 mL of ultrapure water and vortexed for proper homogenization. Subsequently, 2.5 mL of MeCN were added, which was the minimum volume able to extract the studied compounds with acceptable recoveries from this amount of sample.

The ionic strength was studied because the addition of salts to the aqueous phase may have a salting-out effect decreasing the analyte solubility in water and favoring their transference to the organic phase. In this sense, as shown in **Figure 3a**, several salts such as $MgSO_4$, Na_2SO_4, $(NH_4)_2SO_4$, and NaCl were evaluated. Thus, after adding the extraction solvent to the aqueous sample, 200 mg of each salt were also added, and the tube was shaken for 2 min and centrifuged for 5 min at 9000 rpm and 4 °C. It must be mentioned that NaCl was incompatible with the MEKC separation since the electrophoretic current was no stable, therefore, it was discarded. The best results in terms of recoveries (above 75 % in all cases) were obtained when $MgSO_4$ was employed, so it was selected as salting-out agent. Subsequently, the amount of this salt was also tested from 100 to 400 mg (**Figure 3b).** It was observed that 100 mg was not enough to obtain a well-defined phase separation, leading to poor recoveries. On the other hand, above 200 mg, recoveries decreased in all cases. Therefore, 200 mg of $MgSO_4$ was selected as salting-out agent.

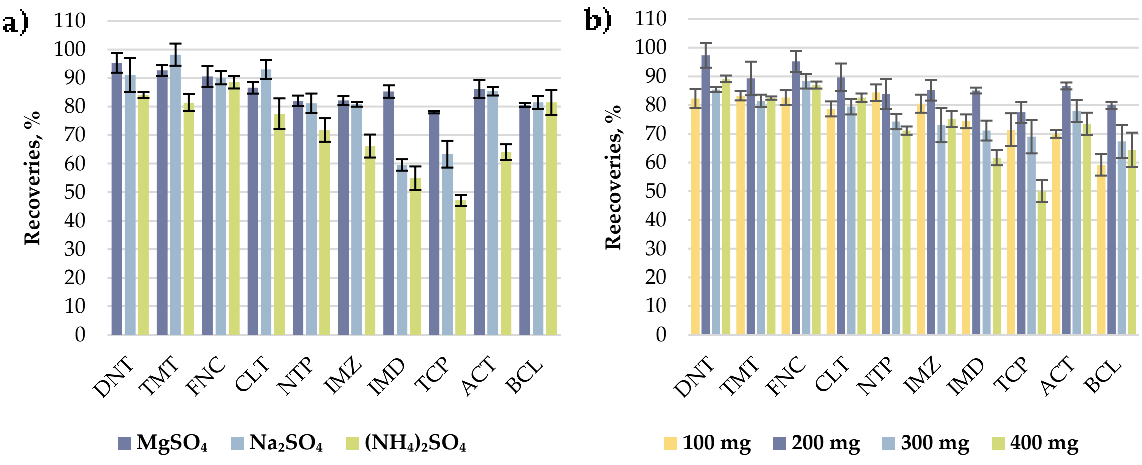


Figure 3. Optimization of salting-out agent in the extraction of the analytes from a spiked pollen sample. **a)** Effect of different salts on the extraction recovery; **b)** Effect of the amount of $MgSO_4$ on the extraction recovery. Error bars represent relative standard error (n=4).

Afterwards, in order to reduce the matrix effect (ME), different dispersive sorbents were evaluated in the d-SPE step such as Z-Sep+, EMR lipids, PSA, C18 and a mixture of PSA/C18 (1:1) as it is shown in **Figure 4**. In all cases an amount of 80 mg of sorbent was used together with 100 mg of $MgSO_4$ anhydrous to remove possible traces of ultrapure water in the organic extraction solvent. In general, recoveries were above 70 % in most cases except when the EMR lipids sorbent was used. In addition, the recovery for nitenpyram significantly decreased when Z-Sep+ was employed, being around 40 % **(Figure 4a).** On the other hand, this sorbent provided the best results in terms of ME **(Figure 4b)**.

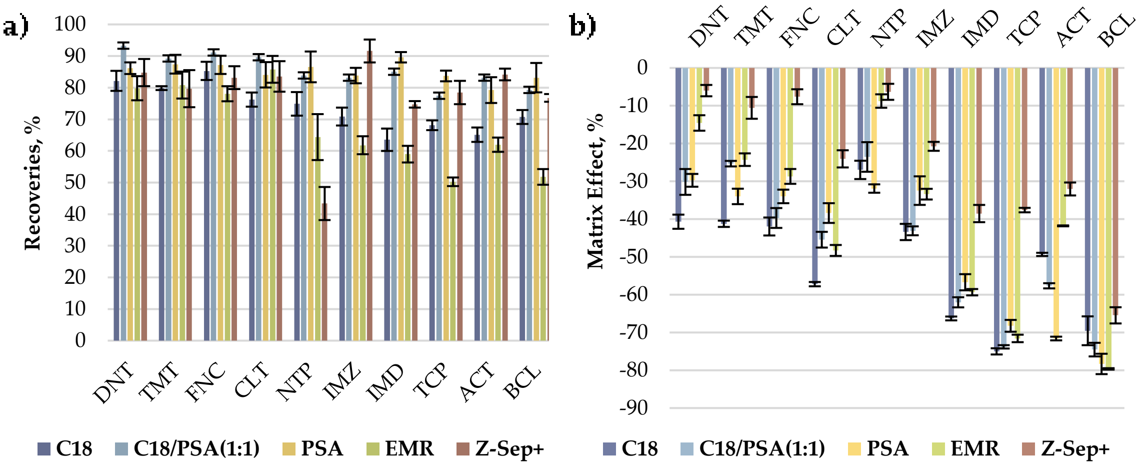


Figure 4. Optimization of dispersive sorbents in the d-SPE step of the sample treatment procedure for the extraction of the analytes from a spiked pollen sample. **a)** Effect on the extraction recoveries; **b)** Effect on the matrix effect.

Then, the amount of Z-Sep+ was reduced to improve nitenpyram recovery. As can be seen in **Figure 5a**, reducing the amount of this sorbent to 30 mg, recoveries around 70 % for nitenpyram were achieved. Decreasing the amount of sorbent led to ME slightly higher for all analytes, but still better than those obtained with the other sorbents (**Figure 5b**). This sorbent, despite its high potential to clean complex extract, has not been explored in d-SPE of honeybee products and NNIs determination where PSA sorbent has been traditionally used [3,5].

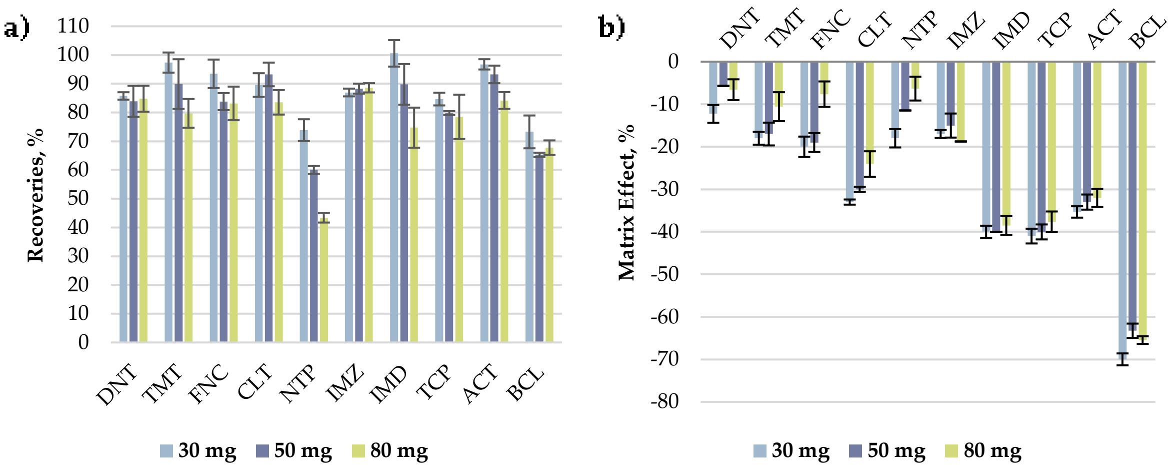


Figure 5. Optimization of the amount of Z-Sep+ in the d-SPE step of the sample treatment procedure for the extraction of the analytes from a spiked pollen sample. **a)** Effect on the extraction recoveries; **b)** Effect on the matrix effect.

Finally, different syringe filters were tested through the filtration of a standard solution with each one. Then, the results obtained were compared with a standard solution without filtering at the same concentration. The best results, in terms of recoveries, for most analytes were obtained with hydrophilic-PTFE filters. Unfortunately, even with this filter, around 50 % of boscalid was lost during filtration (**Figure 6**).

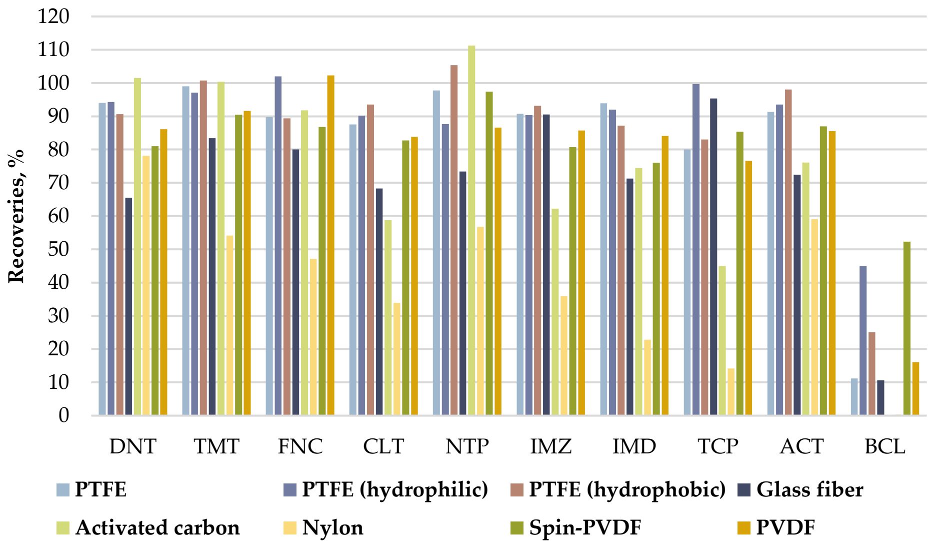


Figure 6. Evaluation of different filters for the sample filtration step before the injection into the MEKC-MS/MS system.

An electropherogram of a pollen sample spiked with the studied analytes submitted to the optimized sample treatment and analyzed by the proposed MEKC-MS/MS method is shown in **Figure 7.**

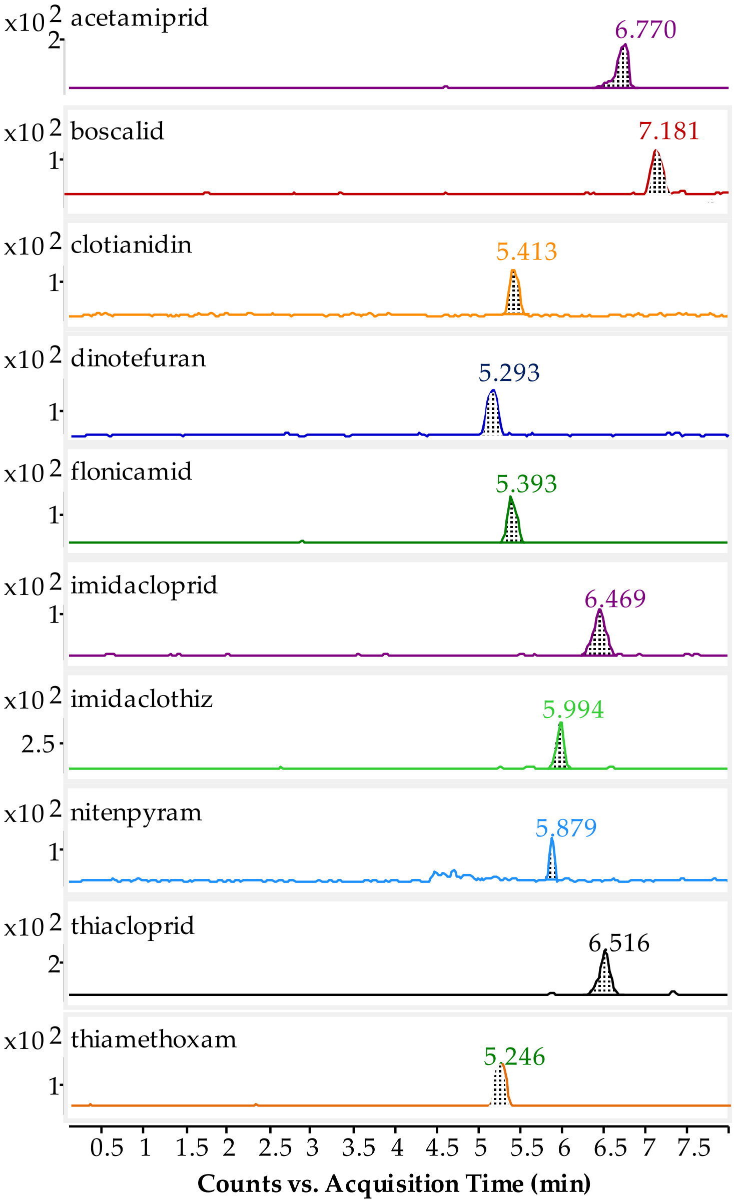


Figure 7. Electrophoretic separation of a blank pollen sample spiked with the standard mixture solution of NNIs and boscalid at a concentration of 200 µg kg^{-1}.

4. METHOD CHARACTERIZATION

The optimized scaled-down QuEChERS-MEKC-MS/MS method was evaluated in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), extraction recovery, matrix effect, and precision (i.e., repeatability and intermediate precision) in pollen and honeybee samples. Both samples were previously analyzed using the proposed method and neither analytes nor interferences were found.

4.1 Calibration curves and performance characteristics

Procedural calibration curves for commercial pollen and honeybee samples were performed at different concentration levels; 5, 10, 25, 50, 100, and 200 μg kg^{-1} for pollen samples and 2, 5, 10, 25, 50, 100, and 200 μg kg^{-1} for honeybee samples. Procedural calibration involves the analysis of samples fortified before the sample treatment. Two samples were spiked at each concentration level, treated according to the scaled-down QuEChERS procedure, and analyzed in triplicate by the proposed MEKC-MS/MS method. Peak area was selected as analytical response and considered as a function of the analyte concentration on the sample. LODs and LOQs were calculated as the minimum analyte concentrations yielding a S/N ratio equal to three and ten times, respectively. As shown in **Table 4**, a satisfactory linearity was confirmed at the concentration range studied ($R^2 > 0.9900$) with LODs and LOQs below 3.5 μg kg^{-1} and 11.6 μg kg^{-1} respectively, for pollen samples, and below 4.0 μg kg^{-1} and 12.5 μg kg^{-1}, respectively, for honeybee samples. These results highlight that the proposed method allows the determination of NNIs and boscalid in pollen samples at levels below their MRLs established in apiculture products by the European Legislation [6].

Table 4. Statistical and performance characteristics of the proposed method for the determination of NNIs and boscalid in pollen and honeybee samples.

Analyte	Linear regression equation	Linear Range (μg kg^{-1})	Linearity (R^2)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	MRL (μg kg^{-1})
Pollen samples						
DNT	$y = 16.902x + 75.7$	9.7-200	0.9915	2.9	9.7	♦
TMT	$y = 22.533x – 39.225$	6.5-200	0.9904	1.9	6.5	50*
FCM	$y = 13.244x – 25.013$	3.8-200	0.9915	1.1	3.8	50*
CLT	$y = 13.38x + 8.885$	9.7-200	0.9902	2.9	9.7	50*
NTP	$y = 2.458x + 7.149$	9.0-200	0.9906	2.7	9.0	♦
IMZ	$y = 35.417x – 23.187$	8.0-200	0.9900	2.4	8.0	♦
IMD	$y = 10.372x – 8.522$	6.1-200	0.9906	1.8	6.1	50*
TCP	$y = 25.305x – 45.832$	5.7-200	0.9911	1.8	5.7	200
ACT	$y = 19.975x + 32.224$	6.0-200	0.9930	1.8	6.0	50*
BCL	$y = 5.303x – 28.086$	11.6-200	0.9923	3.5	11.6	150
Honeybee samples						
DNT	$y = 29.929x + 158.49$	12.5- 200	0.9920	3.75	12.5	-
TMT	$y = 45.764x + 77.917$	4.3 - 200	0.9906	1.28	4.3	-
FCM	$y = 44.17x – 107.67$	2.6 - 200	0.9918	0.77	2.6	-
CLT	$y = 30.028 – 88.054$	8.6 - 200	0.9926	2.57	8.6	-
NTP	$y = 8.1773x + 184.69$	11.1-200	0.9868	3.33	11.1	-
IMZ	$y = 48.276x + 247.73$	10.3-200	0.9947	3.08	10.3	-
IMD	$y = 24.398x – 106.31$	6.3 - 200	0.9915	1.88	6.3	-
TCP	$y = 45.856x – 54.305$	4.1 - 200	0.9931	1.22	4.1	-
ACT	$y = 54.047x – 289.22$	3.9 - 200	0.9920	1.15	3.9	-
BCL	$y = 4.811x + 151.2$	12.5-200	0.9916	4.04	12.5	-

♦ MRL non-established. Default value of 10 μg kg^{-1}. *Indicates lower limit of analytical determination.

4.2 Repeatability and intermediate precision assays

Precision of the proposed method was evaluated in terms of repeatability (i.e., intra-day precision) and intermediate precision (i.e., inter-day precision) by the application of the method to pollen and honeybee samples spiked at two concentration levels in the linear range (10 and 50 μg kg^{-1}). For repeatability, three samples were submitted to the sample treatment procedure (experimental replicates) and injected in triplicate (instrumental replicates) the same day under the same conditions (n=9). In the case of intermediate precision, it was evaluated with a similar procedure, but analyzing one sample prepared each day during three different days (n=9). The obtained results, expressed as RSD (%) of peak areas, for pollen and honeybee samples are summarized in **Table 5.** Satisfactory RSD were achieved for both samples, being lower than 11.3 % and 15.5 % for repeatability and intermediate precision, fulfilling the EU recommendations concerning the performance of analytical methods for the determination of contaminants, which set an upper limit for RSD of 20 % [7].

Table 5. Precision of the proposed method for the determination of NNIs and boscalid in pollen and honeybee samples.

	Repeatability, % RSD (n=9)		**Intermediate Precision, % RSD (n = 9)**	
	10 µg kg^{-1}	**50 µg kg^{-1}**	**10 µg kg^{-1}**	**50 µg kg^{-1}**
		Pollen samples		
DNT	8.3	5.7	12.9	9.6
TMT	10.0	10.4	14.4	13.8
FCM	9.4	8.2	13.6	8.7
CLT	10.3	8.5	13.9	9.8
NTP	10.1	9.0	14.8	12.7
IMZ	8.3	8.9	14.2	9.2
IMD	10.6	8.3	13.6	8.6
TCP	10.8	9.6	13.7	12.2
ACT	9.0	7.5	12.0	11.4
BCL	11.3	9.3	15.5	13.5
		Honeybee samples		
DNT	6.8	9.7	6.3	8.8
TMT	6.2	5.0	7.2	7.3
FCM	7.7	5.4	8.3	9.3
CLT	6.3	6.2	12.6	8.8
NTP	11.1	6.1	12.6	8.5
IMZ	8.1	7.4	9.2	10.3
IMD	10.3	7.6	13.1	11.8
TCP	7.2	6.2	9.0	10.8
ACT	4.9	5.5	7.3	5.9
BCL	9.9	6.1	8.7	7.8

4.3 Trueness

The trueness of the developed method was assessed in both honeybee and natural pollen samples by their fortification at two different concentration levels (10 and 50 µg kg^{-1}). In both cases, the samples used through this study were different that those used to perform the calibration curves in each case. Fortified samples were processed following the scaled-down QuEChERS procedure and analyzed in triplicate by the proposed MEKC-MS/MS method. Trueness, expressed in percentage, was calculated interpolating the peak areas obtained for each target compound in the corresponding procedural calibration curve previously obtained with pollen and honeybee samples and comparing the obtained concentration with the expected value. As can be seen in **Table 6**, values for trueness between 89.2 and 106.7 % for all compounds in pollen samples and, between 93.5 and 107.1 % for honeybee samples. These values meet the EU requirements for quantitative methods of analysis [7].

Table 6. Trueness of the proposed method for the determination of NNIs and boscalid in pollenand honeybee samples

	% Trueness, 10 µg kg^{-1}	% RSD(n=9)	% Trueness, 50 µg kg^{-1}	% RSD (n=9)
		Pollen samples		
DNT	99.0	5.9	101.8	6.3
TMT	101.8	6.4	95.0	5.9
FCM	99.8	3.8	99.6	3.3
CLT	94.5	6.8	89.2	5.6
NTP	99.4	5.5	99.5	6.4
IMZ	102.1	5.5	90.8	5.0
IMD	106.7	6.3	94.3	6.6
TCP	105.8	6.0	93.2	5.1
ACT	98.6	5.5	95.4	5.1
BCL	111.2	7.4	98.8	6.2
		Honeybee samples		
DNT	103.0	4.8	90.1	6.4
TMT	105.2	4.0	99.1	5.9
FCM	107.1	5.8	102.3	4.7
CLT	99.2	4.4	103.9	3.4
NTP	106.6	7.2	100.8	6.2
IMZ	95.5	4.0	106.2	4.4
IMD	103.6	4.9	89.1	3.6
TCP	96.	6.5	98.5	4.2
ACT	99.3	5.7	101.4	5.3
BCL	93.5	5.3	95.1	5.1

4.4 Recovery studies

In order to evaluate the efficiency of the proposed scaled-down QuEChERS, recovery experiments were carried out. Three blank samples of each matrix were fortified at two different concentration levels (10 and 50 μg kg^{-1}), treated following the sample treatment procedure and analyzed in triplicate by MEKC-MS/MS. The data, in terms of peak area, were compared with those obtained by analyzing extracts of blank samples submitted to the sample treatment and fortified at the same concentration levels just before the injection. As can be seen in **Table 7,** recoveries over 80% were obtained except for nitenpyram and boscalid in pollen samples, which showed recovery values above 70 %**.** In any case, these results suggest that the proposed sample treatment method could be satisfactorily applied to determine NNIs and boscalid in these matrixes.

4.5 Evaluation of matrix effect

This is known as matrix effect (ME), which can be attributed to many factors, affecting analyte ionization in MS and, therefore, resulting in ion suppression or signal enhancement. ME can be estimated by comparing the analytical response provided by blank extracts spiked after the sample treatment with the response that results from a standard solution at the same concentration. The following equation is used for this comparison:

$$\mathrm{ME}(\%) = \frac{\text{signal of extract spiked after extraction} - \text{signal of standard solution}}{\text{signal of standard solution}} \text{ x } 100$$

The ME was evaluated in pollen and honeybee samples at two concentration levels (10 and 50 μg kg^{-1}). As shown in **Table 7,** some analytes presented a negligible ME (<|20%|), particularly in honeybee samples. However, higher signal suppression (i.e., ME below 0%) was observed for most analytes in pollen samples. Nevertheless, procedural calibration curves were established for both matrices to compensate both ME and losses during the sample treatment procedure.

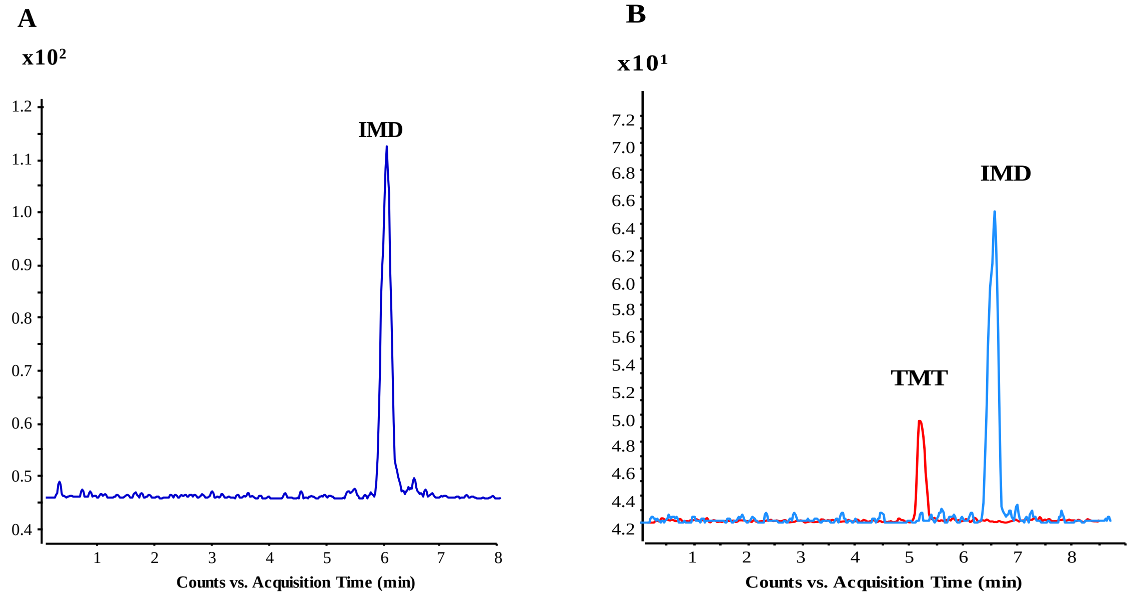
Table 7. Matrix effect and recovery studies of the proposed method for the determination of NNIs and boscalid in pollen samples by MEKC-MS/MS.

	Matrix Effect (%, n=9)		**Recovery (%, n=9)**	
	10 μg kg^{-1}	**50 μg kg^{-1}**	**10 μg kg^{-1}**	**50 μg kg^{-1}**
		Pollen samples		
DNT	-15.4	-11.3	80.1	85.5
TMT	-21.4	-19.6	87.3	90.1
FCM	-22.0	-18.7	86.1	88.2
CLT	-33.7	-30.1	80.8	83.9
NTP	-17.9	-16.7	70.6	74.2
IMZ	-16.8	-16.2	85.4	86.4
IMD	-41.9	-38.4	91.5	94.2
TCP	-42.8	-37.2	80.5	85.9
ACT	-37.6	-34.7	92.6	95.2
BCL	-70.1	-66.1	75.2	79.4
		Honeybee samples		
DNT	-21.2	-15.7	81.6	87.8
TMT	-10.6	-4.8	85.6	85.1
FCM	-10.2	-17.0	89.3	90.8
CLT	-27.1	-14.8	85.1	87.0
NTP	-19.4	-17.9	82.7	81.3
IMZ	-9.2	-16.8	86.4	87.9
IMD	-35.4	-15.5	83.3	90.5
TCP	-28.2	-19.5	81.9	83.5
ACT	-18.7	-26.3	87.4	90.8
BCL	-28.1	-19.5	80.9	84.5

5. ANALYSIS OF REAL SAMPLES

Three pollen samples collected from almond blossoms in three different locations and one sample of honeybee bodies (found dead under suspicious circumstances since hundreds of these specimens died suddenly in the same area) were analyzed in triplicate in order to demonstrate the applicability of the validated method. Both sampling points (pollen and honeybees) were less than 100 m apart from each other.

The criteria set for the positive identification of NNIs in the samples was that a peak should have a S/N ratio of at least 3 and the relative ion intensities for detection and quantification ions must correspond to those of these ions in the solutions of standards. Thereby, samples which met these requirements and also exceeded the corresponding LOQs, were considered as positives. Hence, the results revealed that imidacloprid was found in two of the three analyzed pollen samples, in concentrations of 61.2 µg kg^{-1} (1.7 % RSD, n= 3) and 20.1 µg kg^{-1} (0.9% RSD, n=3), respectively (**Figure 8A,B**). The first sample exceeded the "limit of analytical determination" established for this compound in honey and other apiculture products (50 µg kg^{-1}). In addition, thiamethoxam was also found in the second sample with a concentration of 10.7 µg kg^{-1} (1.1 % RSD, n= 3). The results also indicated that honeybees were contaminated with 8.4 µg kg^{-1} (0.7 % RSD, n=3) of imidacloprid (**Figure 8C**). These results suggest that some NNIs could have been applied as a control insecticide in near agricultural fields leading to the presence of residues in the pollen of almond blossoms. Additionally, the presence of imidacloprid in honeybee samples suggests that honeybees could have been in contact with this insecticide despite of being banned for autdoor uses. This fact suggests a possible causal link between the presence of this insecticide and the death of the honeybees analyzed in this study.



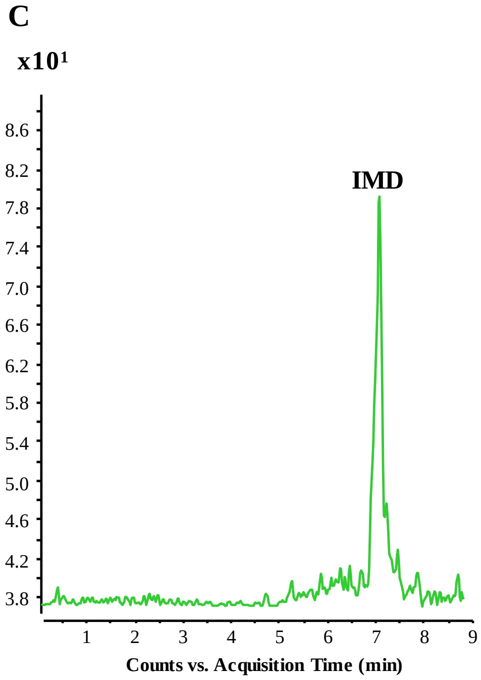


Figure 8. Electropherograms of naturally contaminated samples of pollen: **A)** IMD (61.2 µg kg¹); **B)** IMD (20.1µg kg-1) and TMT (10.7 µg kg-1), and honeybees **C)** IMD (8.4 µg kg-1).

6. CONCLUSIONS

To the best of our knowledge, this is the first time that MEKC coupled to tandem MS detection has been applied for monitoring NNIs and boscalid. The proposed MEKC-MS/MS method offers shorter analysis time, higher resolution and higher selectivity and sensitivity than the only previous method for the control of NNIs in beeswax using CZE-MS [8]. Furthermore, MEKC enables an on-line pre-concentration strategy such as sweeping that made possible to achieve SEFs between 1.6 and 5.6 for the studied compounds. Regarding sample treatment, a scaled-down QuEChERS has been optimized. Different dispersive sorbents were evaluated and Z-Sep+, although less commonly employed than C18 and PSA, provided better results in terms of matrix effect. In addition, unlike traditional QuEChERS methods, sample is not diluted, which improves method sensitivity. LOQs in the range of low μg kg^{-1} were obtained for pollen and honeybee samples. The usefulness of the proposed method was proved by its application to pollen and honeybee samples suspected of being contaminated. Results suggest that NNIs could be the reason of the sudden death of hundreds of honeybees close to a field of almond trees. This method is also in compliance with the principles of green analytical chemistry, combining the low solvent consumption during sample treatment with the reduced volume of BGE used in CE and the lower waste production. In addition, this method involves a low amount of sample and lower cost than LC methods. To conclude, the proposed scaled-down QuEChERS-MEKC-MS/MS method can be a real alternative to LC methods to monitor NNIs and boscalid in pollen and honeybee samples.

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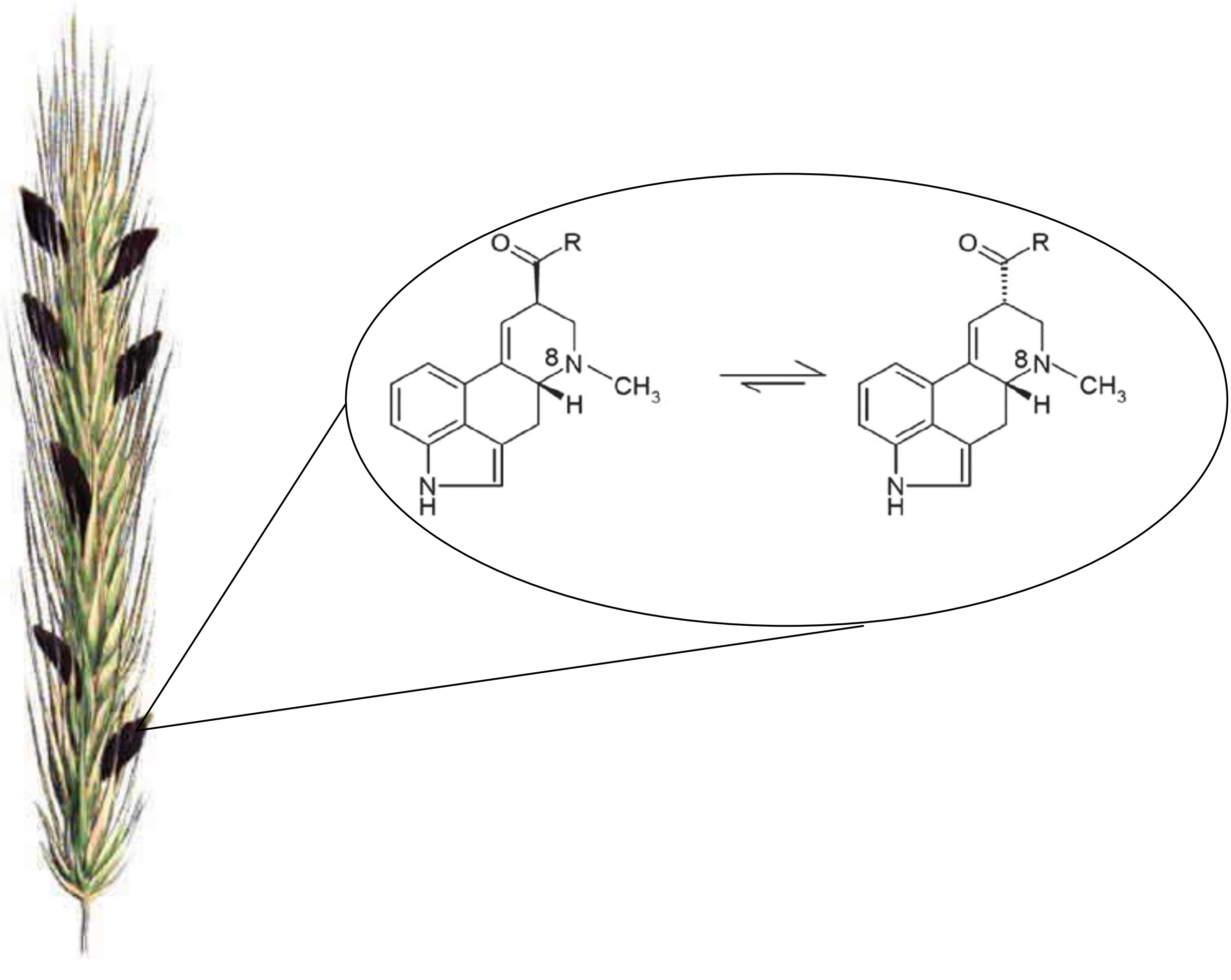
PART II

DETERMINATION OF ERGOT ALKALOIDS

- **Introduction**
- **Chapter 6**
  Determination of the main ergot alkaloids and their epimers in oat-based functional foods by ultra-high performance liquid chromatography tandem mass spectrometry
- **Chapter 7**
  Occurrence of ergot alkaloids in barley and wheat from Algeria
- **Chapter 8**
  Ion mobility-mass spectrometry to extend analytical performance in the determination of ergot alkaloids in cereal samples

INTRODUCTION



1. ERGOT ALKALOIDS

Ergot alkaloids (EAs) are mycotoxins produced mainly by fungi of the *Claviceps* genus, most notably by *Claviceps purpurea*, which can parasitize over 600 monocotyledonous plants of the families Poacea, Juncaceae and Cyperaceae, including grasses, rye, triticale, wheat, oat and barley [1]. Fungal infection starts in spring during flowering when the ascospores are transported by the wind landing in susceptible host plants. Fungal hyphae invade the ovule of the host plant and colonize the whole ovary from the base upwards. Infected florets produce masses of spores that are exuded into a syrupy fluid (honeydew). Insect vectors, rainplash or head-to-head contact transfer this contaminated honeydew to other booming florets provoking the spread of the ergot fungi. After 3-4 weeks, the growing grain or seed is replaced with fungal structures known as sclerotia that contain alkaloids substances. In winter the mature pigmented sclerotia, which has a dark brown color and horn-shaped, falls and overwinter in the ground producing ascospores, and thereby, completing the ergot cycle (**Figure 1**) [1,2].

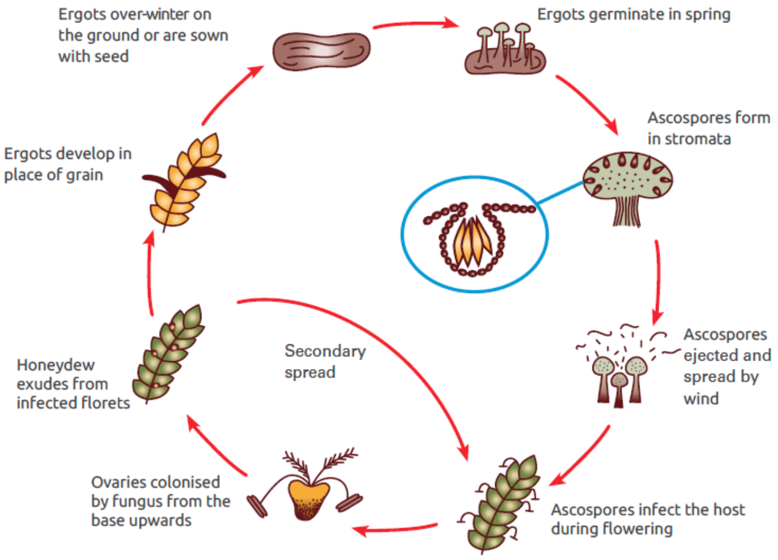


Figure 1. Ergot cycle [3]

The sclerotia can be harvested together with the cereals or grass contaminating the cereal-based foods and feeds. The ingestion of these contaminated products might cause intoxications in humans and animals leading to the illness known as *ergotism* characterized by symptoms such as abdominal pain, vomiting, insomnia and hallucinations. In other cases, the symptoms are related with a pronounced peripheral vasoconstriction of the extremities, burning sensation of the skin and gangrenous limbs (usually feet), which can provoke the loss of it or at worst, the death of the individual [4,5]. This illness appeared in Middle Ages when several epidemics of ergotism (e.g., St. Antony's fire) devastated continental Europe due to the ingestion of contaminated flour, grain or bread [6,7]. In modern times, with the use of grain cleaning equipment such as sieves and separators during the harvesting process, the risk of epidemic has been avoided. Nevertheless, although improvements in agricultural practices and milling techniques have significantly reduced the presence of EAs, being possible to remove up to 82 % of them, EAs can still be found in cereal-based foods and feeds. The cleaning process is not reliable when the sclerotia and the grains are similar in size. This fact can be due to the possible sclerotia breaking into small fragments during the harvest process, or because the sclerotia grew up in dry conditions, having therefore, a smaller size than usually [8].

In 1920, ergotamine was the first EA reported in the literature and used for therapeutic purposes. Since then, more than 80 EAs have been found and determined having as common structural feature, the ergoline ring with a nitrogen atom at position 6. EAs are classified into four biogenetically related classes based on the substitutions at C8 and the structure of D-ring in the tetracyclic ergoline ring system: clavine-type alkaloids, simple lysergic acid derivatives or ergoamides, ergopeptines, and ergopeptams [9].

The high variability of EAs involves a wide range of physicochemical properties although most of them are soluble in various organic solvents and insoluble or only slightly soluble in water. Moreover, EAs are neutral at higher pH values and positively charged at N6 in acidic solutions [2]. Apart from the toxics effects above-mentioned, due to the potent bioactivity of EA, they have also valuable pharmacological properties. Since three important neurotransmitters such as noradrenaline, serotonin, or dopamine (5-hydroxytryptamine, 5-HT) are derived from aromatic L-amino acids, they present similarities with the D-lysergic acid ring system mapping almost entirely onto its structure. For this reason, EAs can interact with the receptors for these neurotransmitters involving pharmacological properties, which are mediated by them [10,11]. These properties include uterotonic action or vasoconstriction, indirect peripheral effects as serotonin antagonism or adrenergic blockade, and central nervous effect as induction of hypothermia and emesis or control of the secretion of pituitary hormones [**2**,12]. However, due to the unpredictable side effects and high instability of EAs, their medical applications have been reduced and replaced by synthetic safer analogs.

The biosynthetic pathway to generate EAs depends on the producer fungi [13]. For instance, the main EAs produced by *Claviceps* species are ergometrine (Em), ergotamine (Et), ergosine (Es), ergocristine (Ecr), ergokryptine (Ekr), and ergocornine (Eco) and their corresponding epimers; ergometrinine (Emn), ergosinine (Esn), ergotaminine (Etn), ergocorninine (Econ), ergokryptinine (Ekrn) and ergocristinine (Ecrn) (**Figure 2**), defined by the European Food Safety Authority (EFSA) as among the most common and physiologically the most active [22]. In general, EAs suffer for a rapid epimerization with respect to the center of symmetry at C8, resulting in the right rotating (8S) and left rotating (8R) isomers. They can epimerize from R to S forms and vice versa, especially in strong aqueous acidic or alkaline solutions [1, 8]. Although the ratio of epimerization depends mainly on the nature of the amide substituent, this process is enhanced by exposure to light or prolonged storage. As in nature, the epimers always are together the main EAs, it is important to consider both main EAs and their epimers when the EA contamination level of a cereal or cereal product has to be determined.

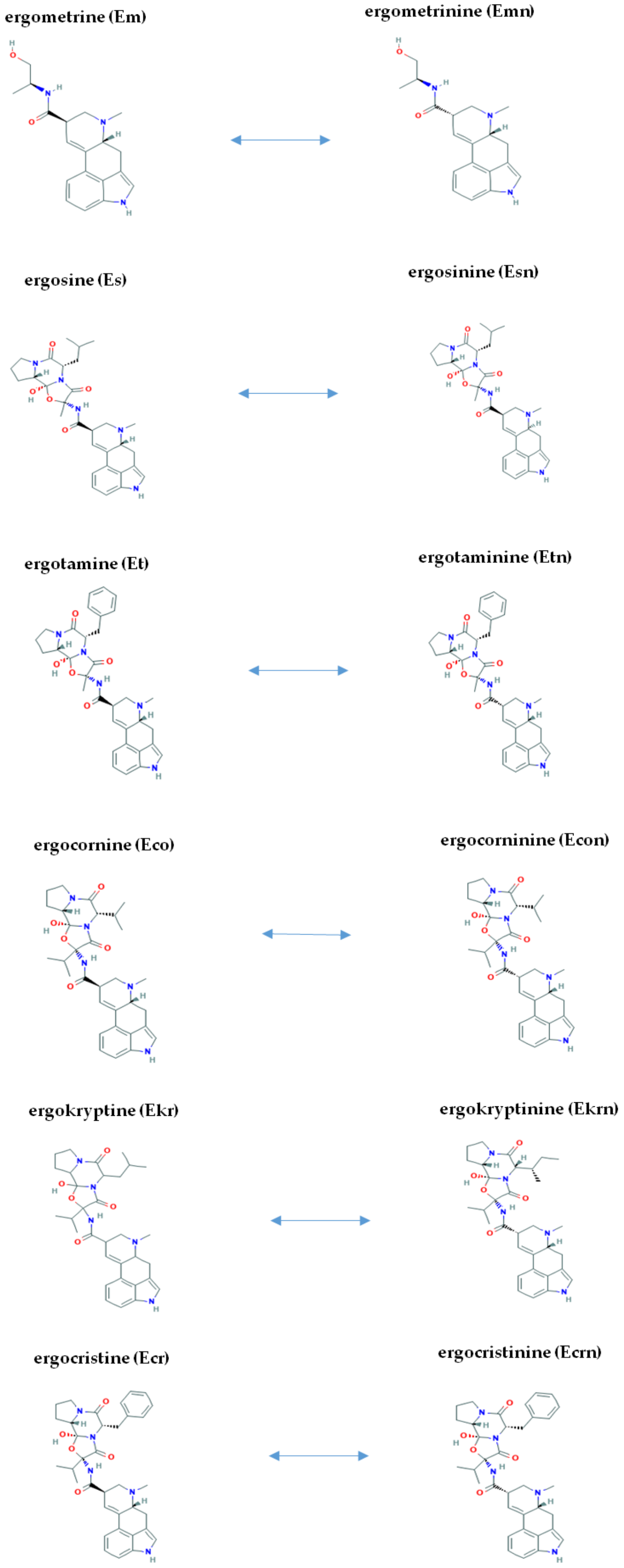


Figure 2. Chemical structures of the main EAs and their epimers.

However, although the European Commission has established a maximum content of 0.5 g kg^{-1} of ergot sclerotia in unprocessed cereals (with the exception of corn and rice) [14], no regulatory limits for either sclerotia or EAs have been set for grain intended for human consumption. In the meantime, a tolerable daily intake (TDI) for total EAs of 0.6 μg kg^{-1} of body weight/day was an acute reference dose of 1 μg kg^{-1} body weight suggested by EFSA [15] and it is likely that limits for EAs will be included in future mycotoxin legislation.

Moreover, food supplements (that is, products intended to provide a concentrated source of nutrients and other substances), including cereal extracts or cereal grass juices in their composition, are available on the market. These products can also suffer from fungi and mycotoxin contamination, and this aspect must be a matter of concern, as highlighted in several research works and reviews [16,17,18,19]. The EFSA has specifies that, regarding to mycotoxin contamination, herbal products used in food supplements must comply with the current food legislation of the EU [20]. In addition, EFSA has stated that is necessary paying special attention to processed foods [15]. However, to the best of our knowledge, the presence of EAs in these kinds of cereal-derived products has not been studied to date. Therefore, in **Chapter 6**, a method based on UHPLC-MS/MS to determine the main EAs and its epimers in oat-based functional foods have been developed [21].

Regarding to the determination of the contamination rate of EAs, according to EFSA, physical techniques are often inaccurate as size, weight, and composition of the sclerotia may vary considerably, so it was encouraged the use of chemical analysis instead [22]. However, the total content of EAs in sclerotia show large differences in the patterns of EAs produced depending on several factors such as the maturity of the sclerotia, the fungal strain, the host plant, the geographical region and weather conditions [23, 24, 25,26]. For that reason, EFSA has stated that it is necessary more analytical data in order to define the variability of EAs patterns in European food and feed commodities [15]. In this regard, recently, more works evaluating the presence of EAs in European foods and the dietary exposure to human and animals have been reported [5,27,28]. Nevertheless, to the best of our knowledge, there are no reports about the occurrence of EAs in African countries. Therefore, in **Chapter 7**, the EA contamination of cereals destined for human consumption in Algeria has been evaluated. This country has been considered because of the extensive consumption of cereals by Algerian population, the available separation techniques shortage for cleaning the sclerotia, the dry weather that favor the formation of these structures and the lack of food control and legislation established.

EFSA have also recommended that more analytical methods with the appropriate sensitivity should be used to reduce the uncertainty associated with the different occurrence scenarios and, consequently, with dietary exposure estimations. Moreover, they remark that the collection of analytical data on EAs in relevant food and feed commodities should continue, with special attention to processed foods [5].

Consequently, considering all of the above-mentioned aspects, in order to protect consumer health, the development of sensitive analytical methods for the determination of the main EAs in cereals and cereal-based products (including food supplements) is of great importance. In this framework, different analytical methodologies have been evaluated demonstrating their applicability to a high variety of cereal-based foods.

2. ANALYTICAL METHODS FOR THE DETERMINATION OF EAs

Numerous analytical methods have been proposed for the determination of EAs, particularly considering the major EAs and their corresponding –inine forms, from diverse matrices in pharmaceutical, forensic and food areas.

High-performance liquid chromatography (HPLC) coupled to fluorescence detection (FLD) or tandem mass spectrometry (MS/MS) have been the most widely used techniques; however, capillary electrophoresis (CE), immunoassays and gas chromatography (GC) have been also reported [2].

Normally, the analysis of contaminants and residues requires a cleaning step in order to minimize matrix interferences without entailing a loss of target compounds at trace levels. This is crucial in the food safety field where high selectivity and sensitivity are demanded to allow the determination of target compounds in matrices with high complexity. In this respect, the sample treatments chosen for EAs isolation involves liquid-liquid extraction (LLE), dispersive solid-phase extraction (d-SPE) or solid-phase extraction (SPE), as clean-up strategies, after a solid- liquid extraction (SLE) of the sample. The SLE is needed due to the nature of the samples, i.e., cereals or cereal-based products. In addition, in some cases, these techniques also allow the preconcentration of the analytes being helpful to reduce the LODs of EAs since a harmonized legislation is not yet established for them.

Taking into consideration the EFSA scientific opinion on EAs in food and feed, and the recent works reported including EAs determination, the most relevant contributions about EAs analysis will be commented in next sections.

2.1 Liquid chromatography methods

Recent reviews of available analytical methods for the determination of EAs show that the most frequently employed technique is LC, particularly HPLC [29,2]. **Table 1** summarizes the most relevant methods reported using this technique as well as their main characteristics.

Reverse phase-based chromatography is the mode of choice for the separation of EAs, mainly using C8 and C18 columns. Regarding to the mobile phase, systems of methanol-water or acetonitrile-water have been reported. Alkaline mobile phases are preferred to maintain the stability of both epimers, to avoid protonation and to improve separation. Thus, ammonium hydroxide, ammonium carbonate, ammonium carbamate or triethylamine have been used to provide alkaline pH conditions [29]. In addition, although isocratic mode was used in first attempts [30], recently, gradient separation mode has been selected to improve the separation resolution among epimers.

The sample solvents have been also studied in order to avoid the epimerization of the EAs. In this regard, it was noticed that the epimerization is minimal when standard solutions are prepared in aprotic solvents such as chloroform or acetonitrile [31], however, the use of a sample solvent stronger that the mobile phase leads to peak distortion [32]. Thus, the sample solvent may have a similar composition that the starting mobile phase.

Regarding to the detections modes, ergopeptines and ergopeptinines can both be measured with ultraviolet (UV) detector [30]. However, due to the native fluorescence attributed to most EAs, FLD has been used instead offering an enhancement in sensitivity and selectivity if compared to LC-UV methods [33,34,35]. The 12 major EAs can be separated by HPLC-FLD with sufficient chromatographic resolution with typical run times around 40 min [36]. However, when it is not possible and they co-eluted, especially in the case of Ecr and Ekr, and their corresponding epimers, they may be reported as single compounds [29]. Recently the use of an internal standard such as lysergic acid diethylamide (LSD) for EA quantification by LC-FLD was reported for the first time in order to improve accuracy and precision of the method [37].

LC coupled to mass spectrometry (LC-MS) and LC tandem MS (LC-MS/MS), have been mainly employed for the quantification of EAs due to its capacity for an unambiguous compound identification. In particular, determination by HPLC-MS/MS with electrospray ionization operated in the positive mode (ESI^+) as the preferred ionization source. ESI^+ provides more intense ions (protonated molecular ions $[M+H]^+$) than the obtained in negative mode (deprotonated molecular ions ($[M-H]^-$)[38]. Moreover, ESI and atmospheric pressure chemical ionization (APCI) were compared for the analysis of main EAs. The use of ESI resulted in a slight signal suppression while using APCI a very high signal enhancement for most EAs was observed, so ESI was finally selected [39]. Then, ESI and atmospheric pressure photoionization (APPI) were also compared for the analysis of lysergic acid amide (LSA) and Em in grass samples, obtaining similar results [40].

Most of the methods use triple quadrupole [38,39,40] or less frequently, ion trap (IT) [41] as mass analyzers. In quantitative analysis the selection and monitoring of relatively intense and specific fragment ions in LC-MS/MS is used to both confirm the identity of the EA, and confirm the quantification. Thus, the ratios of the selected fragment ions (quantification and identification ions) should be equal to the ratios for the same ions in standard solutions. Detailed information about the known and postulated fragmentation routes for EAs has been provided. Specific transitions typically used include $[M+H]^+ \rightarrow m/z = 268$, 223 and/or 208. The fragment ion with m/z=223 is frequently used as quantifier ion while the fragment with m/z=208 is used as qualifier. For Ekr and Ecr the transition of $[M+H]^+$ to m/z 268 as qualifier is more appropriate [29,42,43].

High resolution MS (HRMS) instruments, such as Orbitrap or Time of Flight (TOF) MS analyzers, provide a low level of background noise, improving the S/N ratio and therefore, the sensitivity of the method. HRMS is becoming more and more popular for identification purposes in natural products analysis and very useful for retrospective studies in order to look for novel EAs or metabolites [44]. In this regard, Arroyo-Manzanares *et al*. developed a method based on HRMS and IT-MS technology for the study of the fragmentation pattern of EAs and established a simple strategy for the identification of novel EA derivatives [43]. Moreover, a modern hybrid Q-TOF-MS instrument was used to provide simultaneously a quantitative analysis of common EAs and the screening, detection, and identification of unexpected or novel EAs in rye samples [45].

Due to the lack of available standards, most of the reported LC–MS/MS methods have been developed for the determination of the six major EAs and their corresponding epimers. However, more recently, studies involving a higher number of EAs have been also reported [46,27]. Furthermore, EAs are increasingly being incorporated into multi-mycotoxin methods which are very useful to estimate absolute mycotoxin concentration and human and animal exposure to them. Nevertheless, unspecific sample treatment may be used which can lead to poor recovery of some compounds. To overcome this drawback, very high sensitivity techniques should be selected for detection. Therefore, multi-mycotoxins methods including EAs and using the UHPLC-Orbitrap MS methodology have been reported to determine four different groups of mycotoxins in cereal-based products [47] as well for the analysis of 32 mycotoxins in beer samples [48].

Regarding sample treatments used prior the analysis of EAs by LC coupled to FLD and MS detection, SPE and QuEChERS have been mostly employed. The selection of a proper extraction solvent as well as the clean-up strategy used allows minimizing the epimerization of the EAs during sample treatment procedure. In addition, one on the main drawbacks of MS detection is the matrix effect (signal suppression/enhancement) due to matrix components that can be significantly reduced by the application of the proper sample treatment.

2.1.1 Solid-phase extraction

EAs have been extracted from dry samples such as sclerotia, grasses, cereals or grain-based foods and feeds using either non-polar organic solvents under alkaline conditions or with polar solvents under acidic conditions. In 2008 Krska *et al*., proposed for the first time the mixture of MeCN-alkaline aqueous solvent in the ratio 84:16 (v/v) for extraction of EAs from several cereals and foodstuff samples. The extraction efficiency of this mixture was compared with acidic mixture MeOH-0.25% H_3PO_4 40:60 and with neutral MeCN-ammonium acetate 1:2, obtaining the highest extraction recoveries when MeCN-alkaline aqueous was employed [49]. Since then, this extraction mixture has been widely applied for the extraction of EAs from different cereal matrices such as rye-based food products (flour, bran and flakes) [41], rye and wheat flour, bread and noodles [46], wheat and rye [50], and more recently, in feed samples [51].

The extracts obtained have been subsequently treated to separate the EAs from other matrix components extracted by the solvent. This clean-up step offer several advantages such as to reduce the compounds that will arrive to the column, and therefore, to the detector affecting the chromatography separation and the sensitivity, respectively. Moreover, with this step a preconcentration of the target analytes can be also possible as well as the change of the sample injection solvent if necessary.

This clean-up step has been widely carried out by the application of SPE, especially before HPLC-FLD analysis. Different cartridges including C18 reversed phase, Hydrophilic-Lipophilic Balance (HLB), strong cation exchange (SCX), and mixed-mode cation exchange (MCX) cartridges have been evaluated. For instance, the main 12 EAs were extracted under basic conditions from rye and rye products followed by a SPE using alumina cartridges prior their determination by HPLC- FLD method [35]. SCX was successfully applied after a SLE under acidic conditions from rye flour [33] and cereals for animal feed [52]. Köppen *et al*., proposed an improved SPE method for the clean-up of rye flour and wheat germ oil based on sodium-neutralized strong cation exchange (Na+-SCX) where EAs (in their protonated form) were eluted from the column by forming ion pairs with sodium hexanesulfonate, delaying epimerization for over 96 h [36]. C18 cartridges have been also evaluated [38]. However, poor recoveries were obtained using HLB cartridges [32]. In addition, molecularly imprinted polymers (MIPs) have been developed for use in SPE for cleaning-up barley samples prior LC-MS/MS. Metergoline was used as template in the production of suspension polymerized beads used as selective sorbent for EAs, obtaining recoveries between 65 % and 79 % [53]. A selective SPE column commercially available for EAs known as MycoSep® 150 Ergot has been also applied for the clean-up of EAs prior their determination by UHPLC-MS/MS [50].

A neutral alumina-based SPE was selected for EA clean-up in rye-based food products and ergot sclerotia isolated from rye grains prior their analysis by LC–IT–MS. In this case, the clean-up step removed the problems related with matrix ions that may easily degrade the performance of the IT [40]. In a comparative study of different samples treatments including LLE, d-SPE using primary secondary amine (PSA), and SPE, different sorbents, such as SCX, MycoSep® Ergot multifunctional, and MIP, were evaluated. As a result, MycoSep® and SCX cartridges were useful in minimization signal suppression.

2.1.2 QuEChERS procedure

QuEChERS has been the method of choice when multi-analyte mycotoxins determination is carried out. The inclusion of very many analytes of different structure into one method means that compound-specific clean-up step cannot be used. Thus, to perform multi-mycotoxin determinations the sample preparation procedure must be as basic and simple as much as possible even although it might lead to poor recovery of some compounds. A QuEChERS based method was first applied to EAs in a multi-toxin analysis developed by Malachova *et al.*, for cereal-based products [47]. Recoveries of Eco, Ecr, Ekr, and Es ranged from 60 to 70 %.

A QuEChERS method was preferred for multi-toxin analysis after its comparison with other three different extraction procedures for the simultaneous determination of 32 mycotoxins including EAs in barley. The compared methods were a modified QuEChERS procedure, matrix solid-phase dispersion (MSPD), SLE and SPE. The extracts were then analysed by UHPLC-Orbitrap mass spectrometry and recoveries over 60 % for most mycotoxins were achieved when using the QuEChERS method. In addition this method could easily be modified and adapted [54].

In addition, a QuEChERS-based extraction has been proposed as sample treatment for the determination of ergovaline in tall fescue seed and straw followed by HPLC–FLD determination. In this work, 14 extraction solvents were tested selecting ammonium carbonate/MeCN (50/50, v/v) since it provided the highest and most consistent recovery (91–101 %). Moreover, this method did no require a clean-up step, eliminating the need for halogenated/chlorinated solvents in contrast with the sample treatment typically used for this kind of samples based on the use of Ergosil® silica gel SPE (a modified silica gel designed for the analysis of ergopeptine) [55].

In order to estimate absolute mycotoxins concentration and animal exposure to them, a broad spectrum of mycotoxins including the 12 main EAs was analyzed in a large set of feeding stuffs (non-fermented or fermented feeding stuffs, feeding stuff supplements, and complex compound feeds) using a QuEChERS previous to the analysis by UHPLC–MS/MS. [56] The QuEChERS procedure involved a d-SPE clean-up step using C18 as dispersive sorbent after the extraction with MeCN.

This sorbent have been also employed in other QuEChERS based methods. Guo *et al.*, compared the efficiency of PSA and C18 removing fat, protein and carbohydrates present in the cereal matrix with a one-step cleanup MycoSep 150® Ergot column. Similar recovery results were obtained for the MycoSep 150® Ergot column and the C18 dispersive sorbent. To shorten the cleanup process and reduce the cost of sample preparation, C18 was selected as alternative [46].

Recently, three different methods for the EAs were compared in terms of recoveries and matrix effects, including the method proposed for Guo *et al*., comment before. This method finally selected to be modified in order to improve matrix effects. For that purpose different sorbents such as C18, Z-sep+ and a polymer known as EMR (Enhanced matrix removal) both recommended for high content of fat. Z-sep+ was selected as it provided the best matrix effect for the 12 EAs studied [21].

2.1.3 Other sample treatments

In some cases, the cleaning-up of the extracts is unnecessary. Modern LC-MS/MS instruments have such sensitivity and selectivity that instead of using sometimes complicated techniques to clean up the extract it can be injected directly after considerable dilution to reduce matrix effect (dilute-and-shoot strategy). Rubert *et al.,* observed that the highest recoveries (about 80 to 91 %) were obtained with the SLE method used without SPE clean-up [54]. Usually, the extracts obtained from SLE are dried and reconstituted using LC-MS mobile phase. Before the injection, these extracts are filtered to avoid the obstruction of the system as it was reported for beer samples [48].

An ultra filtration was also done after a SLE using MeOH-acidic conditions for the isolation of 20 EAs from bread samples with different cereal composition. Subsequently, the solvent was directly injected into the LC-MS/MS [57].

For the first time, Planar solid-phase extraction coupled to FLD was applied for the screening of the total EAs content in rye and rye products after a extraction in alkaline conditions [34]. This technique, which was initially developed for pesticide residue analysis, allows to separate co-extracted matrix compounds from target analytes and focusing them into a sharp zone which can then be analysed by high-performance thin-layer chromatography (HPTLC).

2.2 Capillary elecgtrophoretic methods

CE analysis has been scarcely applied for determination of EAs. Most of these works were developed in the 1990s using UV as detection mode and involving just a few of EAs [58,59]. In those years, CE emerged as a promising analytical technique with a high potential for the analysis of a wide range of compounds due to its advantages over LC. Among them, high efficiency, reduced analysis time, and low sample and reagent consumption, have been reported. Nevertheless, a lack of sensitivity has been also reported depending on the analyte, particularly when it is coupled to UV detection.

Frach *et al.*, proposed a capillary zone electrophoresis (CZE) with laser-induced fluorescence (LIF) method to determine EAs from sclerotia samples as an alternative to UV detection. This method involved the use of β- and γ-cyclodextrins, urea and poly(vinyl alcohol) in phosphate buffer at pH 2.5 as background electrolyte (BGE) with a fused-silica capillary at 25 kV. The separation of 9 EAs was achieved improving the LODs about 30-fold if compared with UV [60]. Later on, CZE was also investigated for the separation of lysergic, isolysergic, and paspalic acid in pharmaceutical samples taken during the manufacturing process. [61] The method was carefully optimized in terms of selectivity and analysis time as well as to made it compatible with TOF and UV detection. The optimum BGE was based on MeOH with asparagine, sodium tetraborate, or ammonium acetate at alkaline pH. The LODs were below 0.5 mg L^{-1} and 0.1 mg L^{-1} with UV and TOF detections, respectively. These LODs are in the same range as those of LC-MS/MS-based methods. In these methods, sample treatment was based mainly on SLE using mixtures of different solvents.

However, recently a new sample treatment based on cloud point extraction (CPE) prior to CE-UV for determination of ergotamine and ergometrine in cereal samples has been proposed [62]. The methodology involves extraction under acid conditions and subsequent preconcentration of the analytes using a low volume of a nonionic surfactant as extraction solvent. The surfactant, known as PONPE 7.5 (olyoxyethylene(7.5)nonylphenylether), was able to extract the EA into micelles achiving a preconcentration factor of 22 of total EAs.

2.3 Other analytical methods

Other techniques such as immunoassay methods including Enzyme Linked Immunosorbent Assays (ELISA) and gas chromatography (GC) have also been evaluated for the analysis of EAs.

GC was not very useful for the determination of peptide EAS since they are non-volatile and thermolabile. Thus, most of the applications of GC–MS were published during the 1990s and concern the monitoring of LSD, proposing several derivatization strategies in order to improve volatility and stability and to reduce the peak tailing. On the other hand, ELISA methods were proposed as a rapid and inexpensive alternative for EA determination. ELISA was frequently applied to determine the total EAs [2]. However, these methods are less specific and less accurate than HPLC–FLD or LC–MS methods [29].

Ergot sclerotia can be detected and quantified in cereals using near-infrared (NIR) hyperspectral imaging and multivariate image analysis [63]. Discrimination is based on the fat and starch content ergot bodies, which differ from those of cereals. The method is intended for use in cereal convey or belt systems at industrial level and has identified a sclerotia content of 0.02 %, which is below the European Commission limits of 0.1 % for feedstuffs containing unground cereals, and 0.05 % in "intervention" cereals destined for human consumption.

3. ION MOBILITY

Ion mobility spectrometry (IMS) has been recently emerged as a powerful analytical technique to improve the performance characteristics of LC-MS methods applied in the analysis of residues and contaminants in feed and food-related matrices.

IMS is a gas-phase technique in which ionized molecules are separated based on their mobility in a carrier buffer gas through the drift tube (or mobility cell) under an electric field at atmospheric pressure or near to atmospheric pressure. The mobility of ions depends on their size, shape and charge. Thus, differences in these molecular characteristics lead to faster or slower movement of the ions in the drift tube and allow their separation [64]. Normally, ion mobility is measured in terms of drift time that corresponds with the time that the ions spend travelling through the mobility cell. Nevertheless, the drift time is an instrument dependent parameter, so the reporting of the so-called 'collision cross section' (CCS) is preferred to allow instrument comparison, as it is an intrinsic characteristic of each molecule [65].

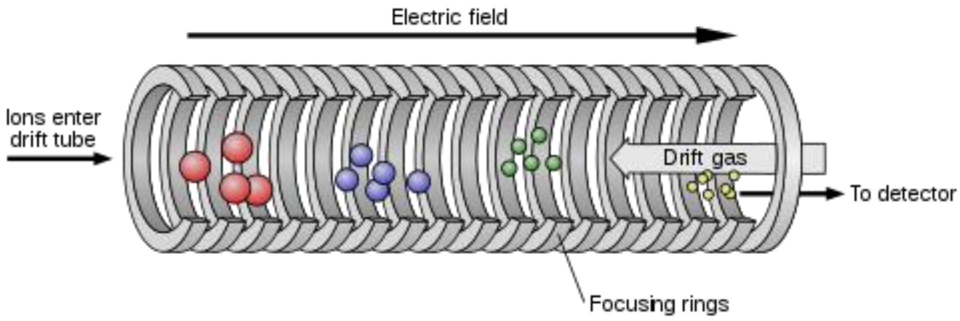


Figure 3. Drift tube ion mobility spectrometer

The CCS provides additional information to retention index and mass spectra, so it can be used as an identification parameter. Thus, the integration of IMS into traditional LC-MS workflows provides an additional separation dimension, improving peak resolution, particularly useful to separate isomeric and isobaric compounds [66,67]. Furthermore, the implementation of IMS in LC-MS methods contributes to reducing background noise, improving signal-to-noise ratio (S/N), and therefore, the sensitivity, providing higher quality mass spectra for compound identification [68]. As a consequence, a growing number of CCS databases have recently been created and are increasingly used for this purpose [69,70,71]. However, there is still a lack of CCS databases for food contaminants and residues. In the last years, several studies addressing the CCS characterization of a great number of mycotoxins have been reported [72,73,74]. Nevertheless, EAs have been scarcely studied by IM-MS [75].

On the other hand, the enhancement in sensitivity provided by IMS hyphenation can be very helpful for the analysis of complex matrices such as food samples. However, to the date, this fact has hardly been exploited for this sort of samples and usually involving the analysis of pesticides. Only few methods have been developed for the analysis of natural toxins in real samples [70]. IMS-MS hyphenation was firstly investigated for the analysis of zearalerone in cornmeal [76], and recently, for the screening of multitoxins in fruits [77], achieving great results in terms of S/N ratio and matrix interferences clean-up.

As it was discussed before, most of the analysis reported for EA analysis have been developed by LC-MS/MS. However, unequivocal identification of major EAs is not easy due to the presence of epimers that have the same accurate mass and lead to the same fragment ions. In addition, they tend to present similar retention times in LC separations, which can lead to their misidentification as they are often found together in naturally contaminated samples. In view of these drawbacks, the implementation of IMS could improve the performance characteristics of current LC-MS methods. Thus, in **Chapter 8** the potential applicability of IMS for the analysis of the main EAs together with their epimers in cereal samples has been evaluated.

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CHAPTER 6

DETERMINATION OF THE MAIN ERGOT ALKALOIDS AND THEIR EPIMERS IN OAT-BASED FUNCTIONAL FOODS BY ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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1. SUMMARY

An ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) method is proposed for the determination of the major ergot alkaloids (EAs): ergometrine, ergosine, ergotamine, ergocornine, ergokryptine, ergocristine, and their epimers; ergometrinine, ergosinine, ergotaminine, ergocorninine, ergokryptinine, and ergocristinine, in oat-based foods and food supplements. A modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) procedure was applied as sample treatment, reducing the consumption of organic solvent and increasing sensitivity. This method involved an extraction with acetonitrile and ammonium carbonate (85:15, *v/v*) and a clean-up step based on dispersive solid-phase extraction, employing a mixture of C18/Z-Sep+ as sorbents. Procedural calibration curves were established, and limits of quantification were below 3.2 μg kg^{-1} for the studied compounds. Repeatability and intermediate precision (expressed as RSD) were lower than 6.3 % and 15 %, respectively, with recoveries ranging between 89.7 % and 109 %. The method was applied to oat-based products (bran, flakes, flour, grass, hydroalcoholic extracts, juices and tablets), finding a positive sample of oat bran contaminated with ergometrine, ergosine, ergometrinine, and ergosinine (total content of 10.7 μg kg^{-1}).

2. EXPERIMENTAL

2.1 Materials and reagents

All reagents were of analytical reagent grade and solvents were LC–MS grade. Acetonitrile (MeCN), methanol (MeOH), and ammonium carbonate were obtained from VWR (Barcelona, Spain). Formic acid eluent additive for LC–MS was supplied by Sigma Aldrich (Madrid, Spain). Z-Sep+ sorbent for clean-up was obtained from Supelco (Bellefonte, PA, USA), while C18 and PSA sorbents were supplied by Agilent Technologies (Madrid, Spain). Ultrapure water used throughout the work was obtained from a Milli-Q water purification system (18.2 MΩ cm, Milli-Q Plus system, Millipore Bedford, MA, USA).

Standards of the ergot alkaloids (EAs) ergosine (Es), ergocornine (Eco), ergokryptine (Ekr), ergocristine (Ecr), and the corresponding epimers, ergosinine (Esn), ergocorninine (Econ), ergokryptinine (Ekrn), ergocristinine (Ecrn), were purchased from Techno Spec (Barcelona, Spain), whereas ergometrine (Em), ergotamine (Et), ergometrinine (Emn), and ergotaminine (Etn) were obtained from Romer Labs (Getzersdorf, Austria). Following the indications of the manufacturer, the standards were reconstituted in 5 mL of MeCN, to achieve concentrations of 500 μg mL^{-1} for the main EAs and of 125 μg mL^{-1} for the epimers. Immediately after this reconstitution, to avoid the rapid epimerization of EAs in the solution, intermediate dried stock solutions were prepared. For that, aliquots of individual or mixed standard solutions were placed into amber glass tubes, evaporated to dryness under a gentle stream of N_2, and stored at −20 °C. These intermediate stock solutions were reconstituted in the required amount of MeCN just before use.

2.2 Instrumentation

UHPLC–MS/MS experiments were performed in an Agilent 1290 Infinity LC (Agilent Technologies) coupled to an API 3200 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with electrospray ionization (ESI). The chromatographic separation was carried out using an Agilent Zorbax Eclipse Plus RRHD C18 column

(50 × 2.1 mm, 1.8 μm). Analyst software (Version 1.6.3, AB Sciex) was used for acquisition and data analysis.

During the sample treatment procedure, a high-speed solid crusher (Hukoer, China), an evaporator system (System EVA-EC, from VLM GmbH, Bielefeld, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), and a universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) were used.

2.3 Chromatographic separation

The chromatographic separation of EAs was carried out using a C18 Zorbax Eclipse Plus RRHD column (50 × 2.1 mm, 1.8 μm). The mobile phase consisted of 0.3% formic acid aqueous solution (solvent A) and MeOH with 0.3% formic acid (solvent B) at a flow rate of 0.4 mL min^{-1}. The eluent gradient profile was as follows: 0–6 min 30–60% B; 6–9 min 60% B; 9–10 min 60–30% B; 10–12 min 30% B. The column temperature was set at 35 °C and the injection volume was 5 μL. In order to minimize epimerization, the injection sample sequence was limited to 12 h. Moreover, control standard solutions of EAs were injected at the beginning, middle, and end of each analysis sequence.

The mass spectrometer operated in the positive electrospray ionization (ESI+) mode under multiple reaction monitoring (MRM) conditions, shown in **Table 1**. MS parameters for the analysis were established as follows: temperature of the source 500 °C; collision gas (nitrogen) 5 psi; voltage of the ion spray 5 kV; curtain gas (nitrogen) 30 psi; nebulizing gas (GAS 1), and drying gas (GAS 2), in both of them, nitrogen was set at 50 psi. In all cases, a precursor ion and two product ions were studied. The monitored ions were the protonated molecules $[M + H]^+$, except for Esn, Etn, Econ, and Ecrn, where $[M - H_2O + H]^+$ ions were monitored.

Under optimum conditions, EAs and their epimers were separated and detected in less than 7 min.

Table 1. MS parameters for the different target analytes studied in the proposed UHPLC-MS/MS method.

	Precursor ion (m/z)	Molecular ion	DP[a]	EP[a]	Product ions[b]	CE[a]	CXP[a]
Em	326.0	$[M+H]^+$	51	5.0	223.0 (Q)	23	4.0
					208.1 (I)	37	4.0
Emn	326.0	$[M+H]^+$	46	6.0	208.1 (Q)	39	6.0
					223.1 (I)	33	6.0
Es	548.2	$[M+H]^+$	61	5.0	208.2 (Q)	57	4.0
					223.1 (I)	45	8.0
Esn	530.2	$[M-H_2O+H]^+$	66	6.5	223.2 (Q)	37	6.0
					263.1 (I)	33	6.0
Et	582.2	$[M+H]^+$	56	7.0	208.2 (Q)	55	4.0
					223.2 (I)	45	4.0
Etn	564.2	$[M-H_2O+H]^+$	66	6.0	223.0 (Q)	41	6.0
					297.1 (I)	33	6.0
Eco	562.2	$[M+H]^+$	46	4.5	268.1 (Q)	33	6.0
					208.2 (I)	55	4.0
Econ	544.2	$[M-H_2O+H]^+$	61	8.5	277.1 (Q)	31	6.0
					223.1 (I)	37	6.0
Ekr	576.2	$[M+H]^+$	86	6.0	208.3 (Q)	59	6.0
					268.1 (I)	31	6.0
Ekrn	576.2	$[M+H]^+$	36	7.0	223.0 (Q)	45	6.0
					558.0 (I)	21	6.0
Ecr	610.2	$[M+H]^+$	56	6.5	268.2 (Q)	35	6.0
					208.1 (I)	57	6.0
Ecrn	592.2	$[M-H_2O+H]^+$	71	7.5	305.1 (Q)	33	4.0
					223.2 (I)	39	6.0

[a] Declustering potential (DP), Entrance potential (EP), Collision Cell Exit Potential (CXP) and Collision Energy (CE). All expressed in voltage.

[b] Product ions: (Q) Transition used for quantification, (I) Transition employed to confirm the identification.

2.4 Modified QuEChERS procedure for the analysis of oat-based products

A total of 25 oat-based samples, including some food supplements, were randomly purchased from different local markets in Granada (Spain). Oat-based products can be found in different presentations, so the following products were selected: oat bran (11), flakes (5), flours (2), hydroalcoholic extracts (2), juices (2), tablets (2), and grass (1). In order to obtain representative samples, several portions were taken from each unit, being thoroughly mixed. Samples were milled (when necessary) and/or homogenized and stored at room temperature prior to the extraction step.

Briefly, a portion of 1.0 g of the homogenized sample (bran, flakes, flour, tablets, and grass) was placed into a 50-mL falcon tube with a conical bottom, and then 4 mL of MeCN and 5 mM ammonium carbonate (85:15, *v/v*) were added. The mixture was shaken by vortex for 1 min, and afterwards, the sample was centrifuged at 9000 rpm for 5 min at 4 °C. Subsequently, the whole upper layer was collected and placed into a 15-mL falcon tube containing 150 mg of a mixture of C18:Z-Sep+ (1:1) as dispersive sorbent for the clean-up step. Then, the 15-mL tube was vigorously shaken for 1 min and centrifuged at 9000 rpm for 5 min at 4 °C. Finally, the upper layer was fully transferred to a glass tube and evaporated to dryness under a gentle stream of N_2. The residue was reconstituted with 750 μL of a mixture of MeOH:water (50:50, *v/v*) and passed through a 0.22 μm nylon membrane filter before injection into the UHPLC–MS/MS system. The optimized sample procedure for the analysis of the above-mentioned oat-based products is shown in **Figure 2.**

“Direct-injection” and “dilute-and-shoot” methods were used in the case of hydroalcoholic extract samples and juice samples, respectively, as no extraction or clean-up steps were required.

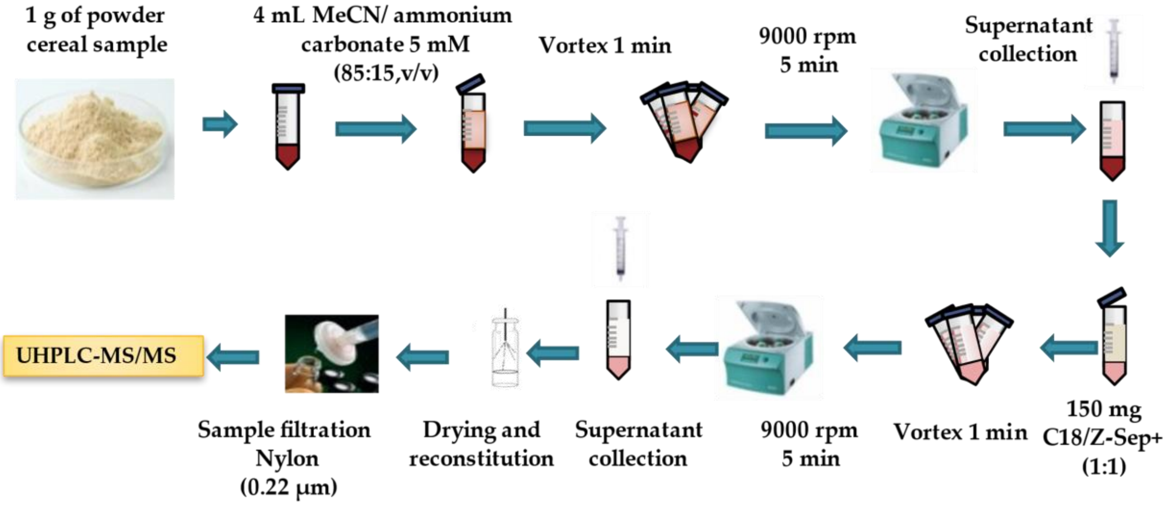

Figure 2. Modified QuEChERS procedure for the analysis of oat-based products by UHPLC-MS/MS.

3. RESULTS AND DISCUSSION

3.1 Optimization of sample treatment

In this work, a QuEChERS procedure reported by Guo et al. for the analysis of EAs in other cereals samples was modified [1] and applied for the analysis of bran, flakes, flour, tablets and grass oat samples. Oat flakes samples were chosen as representative matrix for the optimization of the method. Some important improvements, in terms of reduction of organic solvent consumption and a more efficient clean-up of the matrix, were achieved. Firstly, the volume of the extraction mixture (MeCN: 5 mM ammonium carbonate, 85:15, *v/v*) was reduced from 10 to 4 mL, which was enough to extract the studied EAs from 1 g of sample with satisfactory recoveries. In addition, to increase the sensitivity of the method, all the supernatant volume was collected in all steps, in contrast with the standard QuEChERS procedure, in which only a part of the supernatant was considered.

Subsequently, to improve the extraction efficiency and to reduce the matrix effect (ME), different sorbents were tested in the d-SPE step, such as C18, Z-Sep+, primary-secondary amine (PSA), and a mixture of C18/Z-Sep+ (1:1). In all cases, an amount of 150 mg of sorbent was used. In general, the ME was considerably lower when Z-Sep+ was used (a sorbent recommended to remove lipids, fatty acids, and carbohydrates), but the recoveries were negatively affected in most cases in comparison with the other tested sorbents. On the other hand, when C18 or PSA were employed, better recoveries were obtained (the highest values were obtained with C18 in most cases, especially for Em and Emn), but the ME was also significantly higher for all the analytes. In view of these results, a mixture of C18 and Z-Sep+ (1:1) was tested. A compromise between satisfactory values for recoveries and ME of the studied analytes was achieved when this mixture was employed, as can be seen in **Figure 3**. Therefore, it was selected as a dispersive sorbent for further experiments.

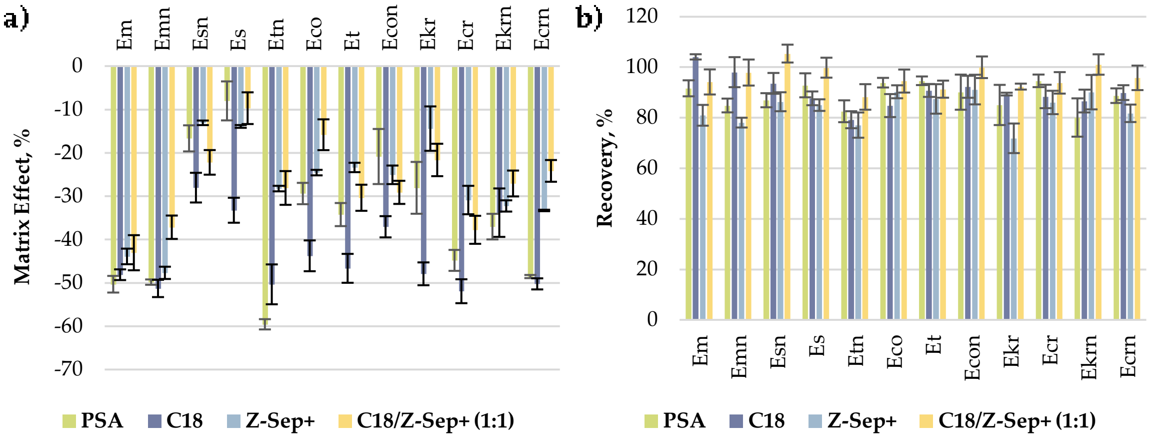

Figure 3. Optimization of the sorbent in the d-SPE step of the modified QuEChERS procedure. Effect of the kind of the dispersive sorbent **a)** on the matrix effect; **b)** on the recovery.

Afterwards, the amount of this mixture was investigated, using 90 and 150 mg. With 90 mg, the ME increased considerably in all cases without affecting the recovery results, meaning that a lower amount of dispersive sorbent was not enough for cleaning-up the extract, so 150 mg of C18:Z-Sep+ (1:1) was selected as optimum (**Figure 4**).

Finally, the extraction time was kept at 1 min to reduce the time of the sample treatment, also preventing epimerization. Moreover, high temperatures in the evaporation step were avoided, and the final extracts were dried at room temperature under a gentle stream of nitrogen.

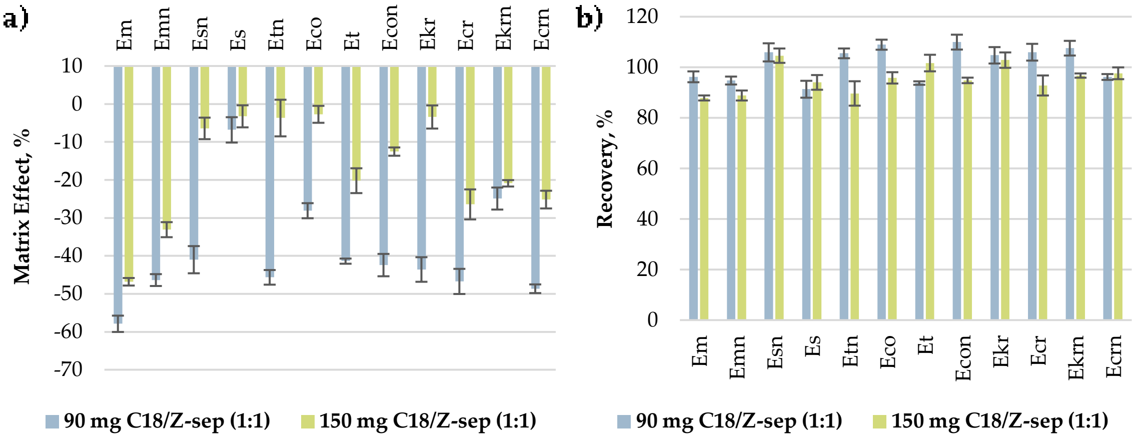

Figure 4. Optimization of the amount of dispersive sorbent Effect of the amount of dispersive sorbent **a)** on the matrix effect; **b)** on the recovery study

A chromatogram of a blank oat flakes sample spiked with EAs at 10 μg kg^{-1}, treated and analyzed under optimum conditions is shown in **Figure 5.**

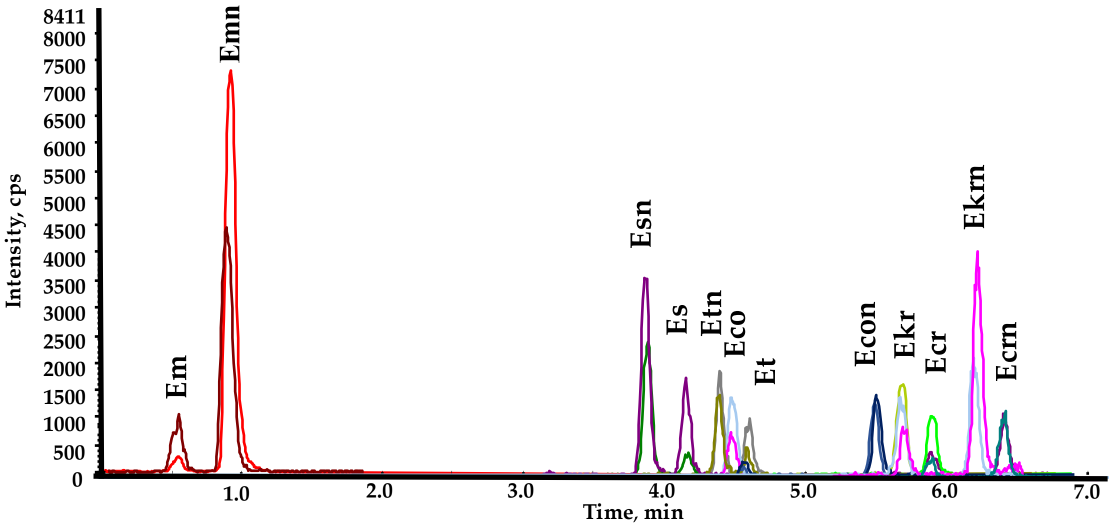

Figure 5. UHPLC-ESI (+)-MS/MS extracted ion chromatogram of an oat flakes sample.

4. METHOD CHARACTERIZATION

For the characterization of the method, procedural calibration curves and statistical parameters, such as limits of detection (LODs) and quantification (LOQs), ME, precision and recoveries were studied using oat flake samples as representative matrix.

4.1 Calibration curves and performance characteristics

In order to compensate ME and the losses in the sample treatment, procedural calibration curves were established in blank oat flake samples, spiked at six different concentration levels (2, 5, 10, 25, 50, and 100 μg kg^{-1}), processed in duplicate, and injected in triplicate. The peak area was considered as a function of the analyte concentration. LODs and LOQs were calculated as the minimum analyte concentrations with a signal-to-noise ratio equal of 3 and 10, respectively. The statistical parameters calculated by least-square regression, LODs and LOQs) are shown in **Table 2**. The satisfactory determination coefficients ($R^2 > 0.994$) confirmed that the analytical responses for the studied EAs were linear over the studied ranges. Satisfactory LOQs (from 0.2 μg kg^{-1} for Emn to 3.2 for Em μg kg^{-1}) were obtained, being lower than those obtained with other QuEChERS–LC–MS/MS methods applied to the quantification of EAs in cereal samples [2,3].

Table 2. Statistical and performance characteristics of the proposed method for the determination of main EAs and their epimers in oat flake samples.

Analyte	Linear regression equation	Linear Range (μg kg^{-1})	Linearity (R^2)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
Em	$y = 152.0x - 112.6$	3.2–100	0.995	1.0	3.2
Emn	$y = 4971.3x - 1979.2$	0.2–100	0.996	0.1	0.2
Es	$y = 1590.4x - 355.9$	1.0–100	0.996	0.5	1.0
Esn	$y = 941.7x + 506.3$	0.9–100	0.994	0.6	0.9
Et	$y = 565.3x - 1045.3$	2.0–100	0.995	0.6	2.0
Etn	$y = 543.5x + 399.4$	1.7–100	0.996	0.5	1.7
Eco	$y = 867.4x + 17.45$	1.4–100	0.998	0.4	1.4
Econ	$y = 692.9x + 373.2$	1.4–100	0.998	0.4	1.4
Ekr	$y = 935.9x - 1148.7$	1.5–100	0.996	0.5	1.5
Ekrn	$y = 935.9x - 1148.7$	1.9–100	0.996	0.6	1.9
Ecr	$y = 546.0x - 223.4$	1.9–100	0.996	0.6	1.9
Ecrn	$y = 677.3x - 304.9$	1.6–100	0.994	0.5	1.6

4.2 Repeatability and intermediate precision assays

The precision of the proposed method was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision). Repeatability was assessed in blank oat flake samples by application of the whole procedure to three samples (experimental replicates) spiked at two different concentration levels of each EA (5 and 50 μg kg^{-1}). All samples were analyzed on the same day and each extract was injected in triplicate (instrumental replicates). Intermediate precision was evaluated with a similar procedure but analyzing one spiked sample in triplicate and per day, for three consecutive days. The results of the precision study, expressed as relative standard deviation (RSD, %) are shown in **Table 3**. In all cases, RSD values lower than 6.3 % for repeatability and 15.0 % for intermediate precision were obtained, in agreement with the recommendations for determination of the contaminants [4].

Table 3. Precision of the proposed method for the determination of main EAs and their epimers in spiked oat flake samples.

	Repeatability, % RSD (n = 9)		**Intermediate Precision, %RSD (n = 9)**	
	5 μg kg^{-1}	**50 μg kg^{-1}**	**5 μg kg^{-1}**	**50 μg kg^{-1}**
Em	5.0	3.2	15.0	10.9
Emn	4.2	2.1	13.6	9.8
Es	3.7	3.2	7.9	6.9
Esn	5.7	3.2	14.3	10.8
Et	6.2	3.8	15.0	11.7
Etn	5.5	4.8	14.0	8.2
Eco	6.3	3.2	12.0	10.4
Econ	4.6	2.2	10.2	7.0
Ekr	5.4	3.7	12.8	7.3
Ekrn	4.2	3.7	14.7	9.4
Ecr	6.2	4.0	11.3	6.7
Ecrn	5.1	4.6	14.3	7.4

4.3 Recovery studies

Recovery experiments were carried out in blank oat flake samples previously analyzed to check the absence of detectable EAs. None of them gave a positive result above the LODs of the method. Recovery experiments were carried out in three samples spiked at two different concentration levels (5 and 50 μg kg^{-1}), and injected in triplicate. The results were compared with those obtained for extracts of blank samples submitted to the sample treatment and spiked with EAs at the same concentration levels just before the measurement. As summarized in **Table 4**, recoveries between 89.7 and 109 % were obtained in all cases, fulfilling requirements of the performance criteria for quantitative methods of analysis [4].

4.4 Evaluation of matrix effects

In order to evaluate the influence of ME on MS detection, cleaned-up extracts of blank samples were spiked with a solution mixture of EAs at two levels of concentration (5 and 50 μg kg^{-1}) and analyzed by UHPLC–MS/MS. Standard solutions of the EAs were prepared at the same levels of concentration and were also analyzed. The ME was calculated as 100 × [(signal of spiked extract − signal of standard solution)/signal of standard solution]. A ME of 0 % indicates the absence of the matrix effect, a ME below 0 % involves signal suppression, while a ME above 0 % reveals signal enhancement. **Table 4** shows the values of the ME and, as can be seen, signal suppression was not significant for most EAs, being lower than |20 %|, except for Em, Emn (two levels), and Ecr and Ecrn (only for the lowest tested level). Anyway, procedural calibration curves were performed to compensate this ME as indicated before.

Table 4. Matrix effect and recovery studies of the proposed method for the determination of main EAs and their epimers in spiked oat flake samples.

	Matrix Effect (%) (n = 9)		**Recovery (%) (n = 9)**	
	5 μg kg^{-1}	**50 μg kg^{-1}**	**5 μg kg^{-1}**	**50 μg kg^{-1}**
Em	−40.4	−39.1	92.4	101
Emn	−36.6	−28.2	90.5	97.1
Es	−8.8	−7.2	105	102
Esn	−8.6	−8.1	97.2	102
Et	−15.5	−10.7	89.7	91.6
Etn	−9.3	−6.2	109	97.9
Eco	−9.6	−5.3	109	90.7
Econ	−13.4	−9.0	105	98.3
Ekr	−14.0	−8.5	106	91.6
Ekrn	−19.7	−11.7	93.1	98.7
Ecr	−22.2	−18.7	90.0	92.1
Ecrn	−24.1	−18.6	103	106

5. ANALYSIS OF OAT-BASED PRODUCTS

In order to check the applicability of the validated method, a total of 25 oat-based products—bran (11), flakes (5), hydroalcoholic extracts (2), juices (2), flours (2), tablets (2), and grass (1)—were analyzed to monitor the natural occurrence of EAs in this sort of products. For identification of EAs in the samples, the recommendations of the SANTE guideline for mass spectrometric detection were followed [5]. Thus, the samples, that presented two ions for the specific analyte and whose ion ratio (defined as the response of the 3 peak with the lower area divided by the response of the peak with the higher area) was within ±30 % (relative) to that obtained from the average of the calibration standards. The contained EAs at levels exceeding the LOQs would be considered positives. Only one sample of oat bran was positive for Em (7.2 μg kg^{-1}, 0.3 % RSD), Emn (1.1 μg kg^{-1}, 0.1 % RSD), Es (1.3 μg kg^{-1}, 0.1 %RSD), and Esn (1.1 μg kg^{-1}, 0.2% RSD), showing, therefore, a total content of EAs of 10.7 μg kg^{-1}. A chromatogram of this naturally contaminated sample is shown in **Figure 6**.

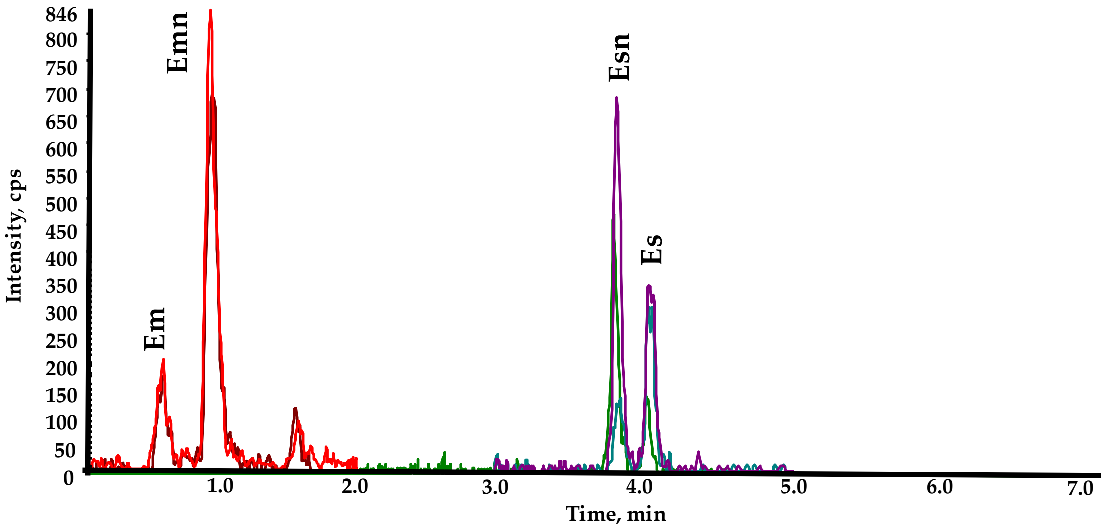

Figure 6. UHPLC-ESI (+)-MS/MS extracted ion chromatogram of naturally contaminated sample of oat bran with Em, Emn, Es and Esn.

6. CONCLUSIONS

In this chapter, an effective and sensitive QuEChERS–UHPLC–MS/MS method, which enabled the quantification of the six major EAs, as well as their corresponding epimers, was validated and applied to the analysis of a variety of oat-based samples. The modifications carried out in the standard QuEChERS procedure improved the sensitivity and the effectiveness of the method. On the one hand, the reduction of the extraction solvent volume resulted in an increase in the sensitivity, since the LOQs obtained (below 3.2 μg kg^{-1}) were significantly lower when compared with those obtained with similar procedures for EAs determination in cereal samples. On the other hand, using a mixture of C18/Z-Sep+ (1:1) as dispersive sorbent, the ME was reduced, being below |20 %| for most analytes studied in such complex matrixes. Moreover, analyzed samples were representative of the wide range of presentations in the market of oat-based products. To the best of our knowledge, this is the first time that EAs have been explored in such a variety of oat-based functional foods, including food supplements. Although only one sample of oat bran was contaminated with EAs, it shows that despite the improvements in industrial grain processing, contamination by EAs must be considered, especially in cereal-based processed foods.

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CHAPTER 7

OCCURRENCE OF ERGOT ALKALOIDS IN BARLEY AND WHEAT FROM ALGERIA

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1. SUMMARY

The natural occurrence of six major ergot alkaloids, ergometrine, ergosine, ergotamine, ergocornine, ergokryptine and ergocristine, as well as their corresponding epimers, was investigated in 60 cereal samples (barley and wheat) from Algeria. Ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) and a modified QuEChERS extraction method were used for sample analysis. The results revealed that 12 out of 60 samples (20 %) were contaminated with ergot alkaloids. Wheat was the most contaminated matrix, with an incidence of 26.7 % (8 out of 30 samples). The concentration of total ergot alkaloids ranged from 17.8 to 53.9 μg kg^{-1} for barley and from 3.66 to 76.0 μg kg^{-1} for wheat samples. Ergosine, ergokryptine and ergocristine showed the highest incidences in wheat, while ergometrine was the most common ergot in barley.

2. EXPERIMENTAL

2.1 Materials and reagents

LC-MS grade methanol (MeOH), acetonitrile (MeCN) and ammonium carbonate ($(NH_4)_2CO_3$) were purchased from VWR International (Barcelona, Spain), and formic acid, eluent additive for LC–MS, from Sigma Aldrich (St. Louis, MO, USA). Z-Sep+ sorbent was supplied by Supelco (Bellefonte, PA, USA), and C18 sorbent by Agilent Technologies (Madrid, Spain). Ultrapure water used throughout the work was obtained from a Milli-Q water purification system (18.2 MΩ cm, Milli-Q Plus system, Millipore Bedford, MA, USA).

Standards of the ergot alkaloids (EAs) ergosine (Es), ergocornine (Eco), ergokryptine (Ekr), ergocristine (Ecr), and the corresponding epimers, ergosinine (Esn), ergocorninine (Econ), ergokryptinine (Ekrn), ergocristinine (Ecrn), were purchased from Techno Spec (Barcelona, Spain), whereas ergometrine (Em), ergotamine (Et), ergometrinine (Emn), and ergotaminine (Etn) were obtained from Romer Labs (Getzersdorf, Austria). The standards were, as indicated by the manufacturer, reconstituted in 5 mL of solvent (MeCN), to give certified concentrations of 500 $\mu g\ mL^{-1}$ and of 125 $\mu g\ mL^{-1}$ for the -ine and -inine isomers, respectively. To prevent rapid epimerization of EAs in the solution, defined volumes of freshly prepared individual or mixed standard solutions were pipetted into amber glass tubes, evaporated to dryness under a stream of nitrogen, and kept at −20 °C. These frozen standards were reconstituted in the proper volume of solvent immediately before use.

2.2 Instrumentation

Separation of EAs was carried out in an Agilent 1290 Infinity LC (Agilent Technologies, Waldbronn, Germany), while detection and quantification were performed in an API 3200 triple quadrupole mass spectrometer (ABSciex, Darmstadt, Germany) with electrospray ionization (ESI) and Analyst version 1.6.3 software. An Agilent Zorbax Eclipse Plus RRHD C18 (50 × 2.1 mm, 1.8 μm) was used as chromatographic column.

A universal 320R centrifuge (Hettich ZENtrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), a multi-tube vortexer BenchMixer™

XL (Sigma-Aldrich, St. Louis, MO, USA), an evaporator System (System EVA-EC, from VLM GmbH, Bielefeld, Germany) and a high-speed solid crusher (Hukoer, China) were used to process samples.

2.3 Chromatographic separation

The chromatographic separation of EAs was carried out using a C18 Zorbax Eclipse Plus RRHD column (50 × 2.1 mm, 1.8 µm) at 35 °C. Mobile phase consisted of a mixture of water (A) and MeOH (B), both containing 0.3 % formic acid. The gradient elution program was as follows: 0 min: 30 % B; 6 min: 60 % B; 9 min: 60 % B; 10 min: 30 % B; 12 min: 30 % B. The flow rate was 0.4 mL min^{-1} and the injection volume was 5 µL. The column temperature was set at 35 °C and the injection volume was 5 µL. In order to minimize epimerization, the injection sample sequence was limited to 12 h. Moreover, control standard solutions of EAs were injected at the beginning, middle, and end of each analysis sequence.

The mass spectrometer operated in the positive electrospray ionization (ESI$^+$) mode under multiple reaction monitoring (MRM) conditions (*See table 1 Chapter 6*). MS parameters for the analysis were established as follows: temperature of the source 500 °C; collision gas (nitrogen) 5 psi; voltage of the ion spray 5 kV; curtain gas (nitrogen) 30 psi; nebulizing gas (GAS 1), and drying gas (GAS 2), in both of them, nitrogen was set at 50 psi. In all cases, a precursor ion and two product ions were studied. The monitored ions were the protonated molecules $[M+H]^+$, except for Esn, Etn, Econ, and Ecrn, , where $[M-H_2O+H]^+$ ions were monitored.

2.4 Modified QuEChERS procedure for the analysis of cereal samples

The sample treatment previously described in Chapter 6 for the analysis of oat-based products [1] was employed for the analysis of cereal samples (barley and wheat) intended for human consumption in Algeria. This study was carried out in collaboration with the University of Oran (Oran, Algeria) with the participation of Dr. Choukri Khelifa Mahdjoubi, who provided the samples (a total of 60 samples (30 samples of barley and 30 of wheat)).

Briefly, 1 g of homogenized sample was placed in a 50-mL falcon tube, 4 mL of MeCN and ammonium carbonate 5 mM (85:15, *v/v*) were added and the mixture was vortexed (1 min) and centrifuged (5 min, 9000 rpm, 4 °C). Then, the whole supernatant was transferred to a 15-mL falcon tube containing 150 mg of a mixture of C18/Z-Sep$^+$ (50/50) dispersive sorbents for cleaning-up. Next, the mixture was vortexed (1 min) and centrifuged (5 min, 9000 rpm, 4 °C). The supernatant was transferred to a 4-mL vial, evaporated to dryness with nitrogen and reconstituted to a final volume of 750 µL with MeOH:water (50:50, *v/v*). Finally, the samples were filtered through a 0.22 µm nylon filter before their injection into the UHPLC-MS/MS system.

3. METHOD CHARACTERIZATION

For the characterization of the analytical method, procedural calibration curves and parameters, such as limits of detection (LODs), limits of quantification (LOQs), matrix effects (ME), precision, and recoveries were studied in blank barley and wheat samples. Samples were previously analyzed to confirm a negative result.

3.1 Calibration curves and performance characteristics

In order to compensate ME and losses from the sample treatment, procedural calibration curves were established in blank wheat and barley samples, spiked at six different concentration levels within the analytical range from 2 to 100 µg kg^{-1}, processed in duplicate, and injected in triplicate. The peak area was considered as a function of the analyte concentration. Linearity was evaluated with the determination coefficient (R^2), and LODs and LOQs were defined as 3 × S/N and 10 × S/N, respectively. **Table 1** summarizes the results for procedural calibration, linear regression equations, linearity, LODs and LOQs for barley and wheat. The linearity was satisfactory for all the studied EAs over the working range (determination coefficients $R^2 > 0.99$). The LOQs ranged between 0.50–3.92 µg kg^{-1} for barley and 0.49–3.33 µg kg^{-1} for wheat.

Table 1. Statistical and performance characteristics of the proposed method for the determination of main EAs and their epimers in barley and wheat samples.

Analyte	**Linear regression equation**	**Linear Range ($\mu g\ kg^{-1}$)**	**Linearity (R^2)**	**LOD ($\mu g\ kg^{-1}$)**	**LOQ ($\mu g\ kg^{-1}$)**
Barley samples					
Em	y = 86.47x + 285.1	3.9-100	0.997	1.2	3.9
Emn	y = 4352x + 482.4	0.5-100	0.997	0.1	0.5
Es	y = 1042x - 181.7	1.1-100	0.997	0.3	1.1
Esn	y = 1293x + 931.4	0.7-100	0.998	0.2	0.7
Et	y = 739.9x – 491.1	1.2-100	0.996	0.4	1.2
Etn	y = 949.1x + 2036	1.1-100	0.995	0.3	1.1
Eco	y = 967.3x + 1534	1.2-100	0.995	0.4	1.2
Econ	y = 772.7x + 230.3	1.4-100	0.998	0.4	1.4
Ekr	y = 850.2x - 42.54	0.9-100	0.998	0.3	0.9
Ekrn	y = 1676x + 1589	0.7-100	0.996	0.2	0.7
Ecr	y = 605.4x + 490.6	1.5-100	0.996	0.5	1.5
Ecrn	y = 730.1x + 1038	1.0-100	0.996	0.3	1.0
Wheat samples					
Em	y = 108.1x + 364.3	3.3-100	0.992	1.0	3.3
Emn	y = 6607x – 336.0	0.5-100	0.993	0.2	0.5
Es	y = 1321x + 127.4	0.5-100	0.998	0.2	0.5
Esn	y = 1783x + 789.8	0.6-100	0.998	0.2	0.6
Et	y = 768.2x - 288.6	1.0-100	0.996	0.3	1.0
Etn	y = 1169x + 917	1.0-100	0.996	0.3	1.0
Eco	y = 1294x + 324.5	0.9-100	0.998	0.3	0.9
Econ	y = 1032x - 205.7	1.1-100	0.998	0.3	1.1
Ekr	y = 1084x - 797.9	1.1-100	0.997	0.3	1.1
Ekrn	y = 2323x - 611.3	0.7-100	0.998	0.2	0.7
Ecr	y = 796.8x - 414.5	1.1-100	0.996	0.3	1.1
Ecrn	y = 1028.5x - 709.4	1.1-100	0.998	0.3	1.1

3.2 Repeatability and intermediate precision assays

Precision was estimated for both barley and wheat samples at two concentration levels (5 and 50 $\mu g\ kg^{-1}$) and expressed as the relative standard deviation (% RSD) of the results obtained from three samples injected in triplicate on the same day (intra-day precision) and one sample on three different days (inter-day precision). Results of precision study are shown in **Table 2** being the RSDs lower than 11 % in all the cases. The obtained values were considered satisfactory under the recommendations of the performance of analytical methods for determination of contaminants [2].

Table 2. Precision of the proposed method for the determination of main EAs and their epimers in spiked barley and wheat samples.

	Repeatability, % RSD (n = 9)		Intermediate Precision, % RSD (n = 9)	
	5 μg kg^{-1}	50 μg kg^{-1}	5 μg kg^{-1}	50 μg kg^{-1}
		Barley samples		
Em	6.5	3.8	5.6	6.4
Emn	4.1	2.4	9.4	6.2
Es	4.9	6.2	9.4	7.7
Esn	6.0	3.0	7.3	9.2
Et	4.6	6.9	8.7	6.0
Etn	6.3	4.5	9.2	6.5
Eco	6.8	4.4	9.6	5.2
Econ	5.5	6.1	6.4	8.0
Ekr	4.8	4.3	9.0	4.9
Ekrn	3.0	4.8	5.6	6.8
Ecr	6.6	4.6	6.2	4.7
Ecrn	4.2	3.6	6.1	4.5
		Wheat samples		
Em	9.0	5.9	11.0	11.0
Emn	3.6	5.4	4.5	6.3
Es	9.2	6.3	8.3	7.4
Esn	5.2	7.5	6.0	9.6
Et	5.9	7.6	7.3	10.0
Etn	9.3	7.6	11.0	7.7
Eco	7.8	5.0	8.3	6.6
Econ	7.4	4.6	8.5	5.8
Ekr	9.6	5.6	11.0	7.4
Ekrn	6.6	4.6	10.1	10.0
Ecr	8.2	6.9	10.1	7.5
Ecrn	6.0	4.6	7.3	5.6

3.3 Recovery studies

Recovery studies were carried out by fortifying blank samples of wheat and barley at two concentration levels (5 and 50 μg kg^{-1}). Each sample was processed in triplicate and injected three times. The peak area ratios of the sample spiked before extraction to sample spiked after extraction were used to calculate the extraction recovery.

As can be seen in **Table 3**, recoveries were within 86.6–105 % for barley and 84.9–109 % for wheat.

Table 3. Matrix effect and recovery studies of the proposed method for the determination of main EAs and their epimers in spiked barley and wheat samples.

	Matrix Effect (%) (n = 9)		**Recovery (%) (n = 9)**	
	5 µg kg^{-1}	**50 µg kg^{-1}**	**5 µg kg^{-1}**	**50 µg kg^{-1}**
		Barley samples		
Em	-51.7	-43.4	99.0	89.3
Emn	-38.5	-30.5	86.6	88.9
Es	-8.4	-10.7	94.8	96.9
Esn	-8.6	-6.6	101.0	101.1
Et	-14.5	-9.9	105.0	103.0
Etn	-10.3	-4.9	100.2	104.0
Eco	-12.7	-15.7	98.8	99.4
Econ	-1.9	-7.9	104.0	102.0
Ekr	-11.0	-11.3	94.6	95.8
Ekrn	-8.5	-4.7	98.0	97.8
Ecr	-12.6	-4.4	95.8	96.6
Ecrn	-5.7	-4.6	97.7	97.4
		Wheat samples		
Em	-38.8	-26.2	84.9	86.0
Emn	-25.8	-20.1	92.6	89.3
Es	-11.4	-7.8	105.1	103.0
Esn	-15.9	-13.6	99.8	104.2
Et	-13.4	-8.7	102.0	91.6
Etn	-12.3	-8.2	107.2	104.0
Eco	-11.8	-6.9	109.0	99.5
Econ	-8.6	-6.7	106.1	94.6
Ekr	-10.3	-10.7	93.7	92.1
Ekrn	-13.2	-7.4	98.0	95.5
Ecr	-12.4	-8.5	98.4	90.7
Ecrn	-10.8	-6.7	93.3	92.2

3.4 Evaluation of matrix effects

The matrix effect (ME %) for each analyte was evaluated preparing blank samples of barley and wheat and spiking the extracts just before analysis at two concentration levels (5 and 50 µg kg^{-1}). It was calculated according to the following equation:

$$\mathrm{ME}(\%) = \frac{\text{signal of extract spiked after extraction} - \text{signal of standard solution}}{\text{signal of standard solution}} \text{x } 100$$

A ME of 0 % indicates the absence of the matrix effect, a ME below 0 % involves signal suppression, while a ME above 0 % reveals signal enhancement. As shown in **Table 3**, the ME was lower than 16 % (absolute value) for all EAs, except for Em and Emn, which showed significant signal suppression in both matrices. As a consequence, procedural calibration curves were used for quantification as it was mentioned before.

4. OCCURRENCE OF ERGOT ALKALOIDS

A total of 60 samples (30 samples of barley and 30 of wheat) destined for human consumption were randomly collected during the year 2018 from retail shops and supermarkets from three cities in Algeria: Aïn Témouchent (10 samples of barley and 10 of wheat), Oran (10 samples of barley and 10 of wheat) and Tiaret (10 samples of barley and 10 of wheat). No information about the country of production was available for the samples. The sampling was done according to Malysheva et al. and European Commission Regulation [3,4]. Briefly, from each sample lot (bag of 50 kg) of barley and wheat, five sub-samples of 200 g were taken from different positions (from the top, middle and bottom of the bag) and thoroughly mixed to achieve a 1-kg aggregate sample. From the aggregate sample, a laboratory sample of 200 g was taken, grinded, homogenized and stored at −20 °C until analysis.

The validated method was applied to monitor the natural occurrence of EAs in the above-mentioned samples which were analyzed in triplicate. For identification of the analytes, SANTE guidelines recommendations were followed [5]. A summary of the positive samples found can be seen in **Table 4.**

Of the 60 analyzed samples, 12 samples (20 %) were found to be positive for EAs. Overall, wheat was the most contaminated matrix, with an incidence of 26.7 % (8 out of 30 samples), with concentrations ranging from 3.66 to 76.0 μg kg^{-1} (total content of EAs, considering only concentrations higher than LOQs) with a mean concentration of 33.1 μg kg^{-1}. On the other hand, in barley, 4 out of 30 analyzed samples (13.3 %) were found to be contaminated with EAs at concentrations ranging from 17.8 to 53.9 μg kg^{-1} (total content of EAs, considering only concentrations higher than LOQ), with a mean concentration of 35.4 μg kg^{-1}.

Table 4. Summary of the EA occurrence in barley and wheat samples.

Sample	I [a] (%)	Mean [b] (μg kg^{-1})	Range [c] (μg kg^{-1})	Distribution (μg kg^{-1})	
				< 10	10–100
Barley (n=30)	4 (13.3 %)	35.4	17.8–53.9	0	4
Wheat (n=30)	8 (26.7 %)	33.1	3.66–76.0	1	7
Total (n=60)	12 (20 %)	34.3	3.66–76.0	1	11

[a]Incidence of positive samples (percentage %); [b]Mean value for positive samples; [c]Minimum concentration value–maximum concentration value.

These results are globally in line with some recent studies on the occurrence of EAs in cereals. Thus, the study developed by the EFSA collecting data from 2011 to 2015 from 15 different European countries revealed that in 76 % cereal-based samples EAs were unquantified [6]. Other surveys revealed the presence of several EAs in barley and wheat samples from Norway, but concentrations were generally low [7]. Additionally, an occurrence study carried out for three years reported low incidence of total EAs in French durum wheat (15–30 %), wheat (23–30 %) and barley (27–39 %) [8], and the analysis of 123 Chinese cereal samples revealed that only five samples were contaminated with EAs at a concentration range of 1.01–593 μg kg^{-1} [9]. Nevertheless, other studies reported higher incidences of EAs: for instance, EAs were found in 54 % of 113 grain-based products for infant and young children from the Netherland, with mean levels of 10.6, 6.2 and 8.6 μg kg^{-1} for 2011, 2012 and 2013, respectively [10]; in Italy, 62 out of 71 samples of wheat and rye were contaminated with at least one EA, with total EA concentrations similar to those reported in our study, although one sample showed a concentration up to 1142.6 μg kg^{-1} [11]. In addition, EAs were detected in 10 of 13 wheat samples from different European countries, with total EA concentrations up to 7654 μg kg^{-1} [12]. Furthermore, 104 out of 122 samples of cereals-based food and feed from Belgium were positives (concentrations ranging from 1 to 1145 μg kg^{-1}) [13]; and 23 out of 39 grain samples from Luxemburg were contaminated with EAs with concentrations from 0.7 to 2530.1 μg kg^{-1}, even after sieving to remove sclerotia [14]. To the best of our knowledge, there are no reports about the occurrence of EAs in African countries.

The occurrence of EAs among the analyzed samples showed some differences from one region to another; thus, 100 % and 37.5 % positive samples of barley and wheat, respectively, were from Tiaret, 50 % positives samples of wheat were from Oran and only 12.5 % positive samples (one out of eight samples) of wheat were from Aïnt Témouchent. These results could be a consequence of the influence of climatic and geographical conditions on the occurrence of EAs in cereals, as Oran and Aïnt Témouchent are close to the coast and have a dry Mediterranean climate, while Tiaret is located inland. However, these are only tentative conclusions, as no information about the cereal growing place was available and could be different from those locations. In this sense, it would be interesting to collect more data about the incidence of EAs in these cereals in different years and locations from Algeria, in order to properly study the influence of climate throughout the country.

4.1 Distribution of individual ergot alkaloids

Among the positive samples, and considering the detected EAs (concentrations higher than LODs), the distribution of individual EAs varied. **Table 5** presents the incidence of the individual EAs in the analyzed samples. Globally, Em was the most frequent EA (detected in six wheat and four barley samples). Considering only concentrations higher than LOQs, Es, Ekr and Ecr in wheat and Em in barley were the most frequent EAs. The highest concentrations of an individual EA were 28.6 $\mu g\ kg^{-1}$ for Ecr and 50.0 $\mu g\ kg^{-1}$ for Em in wheat and barley samples, respectively.

Compared to recent data, the ergot pattern obtained in our study is similar to those reported by other authors. Thus, the EFSA report concluded that the highest average contributions to the total concentration in food samples corresponded to Et (18 %), Ecr (15 %), Es (12 %) and Em (11 %) [6]. Indeed, Es, Ekr and Ecr were also reported in another study as predominant in cereal products in Europe [3]; moreover, Es, Econ and Ekr were the most common EA in cereal samples from Luxembourg [14], while Et, Es and Ecr were the most frequently occurring EAs in French cereals [8]. Contrary to our finding, Em and Emn were prevalent in wheat samples from Italy [11].

Table 5. Summary of individual ergot alkaloid concentrations in positive samples.

Analyte	I [a] (%)	Max [b] (μg kg^{-1})	LOD-LOQ [c]	Min [d] (μg kg^{-1})	Max [e] (μg kg^{-1})
		Barley samples (n=4)			
Em	4 (100%)	33.1	0	17.8	50.0
Emn	0	-	4	-	-
Es	0	-	0	-	-
Esn	0	-	0	-	-
Et	3 (75%)	3.01	0	2.34	3.90
Etn	0	-	0	-	-
Eco	0	-	0	-	-
Econ	0	-	0	-	-
Ekr	0	-	0	-	-
Ekrn	0	-	0	-	-
Ecr	0	-	0	-	-
Ecrn	0	-	0	-	-
		Wheat samples (n=8)			
Em	4 (50%)	13.5	2 (25%)	3.52	24.9
Emn	1 (12.5%)	1.42	3 (37.5%)	1.42	1.42
Es	5 (62.5%)	1.70	0	0.62	3.30
Esn	0	-	3 (37.5%)	-	-
Et	3 (37.5%)	6.2	0	1.15	13.6
Etn	1 (12.5%)	2.91	0	2.91	2.91
Eco	2 (25%)	10.5	1 (12.5%)	8.68	12.40
Econ	2 (25%)	4.4	1 (12.5%)	3.84	4.90
Ekr	5 (62.5%)	12.7	0	1.56	26.2
Ekrn	3 (37.5%)	4.2	2 (25%)	3.28	5.88
Ecr	5 (62.5%)	10.4	0	2.10	28.6
Ecrn	3 (37.5%)	7.2	1 (12.5%)	1.50	12.2

[a]Incidence of samples ≥LOQ; [b]Mean value for samples ≥LOQ; [c]Incidence of samples with concentration ≥LOD and ≤LOQ; [d]Minimum concentration value; [e]Maximum concentration value

The distribution of the -ine and the -inine forms of the EAs was investigated and the results are reported in **Figure 1**. As shown, the frequency of occurrence of the -ine forms was higher than that of the -inine forms. Indeed, Em, Et and Es were highly predominant in the -ine form, while Eco showed an incidence similar than the -inine form (Econ). Moreover, the mean concentrations for -ine forms was higher in all the cases than the concentrations of the -inine forms. These results are similar to other published studies [3, 6, 8,15].

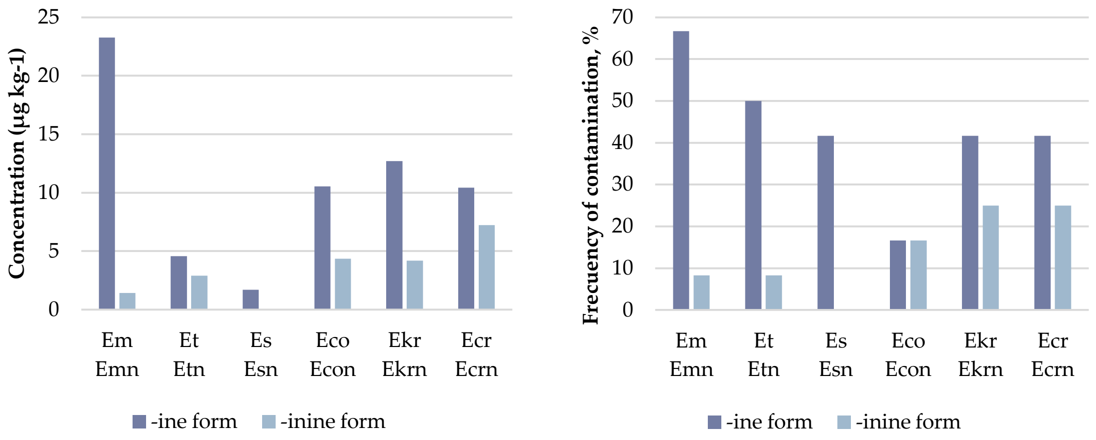

Figure 1. Histograms of incidence and mean concentration of -ine and -inine forms in the positive samples.

4.2 Dietary exposure estimation

With the purpose to evaluate the risk of exposure of the adult population to EAs through the consumption of barley and wheat, the Probably Dietary Intake (PDI) was calculated for the sum of EAs as indicated in the following equation:

$$\mathrm{PDI} = \frac{\mathrm{C\ x\ K}}{\mathrm{bw}}$$

where C is the mean concentration of the EAs in the sample, K is the average consumption of the food (g day^{-1}) and bw is the body weight considered for the adult human population. Once the PDI was calculated, the risk was estimated as the percentage of Tolerable Daily Intake (% TDI), calculated as the ratio of PDI to TDI (µg kg^{-1} bw day^{-1}) as follows [16,17]:

$$\mathrm{TDI\ (\%)} = \frac{\mathrm{PDI}}{\mathrm{TDI}} \mathrm{x\ 100}$$

A tolerable daily intake (TDI) of 0.6 µg kg^{-1} bw day^{-1}, as proposed by the EFSA CONTAM Panel, has been used as a reference dose [16].

In order to substitute the left-censored data, that is, data below the LOD or LOQ, two exposure scenarios were defined: the lower bound scenario (LB) and the upper bound scenario (UB) [18]. In the lower bound scenario (LB), a zero was assigned when EAs were not detected or were detected below the LOQ. In the upper bound (UB) scenario, the LOD was assigned when EAs were not detected, and the LOQ when EAs were detected at levels below LOQ. The population group considered in this study was adult humans (60 kg) [19]. Consumption data of barley (36 g day^{-1}) and wheat (502 g day^{-1}) used in this study derived from data reported by the Food and Agriculture Organization (FAO) regarding the nutrition profile for Algerian population [20]. The obtained results are summarized in **Table 6**. PDI values of EAs through the consumption of barley and wheat were estimated to be 0.003 and 0.074 µg kg^{-1} bw day^{-1} under the LB scenario. However, when the UB scenario was considered, the PDIs were estimated to be 0.005 and 0.105 µg kg^{-1} bw day^{-1} for barley and wheat, respectively. These PDI values obtained for adult population were below the proposed TDI (0.6 µg kg^{-1} bw day^{-1} for the sum of 12 EAs), representing less than 17.6 % of TDI in both the LB and UB approaches.

Table 6. Dietary exposure to EAs through the consumption of barley and wheat (PDI: Probably Dietary Intake; TDI: Tolerable Daily Intake).

Samples	**Mean (µg kg^{-1})**		**PDI (µg kg^{-1} bw day^{-1})**		**TDI %**	
	LB	UB	LB	UB	LB	UB
Wheat (n=30)	8.83	12.59	0.074	0.105	12.32	17.56
Barley (n=30)	4.71	8.68	0.003	0.005	0.47	0.87

This finding suggests that barley and wheat from Algeria could be considered safe for the average adult consumers concerning EAs. However, it is recommended a rigorous control of these toxic compounds as well as a monitoring to preserve the consumers' health, as there are risk groups, such as infant population and consumers with high intake that might suppose an increase of toxicity hazard.

5. CONCLUSIONS

The present study reported the first data about the presence of 12 EAs in cereals (barley and wheat) from Algeria. Wheat showed a higher incidence than barley (26.7 % and 13.3 %, respectively), and the results of our study revealed low contamination of EAs in barley (range for the sum EAs of 17.8–53.9 μg kg^{-1}) and wheat (range for the sum EAs of 3.66–76.0 μg kg^{-1}). The study shows variability in the pattern of EAs among the positive samples, the most frequent being Es, Ekr and Ecr in wheat, and Em in barley, and emphasizes the importance of including the six EAs and their epimers in the risk assessment. In view of the results, there is no evidence of risk linked with the EA intake through the consumption of barley and wheat in Algeria since the levels of the PDI obtained from the studied samples are far below the TDI proposed by the EFSA. Nevertheless, this study includes a limited number of samples and locations, and considering that EA production depends on climate conditions, it would be advisable to collect more data on the incidence of EAs in cereal samples from different locations and in different seasons.

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CHAPTER 8

ION MOBILITY-MASS SPECTOMETRY TO EXTEND ANALYTICAL PERFORMANCE IN THE DETERMINATION OF ERGOT ALKALOIDS IN CEREAL SAMPLES

This work is currently in drafting.

1. SUMMARY

This work evaluates the potential of ion mobility spectrometry (IMS) to improve the analytical performance of current liquid chromatography-mass spectrometry (LC-MS) workflows applied to the determination of ergot alkaloids (EAs) in cereal samples. The simultaneous determination of the EA family is an analytical challenge because it consists of a large number of epimeric compounds with similar retention times and mass-to-charge ratio (*m/z*); therefore, their separation and determination have some limitations with current LC-MS methods. In this regard, taking advantage of the third dimension provided by IMS, which provides the measurement of the rotationally averaged collision cross section (CSS), the first CCS database for EAs has been built using a travelling wave ion mobility spectrometer in order to contribute to their unequivocal identification. The created CCS database has been inter-laboratory cross-validated and compared with predictive CCS values generated by machine-learning models. In addition, slight differences were observed in terms of CCS values for ergotamine, ergosine and ergocristine and their corresponding epimers (ranging from 3.3 to 4%), being sufficient to achieve a satisfactory peak-to-peak resolution for their unequivocal identification. Furthermore, in order to evaluate the advantages that IMS offers in terms of selectivity and sensitivity, a liquid chromatography (LC)-travelling wave ion mobility (TWIM)-time of flight mass spectrometry (TOF-MS) method has been developed for the analysis of EAs in cereal samples. Signal-to-noise ratio (S/N) was improved from 2.5 to 4-fold compared to the analog LC-TOF-MS method; thus, leading to an increase in signal sensitivity. Finally, the proposed method was applied to the determination of EAs in barley and wheat samples and limits of quantification (LOQs) lower than 2.0 μg kg^{-1} were achieved in both cases. Satisfactory precision, expressed as relative standard deviation (RSD), was obtained being lower than 14.4 %. Positive samples were found, whose total EAs content ranged from 8.3 to 36.8 μg kg^{-1} for barley samples, and from 5.2 to 65.0 μg kg^{-1} for wheat samples.

2. EXPERIMENTAL

2.1 Materials and reagents

Standards of Es, Eco, Ekr, Ecr and the corresponding epimers, Esn, Econ, Ekrn, Ecrn, were purchased from Techno Spec (Barcelona, Spain), whereas Em, Et, Emn and Etn were obtained from Romer Labs (Getzersdorf, Austria). The dried standards were reconstituted in acetonitrile (MeCN) to obtain solutions with concentrations of 500 μg mL^{-1} for the main EAs and of 125 μg mL^{-1} for the epimers. Immediately after their reconstitution, intermediate dried stock solutions were prepared by taking aliquots of individual or mixed standard solutions, which were placed into amber glass vials for their evaporation under a gentle stream of nitrogen. Finally, they were stored at -20 °C, in order to avoid EA epimerization, and reconstituted in the required amount of MeCN just before use.

Methanol (MeOH), MeCN, and propan-2-ol (LC-MS Chromasolv® grade) were supplied by Sigma-Aldrich (St. Louis, Mo, USA). Water (HiperSolv Chromanorm® for HPLC) was provided by VWR International (West Chester, PA, USA). Formic acid (eluent additive for LC-MS) was acquired from LGC Standards GmbH (Wesel, Germany). Sodium hydroxide (1 M, Fisher Chemical™) and formic acid (Promochem®) were supplied by Fisher Scientific (Loughborough, UK) and LGC Standards (Wesel, Germany), respectively. Both were used for preparing a solution of sodium formate (0.5 mM in 90/10 (%, v/v) propan-2-ol/water), which was used for mass calibration.

Leucine-enkephalin standard was acquired from Waters® (Manchester, UK) and used for the preparation of leucine-enkephalin (1 μg L^{-1}) in 50/50 (%, v/v) water/MeCN solution containing 0.2 % (v/v) of formic acid which was employed as a lock mass standard. Finally, a Major Mix IMS/TOF Calibration Kit from Waters® ref. 186008113 was employed for CCS calibration.

In addition, for inter-laboratry studies, LC-MS grade MeOH and MeCN were purchased from Scharlab Italia S.r.l (Milan, Italy); bidistilled water was obtained using

Milli-Q System (Millipore, Bedford, MA, USA). Formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, CA, USA) was also used.

2.2 Instrumentation

A high-speed solids crusher (Hukoer, China), an evaporator System (System EVA-EC, from VLM GmbH, Bielefeld, Germany), a universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), and a multi-tube vortexer BenchMixer™ XL (Sigma-Aldrich, St. Louis, MO, USA) were used for sample preparation.

Chromatographic separation was carried out in a Acquity UPLC® System from Waters® using an Agilent Zorbax Eclipse Plus RRHD C18 column (50 × 2.1 mm, 1.8 μm). IM-MS analyses were performed on a hybrid quadrupole-TWIMS-orthogonal acceleration time-of-flight (TOF) mass spectrometer (Synapt G2-S HDMS, Waters®) equipped with an electrospray ionization (ESI) interface.

MassLynx (version 4.2, Waters®) software was used for data acquisition and DriftScope software (version 2.8), which is included in MassLynx software, was used to obtain data related to the CCS of ions and mobility spectra. Chromatograms were analyzed by SkyLine (version 21.1) which is an open-source software that allows processing mobility data and exploring the spectra produced by IMS-enabled mass spectrometers [1].

Inter-laboratory cross-validation of the CCS database was carried out at the University of Parma (UNIR). An ACQUITY I-Class UPLC separation system coupled to a VION IMS-QTOF mass spectrometer (Waters®, Wilmslow, UK) equipped with an ESI interface was employed for travelling wave collision cross section against nitrogen buffer gas ($^{TW}CCS_{N_2}$) database cross-validation. Data acquisition was conducted using UNIFI 1.8 software (Waters®, Wilmslow, UK).

2.3 Chromatographic separation

A concentration gradient program was applied to achieve the chromatographic separation of 12 EAs using a mobile phase consisted of ultrapure water (eluent A) and

MeOH (eluent B), both acidified with 0.3 % (v/v) formic acid. The eluent gradient profile was as follows: 0–2 min 10 % B; 2-4.5 min 10-40 % B; 4.5-9 min 40-45 % B; 9-11 min 45-95 % B; 11-12 95 % B; 12-13 min 95-10 % B; 13-16 min 10 % B. In order to guarantee column equilibration, initial conditions were maintained for 3 min, providing a total run time of 16 min. The temperature of the column was fixed at 35 °C and 5 μL was set as injection volume. In order to avoid EAs epimerization, the injection sample sequence was limited to 12 hours and the temperature of the sample carrousel was kept at 10 °C during the analysis.

For inter-laboratory cross-validation of CCS values, the separation conditions were similar to those mentioned above. Samples were injected (2 μL), and chromatographically separated using a reversed-phase C18 BEH ACQUITY column (2.1 × 50 mm, 1.7 μm particle size) from Waters, (Milford, MA, USA) using the LC conditions described above.

2.4 Ion mobility-mass spectrometry conditions

Analyses were performed in the positive electrospray ionization (ESI$^+$) mode, acquiring continuum data in the range of 50-1200 *m/z* with a scan time of 0.5 s. Nitrogen was used as both cone and desolvation gases at flow rates of 50 and 1000 L h^{-1}, respectively. Nebulizer pressure was fixed at 6.0 bar. Source and desolvation temperature were set at 150 °C and at 350 °C, respectively. Capillary voltage was set at 3.0 kV, cone voltage at 31 V and source offset at 40 V. The TOF analyzer was operated in high-resolution mode for CCS characterization, and in sensitivity mode for the application of the LC-TWIMS-TOF-MS method to the analysis of EAs in barley and wheat samples.

Regarding to IMS conditions, IMS buffer and trap gas consisted of nitrogen, and they were supplied at 90 and 0.4 mL min^{-1}, respectively. The flow rate of gas in the helium cell was 180 mL min^{-1}. IMS wave velocity and height were fixed at 1000 m s^{-1} and 40 V, respectively. In the trap cell, wave velocity and height were established at 311 m s^{-1} and 4.0 V, respectively. In the case of the transfer cell, these parameters were set at 219 m s^{-1} and 4.0 V, respectively. Other high definition- MS (HDMS) settings were trap DC bias, 47 V; and IMS DC bias, 3 V.

A sodium formate and leucine-enkephalin solutions prepared as indicated before were used as mass calibrant and lock mass standard respectively. Leucine-enkephalin solution was employed as mass calibrant and as lock mass standard for accurate mass measurements. Leucine-enkephalin signal was acquired each 20 s for 0.3 s (3 scans to average). CCS calibration was carried out using a Major Mix IMS/ToF Calibration Kit. CCS calibration covered the *m/z* range between 152-800 Da and a CCS range from 130.4 to 271 Å^2.

In respect of cross-validation CCS database studies, mass spectrometry data were collected also in ESI$^+$ mode over the mass range of 50–1000 *m/z*. Source settings were established as follows: capillary voltage, 1.0 kV; source temperature, 150 °C; desolvation temperature, 350 °C and desolvation gas flow, 1000 L h^{-1}. The TOF analyzer was operated in sensitivity mode and data acquired using data independent high-definition MS^E (HDMS^E), which is a data independent approach (DIA) coupled with IMS. The optimized ion mobility settings were as follows: nitrogen flow rate, 90 mL min^{-1} (3.2 mbar); wave velocity 650 m s^{-1} and wave height, 40 V. CCS calibration using the same Major Mix IMS/TOF calibration kit (Waters, Wilmslow, UK) was carried out covering the CCS range from 130 to 306 Å^2. The TOF was also calibrated prior to data acquisition and covered the *m/z* range from 151 to 1013 Da.

2.5 Sample treatment procedure

The sample treatment was based on a modified QuEChERS procedure that we previously optimized for the determination of the main EAs and their epimers in oat-based functional foods (*Chapter 6*) [2]. A portion of 1.0 g of homogenized cereal sample (i.e., barley and wheat from Algeria) was placed into a 50-mL falcon tube with conical bottom. Subsequently, the sample was mixed with 4 mL of the extraction mixture (MeCN:5 mM ammonium carbonate; 85:15, v/v). The sample was agitated by vortex for 1 min and centrifuged for 5 min at 9000 rpm and 4 °C. The whole upper layer was collected and placed into a 15-mL falcon tube containing 150 mg of a mixture of C18:Z-Sep+ (1:1, w/w) as clean-up sorbent. Then, the mixture was vigorously shaken for 1 min and centrifuged for 5 min at 9000 rpm and 4 °C. The entire upper layer was transferred to a 4 mL glass vial and the extraction solvent was evaporated to dryness under a gentle stream of nitrogen. Finally, the residue was reconstituted with 750 µL of a mixture of MeOH:ultrapure water (50:50, v/v) and filtered through a 0.22 µm nylon filter before injection.

3. RESULTS AND DISCUSSION

3.1 CCS characterization

Drift times are instrument-dependent, so these data cannot be extrapolated to other platforms. For this reason, IMS measurements are usually reported in terms of CCS [3]. This parameter is related to the rotationally averaged cross-sectional area of each compound and, consequently, to the movement of the ions through the drift tube. CCS represents the effective area of the ionized molecule that interacts with the buffer gas in the drift cell, which is expressed in Å^2. As it was previously mentioned, the measured CCS contributes to the unequivocal identification of the analyzed compounds. Moreover, CCS data can be combined with MS/MS spectra and retention time to improve the confidence of identification of similar molecules which present the same *m/z* and retention times. This is the case of the six major EAs and their corresponding epimers under study, which have been characterized in terms of *m/z* and CCS for the first time in this work.

All EAs have been characterized in positive ionization mode, as these compounds are generally determined as their protonated form [**¡Error! Marcador no definido.**]. CCS values were measured using nitrogen as drift gas and reported following formalized nomenclature [4]. This chapter does not only provides information about the most abundant ion observed for each compound, but it also reports the CCS of all ions identified for each EA (i.e., $[M+H]^+$, $[M+Na]^+$ or $[M-H_2O+H]^+$), as well as the CCS for the main fragment ions observed. Therefore, a total of 210 CCS measurements were carried out for the 12 EAs including all observed adducts and fragments. All the observed ions were within a *m/z* range between 208 and 610 and presented $^{TW}CCS_{N_2}$ values between 139.9 and 241.8 Å^2. CCS measurements were carried out in triplicate and relative standard deviations (RSDs) lower than 0.45 % were obtained.

Detailed information of the investigated EAs, the ions observed, as well as their *m/z* and CCS can be found in **Table 1**. As previously reported, the CCS of ions is a molecular characteristic closely related to *m/z* [5,6]. **Figure 1** shows a general view of the correlation between both parameters for the main ions characterized. As can be seen, all ions were located within a narrow interval (± 5 %) from correlation curve (i.e., power regression model) represented as a solid line. A satisfactory correlation (R^2= 0.9216) was obtained showing that one single regression model is enough to describe the *m/z* and CCS relation for all characterized ions. According to these results, ± 5 % was observed as the interval of expected CCS for all the observed adducts. When several regression models are needed to explain such correlation, the obtained data could also provide interesting information for identifying the nature of unidentified compounds [**¡Error! Marcador no definido.**]. Thus, the fact that one single regression model can describe the correlation between *m/z* and CCS means that all ions considered present similar basic structure what limits the information offered by this study in this work. The difference in terms of CCS existing between the ions from the same compound can help to distinguish them providing information about its ionization behavior which can be useful for its identification in untargeted approaches [5].

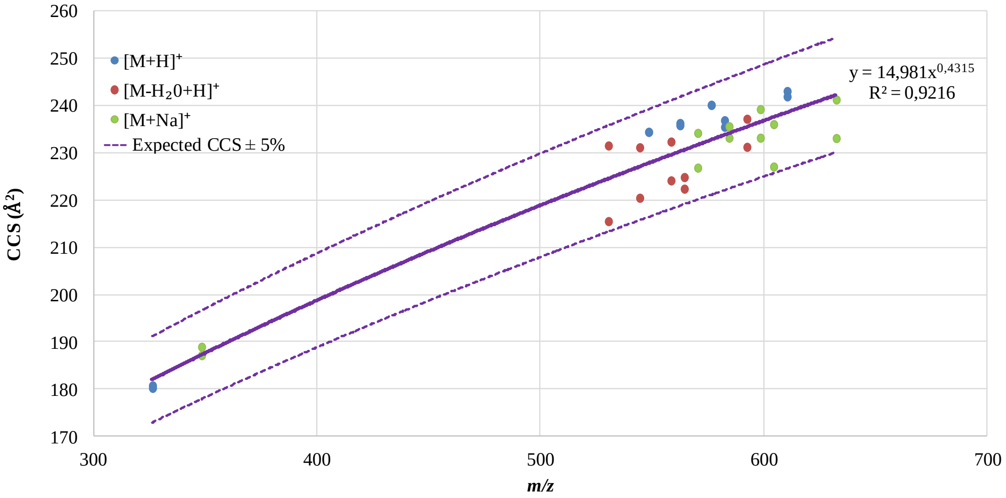


Figure 1. Representation of CCS vs *m/z* for the main ions characterized.

Table 1. $^{TW}CCS_{N_2}$ database for EAs. Cross-validation of $^{TW}CCS_{N_2}$ values and comparison with the values obtained by machine-learning approaches.

EA	**Ions**	***m/z***	**$^{TW}CCS_{N_2}$ LABERCA ***	**%RSD (n = 3)**	**$^{TW}CCS_{N_2}$ UNIPR ****	**%RSD (n = 3)**	**%RSD inter-lab**	**Machine Learning**			
								CCS base	**Error %**	**All CCS**	**Error %**
Em	[M+H]$^+$	326.1868	181.1	0.02	181.1	0.16	0.00	177.1	-2.21	179.7	-0.77
	[M+Na]$^+$	348.1687	186.9	0.01	-	-	-	184.2	-1.44	183.3	-1.93
Emn	[M+H]$^+$	326.1868	182.1	0.05	181.7	0.09	-0.25	177.1	-2.75	179.7	-1.32
	[M+Na]$^+$	348.1687	189.2	0.09	-	-	-	184.2	-2.64	183.3	-3.12
Es	[M+H]$^+$	548.2872	234.2	0.02	234.0	0.49	-0.01	229.2	-2.13	227.6	-2.82
	[M-H$_2$0+H]$^+$	530.2767	214.9	0.01	228.0	0.41	**6.10**	-		226.3	5.30
	[M+Na]+	570.2692	234.2	0.07	232.3	0.26	0.83	230.9	-1.41	229.0	-2.22
Esn	[M+H]$^+$	548.2872	231.3	0.01	232.5	0.28	0.49	229.2	-0.91	227.6	-1.60
	[M-H$_2$0+H]$^+$	530.2767	222.2	0.01	224.7	0.13	**1.12**	-		226.3	1.85
	[M+Na]$^+$	570.2692	226.5	0.04	225.1	0.15	-0.60	230.9	1.94	229.0	1.10
Et	[M+H]$^+$	582.2716	236.0	0.03	236.8	0.31	0.35	235.5	-0.21	235.1	-0.38
	[M-H$_2$0+H]$^+$	564.2610	222.7	0.01	-	-	-	-		234.1	5.12
	[M+Na]$^+$	604.2535	236.3	0.02	235.8	0.41	0.23	239.3	1.27	236.4	0,04
Etn	[M+H]$^+$	582.2716	234.9	0.01	234.3	0.05	-0.27	235.5	0.26	235.1	0,09
	[M-H$_2$0+H]$^+$	564.2610	224.6	0.04	227.6	0.09	**1.31**	-		234.1	4.23
	[M+Na]+	604.2535	227.2	0.01	226.7	0.09	-0.23	239.3	5.33	236.4	4.05
Eco	[M+H]$^+$	562.3029	236.3	0.06	236.6	0.19	0.11	232.3	-1.69	230.0	-2.67
	[M-H$_2$0+H]$^+$	544.2923	220.4	0.03	230.6	0.32	**4.64**	-		228.8	3.81
	[M+Na]$^+$	584.2848	235.9	0.14	234.4	0.03	-0.63	233.5	-1.02	231.4	-1.91
Econ	[M+H]$^+$	562.3029	235.2	0.01	237.2	0.01	0.88	232.3	-1.23	230.0	-2.21
	[M-H$_2$0+H]$^+$	544.2923	224.9	0.02	229.5	0.09	**2.04**	-		228.8	1.73
	[M+Na]$^+$	584.2848	233.1	0.09	233.1	0.87	0.01	233.5	0.17	231.4	-0.73
Ekr	[M+H]$^+$	576.3185	239.5	0.32	240.4	0.41	0.40	236.0	-1.46	233.5	-2.51
	[M-H$_2$0+H]$^+$	558.3080	222.7	0.44	-	-	-	-		232.3	4.31
	[M+Na]$^+$	598.3005	239.1	0.32	238.6	0.08	-0.23	236.8	-0.96	234.8	-1.80
Ekrn	[M+H]$^+$	576.3185	239.2	0.02	241.6	0.23	0.99	236.0	-1.34	233.5	-2.38
	[M-H$_2$0+H]$^+$	558.3080	231.1	0.11	234.1	0.02	**1.30**	-		232.3	0.52
	[M+Na]$^+$	598.3005	233.7	0.05	232.0	0.15	-0.71	236.8	1.33	234.8	0.47
Ecr	[M+H]$^+$	610.3029	242.7	0.06	243.6	0.33	0.38	242.3	-0.16	241.5	-0.49
	[M-H$_2$0+H]$^+$	592.2923	231.2	0.01	234.6	0.45	**1.47**	-		240.6	4.07
	[M+Na]+	632.2848	241.7	0.03	240.4	0.47	-0.53	245.1	1.41	242.6	0.37
Ecrn	[M+H]+	610.3029	241.4	0.01	243.3	0.10	0.79	242.3	0.37	241.5	0.04
	[M-H$_2$0+H]$^+$	592.2923	233.9	0.05	237.0	0.02	**1.31**	-		240.6	2.86
	[M+Na]$^+$	632.2848	233.9	0.05	233.7	0.16	-0.09	245.1	4.79	242.6	3.72

*CCS database was built in LABERCA. **CCS cross-validation was carried out in UNIPR.

As EAs present similar chemical structures, they have an analogous fragmentation pattern and usually lead to the same fragment ions (i.e., *m/z* 208.1, 223.1, 268.1 and 305.1). $^{TW}CCS_{N_2}$ values for these major fragment ions were compared and represented in **Figure 2**. The results showed that similar $^{TW}CCS_{N_2}$ values were obtained for ions with the same *m/z* regardless of the molecular ion from which it comes. This is probably due to the fact that after fragmentation, the molecules did not conserve its isomeric properties giving rise to identical fragment ions for the different EAs, and therefore, same CCS values. In this regard, CCS values for fragment ions cannot offer additional information for the determination of EAs. Nevertheless, the development of a CCS database for fragment ions may help the structural elucidation of unknown EAs so far.

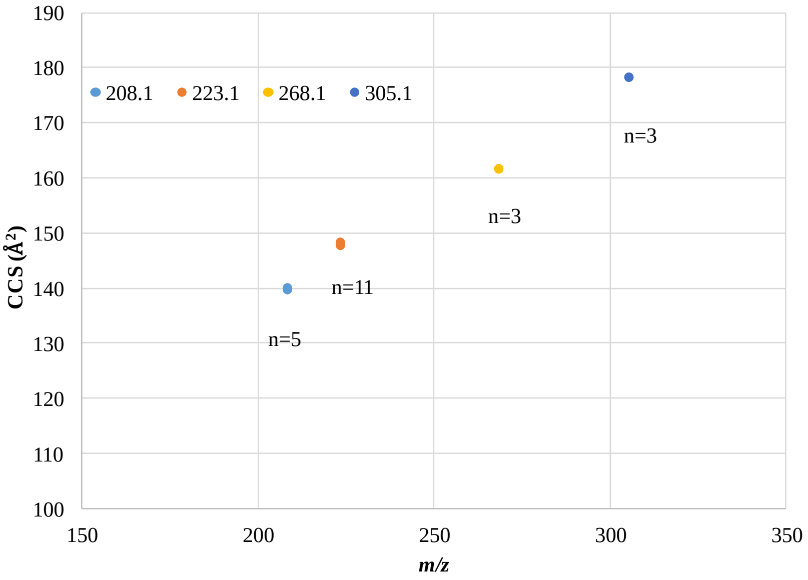


Figure 2. Representation of CCS vs *m/z* for the main fragment ions of EAs.

The aim of generating a CCS database for the main EAs and their epimers is to support their determination, reducing the number of false positive found in classical LC-MS workflows and contributing to develop targeted analysis with high selectivity. This strategy has been already successfully applied for unambiguous identification of targeted pesticides in food samples [7]. On the other hand, it should be pointed out that, in order to use CCS values in EAs analysis with high confidence, the experimental

$^{TW}CCS_{N_2}$ values obtained were cross-validated by an inter-laboratory study. In addition, they were compared with computationally derived CCS values obtained from predictive models, establishing in both cases uncertainty thresholds for CCS measurements.

3.1.1 Cross-validation of the CCS database

One of the challenges associated to the use of CCS values is the demonstration that CCS databases can be used independently from the instrument type and the IMS technology employed [8]. Therefore, in order to evaluate the reproducibility of CCS measurements for EAs, a cross-validation including two different TWIMS platforms (i.e., Synapt and Vion IMS QToF from Waters) has been carried out using the instrumental conditions described in section 2.4.

EA standard mixes prepared at different concentration levels (50, 100, 250 µg L^{-1}) were injected in triplicate, so $^{TW}CCS_{N_2}$ values were the result of nine measurements. RSDs below 1 % were observed for the $^{TW}CCS_{N_2}$ measurement of protonated ions and sodium adducts. In the case of $[M-H_2O+H]^+$ ions, the RSD obtained was below 2 % for most analytes except for Esn, Eco and Econ. Regarding to the CCS deviation between laboratories, a good correlation was found between them; the error was below 1 % in all cases except for the $[M-H_2O+H]^+$ ions of Esn, Eco and Econ (**Table 1**). Although, in Synapt and Vion instruments the position of the TWIMS cell is different, this should not affect molecules conformation because in all TWIMS platforms the ion injection into the mobility cell is preceded by trap and helium cells that guarantee similar injection conditions. However, this fact could have affected to the loss of water of the molecules explaining the deviation in CCS measurements for $[M-H_2O+H]^+$ ions. Just few ions such as $[M+Na]^+$ ions of Em and Emn and $[M-H_2O+H]^+$ of Et and Ekr were not detected by Vion IMS instrument at UNIPR, which is generally due to the fact that ion formation depends on the instrumental configuration and ionization conditions, so different ions can be generated.

To sum up, 73.3 % of the CCS measurements have an error below 1 % and 90 % below 2 % being in accordance with current acceptance criterion threshold of < 2 % for CCS measurements used for CCS data building in order to confirm the identity of molecules [3,6,9]. Thus, the cross-validation of the $^{TW}CCS_{N2}$ values has been properly performed for $[M+H]^+$ and $[M+Na]^+$ ions confirming that the $^{TW}CCS_{N2}$ obtained values for EAs can be used in studies using other TWIMS instruments. However, in the case of $[M-H_2O+H]^+$ ions further investigation is necessary to understand the bias existing between the values obtained by different TWIMS instruments.

To conclude, a cross-laboratory $^{TW}CCS_{N2}$ database was built for 12 EAs (22 ions) with low deviations for the first time. Assays involving a higher number of EAs are interesting in order to develop a larger CCS database for this family of compounds.

3.1.2 Machine learning approach

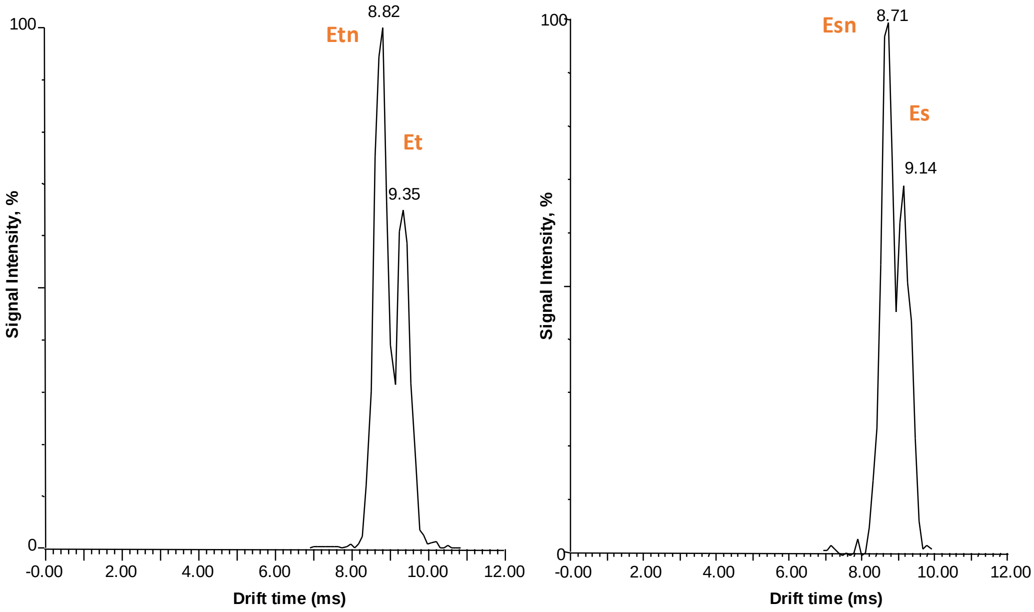
Traditionally, CCS values have been obtained through the experimental measurement of chemical standards or using computational modeling [10]. However, these strategies have some limitations. In the case of experimental measurements, the limited availability of chemical standards and the choice of a suitable calibrant with similar structure to the target analytes for calibrant-dependent methods (e.g., TWIMS methods) determine the accuracy of the experimental CCS data obtained [4]. On the other hand, computational modeling is computationally intensive and is likely to produce large error, especially in molecules with flexible structures [11]. In this context, a new approach namely machine-learning based prediction has emerged to produce CCS values in a large scale. This methodology has been already implemented to generate the CCS values for the identification of small molecules (e.g., in metabolomics studies [12] or in residue analysis [13]), presenting a lower prediction error (i.e., 1-3 %) and being more computationally efficient when compared with computational modeling [14]. Different platforms, such as CCSbase [15], and AllCCS [16], based on machine-learning models have been recently developed to complement CCS empirical data. These platforms provide predicted CCS values for target compounds (known and unknown) taking into consideration their molecular descriptors such as the simplified molecular-input line-entry system (SMILE) for describing the structure of the molecule and the exact mass of each one. In addition, in the absence of experimental or library data, CCS predictions can help to reduce analysis time in identification approaches since they reduce the number of potential candidates [17].

In this chapter, CCSbase and AllCCS web servers were used to compare the predictive CCS provided by them with the experimental $^{TW}CCS_{N_2}$ values obtained for EAs using TWIMS, as included in **Table 1**. With CCSbase, CCS data for $[M-H_2O+H]^+$ ions was not available. The results provided by machine-learning web servers showed a prediction error within 5 % for 94.8 % of all CCS values, and within 3 % for 93.8 % of all CCS values corresponding to $[M+H]^+$ and $[M+Na]^+$ ions, respectively, resulting in a median relative error below 1 %. These results are in the same range that prediction errors previously reported using machine-learning approaches [12,13]. The CCS values with a higher error corresponded to $[M-H_2O+H]^+$ ions as it was also noticed in the CCS cross-validation study. Although these prediction error values were satisfactory, they could be improved providing more data to the machine learning models.

Overall, due to the lack of CCS databases including EAs, the machine-learning based prediction is a useful and user-friendly option to generate CCS values offering more confidence to the CCS values generated experimentally.

3.2 Selectivity enhancement by ion mobility spectrometry

Precision IMS offers a third separation dimension based on the application of the CCS parameter which allows the separation and identification of co-eluting analytes or similar molecules such as isomers and isobars [18]. In this regard, slight differences between CCS values of compounds with similar or equal *m/z,* as it is the case of the main EAs and their epimers, may be enough to distinguish them. Generally, differences of approximately 2 % should be sufficient for identification purposes (peak-to-peak resolution), while more than 5 % is likely to be necessary for quantification (baseline resolution) as it have been theoretically suggested [19]. On this basis, sodium adducts of Et ($^{TW}CCS_{N_2}$ = 236.3 Å^2) and its epimer Etn ($^{TW}CCS_{N_2}$= 227.2 Å^2) could be at least peak-to-peak separated by TWIMS since the CCS difference between them was higher than 2 % (i.e., 4 %). Similarly, sodium adducts of Es ($^{\mathrm{TW}}$CCS$_{\mathrm{N2}}$= 234.2 Å^{2}) and its epimer Esn ($^{\mathrm{TW}}$CCS$_{\mathrm{N2}}$=226.5 Å^{2}) presented a difference of 3.4 % and also sodium adducts of Ecr ($^{\mathrm{TW}}$CCS$_{\mathrm{N2}}$= 241.7 Å^{2}) and its epimer Ecrn ($^{\mathrm{TW}}$CCS$_{\mathrm{N2}}$= 233.9 Å^{2}) with a CCS difference of 3.3 %. As it is shown in **Figure 3**, a partial separation, enough for epimers identification, was achieved in all cases being in accordance with the differences observed in CCS values and the peak resolution expected. Despite the fact that the epimers were not fully separated to baseline, these results highlight the potential of using CCS data as a complementary tool for improving EAs separation and method selectivity in traditional LC-MS workflows. Moreover, considering these results, the use of new IMS technologies such as cyclic ion mobility spectrometry (cIMS) could improve separation resolution in further analysis as it has been recently demonstrated [20].



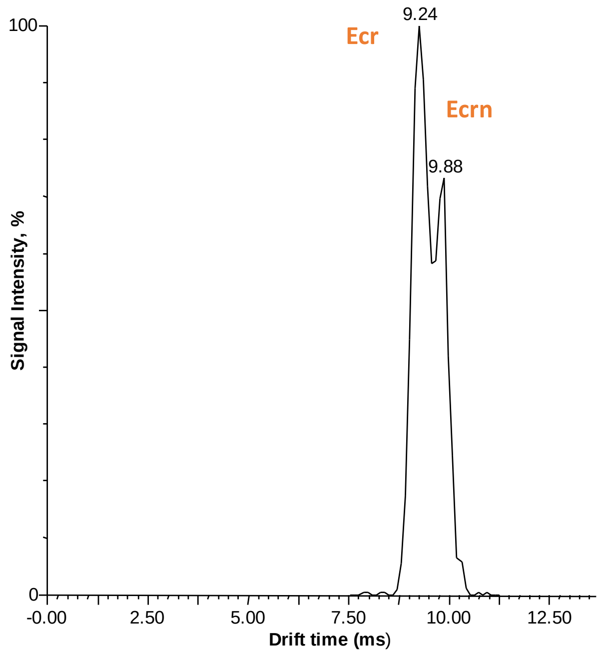


Figure 3. Mobility spectra for the separation of sodium adducts of: **a)** Et and Etn; **b)** Es and Esn; **c)** Ecr and Ecrn by TWIMS. Mobility spectra have been obtained after applying the following signal filters: **a)** *m/z* 604, Rt between 3.8 and 4.3 min, and drift time between 7 and 11 ms; **b)** *m/z* 570, Rt between 3.2 and 3.9 min, and drift time between 7 and 9 ms; **c)** *m/z* 632, Rt between 6.0 and 8.2 min, and drift time between 7.5 and 11 ms.

In addition, the implementation of IMS in LC-MS, allows to separate target compounds from matrix interferences which present similar retention times and *m/z*, leading to an enhancement in selectivity, essential for peak integration and quantification of target compounds. This improvement has been previously demonstrated in the food safety field through the analysis of zearalerone and its metabolites [21], β-adrenergic agonists [22], and steroids isomers [9]. In order to investigate this advantage for the determination of EAs in cereal samples, barley samples were submitted to the QuEChERS sample treatment, and subsequently, the obtained extracts were analyzed by LC-TWIM-MS-TOF. Then, the extracted ion chromatograms (EICs) observed when using the TWIMS as third separation dimension were compared with that obtained without applying this extra separation dimension. As can be seen in **Figure 4**, Em co-eluted with several matrix compounds in the same *m/z* range making impossible its identification in the extracted ion chromatograms (EICs). Nevertheless, when the mobility range of the deprotonated molecule of Em was selected as signal filter, these matrix compounds were avoided making possible the peak integration of Em. Thus, the application of IMS allowed the isolation of this analyte from matrix interferences, granting its unequivocal identification and chromatographic quantification.

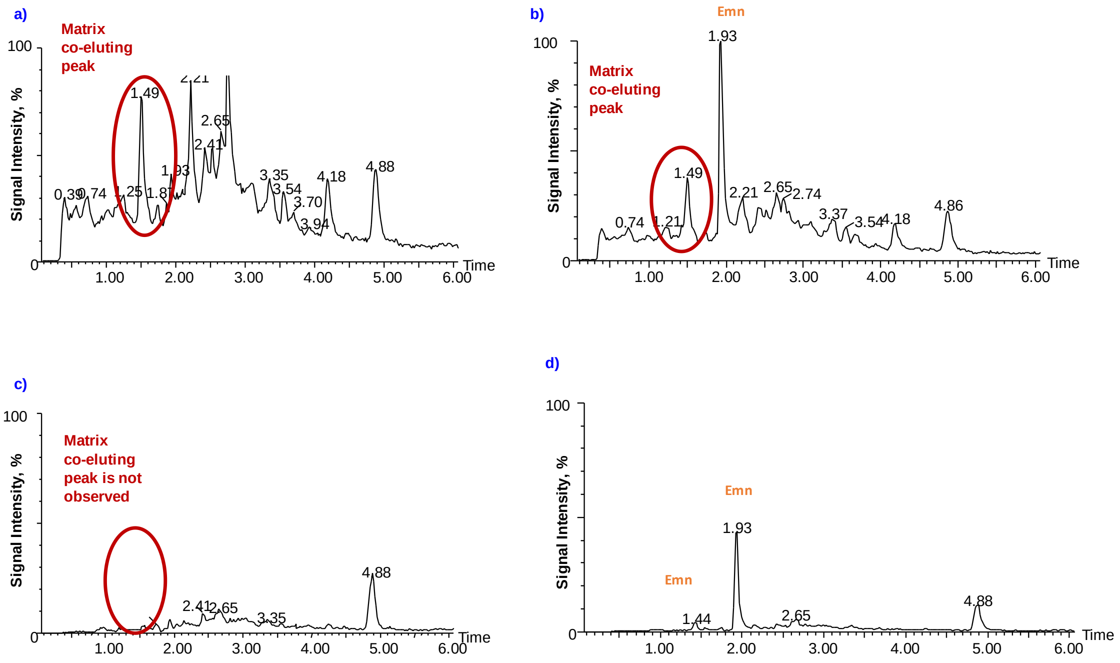


Figure 4. EICs (*m/z* 326.2) resulted from the analysis of: **a)** and **c)** non-spiked barley samples, and **b)** and **d)** barley samples spiked with EAs (5 µg kg^{-1}; [M+H]$^{+}$). Analyses were performed by LC-ESI-TWIM-TOF-MS in positive mode. In **c)** and **d)**, the mobility region of the deprotonated molecule of Em (i.e between 62 and 52 ms) was selected.

Furthermore, in the case of Ekr and Ekrn, although their identification was possible without applying TWIMS dimension, interferences with the same retention times and *m/z* as the target compounds could have been integrated leading to a higher concentration of analyte or even to a false positive report. However, selecting the mobility range of the deprotonated molecule for both analytes, these matrix compounds were eliminated (**Figure 5**). Therefore, the use of TWIMS provided more reliable results for quantification purposes. It is also worth mentioning that these advantages were observed in untargeted workflows where selectivity depends on the mass resolution used through the work (sensitivity, resolution or high-resolution mode). Normally in untargeted studies low resolution is selected so that is why this improvement in terms of selectivity has special interest for the separation of isomer compounds.

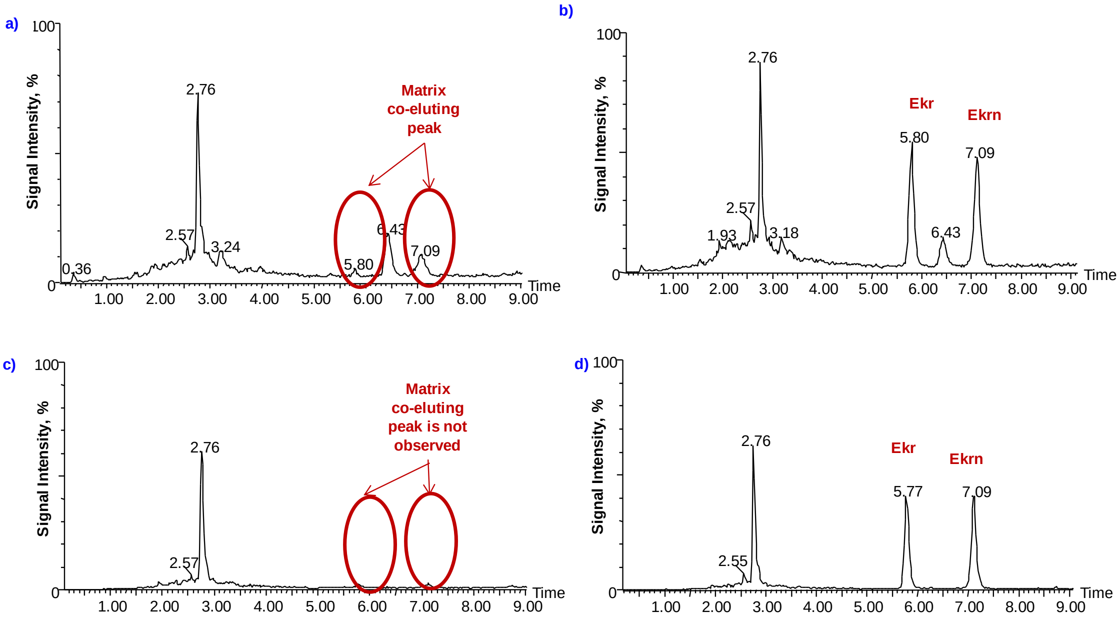


Figure 5. ICs (*m*/*z* 576.3) resulted from the analysis of: **a)** and **c)** non-spiked barley samples, and **b)** and **d)** barley samples spiked with EAs (5 μg kg^{-1}; [M+H]$^{+}$). Analyses were performed by LC-ESI-TWIM-TOF-MS in positive mode. In **c)** and **d)**, the mobility region of the deprotonated molecule of Ekr (i.e between 107 and 87 ms?) was selected.

3.3 Sensitivity enhancement by ion mobility spectrometry

IMS leads to cleaner MS spectra and, as a result, limits of detection (LODs) can be reduced significantly improving the sensitivity of the method [23]. This improvement is especially useful in the analysis of complex matrices composed by a high number of compounds such as food samples, as it has been reported in cornmeal [21], fruits [24] and pistachio samples [25].

In this chapter, this advantage was also evaluated in barley samples, and as can be seen in **Figure 6**, cleaner EICs were observed when the TWIMS dimension was used as signal filter; therefore, facilitating peak integration of EAs and improving S/N ratio.

The S/N ratio improved 2.5 to 4-fold when just the mobility region of target analytes was selected. In all cases, except for Ekr and Ekrn, the analytical signal observed at a concentration level of 5 μg kg^{-1}without applying the mobility region was below the LOQ (S/N=10); however, when the mobility region for those compounds was selected, LOQ was above this value allowing their quantification.

In addition, to evaluate the sensitivity provided when TWIMS is integrated in the LC-MS workflow, barley samples were spiked at different concentration levels (2, 5, 10, 25, 50, 100 μg kg^{-1}), and submitted to the QuEChERS pretreatment. Afterwards, the extracts were injected and analyzed by LC-ESI-TWIM-TOF-MS and LC-ESI-TOF-MS in positive mode using the same LC and ESI conditions in both methods. In addition, in order to improve signal sensitivity, TOF system was operated in sensitivity mode. It was observed that, although, a lack of sensitivity is usually attributed to IM-MS methods, in this case, the sensitivity was not decreased when EAs were analyzed by LC-IMS-MS in comparison to the results obtained by LC-MS (**Figure 6).**

This is probably due to the instrumental improvements developed to overcome low ion transmission in IMS [**¡Error! Marcador no definido.**]. Anyways, this drawback could be overcome with the enhancement in the S/N ratio discussed before.

Taking these results into consideration, the improvement offers by the integration of TWIMS to LC-MS workflow for EAs determination was demonstrated through the reduction of the background noise and the increase in the S/N ratio.

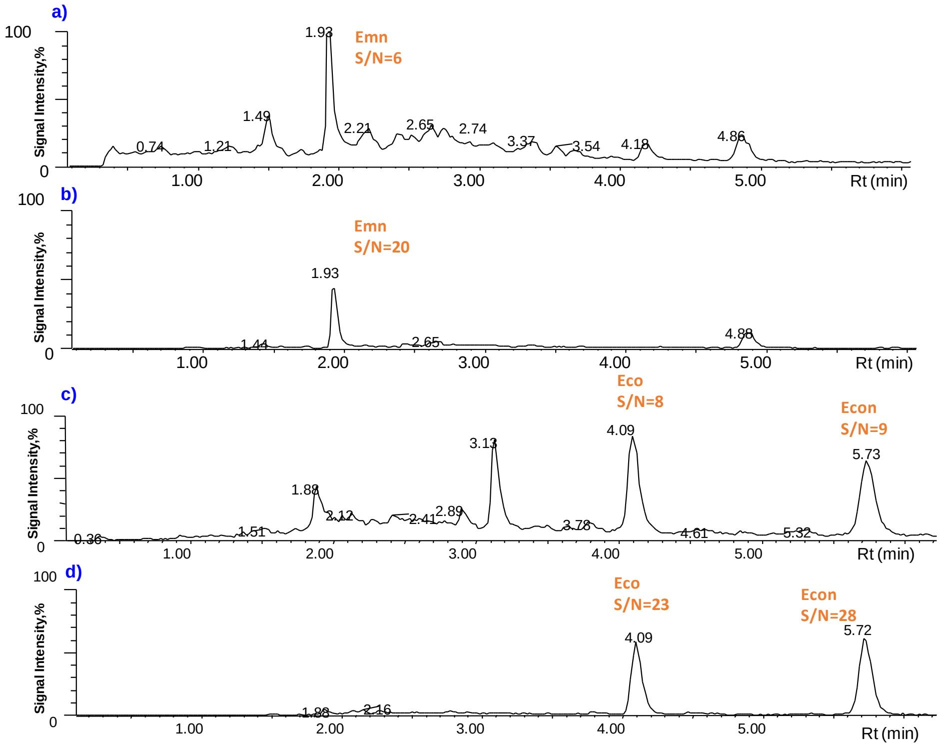


Figure 6. EICs resulted from the analysis of: I) Emn (5 µg kg^{-1}; $[M+H]^+$) and II) Eco and Econ (5 µg kg^{-1}; $[M+H]^+$), in barley samples by LC-ESI-TWIM-TOF-MS in positive mode. The following filters were applied for signal processing of related total ion chromatograms: a) *m/z* 362.2; b) *m/z* 362.2 and drift time range between 52-62 ms; c) *m/z* 562.3; d) *m/z* 562.3 and drift time range between 87-103 ms

3.4 Application of ion mobility spectrometry to determine EAs in cereal samples

The proposed QuEChERS-TWIM-TOF-MS method was validated in terms of linearity, limits of detection and quantification (LODs and LOQs, respectively), and precision in cereal samples.

Firstly, procedural calibration curves for both barley and wheat cereal samples were prepared by spiking blank samples of each matrix at six different concentration levels (2, 5, 10, 25, 50 and 100 µg kg^{-1}). Two samples per each concentration level were processed following the QuEChERS procedure and analyzed in duplicate. Peak area was considered as a function of the analyte concentration. LODs and LOQs were calculated as the minimum analyte concentration with S/N equal to 3 and 10, respectively. The statistical parameters calculated by least-square regression, as well as LODs and LOQs, are shown in **Table 2.** Although, limited dynamic range are associated with LC-IM-MS methods in comparison to LC-MS [26], the same range could be evaluated. In general, satisfactory linearity was achieved for both samples (determination coefficients R^2>0.99) in the studied range. The LOQs obtained ranged between 0.7 and 2.0 μg kg^{-1} for barley samples, and between 0.7 and 2.1 μg kg^{-1} for wheat samples, being comparable and even lower that those obtained by LC-MS for the determination of EAs in similar samples [2,27,28].

Table 2. Statistical and performance characteristics of the proposed method for the determination of the main EAs and their epimers in barley and wheat samples.

Analyte	Linear regression equation	Linear Range (μg kg^{-1})	Linearity (R^2)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
Barley samples					
Em	y=148.58x-383.66	2.0-100	0.9909	0.6	2.0
Emn	y=2140.8x-5747.6	0.9-100	0.9925	0.3	0.9
Es	y=2417.4-6711.9	1.1-100	0.9914	0.3	1.1
Esn	y=1524.2x-5618.8	1.3-100	0.9925	0.4	1.3
Et	y=2565.2x-7002	0.9-100	0.9909	0.3	0.9
Etn	y=5267.2x-13521	0.7-100	0.9943	0.2	0.7
Eco	y=5154.3x-11715	0.8-100	0.9944	0.2	0.8
Econ	y=4090.1x-13377	0.9-100	0.9911	0.3	0.9
Ekr	y=3748.3x-10774	0.6-100	0.9923	0.2	0.6
Ekrn	y=1727.5x-4664.2	0.9-100	0.9906	0.3	0.9
Ecr	y=3711.8x-14007	1.3-100	0.9906	0.4	1.3
Ecrn	y=2185x-6172.2	1.0-100	0.9913	0.3	1.0
Wheat samples					
Em	y=61.207x-234.38	1.6-100	0.9907	0.5	1.6
Emn	y=1594.7x-5443.6	0.7-100	0.9920	0.2	0.7
Es	y=417.73x-2083.7	2.0-100	0.9903	0.6	2.0
Esn	y=503.26x-3227.1	1.7-100	0.9902	0.5	1.7
Et	y=573.41x-2633.8	0.8-100	0.9914	0.2	0.8
Etn	y=3453.6x-15732	0.9-100	0.9910	0.3	0.9
Eco	y=3374.6x-16568	0.7-100	0.9912	0.2	0.7
Econ	y=1898.1x-9306.5	1.5-100	0.9935	0.5	1.5
Ekr	y= 2099.3x-11451	1.1-100	0.9910	0.3	1.1
Ekrn	y=418.85x-971.83	1.3-100	0.9925	0.4	1.3
Ecr	y=685.41x-4182.1	2.0-100	0.9902	0.6	2.0
Ecrn	y=1325.3x-5369.6	0.7-100	0.9939	0.2	0.7

According to SANTE/12682/2019 guideline, one representative sample can be selected from a commodity group for the validation of the method [29]. Since barley and wheat are included in the same group, barley samples were chosen as representative commodity for the precision study. Precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by the application of the proposed QuEChERS-LC-ESI-TWIM-TOF-MS to barley samples spiked at two concentration levels of the linear range (5 and 50 $\mu g\ kg^{-1}$). For repeatability two samples were processed for each level and injected by triplicate on the same day under the same conditions. Intermediate precision was evaluated with a similar procedure, but analyzing one spiked sample in triplicate and per day, for three different days. The obtained results, expressed as relative standard deviation (RSD, %) of peak areas are summarized in **Table 3**. In all cases, RSD values lower than 14.4 % were obtained, being in agreement with the EU recommendations for the performance of analytical methods, which set an upper limit for RSD of 20 % [30].

Table 3. Precision of the proposed method for the determination of the main EAs and their epimers in spiked barley samples.

	Repeatability, % RSD (n = 9)		**Intermediate Precision, % RSD (n = 9)**	
	5 $\mu g\ kg^{-1}$	**50 $\mu g\ kg^{-1}$**	**5 $\mu g\ kg^{-1}$**	**50 $\mu g\ kg^{-1}$**
Em	9.3	6.5	13.6	8.5
Emn	8.0	5.0	12.9	6.3
Es	11.0	6.2	11.3	9.6
Esn	8.5	6.8	12.9	7.4
Et	9.8	4.6	10.2	7.7
Etn	13.0	8.8	9.2	6.6
Eco	7.8	5.6	11.7	9.4
Econ	7.3	6.6	11.5	7.9
Ekr	9.5	7.1	14.4	9.4
Ekrn	8.1	5.9	9.0	7.5
Ecr	10.5	8.7	9.9	8.0
Ecrn	9.3	6.7	13.6	8.5

In addition, in order to demonstrate the applicability of the validated method, wheat and barley samples from Algeria were analyzed to monitor the natural occurrence of EAs in these cereals. A total of 60 cereal samples (30 samples of barley and 30 samples of wheat) were previously investigated by UHPLC-MS/MS [31], founding 8 samples of wheat and 4 samples of barley as positive samples in EAs. These positive samples were immediately aliquoted, dried and kept at 4 °C avoiding direct light exposure until their reconstitution with the appropriate volume of MeOH:water (50:50, v/v), and subsequent analysis by LC-ESI-TWIM-TOF-MS.

The results obtained by the analysis of positive barley and wheat samples are shown in **Tables 4** and **5**, respectively. The results are expressed as concentration of EA higher than LODs (n=6). In the cases where the concentration was below the LOQ for the EA (< LOQ, detected but not quantified) the concentration was considered as equal to zero. The concentration of total EAs ranged from 8.3 to 36.8 μg kg^{-1} for barley and from 5.2 to 65.0 μg kg^{-1} for wheat samples. Em and its epimer Emn were the most common EAs found in barley samples, while Ekr and Ecr showed the highest incidences in wheat.

The following sample code was used: A: samples from Aïn Témouchent; O: samples from Oran; T: samples from Tiaret**.**

Table 4. Results of positive samples in barley samples.

Sample*	Ergot Alkaloid	Concentration (μg/kg)	%RSD
T1	Em	21.7	1.7
	Emn	3	0.1
	Et	2.3	0.1
	Total EAs (T1)	27.0	
T3	Em	32.8	2.4
	Emn	4.0	0.2
	Total EAs (T3)	36.8	
T7	Em	10.6	0.5
	Emn	3.8	0.6
	Total EAs (T7)	14.4	
T10	Em	5.4	0.6
	Emn	2.9	0.1
	Total EAs (T10)	8.3	

Table 5. Results of positive samples in wheat samples.

Sample*	EA	Concentration (µg/kg)	%RSD
O2	Em	5.2	0.4
	Total EAs (O2)	5.2	
T8	Em	16.4	1.8
	Emn	2.8	0.5
	Et	5.5	0.2
	Eco	5.1	0.1
	Ekr	8.7	0.9
	Ecr	18.5	0.9
	Ekrn	<LOQ	1.3
	Ecrn	8.03	0.7
	Total EAs (T8)	65.03	
O7	Esn	<LOQ	---
	Es	7.1	0.1
	Eco	6.3	0.2
	Econ	2.94	0.3
	Ekr	10.3	0.4
	Ekrn	6.5	0.5
	Total EAs (O7)	33.14	
AT1	Et	2.5	0.7
	Ekr	6.85	0.9
	Ecr	1.98	0.4
	Ekrn	<LOQ	---
	Total EAs (AT1)	11.33	
T9	Ekr	6.1	0.2
	Ecr	2.5	0.9
	Ekrn	<LOQ	---
	Ecrn	<LOQ	---
	Total EAs (T9)	8.6	
O4	Em	9.3	0.9
	Emn	3.8	0.1
	Esn	<LOQ	---
	Es	6.8	0.2
	Ecr	3.2	0.6
	Ecrn	1.4	0.3
	Total EAs (O4)	24.5	
T10	Etn	2.3	0.4
	Et	6.5	0.1
	Ecr	30.6	0.9
	Ecrn	12.1	0.7
	Total EAs (T10)	54.41	
O10	Esn	<LOQ	---
	Es	6.2	0.8
	Eco	9.9	0.5
	Econ	4.3	0.7
	Ekr	21.5	0.9
	Ekrn	3.1	0.6
	Total EAs (O10)	50.3	

It was observed that most of the EAs found in both samples matched with the previously reported EAs using the UHPLC-MS/MS method [31]; however, the amount of them presented some variations especially in the case of Em. As it was discussed before, an increase in selectivity was reported by adding TWIMS for the analysis of EAs allowing, for instance, the unequivocal identification of this compound. Otherwise, the co-eluting interferences with the same *m/z* would have been integrated what may explain the higher concentration obtained for this analyte when using UHPLC-MS/MS. This effect was also noticed for Ekrn, for which the concentration found with LC-TWIM-TOF-MS was also lower. This fact could be due to the cleaner EIC achieved for this analyte (**Figure 5**).

In addition, Em was detected in wheat samples using the UHPLC-MS/MS method, however, with the LC-TWIM-TOF-MS method, this analyte was not found in such samples. These results may highlight possible false positive results for this compound when IMS is not integrated in the LC-MS workflow as it has been previously discussed in other works [7,32].

On the other hand, when barley samples were analyzed by UHPLC-MS/MS, Emn was below its LOQ, being just detected. Nevertheless, with the implementation of TWIMS and taking into consideration the S/N improvement allowed for Emn **(Figure 6),** this analyte could be quantified by LC-ESI-TWIM-TOF-MS.

4. CONCLUSIONS

The present chapter shows the advantages provided by the addition of IMS in the LC-MS workflows destined to determine EAs. These advantages are mainly higher separation resolution and higher confidence in analyte identification. In this regard, a $^{TW}CCS_{N_2}$ database for the main EAs and its epimers has been built as this parameter is complementary to *m*/*z* and retention times and useful for the identification of these compounds, especially in non-targeted analysis. It is the first CCS database reported for these compounds, which has been successfully inter-laboratory cross-validated with satisfactory CCS deviations (<2 %). Furthermore, the evaluation of the advantages provided by the implementation of TWIMS in LC-MS workflows, in terms of sensibility and sensitivity, has been demonstrated in cereal samples. The use of CCS as a complementary parameter has been useful for improving the separation of Et, Es and Ecr and its corresponding epimers, as well as, to separate target compounds from matrix interferences leading to an enhancement in selectivity. These results show the usefulness of hyphenated separations in the analysis of complex matrices that requires identification of potential isomers which similar retention times. On the other hand, TWIMS has been an effective tool for reducing background noise, improving the S/N ratio for the studied compounds between 2.5 and 4 times and, consequently, enhancing the sensitivity of the method. In addition, the LC-TWIM-TOF-MS method has been validated in barley and wheat samples obtaining LOQs in the range of low $\mu g\ kg^{-1}$. Finally, the potential of the proposed method was proved by its application to wheat and barley samples previously reported as positive samples. In this regard, the use of IMS has been effective to reduce the number of possible false positive results of EAs in such samples. To sum up, from our point of view, IMS is a powerful technique for enhancing the performance characteristics of LC-MS methods destined to the analysis of contaminants in food and food-related matrices. However, more research involving its application is needed in this field in order to implement this analytical tool in food safety analysis.

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ANNEX I (Chapter 2)

LIS-method for automated nanofibrous online SPE.

Instruction	Comment
Selection valve to position 4 Aspirate 1000 µL at 10 ml/min from head valve position OUT Wait 1 s	Aspiration of acetonitrile
Injection valve to position LOAD Empty at 0.75 ml/min to head valve position MIDDLE Wait 3 s	Washing nanofibers with acetonitrile
Selection valve to position 2 Aspirate 1000 µL at 10 ml/min from head valve position OUT Wait 1 s	Aspiration of water
Empty syringe at 0.75 µL/min to head valve position MIDDLE Wait 3 s	Washing nanofibers with water
Selection valve to position 3 Aspirate 200 µL at 10 mL/min from head valve position OUT	Cleaning holding coil and syringe head valve with buffer
Relay 1 in ON Wait 2 s Relay 1 in OFF	Activate stirring for 2 s
Empty syringe at 10 mL/min to head valve position IN Wait 1 s	Discharge buffer remains from syringe
Selection valve to position 3 Aspirate 300 µL at 10 ml/min from head valve position OUT Wait 2 s	Buffer aspiration
Relay 1 in ON	Activate stirring
Activate stirring Selection valve to position 5 Aspirate 2000 µL at 10 ml/min Wait 3 s	Aspiration of remains of buffer from holding coil and sample, mixing of solution in-syringe
Selection valve to position 8 Aspirate 100 µL at 10 ml/min Wait 1 s	Empty remains of sample from the HC into the syringe with air
Relay 1 in OFF	Deactivate stirring
Empty syringe at 0.5 ml/min to head valve position MIDDLE Wait 3 s	Sample loading onto nanofibers
PORT = 2 Call routine "Syringe cleaning" (2x)	Cleaning the syringe 2x with water
Selection valve to position 3 Aspirate 25 µL at 1 ml/min from head valve position OUT Wait 3 s	Buffer aspiration
Relay 1 in ON	Activation of stirring
Selection valve to position 6 Aspirate 1000 µL at 10 ml/min from head valve position OUT Wait 2 s	Water aspiration for washing
Relay 1 in OFF	Deactivation of stirring
Dispense 2000 µL at 0.75 ml/min to head valve position MIDDLE Wait 3 s	Washing of nanofibers
Injection valve to position INJECT	
Relay 3 in ON for 1 s	Triggering of HPLC
Relay 2 in ON for 1 s	Initiation of Gradient
Wait 300 s	Wait for method restarting

FINAL CONLUSIONS

The different developed methods reported in this Thesis contribute to increase the number of analytical methods available for the control of NNIs, FPN and its metabolites, BCL as well as for the control of EAs, both in food and environmental safety fields. The potential of using miniaturized techniques such as CE and CLC as well as HPLC, UHPLC and IMS coupled to different detection systems such as UV-Vis and MS has been evaluated. In addition, a great variety of sample techniques have been proved and optimized in food and environmental samples providing satisfactory results in terms of recovery and matrix cleaning up, involving just few steps and low solvents consumption. The use of miniaturized techniques and sample treatments is in accordance with the trends in green analytical chemistry.

The conclusions obtained in this Thesis are the following:

- CE and CLC methods for the monitoring of insecticides involve lower solvent consumption and good efficiency in comparison with traditional LC-methods. Among the considered techniques for NNIs, CE is the greenest technique since no organic solvents are needed during the separation due to the use of a very small volume of buffer as BGE and the low waste generation. Also, the analysis time and the use of silica capillaries of low price instead of chromatographic columns is a positive aspect in relation to the cost of the analysis.

- Despite of the fact that low sensitivity has been attributed to CE-UV methods, high sensitivity with limit of detection (LOQs) in the low μg L^{-1} have been reached. This sensitivity enhancement was provided by the combination of on-line (“sweeping” as stacking mode) and off-line preconcentration (SPE) strategies. In addition, the use of an extended light path capillary contributed to improve the sensitivity.

- Although CLC methods presented the longest analysis time, they allowed the determination of seven NNIs in complex matrices such as cereals and honeys using simple and fast sample treatments despite of using UV-Vis detection, usually available in all the laboratories. The sample treatments employed (SLE and DLLME) did not involve cleaning step, reducing therefore, the reagent consumption and sample handling time.

- Polymers nanofibers were for the first time investigated as novel sorbents for NNIs, selecting polyimide as the most suitable material for the on-line SPE procedure proposed. The developed on-line SPE-HPLC-UV method enabled automated sorbent condition, in-system sample mixing with loading buffer, analyte preconcentration, and finally, the separation and detection of NNIs.

- It has been demonstrated for the first time that NNIs and BCL can be determined by MEKC-MS/MS. Moreover the separation resolution was improved in comparison with the only previously reported CZE-MS/MS method, which monitor a lower number of analytes. The coupling of MEKC with MS has been possible because of the use of a volatile surfactant acting as both as BGE and micellar medium, namely APFO. This highlighted the potential of using this surfactant instead of other possible options needed to make compatible the MEKC mode with MS, taking into account that non-volatile surfactants cannot be used because of contamination of the ion source of the mass spectrometer.

- A scaled-down QuEChERS procedure has been demonstrated to be a useful strategy for the extraction of NNIs prior their determination by MEKC-MS/MS when the available amount of sample is reduced. In addition, a dispersive sorbent known as Z-Sep+, specially indicated for the cleaning of lipids and fatty acids, was selected to eliminate matrix interferences in honeybee and pollen samples. This dispersive sorbent reduced significantly the matrix effects if compared with the commonly chosen for these samples, such as C18 and PSA, without decreasing extraction recoveries.

- For the first time several NADESs have been evaluated as dispersive solvents in a DLLME-SFO procedure, applied to the control FPN ant its metabolites together with BLC. The NADES so-called LGH, composed by lactose, glucose and water was chosen as optimum. This solvent was able to disperse the extraction solvent in fine droplets leading to a cloudy solution characteristic of DLLME-based methods without the need of employing toxic organic solvents. This work revealed the potential of NADES in sample treatment procedures.

- In the analysis of EAS by UHPLC-MS/MS, the modification of the traditionally used QuEChERS procedure for the extraction of EAs and clean-up of cereal samples, have led to a reduction of organic solvents as well as an increase in sensitivity. The volume of the extraction solvent in this sample treatment could be reduced avoiding the dilution of the sample without affecting negatively the extraction recoveries of the analytes.

- The validated method for the determination of EAs in oat-based samples including functional foods is a contribution to the food safety field since more analytical methods are needed to evaluate the consumption of EAs in processed foods and food supplements. Although only was sample was positive, it shows that despite the improvements in grain processing, contamination by EAs must be considered, especially in cereal-based processed foods. In the same way, the study of the natural occurrence of EAs in cereal samples from countries in which mycotoxins are not regulated, such as Algeria, contributes to this issue and emphasizes the importance of including EAs epimers in the risk assessment and the variability of the pattern of EAs among positive samples.

- The collaboration with LABERCA (Nantes, France), allow us to use IMS-HRMS to establish a $^{TW}CCS_{N2}$ database for the all ions identified for the main EAs and their epimers as well as for the main fragments observed. This database has been also inter-laboratory cross-validated in collaboration with the University of Parma (Italy), showing satisfactory CCS deviations and demonstrating the applicability of using CCS as a complementary and instrument independent parameter, especially useful in non-target analyses.

- Finally, the advantages of adding IMS in LC-MS workflows destined to determine EAs have been investigated using a LC-TWIM-TOF-MS method. The use of TWIM allows separating target compounds from matrix interferences, leading to an enhancement in selectivity. This extra-dimension also provided more confidence to the determination of the main EAs from their corresponding epimers. In addition, this technique has been useful for reducing background noise, improving the S/N ratio for the studied compounds, and therefore, the sensitivity of the method.

As a summary, the most significant experimental and instrumental characteristics of the developed methods in this Thesis are shown in **Table 1.**

Table 1. Analytical characteristics of the proposed methods.

Analytes	**Sample**	**Sample treatment**	**R (%)**	**Technique**	**Analysis time**	**LOQs**
DNT, TMT, CLT, NTP, IMD,TCP, ACT,	Rice, barley, wheat, maize, oat (1 g) Multi-flower, rosemary, eucalyptus, orange tree honeys (1 g)	**SLE** (1:3 ACN/DCM as extraction solvent) **DLLME**	80- 105 80- 94	**CLC-DAD** **Column:** Zorbax XDB-C18 (150 × 0.5 mm i.d, 5 µm) **Mobile phase:** Water (eluent A), MeCN (eluent B) **Flow**: 10 µL min^{-1}	18 min	9.3-16.7 µg kg^{1} 9.6-22 µg kg^{1}
ACT, CLT, DNT, IMD, TCP, TMT	Environmental water (2 mL)	**on-line SPE** (nanofibers as sorbent)	63-120 PF: 70-82	**HPLC-UV** **Column**: Phenomenex RP-C18 (150 x 4.6, 2.1 µm) **Mobile phase:** 10 % MeCN (v/v) in 0.05% (v/v) aqueous FA (eluent A), 70 % MeCN (v/v) in 0.05% (v/v) FA (eluent B) **Flow**: 1 mL min^{-1}	13 min	1.2-5.4 µg L^{-1}

FPN, FPN-sulfide, FPN-sulfone, BCL	White wine (5 mL) Environmental water (5 mL)	**DLLME-SFO** (NADES as dispersive solvent)	82- 112 84-98	**HPLC-UV** **Column:** Hypersil GOLD C18 (150 x 2.1 mm, 3 µm) **Mobile phase:** Water (eluent A), MeOH (eluent B) Isocratic mode **Flow:** 0.35 L min^{-1}	7 min	2.7-4.5 µg L^{-1} 2.9-4.4 µg L^{-1}
DNT, TMT, CLT, NTP, IMD,TCP, ACT, 6-CNA	Environmental waters (50 mL) Soil (5 g)	**SPE** (Oasis HLB cartridges as sorbent) **SLE** (1:3 ACN/DCM as extraction solvent	80-107 PF:250 73-92 PF: 25	**Sweeping-MEKC-DAD** **Capillary:** 48.5 cm x 50µm i.d (extended light-path capillary) **BGE:** 25 mM Borax + 120 mM SDS +15% MeOH (pH 9.2) **Voltage**: 25 kV	11 min	0.1-1.4 µg L^{-1} 3.2-9.5 µg kg^{1}
DNT, TMT, CLT, NTP, IMD,TCP, ACT, IMZ, FNC, BCL	Pollen (200 mg) Honeybees (200 mg)	**Scaled-down QuEChERS** (Z-Sep+ as dispersive sorbent)	74-95 81-91	**Sweeping-MEKC-MS/MS** **Capillary:** 70 cm x 50 µm i.d **BGE:** 50 mM APFO (pH 9) **Voltage**: 25 kV	7 min	0.1-1.4 µg L^{-1} 3.2-9.5 µg kg^{1}
Em, Et, Es, Eco, Ekr, Ecr, Emn, Etn, Esn, Econ, Ekrn, Ecrn,	25 oat-based products (1g,1 mL)	**modified QuEChERS** (mixture of C18/Z-Sep+ as sorbents)	90-109	**UHPLC-MS/MS** **Column:** C18 Zorbax Eclipse Plus RRHD column (50 × 2.1 mm, 1.8 µm). **Mobile phase:** 0.3% F.A aqueous solution (eluent A), MeOH with 0.3% FA (eluent B) **Flow**: 0.4 mL min^{-1}	7 min	0.2-3.2 µg kg^{1}
Em, Et, Es, Eco, Ekr, Ecr, Emn, Etn, Esn, Econ, Ekrn, Ecrn,	60 cereal samples: 30 of wheat (1 g) 30 of barley (1 g)	**modified QuEChERS** (mixture of C18/Z-Sep+ as sorbents)	86-104 89-104	**UHPLC-MS/MS** **Column:** C18 Zorbax Eclipse Plus RRHD column (50 × 2.1 mm, 1.8 µm). **Mobile phase:** 0.3% F.A aqueous solution (eluent A), MeOH with 0.3% FA (eluent B) **Flow**: 0.4 mL min^{-1}	7 min	0.5-3.3 µg kg^{-1} 0.5-3.9 µg kg^{-1}

Em, Et, Es, Eco, Ekr, Ecr, Emn, Etn, Esn, Econ, Ekrn, Ecrn,	Wheat (1 g)	**modified QuEChERS** (mixture of C18/Z-Sep+ as sorbents)	--	**LC-TWIM-TOF-MS** **Column:** C18 Zorbax Eclipse Plus RRHD column (50 × 2.1 mm, 1.8 μm). **Mobile phase:** 0.3% F.A aqueous solution (eluent A), MeOH with 0.3% FA(eluent B) **Flow**: 0.4 mL min^{-1}	11 min	0.7-2.0 μg kg^{-1}
	Barley (1 g)		--			0.6-2.0 μg kg^{-1}

PF: Preconcentration Factor, FA: Formic Acid

CONLUSIONES FINALES

Los diferentes métodos desarrollados e incluidos en esta Tesis contribuyen a incrementar el número de métodos analíticos disponibles para el control de NNI, FPN y sus metabolitos, BCL así como para el control de EAs, tanto en el ámbito de la seguridad alimentaria como de la medioambiental. Se ha evaluado el potencial de técnicas miniaturizadas como CE y CLC, así como HPLC, UHPLC e IMS acopladas a diferentes sistemas de detección como UV-Vis y MS. Además, se han probado y optimizado una gran variedad de técnicas de tratamiento de muestra en matrices alimentarias y ambientales, proporcionando resultados satisfactorios en términos de recuperación y limpieza de la matriz, con pocos pasos y bajo consumo de disolventes. El uso de técnicas y tratamientos de muestras miniaturizados está de acuerdo con las tendencias de la química analítica verde.

Las conclusiones obtenidas en esta Tesis son las siguientes:

- Los métodos CE y CLC para el control de insecticidas implican un menor consumo de disolventes y una buena eficiencia en comparación con los métodos tradicionales de LC. Entre las técnicas consideradas para los NNIs, la CE es la técnica más “verde” ya que no se necesitan disolventes orgánicos durante la separación debido al uso de un volumen muy pequeño de tampón como BGE y la baja generación de residuos. Asimismo, el tiempo de análisis y el uso de capilares de sílice de bajo precio en lugar de una columna cromatográfica es un aspecto positivo en relación al coste del análisis.

- A pesar de que tradicionalmente se ha atribuido una baja sensibilidad a los métodos CE-UV, en los métodos desarrollados se ha alcanzado una alta sensibilidad, con límites de detección (LOQs) a niveles de pocos μg L^{-1}. Esta mejora de la sensibilidad fue proporcionada por la combinación de estrategias de tratamiento de muestra *on-line* ("barrido" como modo de apilamiento de analitos en el capilar) y de preconcentración *off-line* (SPE). Además, el uso de un capilar con paso de luz extendido contribuyó a mejorar la sensibilidad.

- Aunque los métodos CLC presentaron el mayor tiempo de análisis, permitieron la determinación de siete NNI en matrices complejas como cereales y mieles usando tratamientos de muestra sencillos y rápidos a pesar de utilizar detección UV-Vis, generalmente disponible en todos los laboratorios. Los tratamientos de muestra empleados (SLE y DLLME) no necesitaron de un paso de limpieza, reduciendo por tanto el consumo de reactivos y el tiempo de manipulación de la muestra.

- Por primera vez, diferentes nanofibras poliméricas fueron evaluadas como nuevos sorbentes para la determinación de NNIs, seleccionando la poliimida como el material más adecuado para el procedimiento SPE *on-line* propuesto. El método SPE-HPLC-UV desarrollado permitió el acondicionamiento del sorbente de forma automatizada, la mezcla de muestras con el tampón en el sistema, la preconcentración de analitos y, finalmente, la separación y detección de cinco NNIs.

- Se ha demostrado por primera vez que el acoplamiento MEKC-MS/MS puede usarse para determinar NNI y BCL. Además, la resolución de la separación se mejoró en comparación con el único método CZE-MS/MS publicado que analiza un número menor de analitos. El acoplamiento de MEKC con MS ha sido posible gracias al uso de un tensioactivo volátil que actúa como BGE y medio micelar, conocido como APFO. El potencial de usar este surfactante destacó sobre otras posibles opciones necesarias para hacer compatible la MEKC con la MS, teniendo en cuenta que no se pueden usar surfactantes no volátiles ya que producen contaminación de la fuente de ionización del espectrómetro de masas.

- Se ha demostrado que un procedimiento miniaturizado basado en la metodología QuEChERS es una estrategia útil para la extracción de NNIs and

BCL antes de su determinación por MEKC-MS/MS cuando la cantidad disponible de muestra es reducida. Además, se seleccionó un sorbente para el proceso de SPE dispersiva conocido como Z-Sep+, especialmente indicado para la eliminación de lípidos y ácidos grasos de la matriz, aplicado para eliminar las interferencias en muestras de abejas y polen. Este sorbente dispersivo redujo significativamente los efectos de la matriz si se compara con los comúnmente elegidos para este tipo de muestras, como C18 y PSA, sin perjudicar las recuperaciones de extracción.

- Se han evaluado por primera vez varios NADES como disolventes dispersivos en un procedimiento DLLME-SFO, aplicado al control de FPN y sus metabolitos junto con BCL. Se eligió como disolvente óptimo el NADES denominado "LGH" compuesto por lactosa, glucosa y agua. Este disolvente fue capaz de dispersar el disolvente de extracción en gotitas finas dando lugar a una disolución turbia característica de los métodos basados en DLLME sin la necesidad de emplear disolventes orgánicos tóxicos. Este trabajo reveló el potencial de usar NADESs en procedimientos de tratamiento de muestra.

- En el análisis de EAs por UHPLC-MS/MS, la modificaciones llevadas a cabo en el procedimiento QuEChERS tradicionalmente utilizado para la extracción en muestras de cereales, ha permitido una reducción del uso de disolventes orgánicos así como un aumento de la sensibilidad. El volumen del disolvente de extracción en este tratamiento de muestra podría reducirse evitando la dilución de la muestra sin afectar negativamente las recuperaciones de extracción de los analitos.

- El método validado para la determinación de EAs en muestras a base de avena incluyendo complementos alimenticios es una propuesta útil en seguridad alimentaria, ya que se necesitan más métodos analíticos que involucren alimentos procesados y suplementos a base de cereales y plantas para evaluar sus riesgos en el consumo humano. Aunque solo una muestra fue positiva, esto significa que a pesar de las mejoras en el procesamiento de los granos, se debe considerar la contaminación por EAs, especialmente en los alimentos procesados a base de cereales. De la misma forma, el estudio de la presencia natural de EAs en muestras de cereales de países en los que las micotoxinas no están reguladas, como Argelia, contribuye a esta cuestión y enfatiza la importancia de incluir los epímeros de los EAs en la evaluación de riesgos y la variabilidad de EAs entre las muestras positivas.

- La colaboración con LABERCA (Nantes, Francia), nos permitió utilizar IMS-HRMS para establecer una base de datos con $^{TW}CCS_{N2}$ para todos los iones identificados para los principales EAs y sus epímeros, así como para los principales fragmentos observados. Esta base de datos también ha sido validada mediante un estudio interlaboratorio en colaboración con la Universidad de Parma (Italia), mostrando desviaciones satisfactorias de los valores de CCS y demostrando la aplicabilidad del uso de la CCS como un parámetro complementario e independiente del instrumento, especialmente útil en análisis no dirigido.

- Finalmente, se han investigado las ventajas de considerar la IMS en los flujos de trabajo LC-MS destinados a determinar EAs utilizando un método LC-TWIM-TOF-MS. El uso de TWIM permite separar los compuestos de interés de las interferencias presentes en la matriz, lo que conduce a una mejora en la selectividad. Esta dimensión adicional también proporcionó más confianza para la determinación de los principales EAs con respecto a sus epímeros. Además, esta técnica ha sido útil para reducir el ruido de fondo, mejorando la relación S/N de los compuestos estudiados y, por tanto, la sensibilidad del método.

A modo de resumen, las características experimentales e instrumentales más significativas de los métodos desarrollados en esta Tesis se muestran en la **Tabla 1.**

Table 1. Características analíticas de los métodos propuestos.

Analitos	Muestra	Tratamiento de muestra	R (%)	Técnica	Tiempo de analisis	LOQs
DNT, TMT, CLT, NTP, IMD,TCP, ACT,	Arroz, avena, trigo, maíz y cebada (1 g) Miel mil flores, miel de eucalipto, romero y naranjo (1 g)	**SLE** (1:3 ACN/DCM como extractante) **DLLME**	80- 105 80- 94	**CLC-DAD** **Columna:** Zorbax XDB-C18 (150 × 0.5 mm i.d, 5 µm) **Fase móvil:** Agua (eluyente A), MeCN (eluyente B) **Flujo**: 10 µL min^{-1}	18 min	9.3-16.7 µg kg^{1} 9.6-22 µg kg^{1}
ACT, CLT, DNT, IMD, TCP, TMT	Aguas naturales (2 mL)	**on-line SPE** (nanofibras como sorbente)	63-120 PF: 70-82	**HPLC-UV** **Columna**: Phenomenex RP-C18 (150 x 4.6, 2.1 µm) **Fase móvil:** 10 % MeCN (v/v) en 0.05% (v/v) FA (eluyente A), 70 % MeCN (v/v) en 0.05% (v/v) FA (eluyente B) **Flujo**: 1 mL min^{-1}	13 min	1.2-5.4 µg L^{-1}
FPN, FPN-sulfide, FPN-sulfone, BCL	Vino blanco (5 mL) Agua natural (5 mL)	**DLLME-SFO** (NADES como disolvente dispersivo)	82- 112 84-98	**HPLC-UV** **Columna:** Hypersil GOLD C18 (150 x 2.1 mm, 3 µm) **Fase móvil:** Agua (eluyente A), MeOH (eluyente B). Modo isocrático **Flujo:** 0.35 L min^{-1}	7 min	2.7-4.5 µg L^{-1} 2.9-4.4 µg L^{-1}
DNT, TMT, CLT, NTP, IMD,TCP, ACT, 6-CNA	Aguas naturales (río, pozo y manantial) (50 mL) Suelo (5 g)	**SPE** (cartuchos Oasis HLB) **SLE** (1:3 ACN/DCM como extractante)	80-107 PF:250 73-92 PF: 25	**Sweeping-MEKC-DAD** **Capilar:** 48.5 cm x 50µm i.d (con paso del luz extendido) **BGE:** 25 mM Borax + 120 mM SDS +15% MeOH (pH 9.2) **Voltaje**: 25 kV	11 min	0.1-1.4 µg L^{-1} 3.2-9.5 µg kg^{1}

DNT, TMT, CLT, NTP, IMD,TCP, ACT, IMZ, FNC, BCL	Polen (200 mg) Abejas (200 mg)	**Scaled-down QuEChERS** (Z-Sep+ como sorbente dispersivo)	74-95 81-91	**Sweeping-MEKC-MS/MS** **Capilar:** 70 cm x 50 µm i.d **BGE:** 50 mM APFO (pH 9) **Voltaje**: 25 kV	7 min	0.1-1.4 µg L^{-1} 3.2-9.5 µg kg^{1}
Em, Et, Es, Eco, Ekr, Ecr, Emn, Etn, Esn, Econ, Ekrn, Ecrn,	25 productos a base de avena (1g,1 mL)	**QuEChERS modificado** (mezcla de sorbentes dispersivos C18/Z-Sep+)	90-109	**UHPLC-MS/MS** **Columna:** C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 µm). **Fase móvil:** Agua con 0.3% F.A (eluyente A), MeOH con 0.3% F.A (eluyente B) **Flujo**: 0.4 mL min^{-1}	7 min	0.2-3.2 µg kg^{1}
Em, Et, Es, Eco, Ekr, Ecr, Emn, Etn, Esn, Econ, Ekrn, Ecrn,	60 muestras de cereales: 30 de trigo (1 g) 30 de cebada (1 g)	**QuEChERS modificado** (mezcla de sorbentes dispersivos C18/Z-Sep+)	86-104 89-104	**UHPLC-MS/MS** **Columna:** C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 µm). **Fase móvil:** Agua con 0.3% F.A. (eluyente A), MeOH con 0.3% FA (eluyente B) **Flujo**: 0.4 mL min^{-1}	7 min	0.5-3.3 µg kg^{-1} 0.5-3.9 µg kg^{-1}
Em, Et, Es, Eco, Ekr, Ecr, Emn, Etn, Esn, Econ, Ekrn, Ecrn,	Trigo (1 g) Cebada (1 g)	**QuEChERS modificado** (mezcla de sorbentes dispersivos C18/Z-Sep+)	-- --	**LC-TWIM-TOF-MS** **Columna:** C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 µm). **Fase móvil:** Agua con 0.3% F.A (eluyente A), MeOH con 0.3% F.A (eluyente B) **Flujo**: 0.4 mL min^{-1}	11 min	0.7-2.0 µg kg^{-1} 0.6-2.0 µg kg^{-1}

PF: Factor de preconcentración, FA: Ácido fórmico